

Reevaluating the species status of the Southern Ghost Pipe, *Monotropa brittonii*

Thesis

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By

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## Abstract

Relationships between members of Ericaceae subfamily Monotropoideae have been notoriously difficult to resolve due to convergent evolution in parasitic plants. Ghost pipes (*Monotropa uniflora*, L.) are fully mycoheterotrophic, meaning they obtain nutrients by parasitizing ectomycorrhizal fungi rather than through photosynthesis. The southern Ghost pipe (*Monotropa brittonii*, Small) was a species proposed to be distinct from the closely related and more widespread *M. uniflora* by John K. Small from his study of Florida flora. It has since largely been treated as a synonym of *M. uniflora*. Here we use several lines of evidence including genetics, morphology, host specificity, and habitat to investigate whether there is evidence to treat *M. brittonii* as its own species or if synonymization with *M. uniflora* is supported.

Through morphological and molecular phylogenetic analysis of *Monotropa* collected throughout their range in the US, we determine there is evidence for two separate lineages in Florida, one of which corresponds morphologically to the description of *M. brittonii* put forth by Small. We also discovered a high degree of host specificity in *M. brittonii*, which almost exclusively parasitize fungi in *Lactifluus* subgenus *Lactariopsis* section *Albati*. While *M. uniflora* have been shown to parasitize many species of Russulaceae, most *M. brittonii* were found to parasitize a single species; *Lactifluus deceptivus*. Through principal component analysis we found support for several morphological characters that differ significantly between the two species. Additionally, *M. brittonii* were almost exclusively collected from Florida scrub habitats, which are dry, shrub-dominated environments that differ greatly from the typical moist woodland habitat where *M. uniflora* is primarily found. Our results suggest there is genetic, morphological, and ecological support to recognize *M. brittonii* as a separate species from *M. uniflora*.

## **Dedication**

To my cat, Big Fluffy.

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# Reevaluating the species status of the Southern Ghost Pipe, *Monotropa brittonii*

## Introduction

### *Genetic diversity within Monotropa*

Underestimating species numbers is detrimental to biodiversity as it may allow for the loss of unknown genetically unique species. This is particularly relevant in the modern era when threats such as habitat loss, climate change, and anthropogenic forces are causing a rapid increase in the rate of extinction of organisms (Steffen *et al.* 2007, Hooper *et al.* 2005). It is important to analyze and document biodiversity so we can ask questions about how to best preserve biodiversity in a changing climate. Parasites are compelling organisms to look at when considering species delimitation due to the role host specificity plays in diversification and speciation. Investigating species limits in parasites can help inform our understanding of symbiosis and how these interactions may play a role in diversification.

Ericaceae subfamily Monotropeoideae consists of 11 genera of parasitic plants (Freudenstein *et al.* 2016). These plants are leafless and nonphotosynthetic, and obtain nutrients by parasitizing ectomycorrhizal fungi. *Monotropa uniflora* are white, or occasionally pink herbaceous plants and are typically found in moist forests often dominated by oaks, hemlocks, pines, or beeches. They have a wide distribution occurring throughout the Eastern US as far west as the Great Plains, southern Canada, the Pacific Northwest, Mexico extending south to Columbia, and in eastern Asia in Russia, China, Korea, and Japan (Wallace 1996, Min *et al.* 2012).

In recent years, the monotropoid genus *Monotropa* has been largely considered monotypic with just one widely recognized species, *Monotropa uniflora* (Logacheva *et al.* 2016, Freudenstein *et al.* 2016). Phylogenetic work by Bidartando and Bruns (2001) has called for the transfer of one

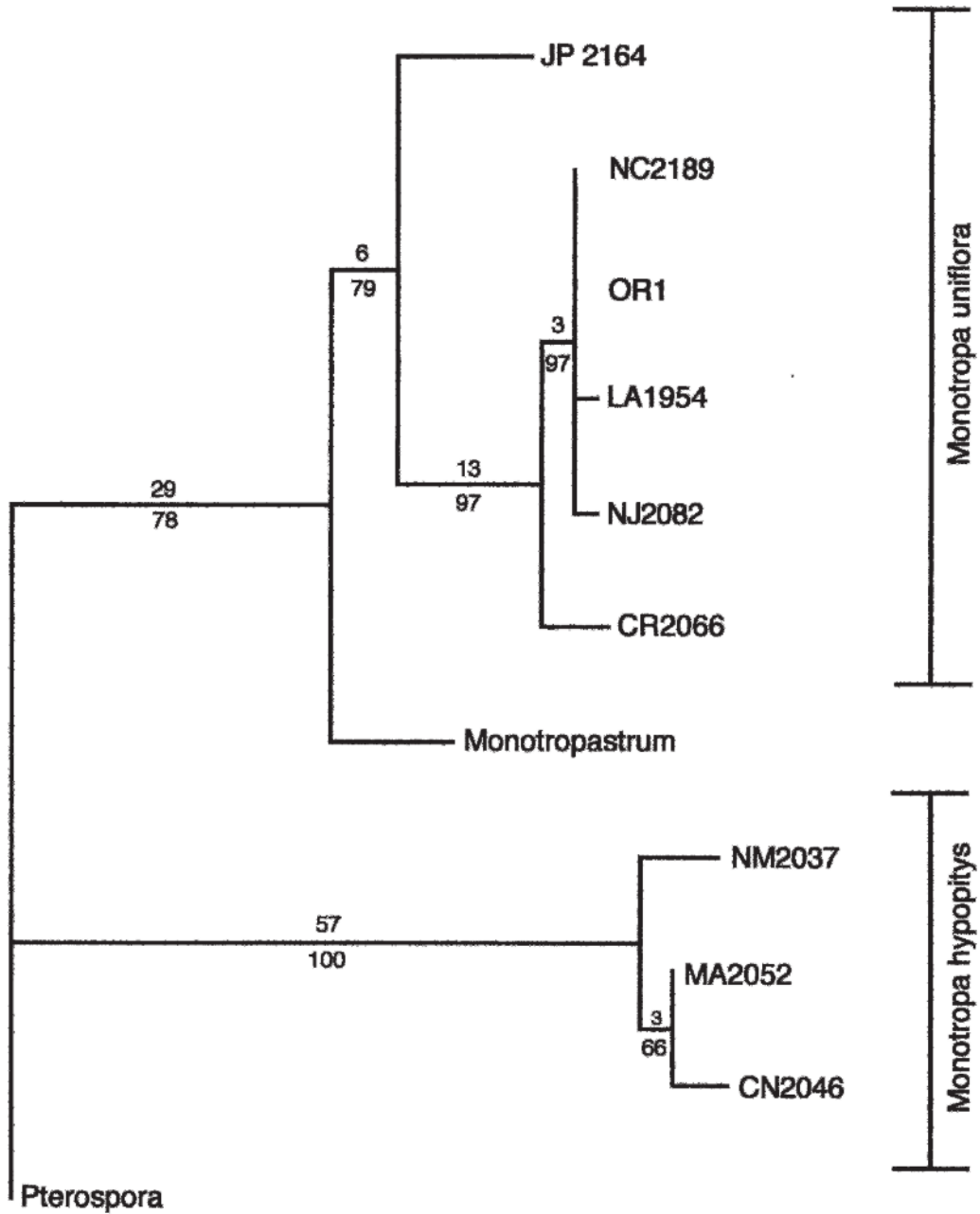
species formerly placed in this genus, *Monotropa hypopitys* L., to its own genus, *Hypopitys*, as it has been shown through phylogenetic analysis that it does not fall into a monophyletic evolutionary lineage with *M. uniflora*, and is more closely related to *Pityopus californicus* (Liu *et al.* 2017). Another species sometimes recognized in this genus is the Red Ghost Pipe (*Monotropa coccinea*, Zucc.), which occurs in Mexico and Central America and is said to differ from *M. uniflora* mainly in color and number of bracts (Zuccarini 1832). It has since primarily been treated as a synonym of *M. uniflora* (Wallace 1975, Neyland and Hennigan 2004). Here we focus on another synonym of *M. uniflora*, *Monotropa brittonii*, the Southern Ghost pipe.

*Monotropa brittonii* was described in 1927 by John K. Small from his exploration of the flora of the southeastern US (Small 1927). It was characterized as different from *M. uniflora* mainly in size, color, scent, and pubescence (Small 1927, Dorman 1958). *Monotropa brittonii* was treated as a synonym of *M. uniflora* by Gary Wallace in his 1975 monographic treatment of Ericaceae subfamily Monotropeoideae. Wallace characterized morphological features originally thought to make *M. brittonii* distinct as simply normal species level variation sometimes exhibited in *M. uniflora* and not sufficient to warrant species status (Wallace 1975). Modern studies have largely followed Wallace's treatment of *Monotropa* and have predominantly treated *M. brittonii* as a synonym (Stevens *et al.* 2004).

Neyland and Hennigan (2009) put this synonymy to the test using phylogenetic analysis of the 26S gene. In their analysis, the *M. brittonii* representative from Louisiana fell into a clade with three other individuals collected from New Jersey, North Carolina, and Oregon (Fig. 1). Their findings were that no synapomorphies distinguish *M. brittonii* from the rest of *Monotropa*. However, in their study they only used six individual *Monotropa uniflora*, including one that was meant to be representative of *M. brittonii*. This individual was used to represent *M. brittonii*

because it “it falls within the range of *M. brittonii* (Dormoa, 1958) and exhibits the morphological characters consistent with Small’s (1927) description” (Neyland and Hennegan 2009). These morphological characters were not elaborated upon, so it is possible this individual was not equivalent to what Small had in mind when he described *M. brittonii*.

In this study, we analyze phylogenetic relationships within the genus *Monotropa*. The purpose of this analysis is to determine if there is evidence for a phylogenetically distinct lineage of *Monotropa* that exists in Florida and is comparable to Small’s description of *M. brittonii*. We use two loci, 26S-ITS and the chloroplast gene *rps2*, and create phylogenetic trees to make inferences about evolutionary relationships within *Monotropa*.



**Figure 1. Phylogeny of monotropoids from Neyland and Hennigan (2009).**

Tree created using 26S, the number of synapomorphies is indicated above branches and the bootstrap values are below. The taxon “LA1954” is their putative *M. brittonii* individual.

### ***Host specificity in Monotropoids***

Fungi and plant roots often engage in mutualistic interactions known as mycorrhizae which are essential to many ecosystems as they help plants improve water and nutrient intake (Allen 1996; Bergemann *et al.* 2005). In exchange for water and nutrients, the fungi receive sugars from the plant that are produced during photosynthesis (Johnson *et al.* 1997). Around 90% of terrestrial plants form these types of mutualistic interactions (Smith and Read 2008). The majority of land plants form arbuscular mycorrhizae (AM) with members of the fungal phylum Glomeromycota (Smith and Read 2008; Brundrett 2004). These fungi enter the plant root cell walls without breaking the cell membrane and form branched hyphae within the cells. The branched hyphae are referred to as “arbuscules” and they facilitate the transfer of sugars and nutrients between the plants and fungi. Interactions between plants that form arbuscular mycorrhizae and their fungal partner tend to be non-specific, with many fungal species able to associate with many plant species and vice versa (Saari *et al.* 2005, Selosse *et al.* 2007).

Other plants form a different type of mycorrhizae: ectomycorrhizae (ECM). Fungi that form ECM are Ascomycetes or Basidiomycetes and they typically partner with woody trees or shrubs (Smith and Read 2008). In ectomycorrhizae, unlike arbuscular mycorrhizae, fungi do not penetrate the cell walls and instead form a sheath that covers the outside of the root and a network of hyphae that pass between the cells forming a structure called a Hartig net (Halling 2001, Lepage *et al.* 1997). ECM are particularly important in boreal forests as the dominant coniferous trees depend on mycorrhizal fungi to help them survive harsh conditions (Lepage *et al.* 1997). ECM have also been found to be important in the tropics as they can help trees compete in highly competitive environments (Halling 2001, Smith and Read 2008).

Plants in Ericaceae exhibit a wide variety of mycorrhizal interactions which differ in terms

of their structure and fungal partnerships (Cullings 1996, Smith and Read 2008, Brundrett and Tedersoo 2018). Mycorrhizal interactions in Ericaceae differ from typical AM and ECM and have been given their own unique classifications such as ericoid, arbutoid, pyroloid and monotropoid (Freudenstein *et al.* 2016). These varied mycorrhizal types are thought to have evolved from an arbuscular mycorrhizal ancestor (Selosse *et al.* 2007, Freudenstein *et al.* 2016). Monotropoid mycorrhizae are morphologically similar to ectomycorrhizae, with an ascomycete or basidiomycete fungal partner, hartig net, and mantle, however they differ from ECM in that they form structures known as “fungal pegs” which penetrate the epidermal root cells (Smith and Read 2008, Freudenstein *et al.* 2016).

While mycorrhizal interactions are ordinarily beneficial to all participants, some plants have adapted to take advantage of these mutualisms. This parasitic lifestyle, known as full mycoheterotrophy, has evolved many times in the plant kingdom and occurs in at least 17 plant families including monocots, dicots, ferns, liverworts, and even one gymnosperm (Merckx *et al.* 2009, 2013, Field and Brodribb 2005).

Members of Ericaceae subfamily Monotropeae are mycoheterotrophic, meaning they obtain carbon and other nutrients by parasitizing mycorrhizal fungi. Monotropoids associate with basidiomycetes and form morphologically distinctive mycorrhizae referred to as “monotropoid” mycorrhizae (Cullings 1996). In contrast to some plants engaged in mycorrhizal interactions, monotropoids tend to be highly specialized in their mycorrhizal interactions and can be limited in their fungal hosts at the level of family, genus, or even section within a genus in some extreme situations (Fig. 2), (Bidartando and Bruns 2001, Yang and Pfister 2006, Smith and Read 2008). It has been established through widespread sampling and molecular analysis that *M. uniflora* exclusively parasitize Russulaceae (Young *et al.* 2002, Yang and Pfister 2006, Bidartando and

Bruns 2001, 2002, Cullings 1996, Min *et al.* 2012). Additionally, *M. uniflora* in the Pacific Northwest seem to be somewhat regionally specific on a single species of fungi, *Russula brevipes* (Bidartando and Bruns 2001, 2005, Young *et al.* 2002).

Some early reports claimed that *M. uniflora* parasitizes ascomycete hosts (Kernan and Finnochio 1983, Riley and Eichenmuller 1970). These studies were published before the widespread use of molecular data and were based solely on the morphological characteristics of the fungal hyphae. It is possible that these fungi were soil saprotrophs or endophytes rather than the actual hosts of *M. uniflora*.

Speciation can occur in parasites when a portion of the parasite population shifts to a different host (Norton and Carpenter 1998). Host switching may result in a lack of gene flow between parasites of each host, which can lead to diversification (Norton and Carpenter 1998, Forbes *et al.* 2017). In this study, we investigate the identity of the fungal hosts of *Monotropa uniflora* to see if there is evidence of host switching or regional host specificity that supports the hypothesis that *M. brittonii* is distinct from *M. uniflora*. We also compare the fungal host phylogeny to the phylogeny of *M. uniflora* to see if differences in host identity are reflected in patterns of diversification in *M. uniflora*.



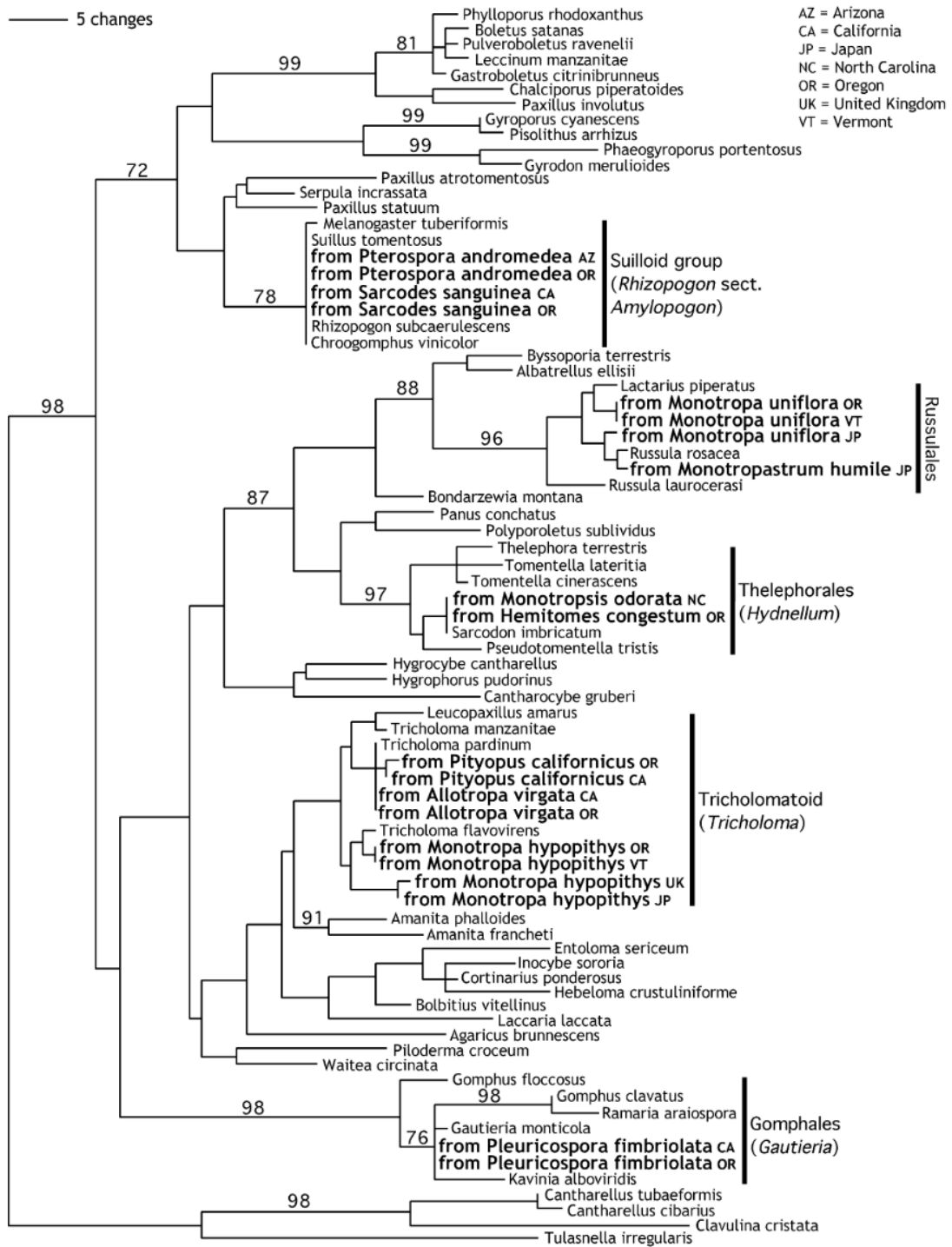


Figure 2. Phylogeny of Monotropoid hosts from Bidartando and Bruns (2001)

### ***Morphology of Monotropa***

Small described *M. brittonii* as orcherous in color and having a larger flower, more pubescent filaments and petals, and less saccate petals than *M. uniflora*. Preliminary assessment of morphological features revealed there were several individuals collected from Florida that had quite large flowers and displayed a yellow-orange coloration and nectaries which were upturned towards the opening of the flower. When descriptions of the nectaries are included, *Monotropa uniflora* is consistently described as having nectaries that are spur-like and paired at the base of filaments and point downwards, away from the opening of the flower and into the saccate bases of the petals (Luteyn *et al.* 1996). Here we investigate these and other morphological characters to evaluate phenotypic dissimilarities between *M. uniflora* and *M. brittonii*.

### ***Habitat fidelity in Monotropa***

When *M. brittonii* was described by Small, he stated the type locality as “Scrub near Pompono”. Florida scrub is a relatively rare and unique ecosystem that is characterized by well drained nutrient-poor sands and dominated by shrubby oaks, Florida rosemary (*Ceratiola ericoides*) and scattered canopies of sand pine (*Pinus clausa*) (Menges and Hawkes 1998). This habitat houses a high number of endemic flora and fauna and is extremely fragmented (Hokit *et al.* 1999).

Florida scrub, a dry habitat with little or no canopy, is considerably different from the typical mesic wood habitat where *M. uniflora* are typically found. We wanted to investigate if populations of *Monotropa* in Florida scrub habitat have diverged from those in moist oak or pine forests, which are the commonly recognized habitat of *M. uniflora* (Luteyn *et al.* 1996). By investigating the fidelity of *Monotropa* to this habitat compared to other habitats, we may be able

to determine if there is evidence to support allopatric speciation in the past.

## Methods

### *Sample collection*

Floral material was collected between 2010 and 2018 from across the distribution of *Monotropa uniflora*, mainly in the eastern United States (Fig. 3, Appendix A). Vouchers were deposited in the Ohio State University Herbarium. When multiple stems were present some flowers were preserved in 70% ethanol for morphological analysis and/or silica gel for further DNA analysis. Roots of *Monotropa* were also collected between 2017 and 2018 to analyze fungal hosts. Roots were dried in silica gel before DNA isolation.

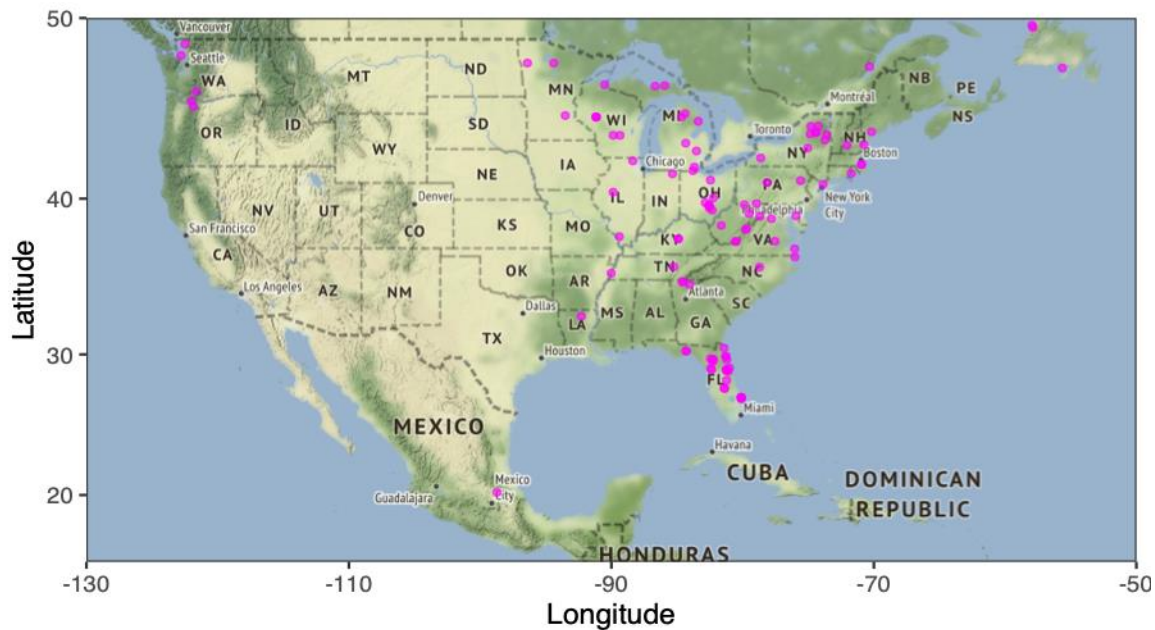


Figure 3. Collection sites for this study

### *Phylogenetic analysis of Monotropa DNA*

DNA of plant material was isolated using a modified CTAB procedure (Doyle and Doyle 1987). For specimens collected without fresh flower material, plant DNA was isolated from root material using a DNeasy plant mini kit (Qiagen).

We amplified two loci, the rDNA ITS-26S region and the chloroplast gene *rps2* using PCR. These two loci were chosen for ease of amplification as well as for their high levels of variation and utility in resolving species level relationships.

For the ITS-26S region, we used the primers 17SE, 641R and 1229R to amplify a region of around 1700bp (Sun *et al.* 1994, Kuzoff *et al.* 1998). The two internal transcribed spacer regions (ITS1 and ITS2) are highly variable and flanked by highly conserved regions. This, along with the fact that they exist in a high number of copies makes this region useful and easy to work with in phylogenetic analysis (Baldwin *et al.* 1995). In our 26S-ITS tree we also included GenBank sequences of *M. uniflora* used in Neyland and Hennigan's (2009) investigation of the Southern Ghost Pipe to establish where their putative *M. brittonii* fell in relation to the *M. uniflora* collected in this study.

*rps2* is a chloroplast gene and therefore also occurs in high copy number in each cell. Monotropoids and other nonphotosynthetic plants experience reductions in their plastomes (Braukmann and Stefanovic 2012, Braukmann *et al.* 2017). However, it has been shown that *rps2* is retained in *Monotropa uniflora* (Braukmann and Stefanovic 2012, Braukmann *et al.* 2017), and it may show enough differentiation between individuals to reveal species level variation (Bidartando and Bruns 2001, Broe 2014).

Two mitochondrial loci were investigated as well, *nad1* and *nad5*, part of the NADH dehydrogenase complex. However, these two mtDNA genes had insufficient variation to reveal

species level variation and the resulting tree had very little resolution. Thus, these genes were not included in this analysis.

Sequences were aligned in Geneious v11.1.15 using MUSCLE v3.8.425 (<http://www.geneious.com>, Kearse *et al.*, 2012, Edgar 2004). We found the optimal substitution models using ModelFinder in IQ-TREE (Kalyaanamoorthy *et al.* 2017) for maximum likelihood analysis and jModeltest2 using three possible substitution schemes for the Bayesian inference analysis in MrBayes (Darriba *et al.* 2012, Guindon and Gascuel 2003). In all cases the model suggested by the BIC criterion was used (Table 1). Trees were created using 26S-ITS, *rps2*, and concatenated 26S-ITS/*rps2* data. We created trees using maximum likelihood in IQ-TREE version 1.6.12 with 1000 ultrafast bootstrap replications (Hoang *et al.* 2018, Nguyen *et al.* 2015). We also performed Bayesian inference analysis using MrBayes v3.2.6 x64 for 5,000,000 generations (Huelsenbeck and Ronquist 2001, Ronquist and Huelsenbeck 2003). Sampling was conducted every 1,000 generations with a 10% burn-in.

IQ-TREE			
	Fungal ITS	26S-ITS	<i>rps2</i>
BIC	TIM2+F+R8	TIM3e+G4	K3Pu+F+I
MrBayes			
BIC	GTR+I+G	SYM+G	GTR+G

**Table 1. Substitution models**

### ***Phylogenetic analysis of fungal host***

DNA was isolated from root tissue using a DNeasy Plant minikit (Qiagen, Valencia, CA, USA). We used PCR to amplify the fungal ITS region and identify the fungal species. The ITS region is frequently used as a standard barcoding locus when working with fungi (Schoch *et al.* 2012). Fungal DNA was amplified using the ITS1OFa-b/ITS4OF primer pair to insure amplification of Basidiomycota fungal DNA (Taylor and McCormick 2008).

We performed BLAST searches on the fungal ITS sequences to determine the range of

fungal species that *Monotropia* parasitize. In our search, we included both the percent identity match and the max score from BLAST. Because of the wide array of works confirming the specialization of *Monotropia uniflora* on russulacean fungi (Young *et al.* 2002, Yang and Pfister 2006, Bidartando and Bruns 2001, 2002, Cullings 1996, Min *et al.* 2012), any fungi found to match species outside of this family were presumed to be other types of fungal endophytes or soil saprotrophs and therefore were not included in this phylogenetic analysis.

We downloaded 383 fungal ITS sequences from GenBank. We chose representatives from each subgenus of *Lactarius*, *Lactifluus*, and *Russula* according to some of the most recent taxonomic treatments (Lee *et al.* 2019, Buyck *et al.* 2008, Buyck *et al.* 2018, De Crop *et al.* 2017, Appendix A) as well as representatives from *Multifurca* and several outgroups. Also included in this analysis were fungal sequences from other studies investigating *Monotropia* host specificity to better observe the full range of hosts these plants parasitize (Bidartando and Bruns 2005, 2001, Kong 2015, Yang and Pfister 2006, Young *et al.* 2002). We aligned sequences, determined the best substitution model, and created trees using maximum likelihood and Bayesian inference methods as previously described for the analysis of the *Monotropia* molecular data.

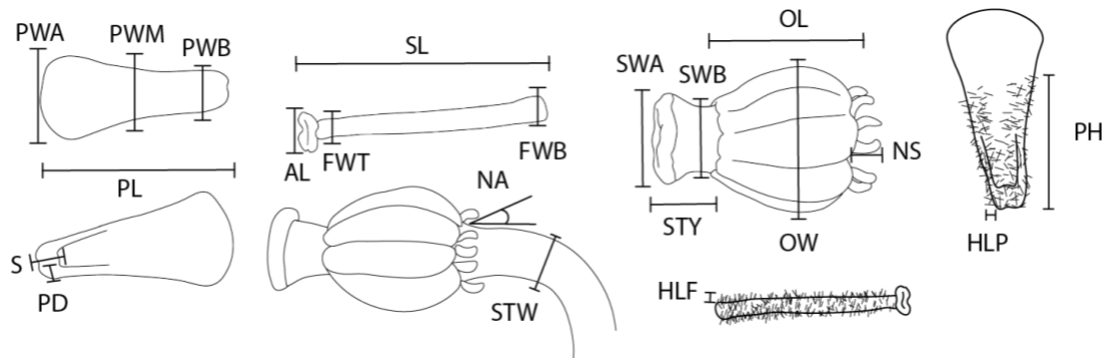
### ***Morphological analysis***

Flowers were preserved in 70% alcohol when fresh and abundant at a site. We used calipers to measure diagnostically significant morphological characters based on descriptions of *M. brittonii* (Small 1927) and *M. uniflora* (Luteyn *et al.* 1996, Wallace 1996). All measurements were taken in millimeters. Depictions of all measurements can be seen in Figure 4. The degree of petal saccateness was measured in two ways. First, by placing the petal on its side and measuring the thickness at the base of the petal, hereafter referred to as “petal depth”. Second, by measuring the

length of the tissue on the interior of the petal base.

When distinguishing *M. brittonii* from *M. uniflora*, Small made note of “ciliate sepals and petals” and “copiously pubescent filaments” (Small 1927). For this reason, we made several measurements to quantify hairiness. Hair length was measured at the base for both the filaments and petals. We also measured how far the pubescence extended along the length of the petal, hereafter referred to as petal hairiness.

To measure the nectar spur angle, the stem was used as the axis and the measurement was taken using the side of the nectar spur closest to the stem. Some individuals had nectar spurs that curled downward back towards the stem creating a negative angle. To avoid negative values for this measurement we added 21 degrees to all angle measurements (the lowest negative angle measurement).



**Figure 4. Morphological measurements.**

PWA – Petal width at apex, PWM – Petal width at middle, PWB – Petal width at base, SL – Stamen length, AL – Anther length, FWT – Filament width at top, FWB – Filament width at bottom, SWA – Stigma width at apex, SWB – Stigma at width base, STY – Style length, OL – Ovary length, OW – Ovary width, NS – Nectar spur length, HLP – Hair length petal, PH- Petal hairiness, S – Saccateness, PD – Petal depth, NA – Nectar spur angle, STW – Stem width, HLF – Hair length filament

To test for significant differences between *brittonii* individuals and *uniflora* individuals, we first assigned them to groups based on their placement in our phylogenetic tree, discussed further in the upcoming sections. Individuals that fell into the *brittonii* clade were assigned to one group, and all others were assigned to a second group, the “*uniflora*” clade. We performed Shapiro-



Wilk tests in R to determine which variables had normal distributions. T-tests were done on variables with normal distributions and Wilcoxon rank sum tests were done on those with non-normal distributions. Any significant variables ( $p < 0.05$ ) were included in the PCA. Principal component analysis of morphological characters was done using the R packages factoextra (Kassambara and Mundt 2017).

## Results

### *Phylogenetic analysis of Monotropa uniflora*

While the trees have different amounts of resolution, all display the same basic structure with a large unresolved clade, and several smaller highly supported clades near the base of the tree (Fig. 5-7). The majority of individuals fell into the large, unresolved clade labeled here as the *uniflora* clade. Most individuals in this clade had very few sequence differences and were collected from a variety of sites across the geographical range of *M. uniflora*.

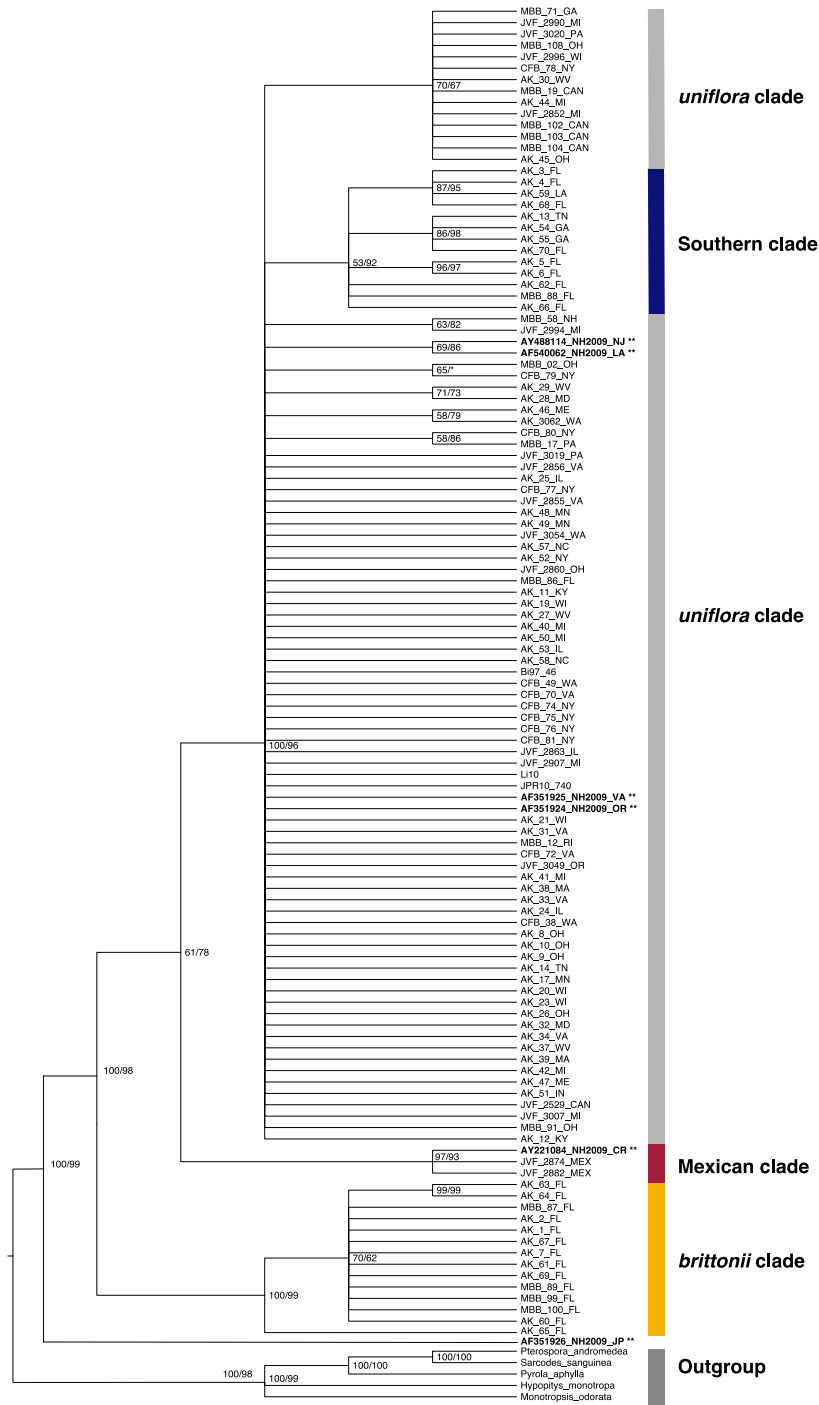
Each tree shows several smaller clades with moderate to high support. One clade of mainly individuals collected from southern states (labeled in the trees as the Southern clade) appears unresolved within the *uniflora* clade in the 26S-ITS tree. It is sister to the *uniflora* clade in the other two trees. This clade was most highly supported in the concatenated 26S-ITS/*rps2* tree. There are 12 individuals that consistently fall into this clade and several others that occur in this clade in only one or two of the trees. Due to the low resolution of this clade, it is grouped with the *uniflora* clade in the morphological analysis.

In all trees, there is a highly supported clade of two Mexican individuals, referred to here as the Mexican clade. In the 26S-ITS tree, which includes several accessions from Neyland and Hennigan (2009), their *Monotropa* from Costa Rica falls into this clade with high support. Interestingly, though these populations are more geographically distant to the populations in the southern and *uniflora* clades, they appear to be more closely related to these clades than the *brittonii* group.

The *brittonii* clade consists solely of individuals from Florida and is also highly supported in all trees. Most of these individuals were collected from Florida scrub habitats. In the majority

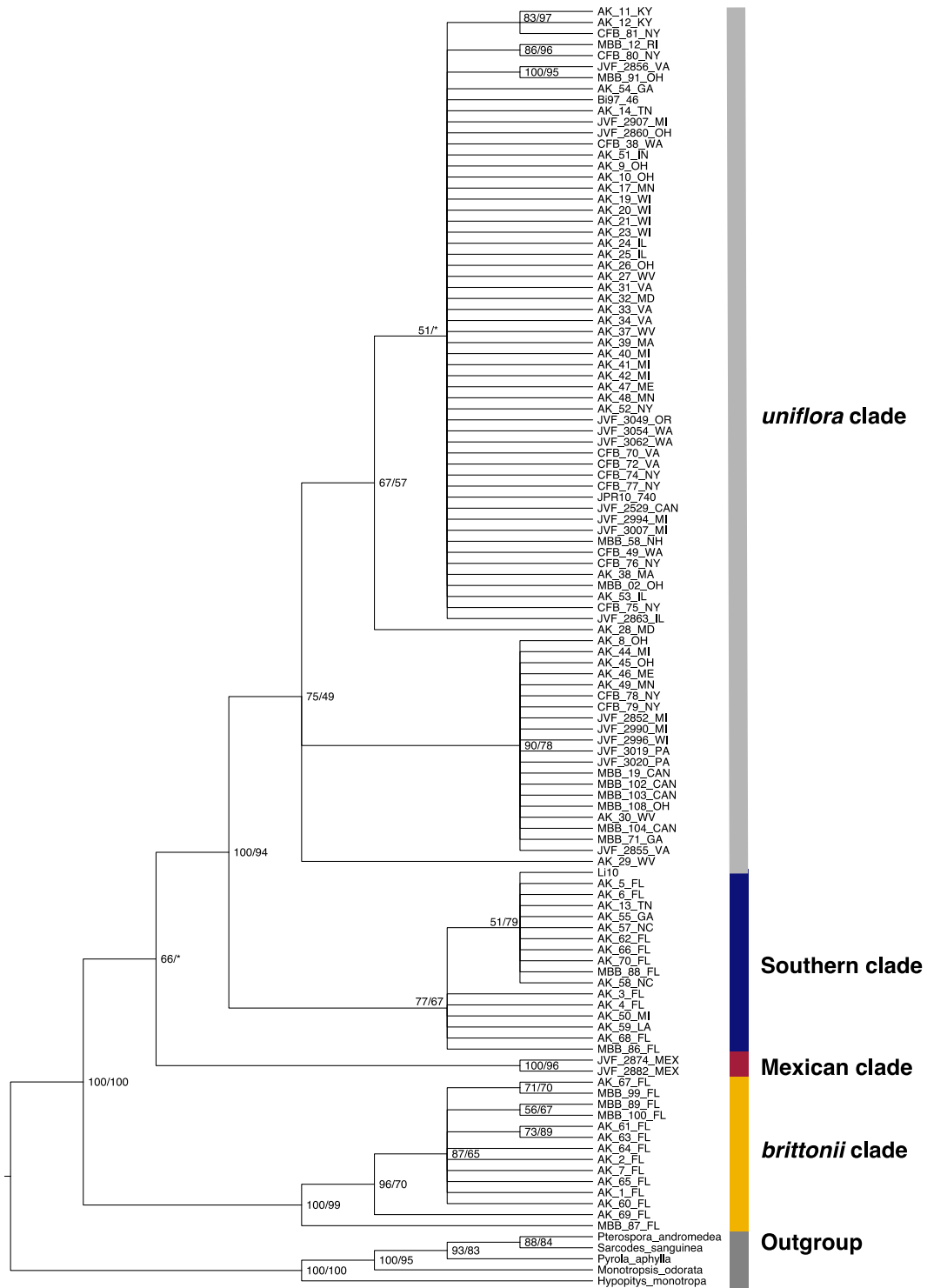
of trees, the *brittonii* clade was sister to the rest of *M. uniflora*, however, in the *rps2* ML consensus tree, the Mexican and *brittonii* clades switch positions so that the Mexican clade is sister to all other clades of *Monotropa* (Fig. 8).

In the 26S-ITS tree, four of the Neyland and Hennigan (2009) *M. uniflora* sequences, including the one that was meant to represent *M. brittonii*, fall within the large unresolved clade. Their specimen from Japan is sister to all other *Monotropa* in this study (Fig. 5).



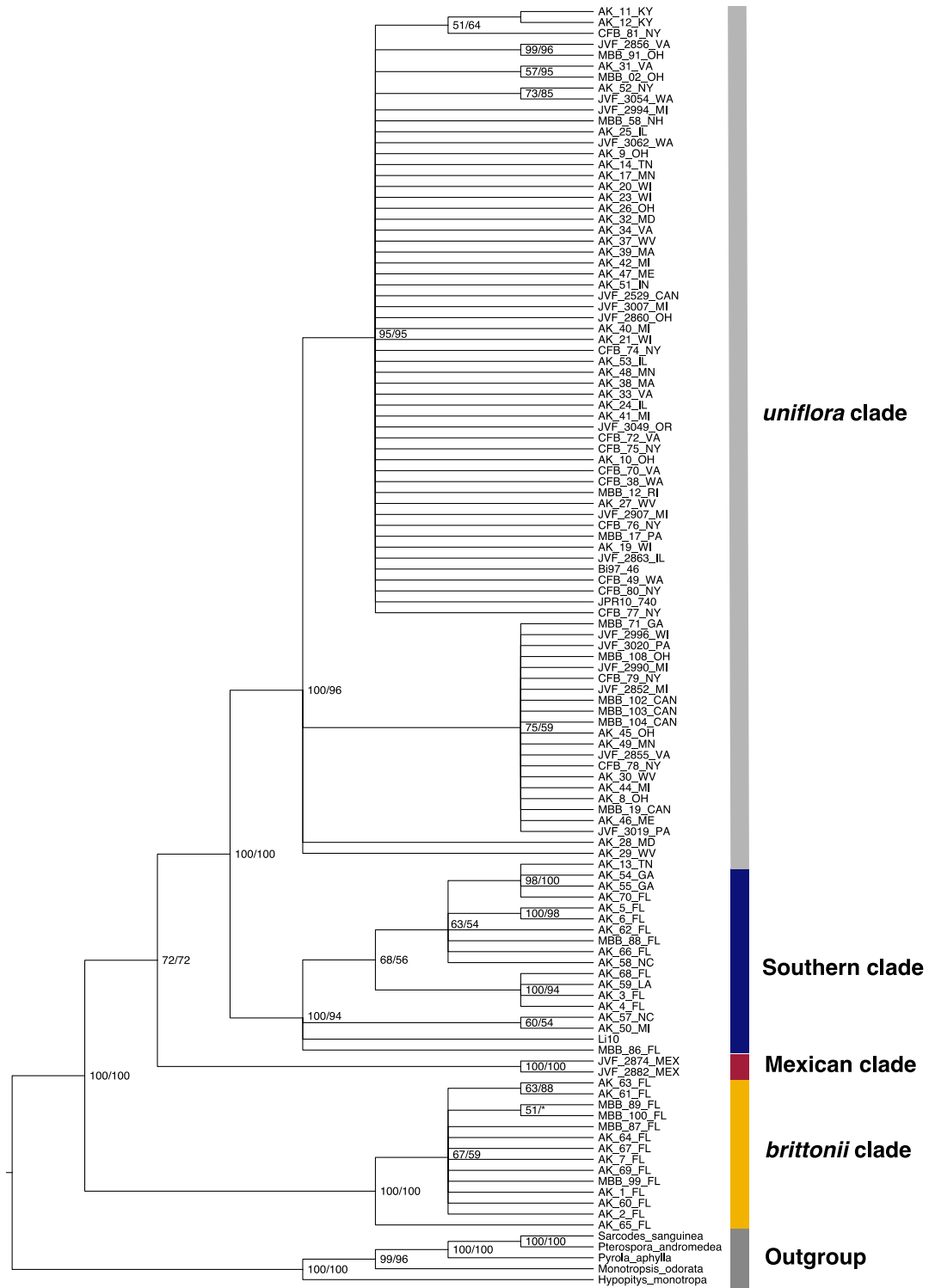
**Figure 5. 26S-ITS BI consensus tree from MrBayes.**

Support is displayed as posterior probability and ultrafast bootstrap approximation via ML in IQ-TREE. Asterisks indicate where a clade did not appear in the ML tree. Taxa that are bold and end with a \*\* indicate accessions from Neyland and Hennigan (2009).



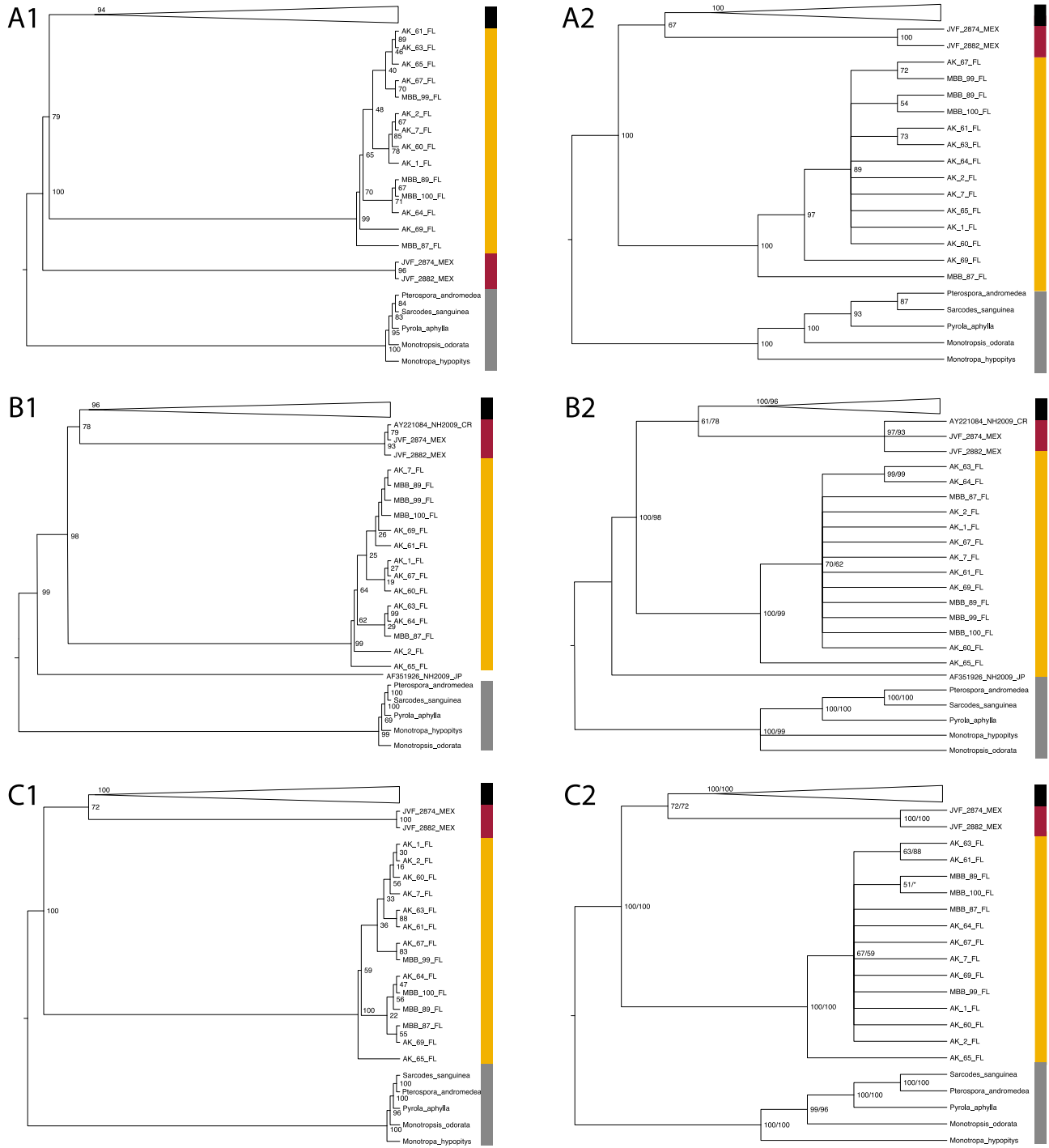
**Figure 6.** *rps2* BI consensus tree from MrBayes.

Support is displayed as posterior probability and ultrafast bootstrap approximation via ML in IQ-TREE. Asterisks indicate where a clade did not appear in the ML consensus tree.



**Figure 7. Concatenated 26S and *rps2* BI consensus tree from MrBayes.**

Support is displayed as posterior probability and ultrafast bootstrap approximation via ML in IQ-TREE. Asterisks indicate where a clade did not appear in the ML consensus tree.



**Figure 8. Condensed phylogenies displaying relationships at the base of the trees.**

Black- Condensed *uniflora* and Southern clades, Red – Mexican Clade, Orange – *brittonii* clade, Grey – Outgroup, A1 – *rps2* IQ-TREE, A2 - *rps2* MrBayes, B1 – 26S-ITS IQ-TREE, B2 – 26S-ITS MrBayes, C1 – Concatenated IQ-TREE, C2 – Concatenated MrBayes

## *Fungal host*

We obtained fungal ITS sequences from roots of 54 individuals collected in this study. A BLAST search revealed *Monotropia* from this study parasitize fungi in three different genera within Russulaceae; *Lactarius*, *Lactifluus* and *Russula* (Table 2). The genus that is most frequently parasitized is *Russula*. As evidenced in Figures 11 and 12, *Monotropia* parasitize species in five out of seven *Russula* subgenera. In *Lactarius* and *Lactifluus*, only one subgenus in each has been shown to contain species that host *Monotropia*; *Lactarius* subgenus *Russularia* (Fig. 9, 10), and *Lactifluus* subgenus *Lactariopsis* (Fig. 13). All figures of the fungal ITS tree are the same tree with certain clades condensed to better view relationships in the tree.

**Table 2. BLAST search results for fungal ITS sequences.**

\* These BLAST search results returned fungal species that do not match the expected result given the other close matches and their placement in the phylogenetic tree, and are possibly misidentified in GenBank.

Coll #	% identity match	Accession	% identity	Max score match	Accession	Max score	County	State
AK1	<i>Lactifluus deceptivus</i>	KR364101	96.9	<i>Lactifluus deceptivus</i>	AY854089	96.8	Polk	FL
AK2	<i>Lactifluus deceptivus</i>	KF937337	97.1	<i>Lactifluus deceptivus</i>	MK069516	97.6	Polk	FL
AK4	<i>Russula dissimulans</i>	MH212004	99.4	<i>Russula nigricans</i>	JQ711972	98.8	Alachua	FL
AK5	<i>Lactarius conglutinatus</i>	MK253488	96.4	<i>Lactarius subdulcis</i>	AJ889964	95	Alachua	FL
AK6	<i>Russula vinosa</i>	JQ888203	89.5	<i>Russula vinosa</i>	JQ888203	94.7	Duval	FL
AK7	<i>Lactifluus deceptivus</i>	MK069514	97.1	<i>Lactifluus deceptivus</i>	MK069516	98.4	Martin	FL
AK8	<i>Lactarius quietus</i>	MG553997	92.2	<i>Lactarius frustratus</i>	MK578698	95.6	Hocking	OH
AK9	<i>Russula decolorans</i>	KX358008	99.3	<i>Russula decolorans</i>	DQ367913	96.1	Hocking	OH
AK10	<i>Russula decolorans</i>	KT933992	92.6	<i>Russula integriformis</i>	KP783458	95.5	Licking	OH
AK11	<i>Russula spagnophila</i>	AY061719	92	<i>Russula decolorans</i>	DQ367913	95.1	Boyle	KY
AK12	<i>Russula nitida</i>	AY061696	91.9	<i>Russula decolorans</i>	DQ367913	95.8	Boyle	KY
AK13	<i>Russula velonovskyi</i>	HM189949	91.6	<i>Russula integriformis</i>	KP783458	95.4	Henderson	TN
AK14	<i>Russula foetens</i>	KX095018	98.3	<i>Russula foetens</i>	FJ845427	98.9	Shelby	TN
AK15	<i>Russula pulverulenta</i>	EU598186	99.8	<i>Russula livescens</i>	JN129398	98.1	Shelby	TN
AK16	<i>Russula decolorans</i>	AY194601	92.5	<i>Russula integriformis</i>	KP783458	96	Polk	WI
AK17	<i>Russula praetervisa</i>	KF245531	96.2	<i>Russula pectinatoides</i>	DQ422026	97.2	Hennepin	MN
AK21	<i>Russula mariae</i>	MF161267	99	<i>Russula mariae</i>	EU819426	98.4	Harsted	WI
AK25	<i>Russula praetervisa</i>	KF245531	96.3	<i>Russula pectinatoides</i>	DQ422026	97.3	Mason	IL
AK26	<i>Russula pusilla</i>	KT933968	99.7	<i>Russula pusilla</i>	KT933968	97.3	Pickaway	OH



AK27	<i>Russula pectinoides</i>	MH212081	99.9	<i>Russula pectinoides</i>	MF773598	99.9	Monongalia	WV
AK28	<i>Lactarius quietus</i> var <i>incanus</i>	MH910574	99.7	<i>Lactarius frustratus</i>	MK578698	99.6	Garrett	MD
AK29	<i>Lactarius oculatus</i>	KF007947	99.9	<i>Lactarius tabidus</i>	MK131494	99	Tucker	WV
AK32	<i>Russula cerolens</i>	MH212101	100	<i>Russula cerolens</i>	HQ604829	98.7	Caroline	MD
AK34	<i>Russula pectinoides</i>	MH212081	100	<i>Russula cerolens</i>	HQ604829	98.6	Chesterfield	VA
AK36	<i>Russula fellea</i>	KF245536	93.7	<i>Russula laccata</i>	HQ604844	95.8	Giles	VA
AK37	<i>Russula pectinatoides</i>	MH212081	100	<i>Russula cerolens</i>	HQ604829	98.8	Kanawha	WV
AK3049	<i>Russula brevipes</i>	KF386757	99.8	<i>Lactifluus subvellereus*</i>	MK575452	97.4	Clackamas	OR
AK40	<i>Russula pectinatoides</i>	EU819493	99.9	<i>Russula pectinatoides</i>	EU819493	99.9	Monroe	MI
AK42	<i>Russula veteriosa</i>	AF418630	94.5	<i>Russula integra</i>	LC192767	95.2	Otsego	MI
AK44	<i>Lactarius oculatus</i>	KF007947	95.2	<i>Lactarius tabidus</i>	KP783447	95.6	Crawford	MI
AK45	<i>Lactarius quietus</i>	MH910574	99.9	<i>Lactarius frustratus</i>	MK578698	99.1	Lucas	OH
AK46	<i>Russula silvestris</i>	KX579800	99.8	<i>Russula bicolor</i>	HQ604845	98.9	York	ME
AK47	<i>Russula compacta</i>	GQ924688	100	<i>Russula compacta</i>	GU229820	98	Cumberland	ME
AK49	<i>Russula decolorans</i>	FJ845432	99.8	<i>Russula decolorans</i>	FJ845432	99.2	Itasca	MN
AK50	<i>Russula nigricans</i>	JQ711972	99.5	<i>Russula nigricans</i>	JQ711972	99.7	Midland	MI
AK51	<i>Russula subtilis</i>	KX358053	99	<i>Russula azurea</i>	KF002763	96.7	Noble	IN
AK52	<i>Russula pectinatoides</i>	MH212081	100	<i>Russula cerolens</i>	HQ604829	98.9	Bronx	NY
AK53	<i>Russula acrifolia</i>	JF834363	99.3	<i>Russula densifolia</i>	HQ439176	98	Mchenry	IL
AK54	<i>Russula lepida</i>	DQ422013	94.6	<i>Russula lepida</i>	DQ422013	97.2	Gilmer	GA
AK55	<i>Russula lepida</i>	MG687359	94.7	<i>Russula lepida</i>	DQ422013	97	Gilmer	GA
AK56	<i>Russula lepida</i>	MG687359	94.2	<i>Russula lepida</i>	DQ422013	96.8	Charleston	SC
AK58	<i>Russula paludosa</i>	KP149057	93	<i>Russula paludosa</i>	KT934000	96.4	Currituck	NC
AK59	<i>Russula acrifolia</i>	DQ421998	99.5	<i>Russula densifolia</i>	HQ439176	98.1	Ouachita	LA
AK60	<i>Lactifluus deceptivus</i>	MH212001	100	<i>Lactifluus deceptivus</i>	MH212001	98.2	S. John's	FL
AK61	<i>Lactifluus deceptivus</i>	MH212001	100	<i>Lactifluus deceptivus</i>	MH212001	98.2	S. John's	FL
AK62	<i>Russula dissimulans</i>	MH212004	99.3	<i>Russula nigricans</i>	JQ711972	98.8	Flager	FL
AK63	<i>Lactifluus deceptivus</i>	MH212001	99.5	<i>Lactifluus deceptivus</i>	MH212001	94	Volusia	FL
AK64	<i>Russula mutabilis</i>	KF810137	98	<i>Russula illota</i>	DQ422024	98.4	Volusia	FL
AK65	<i>Russula mutabilis</i>	KF810137	98	<i>Russula illota</i>	DQ422024	96.8	Volusia	FL
AK66	<i>Russula melliolens</i>	MG680179	94.7	<i>Russula cuprea</i>	KT934010	96	Volusia	FL
AK67	<i>Lactifluus deceptivus</i>	MK069514	97.8	<i>Lactifluus deceptivus</i>	MK069516	96.1	Palm Beach	FL
AK68	<i>Russula dissimulans</i>	MH212004	99.2	<i>Russula nigricans</i>	JQ711972	98.5	Marion	FL
AK69	<i>Lactifluus piperatus*</i>	AB509984	96.3	<i>Lactifluus pilosus</i>	KR364006	95.9	Wakulla	FL
AK70	<i>Russula lepida</i>	MG687359	94.9	<i>Russula lepida</i>	DQ422013	97	Wakulla	FL

*Lactarius*

The taxonomic treatment we used for *Lactarius* was from the recent revision of the genus by Lee *et al.* (2019) which separated it into three subgenera using four loci. For our phylogeny, we used the ITS region only. This may be why the subgenus *Russularia* appears paraphyletic in our tree. An additional discrepancy from the Lee *et al.* (2019) phylogeny is that *Lactarius cucurbitoides* was placed in subgenus *Plinthogalus* in their treatment but appears in subgenus *Lactarius* in our tree. Perhaps with sampling of additional fungal genes these discrepancies would not appear.

Five *Monotropia* collected in this study were found to parasitize fungi in *Lactarius* subgenus *Russularia* (Fig 9, 10). These hosts fall into the same clade as *L. quietus*, *L. tabidus*, and *L. conglutinus*. Other studies have found *Monotropia* fungal hosts to fall into the same subgenus and most closely match these three species of *Lactarius* (Yang and Pfister 2006, Bidartando and Bruns 2001).

Two hosts did not clearly fall into any subgenera in the ML tree, but in the BI consensus tree, AK\_8\_OH and AK\_44\_MI fall into clades with *L. mutabilis* and *L. tabidus* respectively with high support (Fig. 10). Additionally, BLAST searches return 90% identity match with *L. mutabilis* for AK\_8\_19 and 91% match for *L. tabidus* and AK\_44\_MI (Table 3). Both *L. mutabilis* and *L. tabidus* are members of *Lactarius* subgenus *Russularia*, and from the BLAST search and consensus tree from MrBayes, we can conclude it is likely the hosts of AK\_8\_OH and AK\_44\_MI fall into this subgenus.

## ***Russula***

We used the taxonomic treatment of Buyck *et al.* (2018) to delimit subgenera of *Russula*. Their study used five loci to delimit subgenera and reconcile species relationships. While our

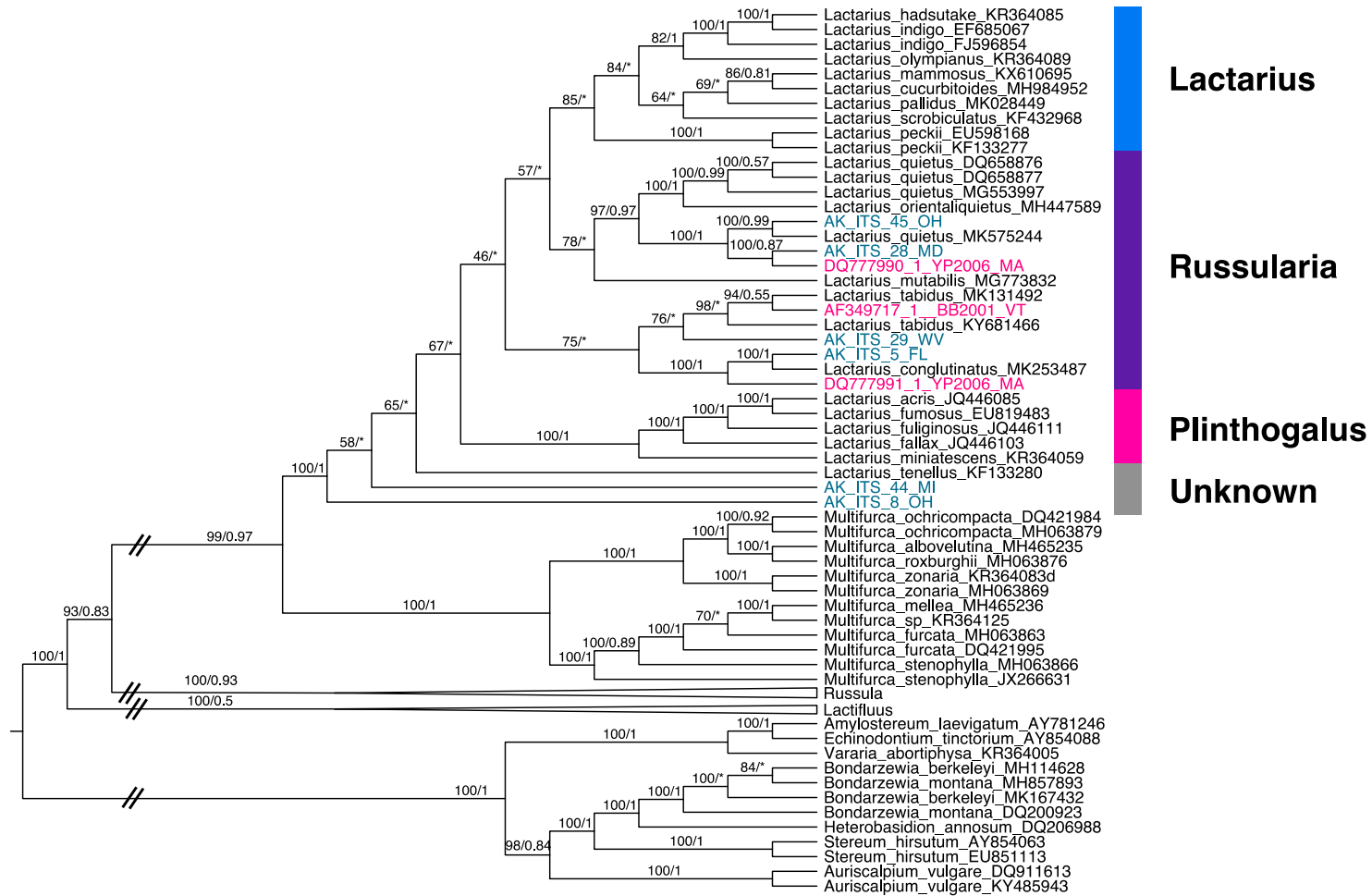
topology does not exactly match that of Buyck *et al.* (2018) in terms of relationships between subgenera, all subgenera were recovered as monophyletic with the exception of *Russula* subgenus *Heterophyllidia*, which appeared in our tree as polyphyletic (Fig. 11, 12). *Monotropa* hosts fell into all subgenera except two, *Crassotunicata* and *Archaea*. This is in line with Buyck *et al.*'s (2018) notes on these two subgenera that state mycorrhizal associations between ericaceous plants and *Crassotunicata* and *Archae* are unknown. They additionally acknowledge that mycoheterotrophic associations have been found to occur in subgenera *Compactae*, *Heterophyllidia*, *Malodora*, *Russula*, and *Brevipes*, which also agrees with our findings.

### ***Lactifluus***

For *Lactifluus* we used the taxonomic treatment put forth by De Crop *et al.* (2017). We found that within *Lactifluus*, *Monotropa* appear to parasitize only one section of a subgenus (*Lactifluus* subg. *Lactariopsis* sect. *Albati*), most of which parasitize a single species, *Lactifluus* *deceptivus*. Interestingly, the *Monotropa* that parasitize *L. deceptivus* all fall into the *brittonii* clade, indicating a high level of specificity in this group (Fig. 13).

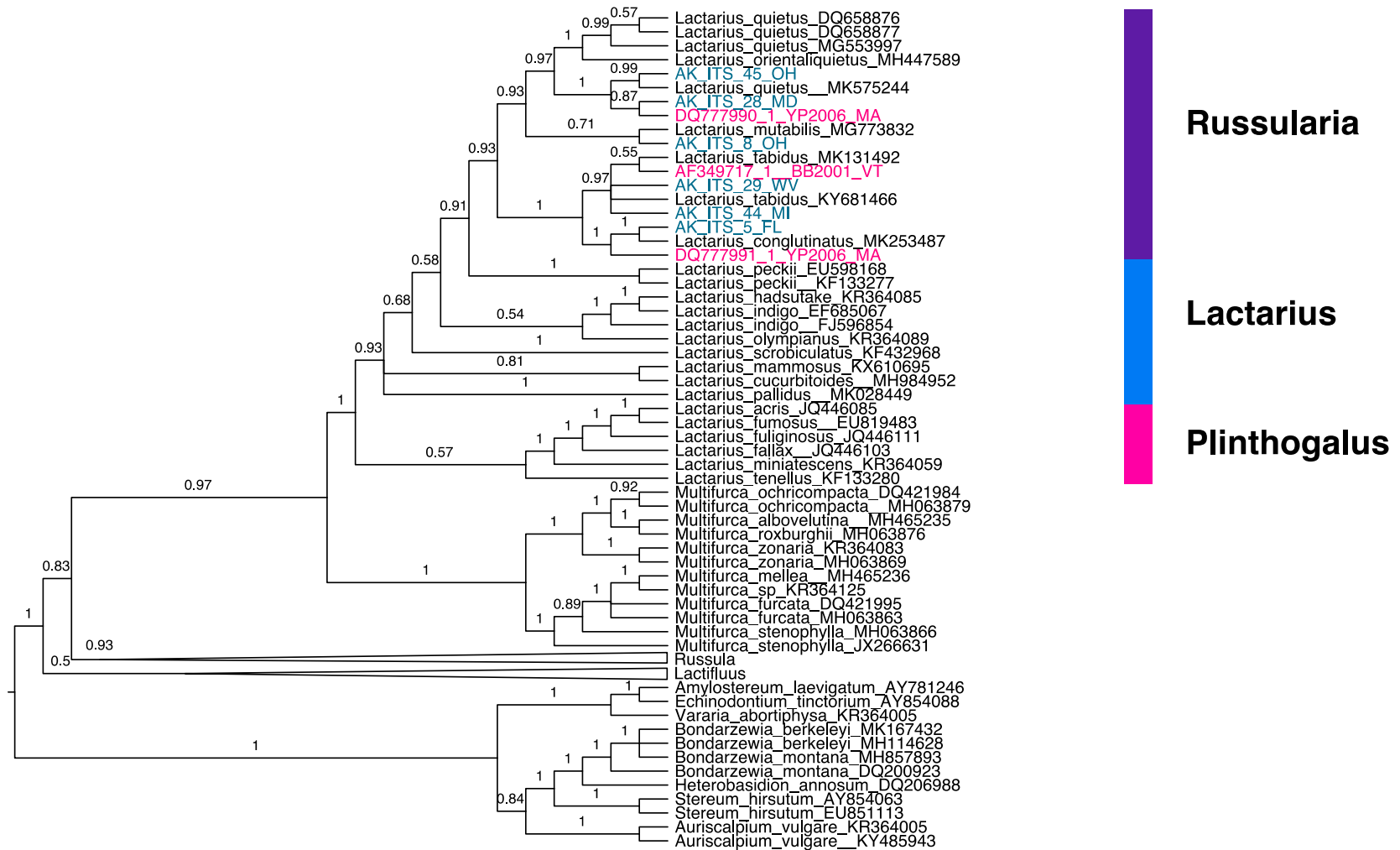
Only two *Monotropa* from the *brittonii* clade for which we have host DNA did not parasitize fungi in *Lactifluus* subg. *Lactariopsis* sect. *Albati*. These two individuals (AK 64, AK 65) were collected from the same site. These two parasitize hosts in *Russula* subgenus *Heterophyllidia*, and BLAST searches suggest they both parasitize *Russula mutabilis*.

The fungal sequence for AK 69 most closely matched *Lactifluus piperatus* (96.3%) however, the other BLAST results showed a high pairwise identity to *L. bertilloni* (94%) and a high max score to *L. pilosus* (95.9%) and it fell into subgenus *Lactariopsis* in the same clade as these two species rather than subgenus *Lactarius* with *L. piperatus*.



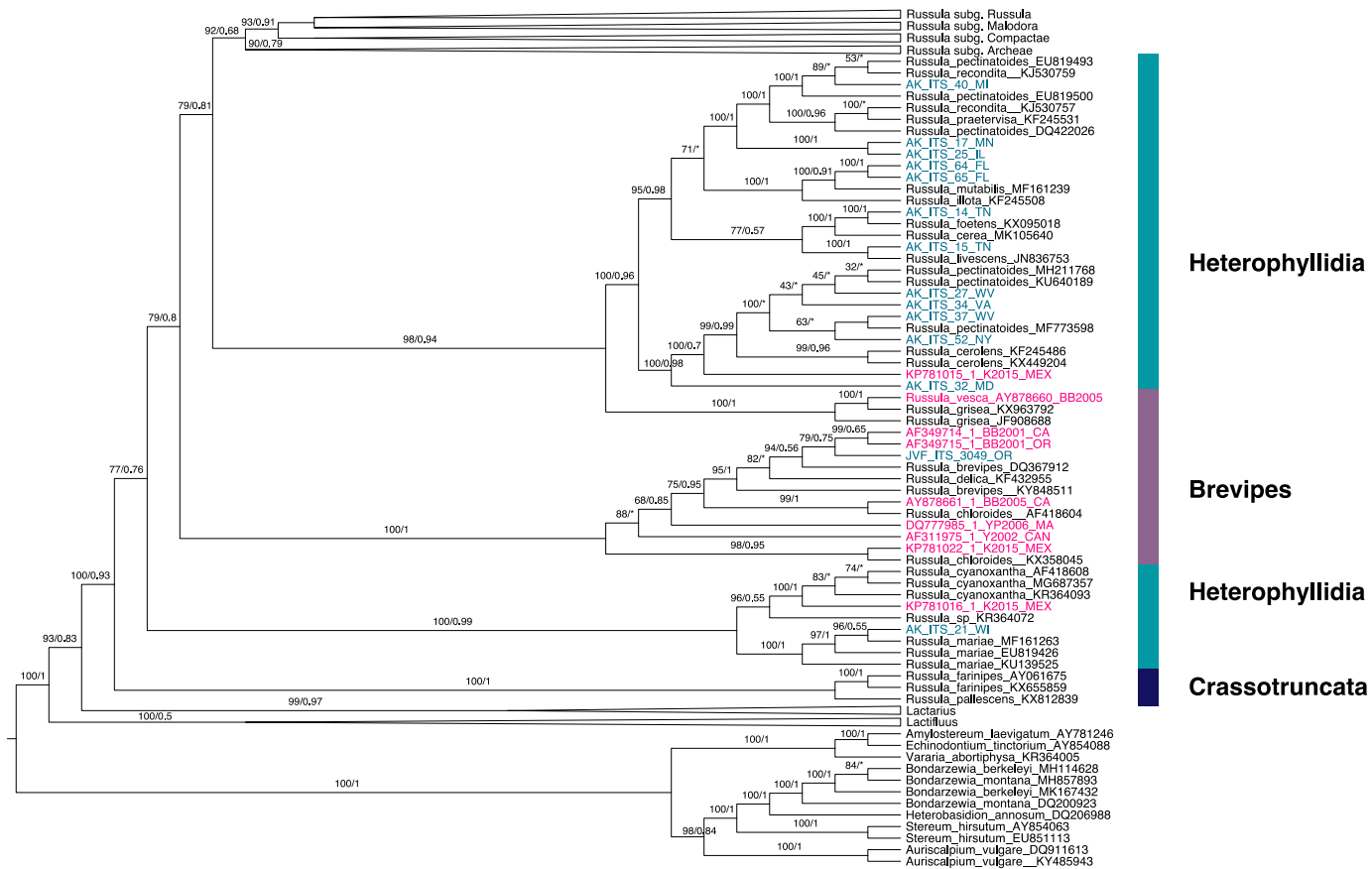
**Figure 9. Fungal ITS host phylogeny, *Lactarius* clade, ML consensus tree**

Support values represent 1000 ultrafast bootstrap replicates in IQ-TREE and 5,000,000 mcmc generations in MrBayes. Asterisks denote clades not seen in the BI consensus tree. Names on the right indicate fungal subgenera. *Monotropia* root fungal isolates from this study are in blue text. *Monotropia* fungal isolates from similar studies are indicated in pink text. (YP2006 = Yang and Pfister 2006, BB2001 = Bidartando and Bruns 2001). *Russula* and *Lactifluus* clades are condensed for easier viewing of the tree.



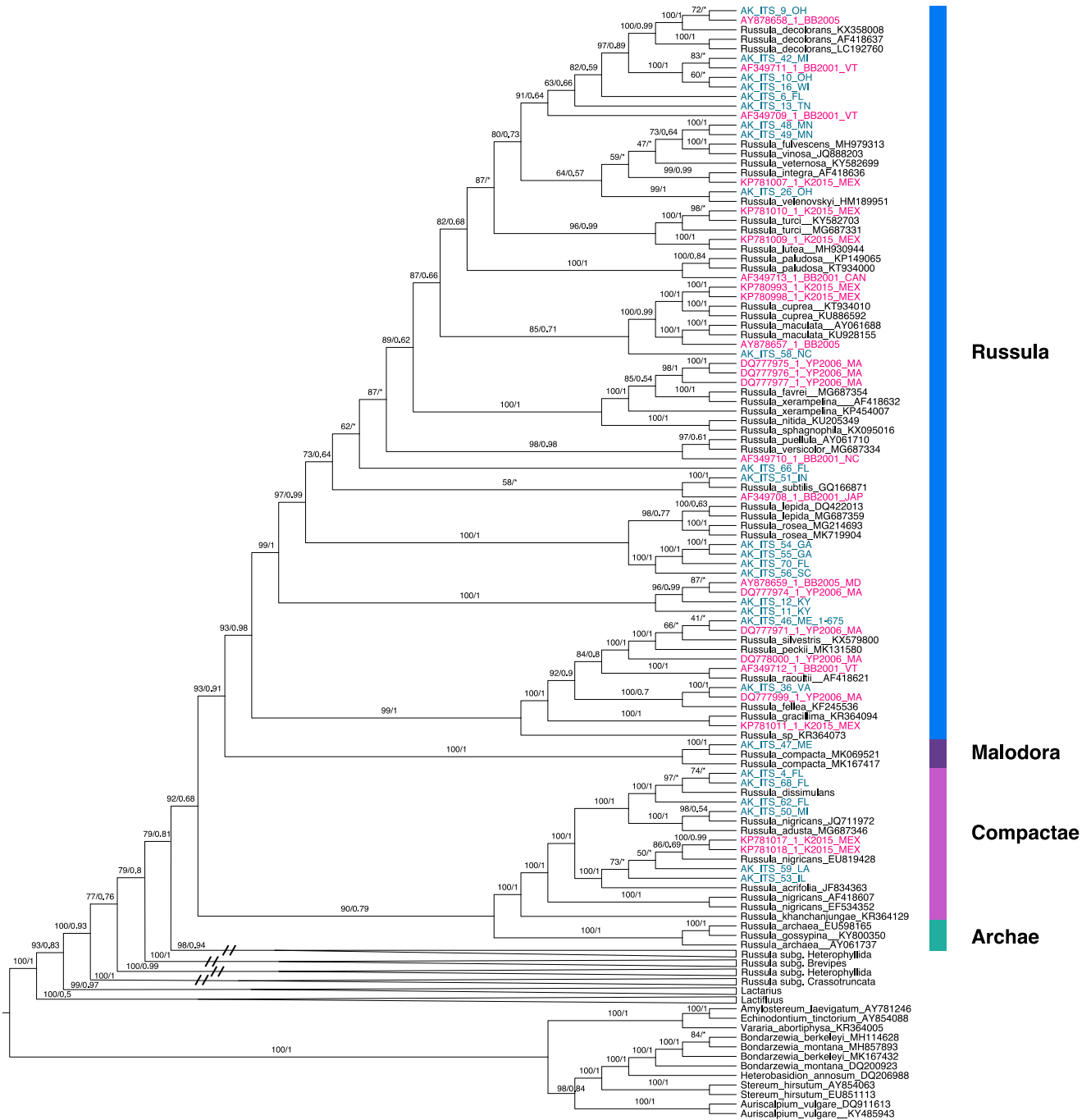
**Figure 10. Fungal ITS host phylogeny, *Lactarius* clade, BI consensus tree**

Support values represent 5,000,000 mcmc generations in MrBayes. Names on the right indicate fungal subgenera. *Monotropa* root fungal isolates from this study are in blue text. *Monotropa* fungal isolates from similar studies are indicated in pink text. (YP2006 = Yang and Pfister 2006, BB2001 = Bidartando and Bruns 2001). *Russula* and *Lactifluus* clades are condensed for easier viewing of the tree.



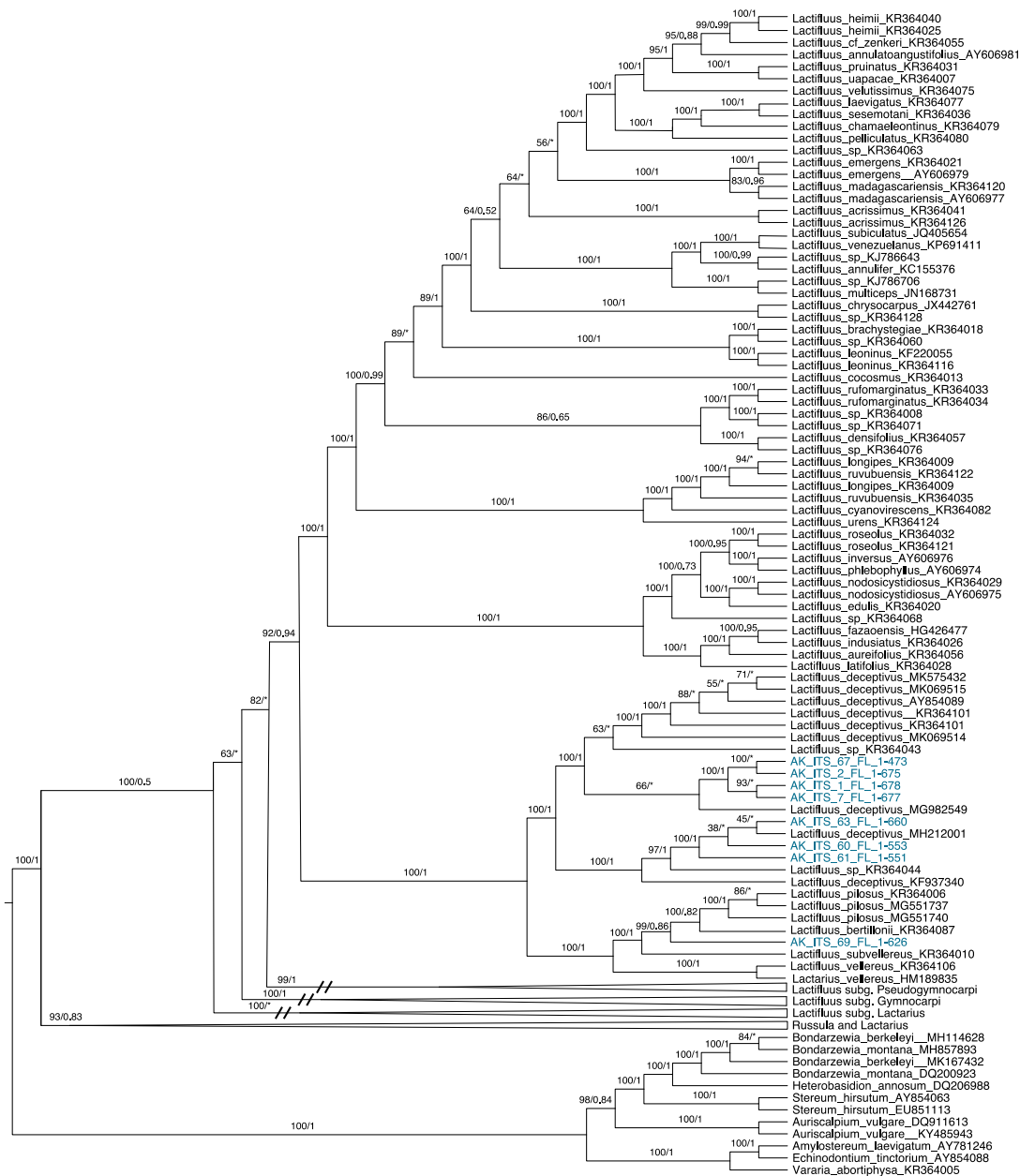
**Figure 11. Fungal ITS host phylogeny *Russula* clade part 1.**

Support values represent 1000 ultrafast bootstrap replicates in IQ-TREE and 5,000,000 mcmc generations in MrBayes. Asterisks denote clades not seen in the BI consensus tree. Names on the right indicate fungal subgenera. *Monotropa* root fungal isolates from this study are in blue text. *Monotropa* fungal isolates from similar studies are indicated in pink text. (K2016= Kong *et al.* 2015, YP2006 = Yang and Pfister 2006, BB2001 = Bidartando and Bruns 2001). Some *Russula* subgenera, *Lactarius* and *Lactifluus* clades are condensed for easier viewing of the tree.



**Figure 12. Fungal ITS host phylogeny *Russula* clade part 2.**

Support values represent 1000 ultrafast bootstrap replicates in IQ-TREE and 5,000,000 mcmc generations in MrBayes. Asterisks denote clades not seen in the BI consensus tree. Names on the right indicate fungal subgenera. *Monotropia* root fungal isolates from this study are in blue text. *Monotropia* fungal isolates from similar studies are indicated in pink text. (K2016= Kong *et al.* 2015, YP2006 = Yang and Pfister 2006, BB2001 = Bidartando and Bruns 2001, BB2005 = Bidartando and Bruns 2005). Some *Russula* subgenera, *Lactarius* and *Lactifluus* clades are condensed for easier viewing of the tree.



**Figure 13. Fungal ITS host phylogeny, *Lactifluus* clade.**

Support values represent 1000 ultrafast bootstrap replicates in IQ-TREE and 5,000,000 mcmc generations in MrBayes. Asterisks denote clades not seen in the BI consensus tree. Names on the right indicate fungal subgenera. Other *Lactifluus* subgenera have been condensed as no fungal hosts from this study fell into those subgenera. *Monotropia* root fungal isolates from this study are in blue text. Some *Lactifluus* subgenera, *Russula*, and *Lactarius* clades have been condensed for easier viewing of the tree



## Morphology

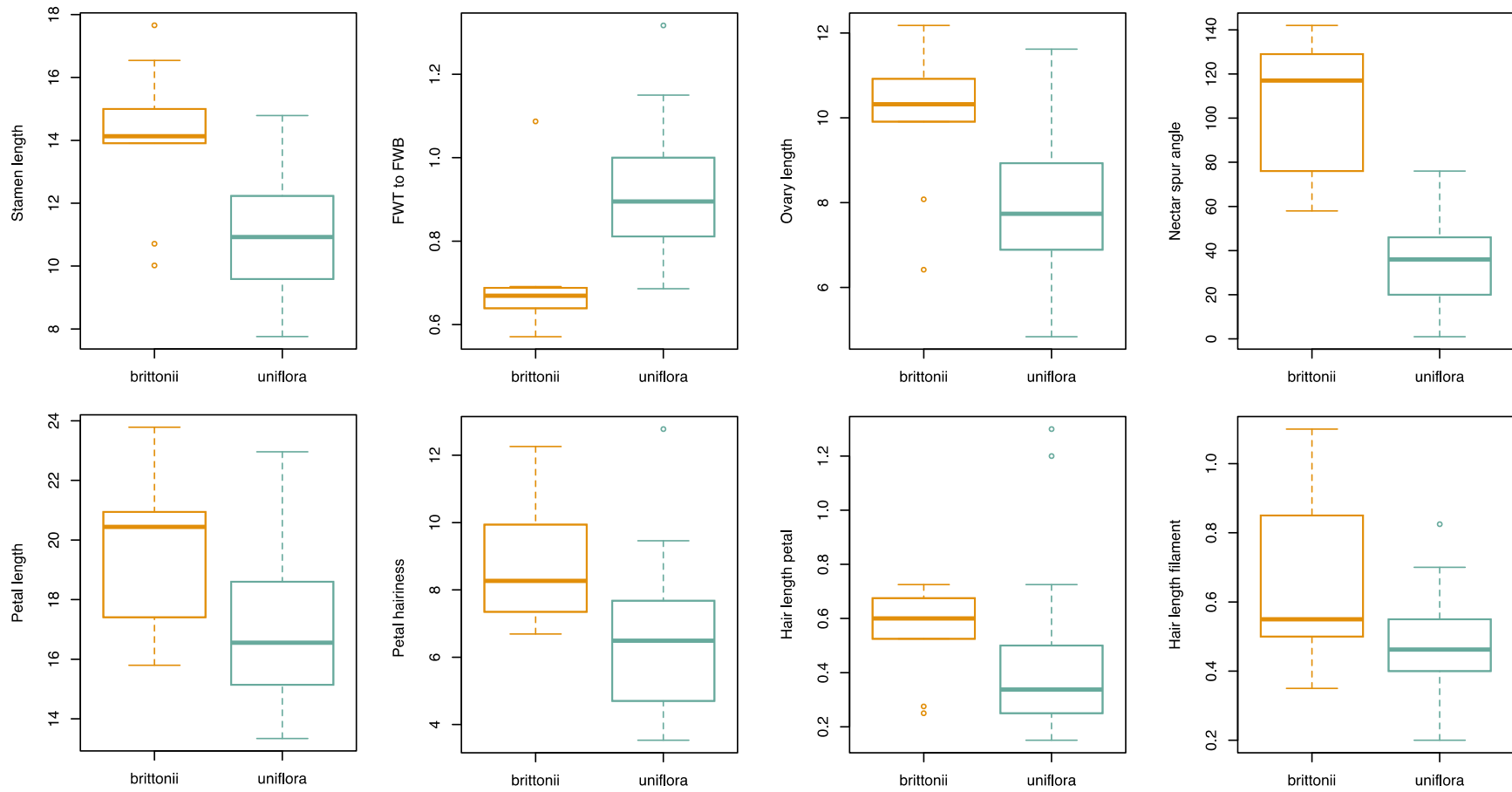
We measured 20 morphological characters for 47 individuals for which we had preserved flowers. We did not have flower material for any individuals in the Mexican clade. Of the individuals analyzed for morphological characters, 9 fell into the *brittonii* clade in our phylogenetic analysis and the rest fell into either the southern or *uniflora* clades. Because of their ambiguous placement in our phylogenetic analysis, individuals in the southern clade were grouped with those in the *uniflora* group for the morphological analysis.

To find where the *brittonii* group and the *uniflora* group differed significantly, we performed Wilcoxon rank sum tests and t-tests. These tests showed significant differences between the *brittonii* group and the *uniflora* group in 8 out of 20 measured characters (Table 3). We created box plots for these variables to visualize the differences between groups in those characters (Fig. 14). In all characters the *brittonii* had a larger average measurement than the *uniflora* group, with the exception of the ratio of filament width from top to bottom (FWT to FWB).

**Table 3. Normality and hypothesis tests for all measured morphological characters.**  
Significant characters are bolded. All measurements except nectar spur angle were done in millimeters

	Shapiro wilk W	Shapiro wilk p	T Test t	T Test df	T Test p	Wilcoxon test W	Wilcoxon test P	Mean <i>brittonii</i> ± SD	Mean <i>uniflora</i> ± SD
Anther length (AL)	0.970	0.272	0.528	12.826	0.606			2.21 ± 0.32	2.15 ± 0.34
<b>FWT to FWB</b>	<b>0.982</b>	<b>0.691</b>	<b>-3.925</b>	<b>11.312</b>	<b>0.002</b>			<b>0.7 ± 0.15</b>	<b>0.92 ± 0.14</b>
<b>Hair length filament (HLF)</b>	<b>0.905</b>	<b>0.001</b>				<b>246</b>	<b>0.043</b>	<b>0.67 ± 0.26</b>	<b>0.47 ± 0.13</b>
<b>Hair length petal (HLP)</b>	<b>0.824</b>	<b>0.000</b>				<b>257</b>	<b>0.021</b>	<b>0.54 ± 0.17</b>	<b>0.41 ± 0.24</b>
<b>Nectar spur angle (NA)</b>	<b>0.882</b>	<b>0.000</b>				<b>336</b>	<b>0.00001</b>	<b>102.67 ± 31.31</b>	<b>34.79 ± 18.18</b>
Nectar spur length (NS)	0.947	0.034				212	0.274	1.91 ± 0.62	1.62 ± 0.48
<b>Ovary length (OL)</b>	<b>0.975</b>	<b>0.400</b>	<b>3.204</b>	<b>11.468</b>	<b>0.008</b>			<b>10 ± 1.74</b>	<b>7.96 ± 1.61</b>
Ovary width (OW)	0.983	0.718	1.986	10.406	0.074			9.24 ± 2.3	7.62 ± 1.79

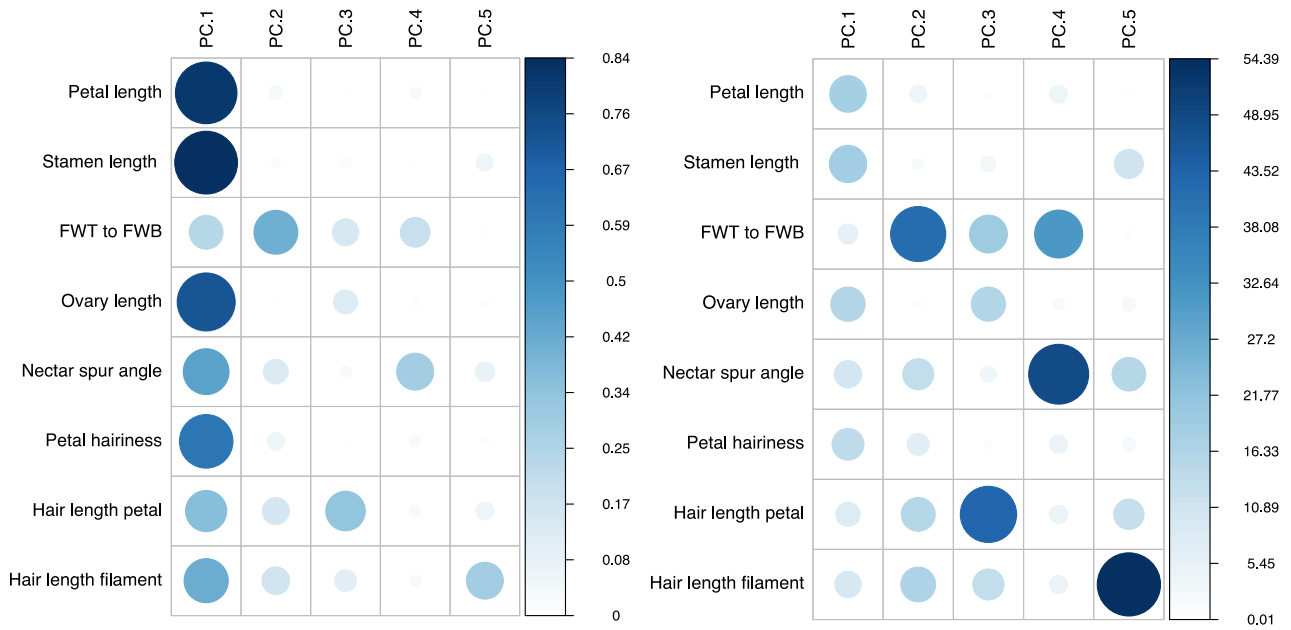
Petal depth (PD)	0.965	0.170	1.642	11.817	0.127			2.58 ± 0.35	2.36 ± 0.34
<b>Petal hairiness (PH)</b>	<b>0.962</b>	<b>0.132</b>	<b>3.217</b>	<b>12.614</b>	<b>0.007</b>			<b>8.75 ± 1.9</b>	<b>6.46 ± 2.01</b>
<b>Petal length (PL)</b>	<b>0.950</b>	<b>0.042</b>				<b>260</b>	<b>0.017</b>	<b>19.51 ± 2.9</b>	<b>16.93 ± 2.45</b>
Petal W at apex (PWA)	0.985	0.810	1.355	10.608	0.204			9.09 ± 2.22	8.01 ± 1.79
Petal W at base (PWB)	0.984	0.776	2.044	14.234	0.060			3.87 ± 0.56	3.43 ± 0.68
Petal W at middle (PWM)	0.974	0.381	1.527	11.036	0.155			6 ± 1.27	5.3 ± 1.1
Saccatness (S)	0.985	0.813	-1.462	18.553	0.161			2.01 ± 0.31	2.2 ± 0.49
<b>Stamen length (SL)</b>	<b>0.972</b>	<b>0.304</b>	<b>3.595</b>	<b>10.043</b>	<b>0.005</b>			<b>14.04 ± 2.44</b>	<b>10.94 ± 1.75</b>
Stem width (STW)	0.979	0.564	1.751	13.954	0.102			2.96 ± 0.52	2.6 ± 0.62
Stigma width apex (SWA)	0.982	0.690	-0.275	13.905	0.788			4.52 ± 0.7	4.59 ± 0.83
Style length (STY)	0.972	0.327	1.470	10.954	0.170			4.05 ± 0.91	3.57 ± 0.78
Style width at base (SWB)	0.984	0.757	0.039	12.738	0.969			3.18 ± 0.67	3.17 ± 0.72



**Figure 14. Boxplot comparisons of significant morphological characters**

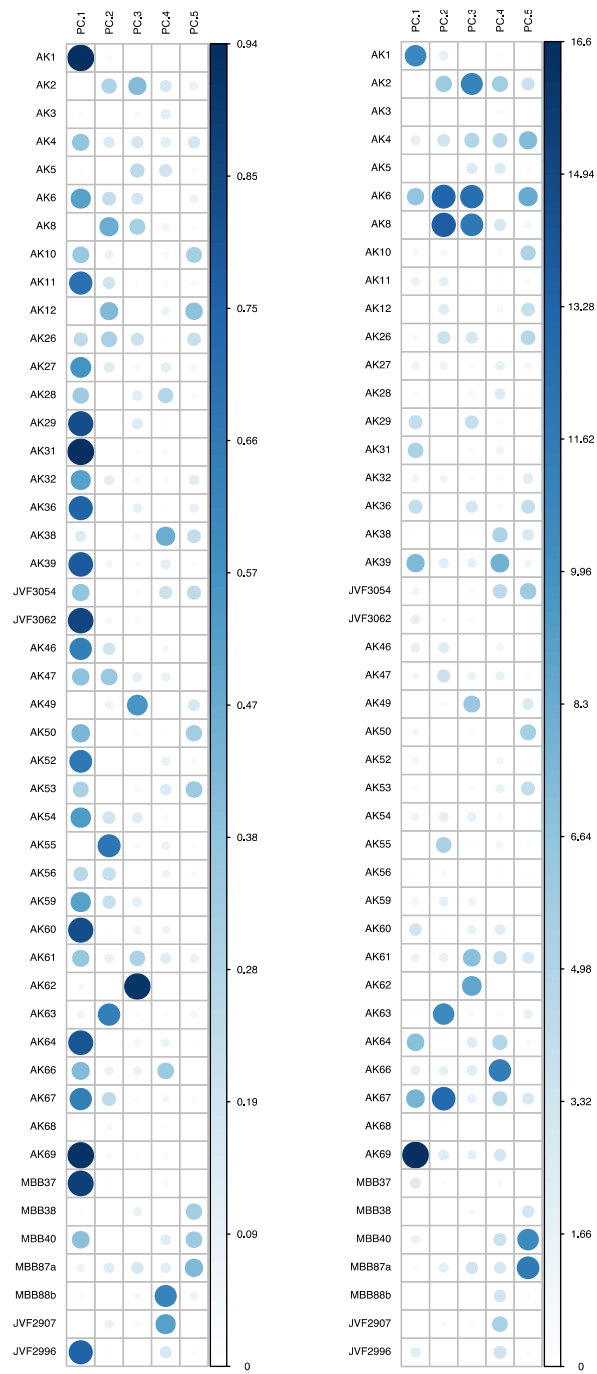
We used the package `corrplot` to visualize how the variables and individuals interacted with the axes of our PCA (Wei and Simko 2017). We visualized the `Cos2`, which is a measure of the quality of representation of a variable, and the contribution of each variable to the axis (Fig. 15). Stamen length and petal length contributed most to the first axes and the filament width ratio (FWT to FWB) contributed the most to the second axis. We also visualized the `Cos2` and contribution of individuals to each axis (Fig. 16). AK69 contributed substantially to the first axis. This individual had the longest petals (23.78 mm) and the longest stamens (17.66 mm). AK39 had the smallest petal (13.34 mm) and stamen length (7.76 mm).

The PCA consisted of 8 characters measured for 47 individuals. We tested for highly correlated characters and only petal length and stamen length were found to be highly correlated (0.883). The first axis explains 55.6% of the variation. The highest contributors to this axis were stamen length (18.9), petal length (18.2), and ovary length (16.0). The second axis explains 12.4% of the variation and the variables contributing to this axis are mainly the filament width ratio top to bottom (41.4) and the length of filament hairs (16.5). The *brittonii* group is clustered in the lower right portion of the PCA, showing it to be somewhat morphologically distinct from the *uniflora* group although there is some overlap (Fig. 17).



**Figure 15. Representation of variables used in PCA**

Left: Corplot of cos2 of each variable for axes 1-5; Right: Corplot of contribution for each variable to axes 1-5



**Figure 16. Representation of individuals in PCA**

Left: Corplot of Cos2 of each individual for axes 1-5; Right: Corplot of contributions for each individual to axes 1-5

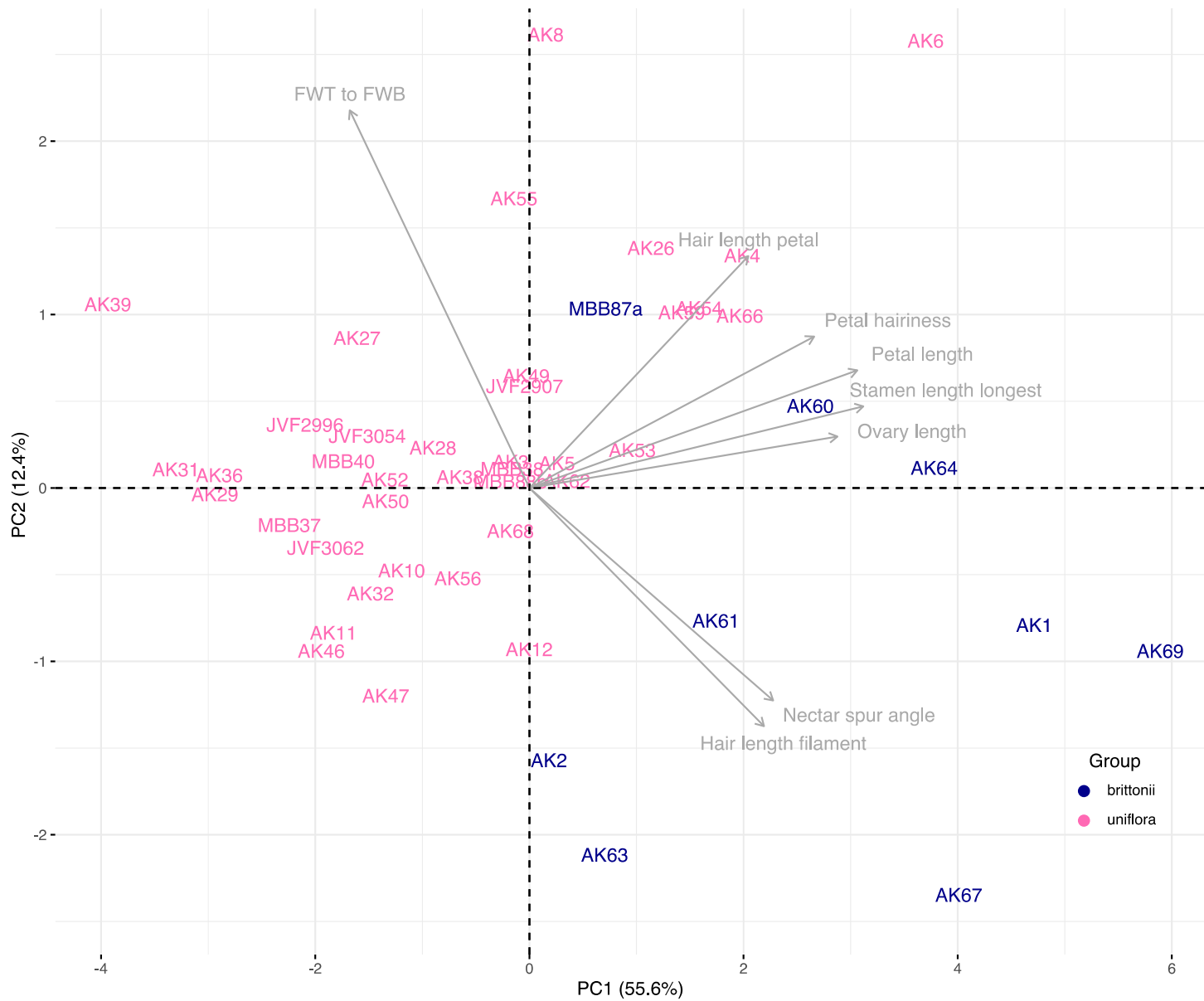


Figure 17. PCA of morphological characters

## Discussion

### *Plant material*

In all trees, the *brittonii* clade appears with high support. While all individuals that fall into the *brittonii* clade were collected in Florida, not all individuals collected in Florida fall into that clade, suggesting the existence of two lineages of *Monotropa* that occur in Florida. This aligns with our hypothesis that there is evidence to support the species status of *M. brittonii*. In all but the ML *rps2* tree the *brittonii* clade appears sister to all other *Monotropa* clades. This is interesting because the *brittonii* clade is geographically much closer to some individuals in the *uniflora* clade than the Mexican clade. For example, specimens AK69 and AK70 were collected at the same park, Edward Ball Wakulla Springs State Park, but they do not appear closely related given their placement on the trees. These two specimens were collected from different habitat types, with AK69 collected from a scrubby flatwoods habitat and AK70 from an upland hardwood forest (Florida DEP 2007).

The 26S-ITS tree included six sequences from Neyland and Hennigan (2009), a study that concluded the synonymization of *Monotropa brittonii* was supported. Our tree shows it is likely the putative *M. brittonii* used in their study does not correspond to what Small described as *M. brittonii*, so their conclusion regarding the synonymization of the Southern Ghost Pipe may have been misguided.

The Mexican clade also appears with high support in every tree. The specimen from Neyland and Hennigan (2009) from Costa Rica also fell into this clade when included in the 26S-ITS analysis. This supports the existence of an additional lineage of *Monotropa* that occurs in



Mexico and Central America. Further collection and analysis of genetics, morphology, and host specificity in *Monotropa* from this area is needed.

While there are several clades that consistently occur with high support in all our trees, the tree is largely unresolved. Additional sampling of more loci would be beneficial to understanding species level relationships on a broader geographic scale. A tree with higher resolution could also reveal more regional patterns of host specificity.

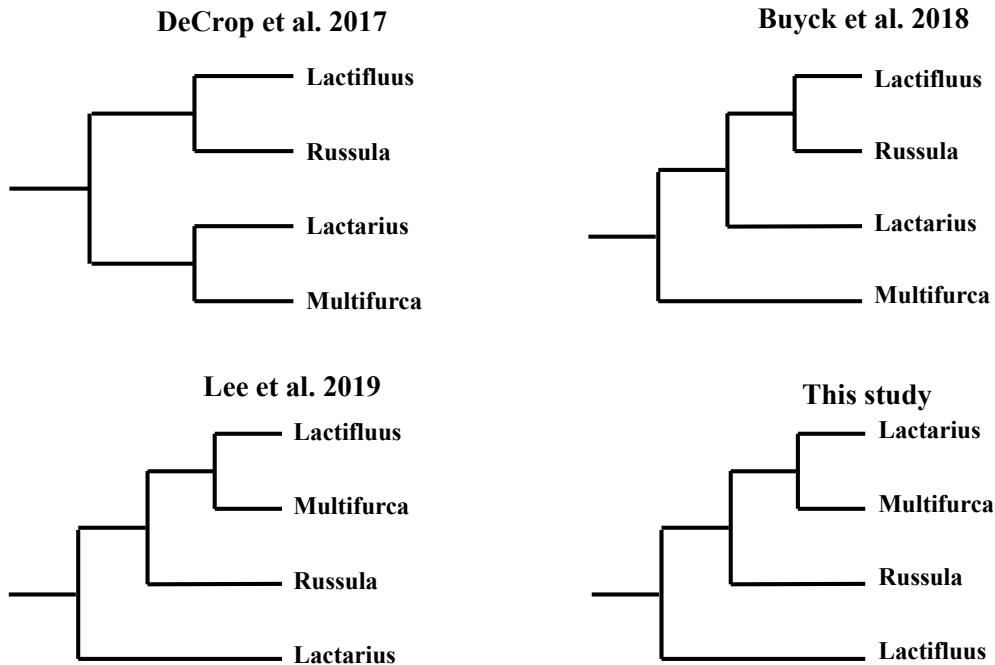
### ***Fungal host***

It has been established that *Monotropa* parasitize mainly fungi in *Russula* with some regional specificity to more narrow groups (Bidartando and Bruns 2001, Kong *et al.* 2015). Our study supports this finding as 40 out of 54 *Monotropa* from this study were found to parasitize *Russula spp.*, five parasitized *Lactarius spp.*, and eight parasitized *Lactifluus spp.* There is a phylogenetic and geographic pattern evident in the *Monotropa* that parasitize fungi from *Lactifluus* subgenus *Lactariopsis* section *Albati* that indicates high levels of regional specificity to these fungi by *Monotropa* that fall into the *brittonii* clade.

To our knowledge this is the first study to find *Monotropa* using fungi in *Lactifluus* as a host. The relation of *Lactifluus* to other genera within Russulaceae is still uncertain as many recent studies have reported conflicting phylogenies (Fig. 18). While recent studies may not agree on the relationships of these groups, all support the monophyly of the genera. Our study was meant to investigate the placement of *M. uniflora* hosts in the Russulacean tree, and used only one locus, (ITS), and our trees should not be taken as a hypothesis of relationships between genera in this family.

*Lactifluus deceptivus*, also known as the deceptive milky, is an edible mushroom common from June to November in the eastern US and Mexico and known to associate with hemlocks, pines, spruces, and oaks (Peck 1885, Montoya 1996, Montoya and Bandala 2005). Interestingly, this species has recently been reported to be a complex potentially consisting of 15 or more species (Delgat *et al.* 2019). More work is needed to settle relationships in this group.

These results contribute to a clearer understanding of the range of host specificity in *M. uniflora* across their range and how hosts differ phylogenetically (or not) across geographic areas. The majority of our *M. uniflora* tree is unresolved, so it is difficult to make inferences about patterns of host specificity for the larger clade with any certainty. One specimen collected from Oregon (JVF\_3049\_OR) was found to parasitize *Russula brevipes* (99.8% identity match from BLAST) which supports the finding of local host adaptation to *R. brevipes* by *M. uniflora* in the Pacific Northwest (Bidartando and Bruns 2001).



**Figure 18. Hypotheses of relationships within Russulaceae from recent publications**

## ***Morphology***

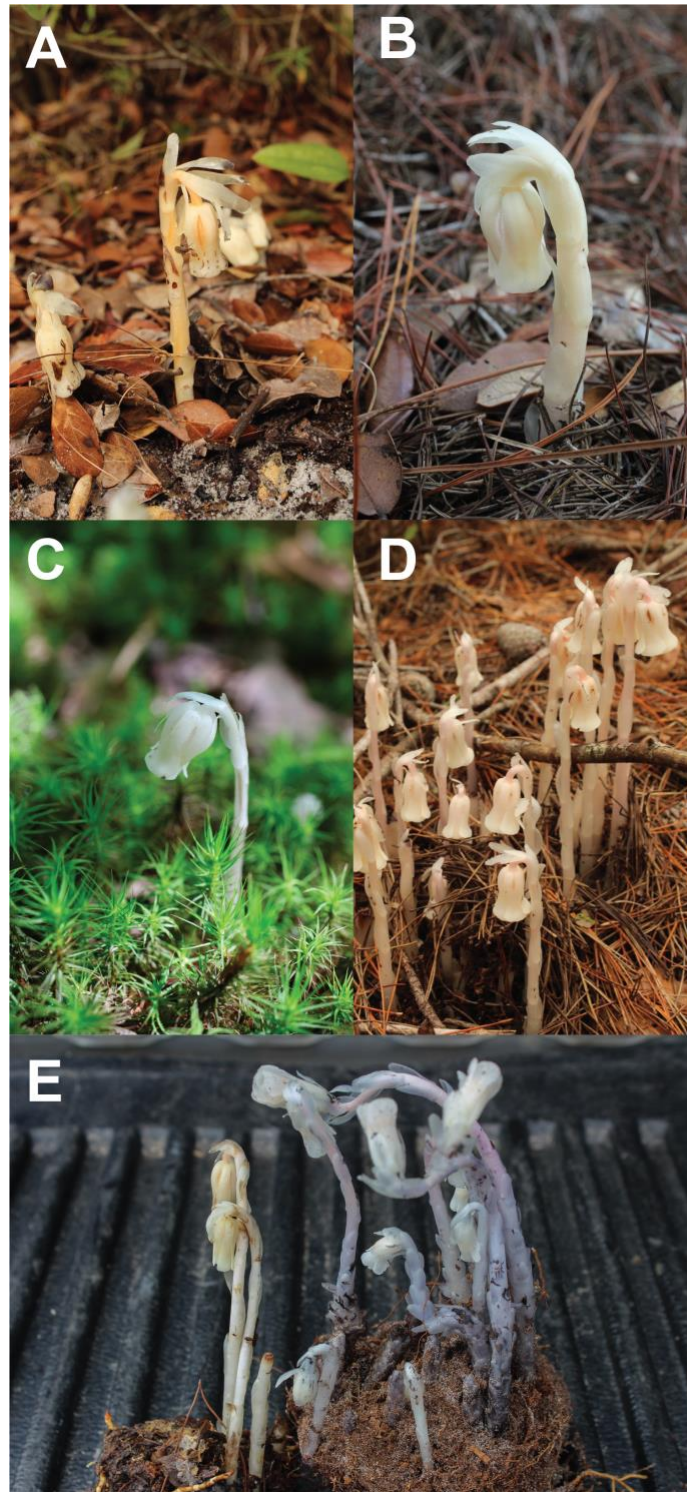
The analysis of morphological data revealed several differences between the *uniflora* group and the *brittonii* group. In the *uniflora* group the nectaries are paired at the bases of the stamens and point downward away from the opening of the flower. In most of the *brittonii* group, the nectaries pointed upwards and were longer than those in the *uniflora* group. This difference in nectary morphology may imply differences in pollinators. *Monotropa uniflora* have been shown to be pollinated largely by bumblebees but it is possible that in another environment they may have adapted to different pollinators (Klooster and Culley 2009). While most of the *brittonii* group had upturned nectaries with an average angle of 82.7 degrees from the stem, there was a wide range with some overlap with range of the *uniflora* group and a few of the *brittonii* group had smaller nectar spur angles more typical of the *uniflora* group, so this nectary morphology is not ubiquitous.

The filaments in the *uniflora* group are fairly uniform in width from top to bottom with an average top to bottom ratio of 0.92 whereas the filaments of the *brittonii* group taper near the tip and have an average ratio of 0.7 from top to bottom. These groups also differed significantly in petal, stamen, and ovary length. In general, flowers in the *brittonii* group tended to be larger than the *uniflora* group. This is in line with Small's original description that characterized *M. brittonii* as larger in size than *M. uniflora*. Small also noted the petals of *M. brittonii* were more ciliate and the filaments were hairier than *M. uniflora*. Our analysis supports this as the length of filament and petal hairs as well as the hairiness of petals was significantly greater in the *brittonii* group.

Some of Small's description was not supported in this analysis. His suggestion that petals of *M. brittonii* are less saccate than *M. uniflora* was not upheld by our analysis. Of our two measurements of saccateness, neither was significantly different between groups. We were also unable to quantify color in this analysis, although through observation it does appear that many of

the individuals in the *brittonii* group had a yellow-orange coloration (Fig. 19). This was not seen in any of the *uniflora* group which range from pure white to quite pink in some individuals.

Though there were several characters found to be significantly different between these two groups, there was overlap in every character. When trying to identify which of the two groups you are observing from morphological characters, it is important to take all characters into account including, as discussed below, the habitat where the individual was seen.



**Figure 19. Photos of *Monotropa* from both groups**

A. AK 64, *brittonii* group, B. AK 67, *brittonii* group, C. AK 21, *uniflora* group, D. AK 68, *uniflora* group, E. Side by side comparison, Left: AK 2, *brittonii* group, Right: AK 6, *uniflora* group

## *Habitat*

It has usually been asserted that *M. uniflora* grow exclusively in mesic woods containing oaks, pines, or other conifers (Table 5). However, many of the *Monotropa* from the *brittonii* group were collected in either Florida scrub or scrubby flatwoods as designated in management plans of those sites (Florida DEP 2012, Florida Forest Service 2018, Epperson 2004, US DOI 2010). Scrub habitats are strikingly different than mesic temperate woods and are characterized by xeric, well-draining soils. Scrub habitats may have no canopy or a scattered canopy of sand pine and are typically dominated by shrubby oaks and Florida rosemary (*Ceratiola ericoides*) (USFWS 1999).

However, several specimens in the *brittonii* group, such as those collected from Anastasia State Park (AK 60, AK 61), Doris Leeper Spruce Creek Preserve (AK 63), and Edward Ball Wakulla Springs State Park (AK 69), were found in the more typical habitat, mesic woods designated by the Florida DNR as maritime hammock, mesic hammock, and upland pine forest respectively. (Florida DEP 2007, 2016, Zev Cohen and Associates Inc., 2011) However, AK 63 was collected close to scrub habitat where there is a transition in habitat types.

The *brittonii* group is sister to all other *Monotropa* in the majority of phylogenetic trees (Fig. 8) indicating an early split from the rest of the genus. The paleogeographic history of Florida is complex as the state experienced repeated periods of submersion and drying out during the glacial periods and subsequent glacial retreats of the Pleistocene (Lane 1994). Florida scrub and sandhill habitats are thought to be remnants of ancient refugia for plants and animals during the last glacial maximum (Germain-Aubrey *et al.* 2014, Trapnell *et al.* 2007, Marshall *et al.* 2000). This hypothesis is supported by patterns of diversification and the high level of endemism seen in these habitats which has been shown to be correlated with ancient, climatically stable refugia (Soltis *et al.* 2006, Harrison and Noss 2017). It is possible these areas acted as refugia for

*Monotropa* during this time, leading to the separation and diversification of the *brittonii* group within these habitats. More analysis of divergence times is needed to support this hypothesis.

**Table 4 Quotes from literature describing the habitat of *M. uniflora***

Quote	Source
“In the Neotropics, it grows in <b>moist forests</b> of <i>Pinus</i> , <i>Abies</i> , and <i>Quercus</i> at elevations of 950-3400 m.”	Luteyn <i>et al.</i> 1996
“Throughout most of its geographical distribution <i>Monotropa</i> grows in <b>moist conifer or hardwood forests.</b> ”	Kong <i>et al.</i> 2015
“North American populations, which typically occur in <b>mesic woodlands</b> , are separated by desert and xeric grasslands from Central American populations, which inhabit moist montane regions”	Neyland and Hennigan 2004
“ <i>Monotropa uniflora</i> L. is an understorey plant of <b>north temperate forests</b> and it is known to associate with fungal species in the family Russulaceae”	Massicotte <i>et al.</i> 2005
“ <b>Moist mixed or coniferous forests</b> , at elevations of 100-11,000 ft; usually growing in shade under other vegetation in litter and humus.”	Wallace 1975 p. 82

## Conclusions

The phylogenetic evidence shows support for two lineages of *Monotropa* in Florida, one that occurs in the typical mesic forest habitat and displays the commonly accepted morphological features of *Monotropa uniflora* and parasitizes a variety of russulacean fungi, and another lineage that occurs mainly in Florida scrub habitats, is larger and hairier than the typical *M. uniflora*, has nectaries that point upward, and parasitizes a small range of fungal species within *Lactifluus* subgenus *Lactariopsis* section *Albati*. By analyzing morphology, genetics, and host specificity, we have shown the southern Ghost Pipe, *Monotropa brittonii*, to be evolutionarily distinct from *Monotropa uniflora*. We propose this evidence is sufficient to acknowledge *M. brittonii* as a species under the phylogenetic, ecological, and morphological species concepts. More work is

needed to evaluate the distribution of *M. brittonii*. Florida scrub habitats are highly fragmented and home to large numbers of endemic species. There may be need for conservation efforts when it comes to preserving populations of *M. brittonii* if its range is restricted. This is crucial to insure genetic diversity persists in this group in the face of climate change and habitat loss.



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## Appendix A. Genbank Accessions

### Fungal species

Species	Genbank Accession Number
<i>Amylostereum laevigatum</i>	AY781246
<i>Auriscalpium vulgare</i>	KR364005
<i>Auriscalpium vulgare</i>	KY485943
<i>Bondarzewia berkeleyi</i>	MH114628
<i>Bondarzewia berkeleyi</i>	MK167432
<i>Bondarzewia montana</i>	MH857893
<i>Echinodontium tinctorium</i>	AY854088
<i>Heterobasidion annosum</i>	DQ206988
<i>Lactarius acris</i>	JQ446085
<i>Lactarius conglutinatus</i>	MK253487
<i>Lactarius cucurbitoides</i>	MH984952
<i>Lactarius fallax</i>	JQ446103
<i>Lactarius fuliginosus</i>	JQ446111
<i>Lactarius fumosus</i>	EU819483
<i>Lactarius hadsutake</i>	KR364085
<i>Lactarius hygrophoroides</i>	KR673574
<i>Lactarius indigo</i>	EF685067
<i>Lactarius indigo</i>	FJ596854
<i>Lactarius mammosus</i>	KX610695
<i>Lactarius miniatescens</i>	KR364059
<i>Lactarius mutabilis</i>	MG773832
<i>Lactarius olympianus</i>	KR364089
<i>Lactarius orientaliquietus</i>	MH447589
<i>Lactarius pallidus</i>	MK028449
<i>Lactarius peckii</i>	EU598168
<i>Lactarius peckii</i>	KF133277
<i>Lactarius piperatus</i>	KF220093
<i>Lactarius quietus</i>	DQ658876
<i>Lactarius quietus</i>	DQ658877
<i>Lactarius quietus</i>	MG553997
<i>Lactarius quietus</i>	MK575244
<i>Lactarius scrobiculatus</i>	KF432968
<i>Lactarius tabidus</i>	KY681466
<i>Lactarius tabidus</i>	MK131492

<i>Lactarius tenellus</i>	KF133280
<i>Lactarius vellereus</i>	HM189835
<i>Lactifluus acicularis</i>	HQ318226
<i>Lactifluus acrisimus</i>	KR364041
<i>Lactifluus acrisimus</i>	KR364126
<i>Lactifluus aff gerardii</i>	EF560685
<i>Lactifluus aff glaucescens</i>	KF220045
<i>Lactifluus aff glaucescens</i>	KF220104
<i>Lactifluus aff glaucescens</i>	KR364107
<i>Lactifluus aff nebulosus</i>	KP691412
<i>Lactifluus aff phlebonemus</i>	KR364062
<i>Lactifluus aff piperatus</i>	KF220105
<i>Lactifluus aff piperatus</i>	KF220106
<i>Lactifluus aff piperatus</i>	KR364103
<i>Lactifluus albocinctus</i>	KR364117
<i>Lactifluus albomembranaceus</i>	KR364064
<i>Lactifluus allardii</i>	KF220016
<i>Lactifluus ambicystidiatus</i>	KR364108
<i>Lactifluus annulatoangustifolius</i>	AY606981
<i>Lactifluus annulifer</i>	KC155376
<i>Lactifluus atrovelutinus</i>	GU258231
<i>Lactifluus aurantiifolius</i>	KR364017
<i>Lactifluus aureifolius</i>	KR364056
<i>Lactifluus auriculiformis</i>	KR364086
<i>Lactifluus bertillonii</i>	KR364087
<i>Lactifluus bicolor</i>	JN388955
<i>Lactifluus brachystegiae</i>	KR364018
<i>Lactifluus brunellus</i>	JN168728
<i>Lactifluus bruneoviolascens</i>	KR364123
<i>Lactifluus brunnescens</i>	KR364019
<i>Lactifluus caribaeus</i>	KP691415
<i>Lactifluus carmineus</i>	KR364131
<i>Lactifluus castaneibadius</i>	KP691417
<i>Lactifluus cf gerardii var fagicola</i>	GU258224
<i>Lactifluus cf longisporus</i>	KR364054
<i>Lactifluus cf ochrogalactus</i>	KR364130
<i>Lactifluus cf pseudogymnocarpus</i>	KR364012
<i>Lactifluus cf pseudovolemus</i>	KR364113



<i>Lactifluus cf pumilus</i>	KR364067
<i>Lactifluus cf tanzanicus</i>	KR364053
<i>Lactifluus cf zenkeri</i>	KR364055
<i>Lactifluus chamaeleontinus</i>	KR364079
<i>Lactifluus chiapanensis</i>	GU258297
<i>Lactifluus chrysocarpus</i>	JX442761
<i>Lactifluus clarkeae</i>	KR364011
<i>Lactifluus cocosmus</i>	KR364013
<i>Lactifluus conchatulus</i>	GU258296
<i>Lactifluus corrugis</i>	JQ753822
<i>Lactifluus crocatus</i>	HQ318243
<i>Lactifluus cyanovirescens</i>	KR364082
<i>Lactifluus deceptivus</i>	AY854089
<i>Lactifluus deceptivus</i>	KF937340
<i>Lactifluus deceptivus</i>	KR364101
<i>Lactifluus deceptivus</i>	MG982549
<i>Lactifluus deceptivus</i>	MH212001
<i>Lactifluus deceptivus</i>	MK069514
<i>Lactifluus deceptivus</i>	MK069515
<i>Lactifluus deceptivus</i>	MK575432
<i>Lactifluus denigricans</i>	KR364051
<i>Lactifluus densifolius</i>	KR364057
<i>Lactifluus dissitus</i>	JN388978
<i>Lactifluus distantifolius</i>	HQ318274
<i>Lactifluus dwaliensis</i>	KF220111
<i>Lactifluus dwaliensis</i>	KR364042
<i>Lactifluus edulis</i>	KR364020
<i>Lactifluus emergens</i>	AY606979
<i>Lactifluus emergens</i>	KR364021
<i>Lactifluus fazaoensis</i>	HG426477
<i>Lactifluus flammans</i>	KR364078
<i>Lactifluus flocktonae</i>	JX266621
<i>Lactifluus foetens</i>	KR364022
<i>Lactifluus foetens</i>	KR364023
<i>Lactifluus genevieveae</i>	GU258294
<i>Lactifluus gerardii</i>	GU258254
<i>Lactifluus glaucescens</i>	KR364105
<i>Lactifluus goossensiae</i>	KR364132

<i>Lactifluus gymnocarpoides</i>	KR364024
<i>Lactifluus gymnocarpoides</i>	KR364074
<i>Lactifluus gymnocarpus</i>	KR364065
<i>Lactifluus heimii</i>	KR364025
<i>Lactifluus heimii</i>	KR364040
<i>Lactifluus hygrophoroides</i>	HQ318285
<i>Lactifluus hygrophoroides</i>	KX358039
<i>Lactifluus hygrophoroides</i>	MK430041
<i>Lactifluus igniculus</i>	JX442759
<i>Lactifluus indusiatus</i>	KR364026
<i>Lactifluus inversus</i>	AY606976
<i>Lactifluus kigomaensis</i>	KR364179
<i>Lactifluus kivuensis</i>	KR364027
<i>Lactifluus laevigatus</i>	KR364077
<i>Lactifluus lamprocystidiatus</i>	KR364015
<i>Lactifluus latifolius</i>	KR364028
<i>Lactifluus leae</i>	KF432957
<i>Lactifluus leonardii</i>	GU258308
<i>Lactifluus leoninus</i>	KF220055
<i>Lactifluus leoninus</i>	KR364116
<i>Lactifluus leptomerus</i>	JN388972
<i>Lactifluus leucophaeus</i>	KR364107
<i>Lactifluus longipes</i>	KR364009
<i>Lactifluus longipes</i>	KR364009
<i>Lactifluus longipilus</i>	HQ318256
<i>Lactifluus longisporus</i>	KR364118
<i>Lactifluus longivelutinus</i>	KR364114
<i>Lactifluus luteolus</i>	KR364016
<i>Lactifluus luteopus</i>	KR364049
<i>Lactifluus luteopus</i>	KR364119
<i>Lactifluus madagascariensis</i>	AY606977
<i>Lactifluus madagascariensis</i>	KR364120
<i>Lactifluus medusae</i>	KR364069
<i>Lactifluus multiceps</i>	JN168731
<i>Lactifluus murinipes</i>	KP691418
<i>Lactifluus nodosicytidiosus</i>	AY606975
<i>Lactifluus nodosicytidiosus</i>	KR364029
<i>Lactifluus nonpiscis</i>	KR364030

<i>Lactifluus nonpiscis</i>	KR364058
<i>Lactifluus oedematopus</i>	KR364100
<i>Lactifluus panuoides</i>	KJ786647
<i>Lactifluus pegleri</i>	KP691416
<i>Lactifluus pelliculatus</i>	KR364080
<i>Lactifluus petersenii</i>	GU258281
<i>Lactifluus phlebophyllus</i>	AY606974
<i>Lactifluus pilosus</i>	KR364006
<i>Lactifluus pilosus</i>	MG551737
<i>Lactifluus pilosus</i>	MG551740
<i>Lactifluus pinguis</i>	HQ318211
<i>Lactifluus piperatus</i>	KF220119
<i>Lactifluus pleurotellus</i>	KR364090
<i>Lactifluus pleurotoideus</i>	KR364111
<i>Lactifluus pruinatus</i>	KR364031
<i>Lactifluus pseudoluteopus</i>	KR364084
<i>Lactifluus pulchrellus</i>	KR364092
<i>Lactifluus putidus</i>	KP691413
<i>Lactifluus reticulatovenosus</i>	GU258286
<i>Lactifluus robustus</i>	KR364047
<i>Lactifluus roseolus</i>	KR364032
<i>Lactifluus roseolus</i>	KR364121
<i>Lactifluus roseophyllus</i>	KF220107
<i>Lactifluus rubiginosus</i>	KR364014
<i>Lactifluus rubiginosus</i>	KR364081
<i>Lactifluus rubrobrunnescens</i>	KR364115
<i>Lactifluus rubroviolascens</i>	KR364066
<i>Lactifluus rufomarginatus</i>	KR364033
<i>Lactifluus rufomarginatus</i>	KR364034
<i>Lactifluus rugatus</i>	KR364104
<i>Lactifluus rugatus</i>	KU885436
<i>Lactifluus ruvubuensis</i>	KR364035
<i>Lactifluus ruvubuensis</i>	KR364122
<i>Lactifluus sesemotani</i>	KR364036
<i>Lactifluus sp</i>	KJ786643
<i>Lactifluus sp</i>	KJ786645
<i>Lactifluus sp</i>	KJ786691
<i>Lactifluus sp</i>	KJ786694

<i>Lactifluus sp</i>	KJ786706
<i>Lactifluus sp</i>	KP691414
<i>Lactifluus sp</i>	KP691436
<i>Lactifluus sp</i>	KR364008
<i>Lactifluus sp</i>	KR364043
<i>Lactifluus sp</i>	KR364044
<i>Lactifluus sp</i>	KR364045
<i>Lactifluus sp</i>	KR364060
<i>Lactifluus sp</i>	KR364061
<i>Lactifluus sp</i>	KR364063
<i>Lactifluus sp</i>	KR364068
<i>Lactifluus sp</i>	KR364070
<i>Lactifluus sp</i>	KR364071
<i>Lactifluus sp</i>	KR364076
<i>Lactifluus sp</i>	KR364088
<i>Lactifluus sp</i>	KR364091
<i>Lactifluus sp</i>	KR364097
<i>Lactifluus sp</i>	KR364102
<i>Lactifluus sp</i>	KR364127
<i>Lactifluus sp</i>	KR364128
<i>Lactifluus sp</i>	KR364177
<i>Lactifluus subclarkeae</i>	KR364095
<i>Lactifluus subgerardii</i>	GU258263
<i>Lactifluus subiculatus</i>	JQ405654
<i>Lactifluus subpruinosis</i>	KR364046
<i>Lactifluus subvellereus</i>	KR364010
<i>Lactifluus subvolemus</i>	JQ753927
<i>Lactifluus sudanicus</i>	HG426469
<i>Lactifluus sulcatipes</i>	KR364109
<i>Lactifluus tanzanicus</i>	KR364037
<i>Lactifluus tenuicystidiatus</i>	KR364048
<i>Lactifluus uapacae</i>	KR364007
<i>Lactifluus urens</i>	KR364124
<i>Lactifluus vellereus</i>	KR364106
<i>Lactifluus velutissimus</i>	KR364075
<i>Lactifluus venezuelanus</i>	KP691411
<i>Lactifluus veraecrucis</i>	KR364112
<i>Lactifluus versiformis</i>	JN388967

<i>Lactifluus vitellinus</i>	HQ318236
<i>Lactifluus volemoides</i>	KR364038
<i>Lactifluus volemoides</i>	KR364098
<i>Lactifluus volemus</i>	HQ318232
<i>Lactifluus volemus</i>	HQ318240
<i>Lactifluus volemus</i>	JN388979
<i>Lactifluus volemus</i>	JQ753944
<i>Lactifluus volemus</i>	JQ753948
<i>Lactifluus volemus</i>	KR364096
<i>Lactifluus volemus</i>	KX095050
<i>Lactifluus wirrabara</i>	GU258287
<i>Lactifluus wirrabara</i>	GU258307
<i>Lactifluus xerampelinus</i>	KR364039
<i>Lactifluus xerampelinus</i>	KR364099
<i>Multifurca albovelutina</i>	MH46523
<i>Multifurca furcata</i>	DQ421995
<i>Multifurca furcata</i>	MH063863
<i>Multifurca mellea</i>	MH465236
<i>Multifurca ochricompacta</i>	DQ421984
<i>Multifurca ochricompacta</i>	MH063879
<i>Multifurca roxburghii</i>	MH063876
<i>Multifurca sp</i>	KR364125
<i>Multifurca stenophylla</i>	JX266631
<i>Multifurca stenophylla</i>	MH063866
<i>Multifurca zonaria</i>	KR364083
<i>Multifurca zonaria</i>	MH063869
<i>Russula acrifolia</i>	JF834363
<i>Russula adusta</i>	MG687346
<i>Russula archaea</i>	AY061737
<i>Russula archaea</i>	EU598165
<i>Russula brevipes</i>	DQ367912
<i>Russula brevipes</i>	KY848511
<i>Russula cerea</i>	MK105640
<i>Russula cerolens</i>	KF245486
<i>Russula cerolens</i>	KX449204
<i>Russula chloroides</i>	AF418604
<i>Russula chloroides</i>	KX358045
<i>Russula compacta</i>	MK069521

<i>Russula compacta</i>	MK167417
<i>Russula cuprea</i>	KT934010
<i>Russula cuprea</i>	KU886592
<i>Russula cyanoxantha</i>	AF418608
<i>Russula cyanoxantha</i>	KR364093
<i>Russula cyanoxantha</i>	MG687357
<i>Russula decolorans</i>	AF418637
<i>Russula decolorans</i>	KX358008
<i>Russula decolorans</i>	LC192760
<i>Russula delicata</i>	KF432955
<i>Russula dissimulans</i>	MH212004
<i>Russula farinipes</i>	AY061675
<i>Russula farinipes</i>	KX655859
<i>Russula favrei</i>	MG687354
<i>Russula fellea</i>	KF245536
<i>Russula foetens</i>	KX095018
<i>Russula fulvescens</i>	MH979313
<i>Russula gossypina</i>	KY800350
<i>Russula gracillima</i>	KR364094
<i>Russula grisea</i>	JF908688
<i>Russula grisea</i>	KX963792
<i>Russula illota</i>	KF245508
<i>Russula integra</i>	AF418636
<i>Russula khanchanjungae</i>	KR364129
<i>Russula lepida</i>	DQ422013
<i>Russula lepida</i>	MG687359
<i>Russula livescens</i>	JN836753
<i>Russula lutea</i>	MH930944
<i>Russula maculata</i>	AY061688
<i>Russula maculata</i>	KU928155
<i>Russula mariae</i>	EU819426
<i>Russula mariae</i>	KU139525
<i>Russula mariae</i>	MF161263
<i>Russula mutabilis</i>	MF161239
<i>Russula nigricans</i>	AF418607
<i>Russula nigricans</i>	EF534352
<i>Russula nigricans</i>	EU819428
<i>Russula nigricans</i>	JQ711972

<i>Russula nitida</i>	KU205349
<i>Russula pallescens</i>	KX812839
<i>Russula paludosa</i>	KP149065
<i>Russula paludosa</i>	KT934000
<i>Russula peckii</i>	MK131580
<i>Russula pectinatoides</i>	DQ422026
<i>Russula pectinatoides</i>	EU819493
<i>Russula pectinatoides</i>	EU819500
<i>Russula pectinatoides</i>	KU640189
<i>Russula pectinatoides</i>	MF773598
<i>Russula pectinatoides</i>	MH211768
<i>Russula praetervisa</i>	KF245531
<i>Russula puellula</i>	AY061710
<i>Russula raoultii</i>	AF418621
<i>Russula recondita</i>	KJ530757
<i>Russula recondita</i>	KJ530759
<i>Russula rosea</i>	MG214693
<i>Russula rosea</i>	MK719904
<i>Russula silvestris</i>	KX579800
<i>Russula sp</i>	KR364072
<i>Russula sp</i>	KR364073
<i>Russula sphagnophila</i>	KX095016
<i>Russula subtilis</i>	GQ166871
<i>Russula turci</i>	KY582703
<i>Russula turci</i>	MG687331
<i>Russula velenovskyi</i>	HM189951
<i>Russula versicolor</i>	MG687334
<i>Russula vesca</i>	AY878660
<i>Russula veterinosa</i>	KY582699
<i>Russula vinosa</i>	JQ888203
<i>Russula xerampelina</i>	AF418632
<i>Russula xerampelina</i>	KP454007
<i>Stereum hirsutum</i>	AY854063
<i>Stereum hirsutum</i>	EU851113
<i>Vararia abortiphysa</i>	KR364005

**Monotropa hosts from similar studies**

Genbank Accession #	Locality	Source
AY878657	CA	Bidartando and Bruns 2005
AF349714	CA	Bidartando and Bruns 2001
AF349713	CAN	Bidartando and Bruns 2001
AF349708	JAP	Bidartando and Bruns 2001
AF349710	NC	Bidartando and Bruns 2001
AF349715	OR	Bidartando and Bruns 2001
AF349717	VT	Bidartando and Bruns 2001
AF349709	VT	Bidartando and Bruns 2001
AF349711	VT	Bidartando and Bruns 2001
AF349712	VT	Bidartando and Bruns 2001
AY878658	MD	Bidartando and Bruns 2005
AY878661	CA	Bidartando and Bruns 2005
AY878659	MD	Bidartando and Bruns 2005
KP781015	MEX	Kong <i>et al.</i> 2015
KP780993	MEX	Kong <i>et al.</i> 2015
KP780998	MEX	Kong <i>et al.</i> 2015
KP781007	MEX	Kong <i>et al.</i> 2015
KP781009	MEX	Kong <i>et al.</i> 2015
KP781010	MEX	Kong <i>et al.</i> 2015
KP781011	MEX	Kong <i>et al.</i> 2015
KP781016	MEX	Kong <i>et al.</i> 2015
KP781017	MEX	Kong <i>et al.</i> 2015
KP781018	MEX	Kong <i>et al.</i> 2015
KP781022	MEX	Kong <i>et al.</i> 2015
AF311975	CAN	Young <i>et al.</i> 2002
DQ777990	MA	Yang and Pfister 2006
DQ777991	MA	Yang and Pfister 2006
DQ777971	MA	Yang and Pfister 2006
DQ777974	MA	Yang and Pfister 2006
DQ777975	MA	Yang and Pfister 2006
DQ777976	MA	Yang and Pfister 2006
DQ777977	MA	Yang and Pfister 2006
DQ777985	MA	Yang and Pfister 2006
DQ777999	MA	Yang and Pfister 2006
DQ778000	MA	Yang and Pfister 2006



**Monotropa Outgroups**

Species	Genbank accessions	Locus
<i>Monotropa hypopitys</i>	KU350177	26S-ITS
<i>Monotropsis odorata</i>	KF707573	26S-ITS
<i>Pterospora andromedea</i>	KU350181	26S-ITS
<i>Pyrola aphylla</i>	KU350182	26S-ITS
<i>Monotropa hypopitys</i>	MF120264	rps2
<i>Monotropsis odorata</i>	AF351946	rps2
<i>Pterospora andromedea</i>	MF120270	rps2
<i>Pyrola aphylla</i>	AF351961	rps2

**Monotropa from this study**

Specimen number	Locality
AK 1	FL
AK 2	FL
AK 3	FL
AK 4	FL
AK 5	FL
AK 6	FL
AK 7	FL
AK 8	OH
AK 9	OH
AK 10	OH
AK 11	KY
AK 12	KY
AK 13	KY
AK 13	TN
AK 14	TN
AK 15	TN
AK 17	MN
AK 19	WI
AK 20	WI
AK 21	WI
AK 23	WI
AK 24	IL
AK 25	IL
AK 26	OH
AK 27	WV
AK 28	MD

AK 29	WV
AK 30	WV
AK 3049	OR
AK 3054	WA
AK 3062	WA
AK 31	VA
AK 32	MD
AK 33	VA
AK 34	VA
AK 36	VA
AK 36	VA
AK 37	WV
AK 38	MA
AK 39	MA
AK 40	MI
AK 41	MI
AK 42	MI
AK 44	MI
AK 45	OH
AK 46	ME
AK 47	ME
AK 48	MN
AK 49	MN
AK 50	MI
AK 51	IN
AK 52	NY
AK 53	IL
AK 54	GA
AK 55	GA
AK 56	SC
AK 57	NC
AK 58	NC
AK 59	LA
AK 60	FL
AK 61	FL
AK 62	FL
AK 63	FL
AK 64	FL

AK 65	FL
AK 66	FL
AK 67	FL
AK 68	FL
AK 69	FL
AK 70	FL
AK 71	LA
AK 72	MA
AK 73	NH
AK 74	AL
AK 75	AL
AK 76	TX
AK 77	AL
AK 78	FL
AK 79	FL
Bi97 46	
CFB 38	WA
CFB 49	WA
CFB 70	VA
CFB 72	VA
CFB 74	NY
CFB 75	NY
CFB 76	NY
CFB 77	NY
CFB 78	NY
CFB 79	NY
CFB 80	NY
CFB 81	NY
JPR10 740	MI
JVF 2529	CAN
JVF 2852	MI
JVF 2855	VA
JVF 2856	VA
JVF 2860	OH
JVF 2863	IL
JVF 2874	MEX
JVF 2882	MEX
JVF 2907	MI

JVF 2990	MI
JVF 2994	MI
JVF 2996	WI
JVF 3007	MI
JVF 3019	PA
JVF 3020	PA
Li10	TN
MBB 100	FL
MBB 102	CAN
MBB 103	CAN
MBB 104	CAN
MBB 108	OH
MBB 12	RI
MBB 19	CAN
MBB 2	OH
MBB 58	NH
MBB 71	GA
MBB 86	FL
MBB 87	FL
MBB 88	FL
MBB 89	FL
MBB 91	OH
MBB 99	FL