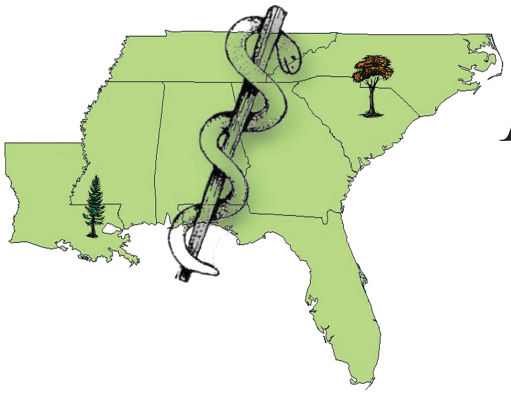

AUBURN UNIVERSITY
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DELADENUS SPECIES ASSOCIATED WITH NATIVE SIRICID WOODWASPS IN ALABAMA

by
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ABSTRACT
Sirex nigricornis woodwasp populations in the southeastern United States have been studied to gain a better understanding of how this species interacts in the pine forest ecosystem. In this study, specimens collected from three forests in Alabama were dissected in order to sample for the mutualistic fungi, *Amylostereum* spp. and parasitic nematode, *Deladenus* spp. Molecular and phylogenetic analyses were performed in order to determine relationships between the woodwasps collected, and fungal and nematode species determined to coexist inside the wasps. *Sirex nigricornis* was found to carry both *A. chailletii* and *A. areolatum*, and was found to be parasitized by both *D. proximus* and *D. siricidicola*. A *Tremex columba* specimen was found to carry *Cerrena unicolor*, and also was parasitized by *D. siricidicola*.

INTRODUCTION
The invasive woodwasp *Sirex noctilio* L. (Hymenoptera: Siricidae) was first discovered in the State of New York in 2004 (Hoebeke et al., 2005). *Sirex noctilio* is known to kill pine trees by using its ovipositor to drill into the xylem of the pine tree where it subsequently deposits a mixture of eggs, fungal spores, and phytotoxic venom resulting in cell death due to reduced moisture present within the impacted tree (Madden, 1968c). This woodwasp is native to Europe and Northern Africa (Spradbery and Kirk, 1978), and has become a pest of significant economic importance in commercial forestry plantations within the Southern Hemisphere (Slippers and Wingfield, 2012). Such nonnative planted pine plantations are usually established by planting a single pine species over vast areas (Carnegie et al., 2006). The economic impact caused by this pest in the Southern Hemisphere has not yet occurred within the mixed pine and hardwood forests of the northeastern United States. It is still unclear as to whether *S. noctilio* could have a significant impact on commercial pine forestry in the southeastern United States, which are comprised predominantly of loblolly pine (*Pinus taeda* L.). Since the initial introduction of *S. noctilio*, researchers throughout the United States have been conducting studies to determine as to how this pest could potentially impact native ecosystems, and other woodwasps found within overlapping geographic areas as to where S. noctilio is found. The complex of the siricid woodwasp in relation to their mutualistic fungi and nematode species has been studied extensively (Bedding and Akhurst, 1974; Madden, 1968c; Hurley et al., 2008). Historically, there was thought to be a strong species specific relationship between *Sirex* spp. and *Amylostereum* spp. This fungal symbiont typically associated with native North American siricids is that of *A. chailletii* Pers. Boidin (Smith and Schiff, 2002; Hajek et al., 2013). Recently, however, several fungal species including *A. areolatum* were found to occur with the North American native *S. nigricornis* F. (Olatinwo et al., 2013). Fruiting basidiocarps are

very rarely seen in nature (van der Nest et al., 2012), so it is unlikely that spores could have been transmitted in this manner.

A sterilizing strain of nematode, *Deladenus siricidicola* Bedding (Tylenchida: Neotylenchidae), is commonly used in the southern hemisphere as a biological control agent on *S. noctilio* (Bedding and Iede, 2005). This parasitic strain of nematode is thought to be species specific thus unlikely to infest *Sirex* spp. native to North America. Currently in North America, there are seven native *Sirex* spp. and one invasive species in addition to *S. noctilio, S. juvencus juvencus* L. (Schiff et al., 2006). This presence of *D. siricidicola* in native siricids was however, found to occur on eggs dissected from female *S. nigricornis* in this study. This association is most likely due to vertical transmission within the tree during the larval stage, when adult *S. nigricornis* wasps carry *A. areolatum* spores internally. It is currently unclear as to whether *D. siricidicola* associated with *S. nigricornis* is the sterilizing strain commercially used in the Southern Hemisphere, or a non-sterilizing strain of *D. siricidicola* found to occur in *S. noctilio* in Canada (Yu et al., 2009). Studies report that there is a strict relationship between the species of nematode and the fungus for which they are associated. Bedding and Akhurst (1978) found that *D. siricidicola* was strictly associated with *A. areolatum*, and *D. canii* Bedding was strictly associated with *A. chailletii*. These linkages support the idea that siricids are associated to the species of nematode that are endemic to the same areas in which they are found. In addition other Siricidae have a strict relationship with associated nematodes and fungus, examples include *Tremex columba* L., the pigeon horntail, a woodwasp native to the United States that generally inhabits hardwoods such as oak, elm, and beech (Schiff et al., 2006). *Tremex columba* is typically associated with *Cerrena unicolor* (Bull.) Merrill, a similar saprophytic white rot (Stillwell, 1965; Pažoutová et al., 2010). This fungus is native to the United States, and is commonly found to colonize dead or dying hardwoods (Enebak and Blanchette, 1989).

MATERIALS AND METHODS

Sampling

Through the duration of trapping, 132 specimens were trapped fortnightly from forested sites throughout the State of Alabama from August 2014 to February 2015. These sites include two National Forests (Tuskegee and Talladega) as well as a site at Auburn University's Solon Dixon Center. In each forest locale, 33 black cross vane panel insect traps (Forestry Distributing Inc., Boulder, CO, U.S.A.) were used for trapping and were placed at each site Tuskegee (August 2014-March 2016), Talladega (Oakmulgee Ranger District) National Forests (February 2015- March 2016) and at Auburn University's Solon Dixon Center in Andalusia, (February 2015- March 2016). Collection cups were attached at the bottom of each trap to catch insects that were lured to the traps. These collection cups were filled with 250 mL of a 30% propylene glycol solution to preserve specimens. Traps were baited using 8 mL glass vials filled with a mixture of 70% α-Pinene and 30% β-Pinene, previously determined by Simpson and McQilkin (1976) to be optimal for attracting *S. noctilio*. This collection method, for surveying woodwasp populations, was undertaken in accordance with Barnes et al. (2014). An additional 36 samples were collected in 2014 from traps placed on the same field sites in the Talladega National Forest, as a part of another study with these traps being baited using turpentine and ethanol.

For live specimen collections during the expected *S. noctilio* flight season (August- January) additional insect traps were deployed in both 2014 and 2015 at three additional localities: 1) a small forest located around School of Forestry and Wildlife Sciences, Auburn University, 2) Mary Olive Thomas Demonstration Forest (MOT) and 3) Louise Kreher Forest Ecology Preserve and Nature Center in Auburn, Alabama. These traps were suitable to capture live females as they had a paper towel crumpled as a means to detain live wasps instead of the usual propylene glycol mixture in collection cups used in the other traps. Traps were checked every other day at all these additional localities. Live specimens were first killed by brushing with ethyl acetate, and then were processed using the same protocol as dead captured specimens. Additional traps were set up in order to capture live females, to obtain cultures of *Amylostereum* spp.

Dissection

Nematode samples were collected from eggs of the female woodwasps. Captured live *Sirex* spp. specimens were stored in the laboratory at 4° C until time of processing. All woodwasp specimens were morphologically identified to species level using the morphological key of Schiff et al. (2006). During the wasp dissection, mycangia (Fig 3.1) were removed and plated on potato dextrose agar (PDA) plates with streptomycin for fungal culturing. Dissection techniques were in accordance with Thomsen and Harding (2011). Eggs, mycangium, and leg samples were all placed in individual glass vials of 95% ethanol for storage and shipment and used for molecular analyses.

Molecular Analyses

For nematode samples, a modified version (Katrin Fitza, pers. comm.) of the DNA extraction protocol from Wilhelm et al. (1992) was used. Identification of the samples were conducted using the COI primers published by Morris et al. (2013), amplifying part of the cytochrome oxidase 1 gene region, as well as the TW81 and AB28 primers published by Morris et al. (2013) to amplify the internal transcribed spacer region (ITS rDNA). The entire extracted DNA was then used for PCR reactions. PCR reactions for both primers were made to a total volume of 21.5 µl, 5 µl of MyTaq™ Reaction Buffer (Bioline USA, Tauton, Massachusetts), 0.1 µg of both COIF and COIR and 0.5 µl of MyTaq™ DNA polymerase (Bioline USA, Tauton, Massachusetts). The following PCR conditions were applied to both COI and ITS: preincubation of 95°C 4 min, 35 cycles of 95°C for 45 s, 56°C for 30 s and 72°C for 1 min, ending with a final extension of 72°C for 10 min. Sequencing was performed on the amplicons using the ABI PrismTM 3500xL automated DNA sequencer (Applied Biosysytems USA, Foster City, California).

To verify the *Amylostereum* isolates DNA was extracted from mycangia stored in 95% ethanol. Each mycangia was added to 50 µl of PrepMan®Ultra Sample Preparation Reagent (Applied Biosystems, Foster City, CA) and homogenized with a ball mill (Retsch MM301) at 30 shakes/ second frequency for 3 minutes. The samples were spun down and heated for 10 min at 100°C in a heating block. The primers used for analysis were the mtSSU rDNA primer pair MS1 and MS2 (White et al., 1990) amplifying a portion of the mitochondrial small subunit, and the internal transcribed spacer region (ITS rDNA) primer pair ITS1 and ITS4 (White et al., 1990). PCR reactions for both primers included around 0.1 µg of the template DNA, 5 µl of MyTaq™ Reaction Buffer (Bioline USA, Tauton, Massachusetts), 0.1 µg of forward and reverse primer and 0.5 µl of MyTaq™ DNA polymerase (Bioline USA, Tauton, Massachusetts). The following PCR protocol was used for MS primers: 95°C for 3 minutes, followed by 35 cycles of 95°C for 45 seconds, 58°C for 30 seconds, 72°C for one minute, one cycle of 72°C for 10 minutes and holding

at 10°C. The protocol for ITS is as follows: 94°C for 7 minutes, followed by 35 cycles of 94°C for one minute, 48°C for 1 minute, 72°C for 2 minutes, one cycle of 72°C for 10 minutes and holding at 10°C. All the amplicons were sequenced following above mentioned protocol.

Single legs of *Sirex* specimens were cut using micro-scissors and homogenized with a ball mill (Retsch MM301) at 30/s frequency for 3 min. The ZyGEM DNA extraction protocol was followed using the *prep*Gem^{TM}Insect (ZyGEM, Hamilton, New Zealand) kit. The barcoding cytochrome oxidase (cox 1) primers LCO1490 and HCO2198 designed by Folmer at al. (1994) were applied to identify the specimens. The PCR protocol described by Dittrich-Schröder et al., (2012) was followed: 95°C for 7 minutes, 35 cycles of 95°C for 30 seconds, 58°C for 30 seconds, 72°C for 2 minutes, followed by one cycle of 72°C for 2 minutes, then holding at 10°C. The 658bp fragment was then sequences as described above.

Phylogenetic analyses

Raw sequences were edited using the software program 4 Peaks and AliView, where they were aligned using Muscle. Sequences were submitted to GenBank (Accession numbers pending). All trees were drawn in Mega 6 (Tamura et al, 2011). Neighbor-joining and Maximum-liklihood trees using 1000 bootstraps were drawn for *Amylostereum* and *Deladenus* samples. Other isolates used in comparison were accessed from GenBank and Fitza et al. (2016).

RESULTS

Phylogenetic relationships of *Deladenus* spp. samples were determined by sequencing on the Cytochrome Oxidase I gene. In some cases, the ITS region also was sequenced to provide greater reliability of identification (Table 3.1). Three distinct species of nematodes were molecularly identified from samples in this study (Figs 3.1, 3.2). These were identified as *Deladenus proximus*, *D. siricidicola* and the third species is thought to be the previously described *D. wilsoni*. From the total of nine nematode samples, obtained from dissected *S. nigricornis* eggs *D. siricidicola* and *D. proximus* were identified. This study found two other woodwasps species to be hosting *D. siricidicola*; *S. nigricornis* and *T. columba*. A total of 131 woodwasps were initially captured in an earlier survey (Wahl, Chapter 2). Out of those, 21.9% were infested with some species of nematode.

Out of the 47 *S. nigricornis* mycangium sampled, 15 specimens were confirmed to be *A. areolatum*, and 20 were confirmed as *A. chailletii* (Table 3.2, Figs. 3.3, 3.4). The mycangia sampled from the singular *T. columba* specimen yielded a result of *C. unicolor* (Fig. 3.4).

DISCUSSION

The relationship observed between *S. nigricornis* and *D. siricidicola* in the present studies is previously unreported. *Deladenus siricidicola* had previously only been associated with *S. noctilio*, even in areas where populations of *S. nigricornis* overlap (Hartshorn et al., 2015). It is still unclear as to whether this strain of *D. siricidicola* is capable of sterilizing eggs, as visual inspection of eggs, prior to sampling, revealed nematodes only present on the outside of egg cases.

Our findings from this study provide new insight as to the relationship between *Sirex* spp. and

Deladenus spp. Previous studies found that there was strong host specificity between *D. siricidicola* and *S. noctilio* (Table 3.1, Figs. 3.1, 3.2). A horizontal transfer of fungal associates in other areas where the two *Sirex* spp. overlap has been hypothesized by Wooding et al. (2013), so it is conceivable that nematode parasites also might be transferred in a similar manner. The tree feasibly could have been attacked by another siricid carrying *D. siricidicola*. An interesting aspect of this study, however, is that there have been no reports of *S. noctilio* in this region of the country.

The association observed between *T. columba* and *D. siricidicola* also is a new finding. *Tremex columba* does not typically colonize the same trees as *Sirex* spp. Even though the preferred hosts of both of these species are found together in the plots surveyed, some form of direct contact would have to be made if *D. siricidicola* did not naturally coexist with *T. columba*. Although the capture of the *T. columba* specimen was within the same time period as *S. nigricornis* flight in Alabama, the flight season of *T. columba* is longer than that of *S. nigricornis*. A previous study showed that *T. columba* can emerge as early as June, well before typical *S. nigricornis* flight (Wahl, Chapter 2). The earlier emergence period observed for *T. columba* might suggest that this species could overlap temporally with *S. noctilio* if it were to be found in the same locality.

Finding that *S. nigricornis* in Alabama is associated with both *A. areolatum* and *A. chailletii* are in accordance with Wooding et al. (2013) and Olantinwo et al. (2013). This finding is unsurprising, but is significant because it supports the possibility that *D. siricidicola* was originally passed to *S. nigricornis* from a *S. noctilio* in the area of the United States where their populations overlap.

One interesting aspect of *S. nigricornis* carrying either *A. areolatum* or *A. chailletti* is the implication that *D. siricidicola* and *D. proximus* may lack fidelity to respective fungal species, as previously believed. Prior reports (Bedding and Akhurst, 1978; Hajeck and Morris, 2014) state that *D. proximus* cannot feed on *A. areolatum.* These sources also claim that *D. siricidicola* cannot subsist on *A. chailletii*. The fungal and nematode relationships determined for specimens 47 and 79 are not in accordance with the previous studies' findings. The ITS tree drawn (Fig. 3.2) suggests that Specimen 79 is *D. proximus*. Further genes need to be sequenced for this specimen, as the COI tree (Fig. 3.1) suggests that it may group with a different species, where no known reference specimens are available.

CONCLUSIONS

This link between *S. nigricornis* and *D. siricidicola* is previously unreported, and has the potential to impact how *Sirex* control methods are implemented in commercial forestry. The fact that *D. siricidicola* is not genus specific, much less species specific, may warrant more research looking at how this nematode is used as a biological control agent. Lesser amounts of research have gone into studying *S. nigricornis* and its symbiotic relationships than the invasive pest *S. noctilio*. If the *D. siricidicola* found in *S. nigricornis* samples in Alabama are capable of sterilization, this has implications of damaging a population of native species in an environment in which they act harmlessly as a vector of decomposing fungi.

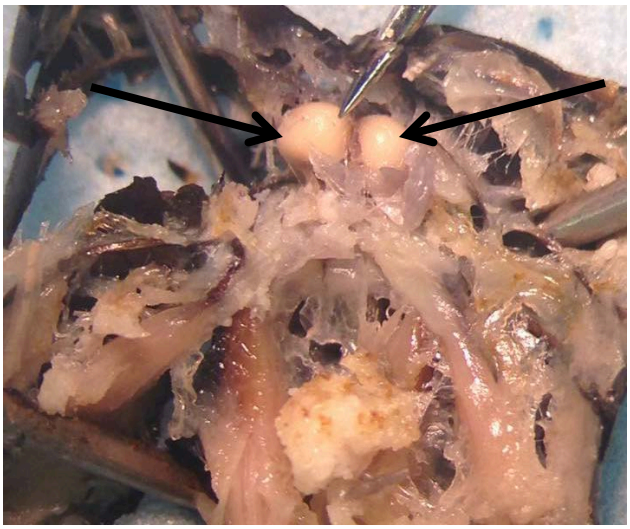

Figure 3.1. Dissected female *S. noctilio*, with arrows pointed at internal mycangia containing *Amylostereum* spp. spores.

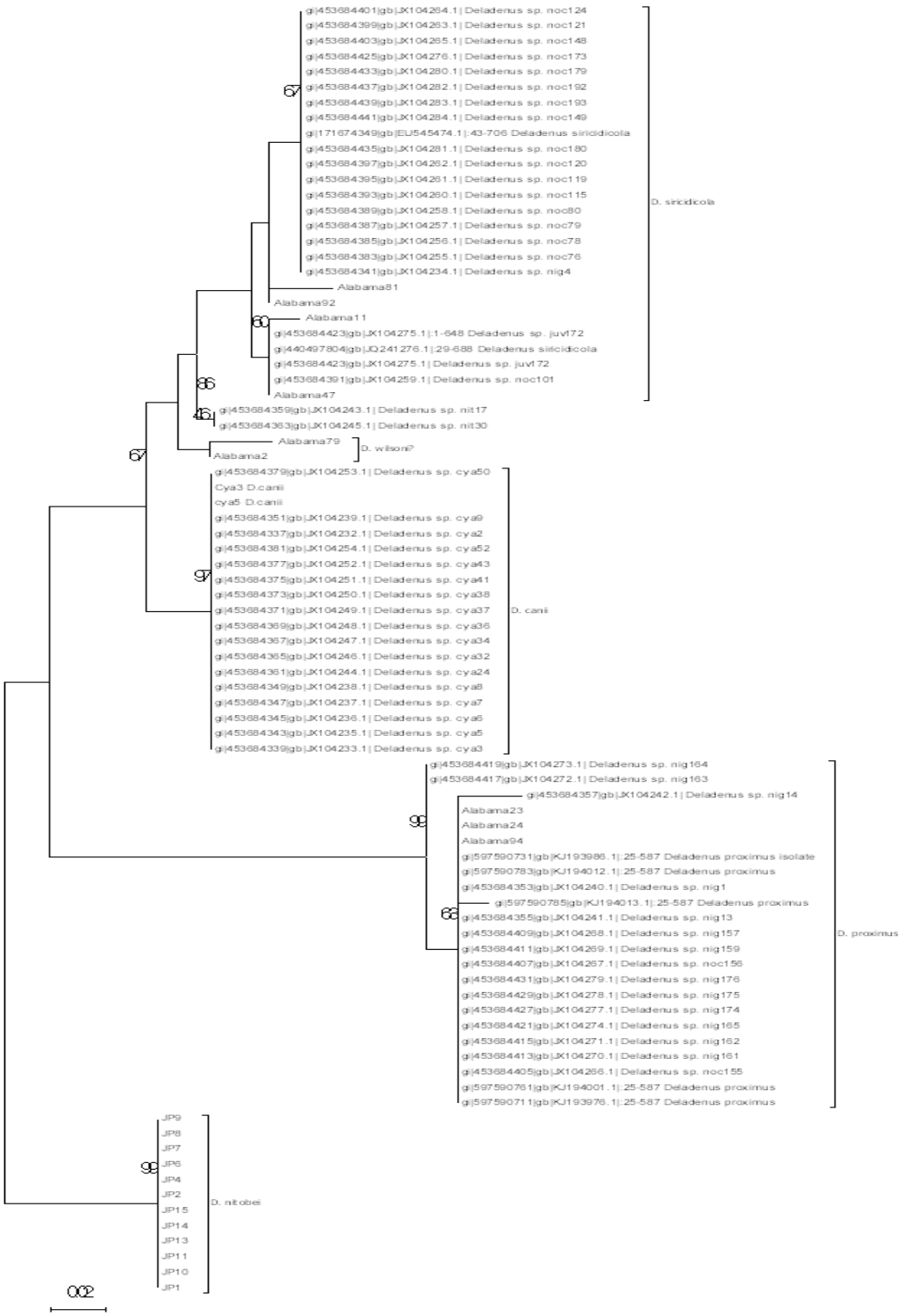

Figure 3.2. Maximum-likelihood tree of COI region of 9 nematode samples dissected from *S. nigricornis* in Alabama. Three specimens (23, 24, 92) fall within *D. proximus*, while 4 more (11,47,81,92) fall within *D. siricidicola*. Specimens 2 and 79 pair by themselves, which could be *D. wilsoni*.

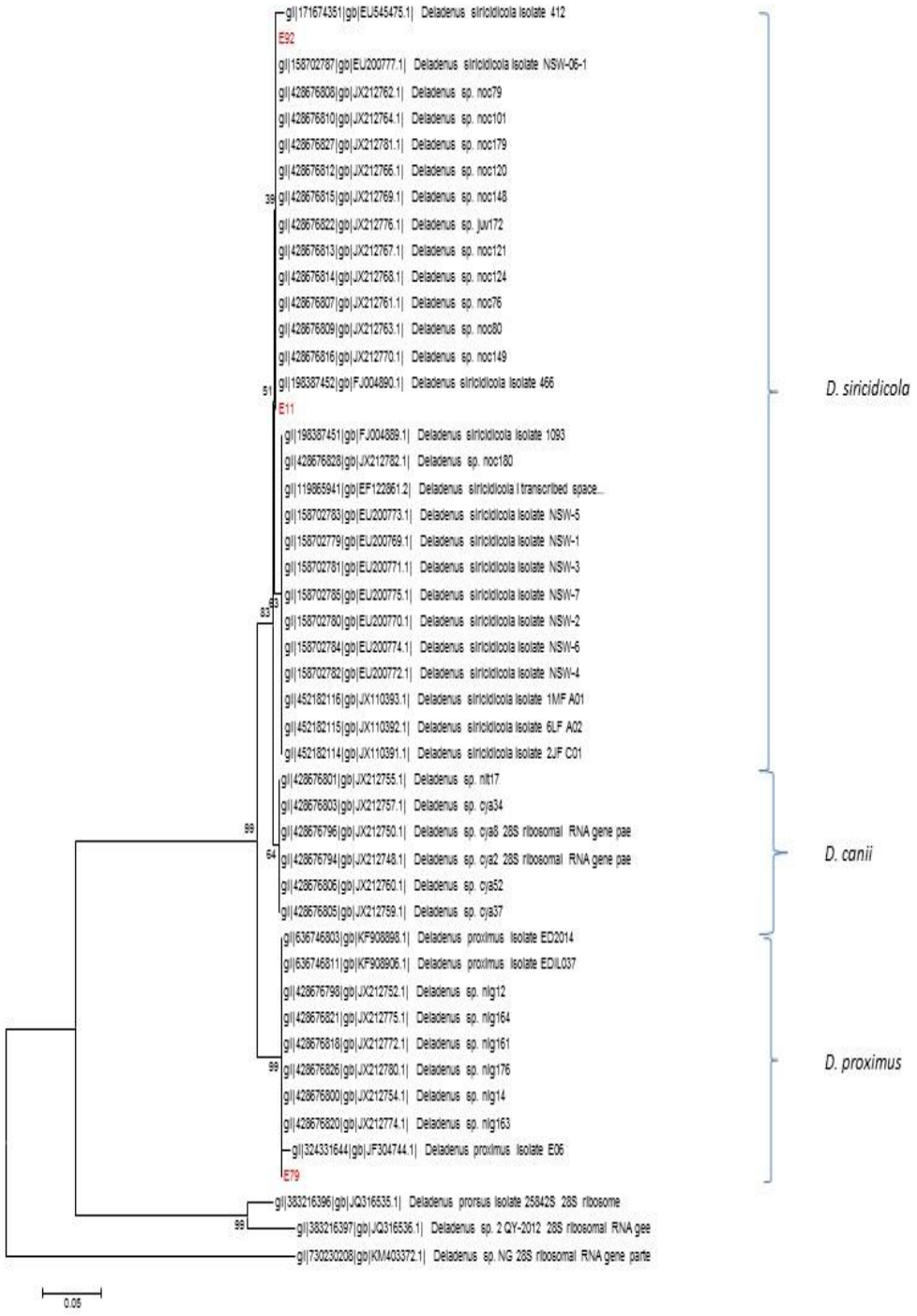

Figure 3.3. Neighbor-Joining tree of ITS region sequenced for *Deladenus* spp. samples. Specimens 11 and 92 fall within *D. siricidicola*, while 79 falls within *D. proximus*.

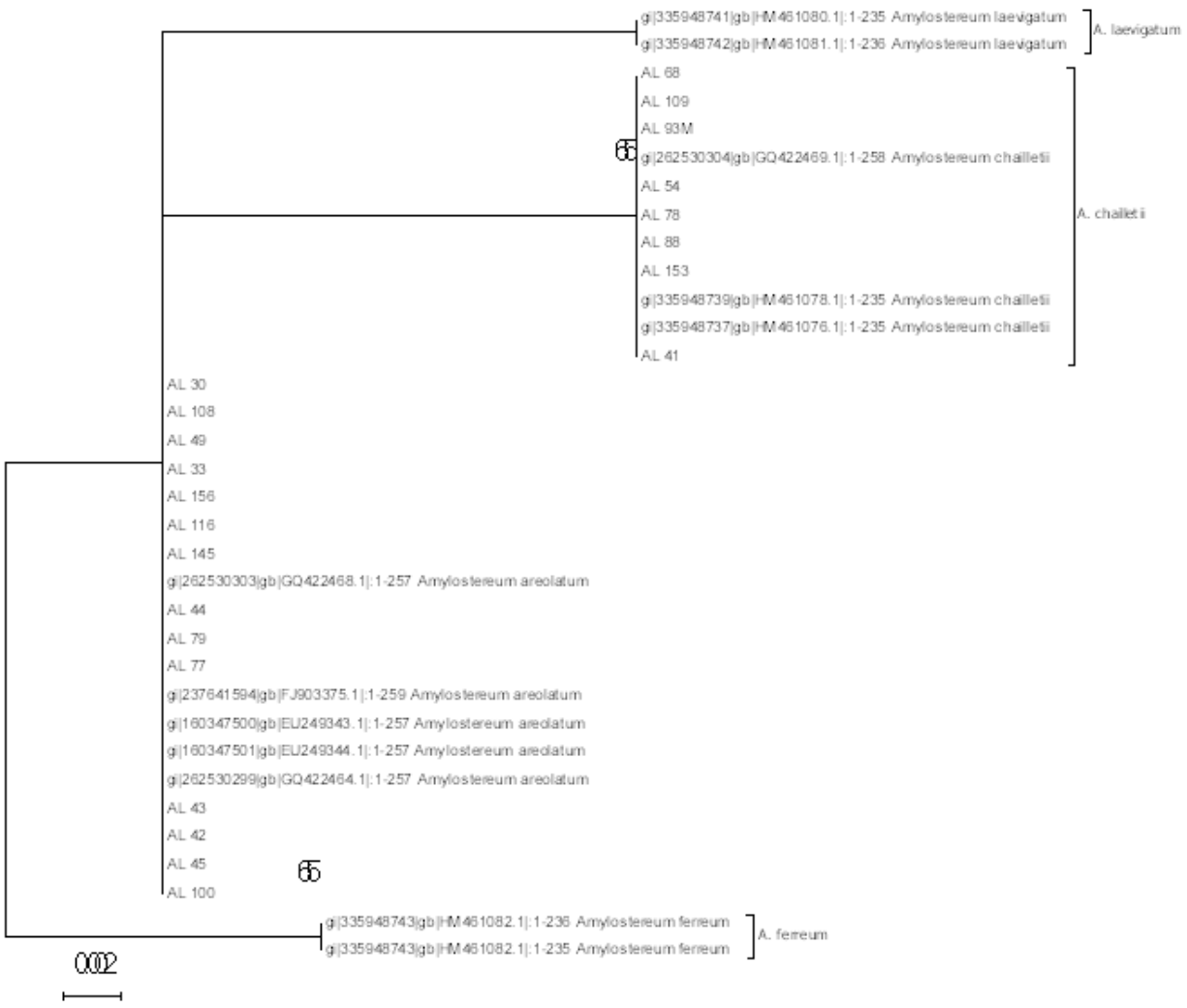

Figure 3.4. Maximum-Likelihood ITS tree of *Amylostereum* spp. samples, showing Alabama isolates grouping as *A. areolatum* (n= 14) and *A. chailletii* (n=8).

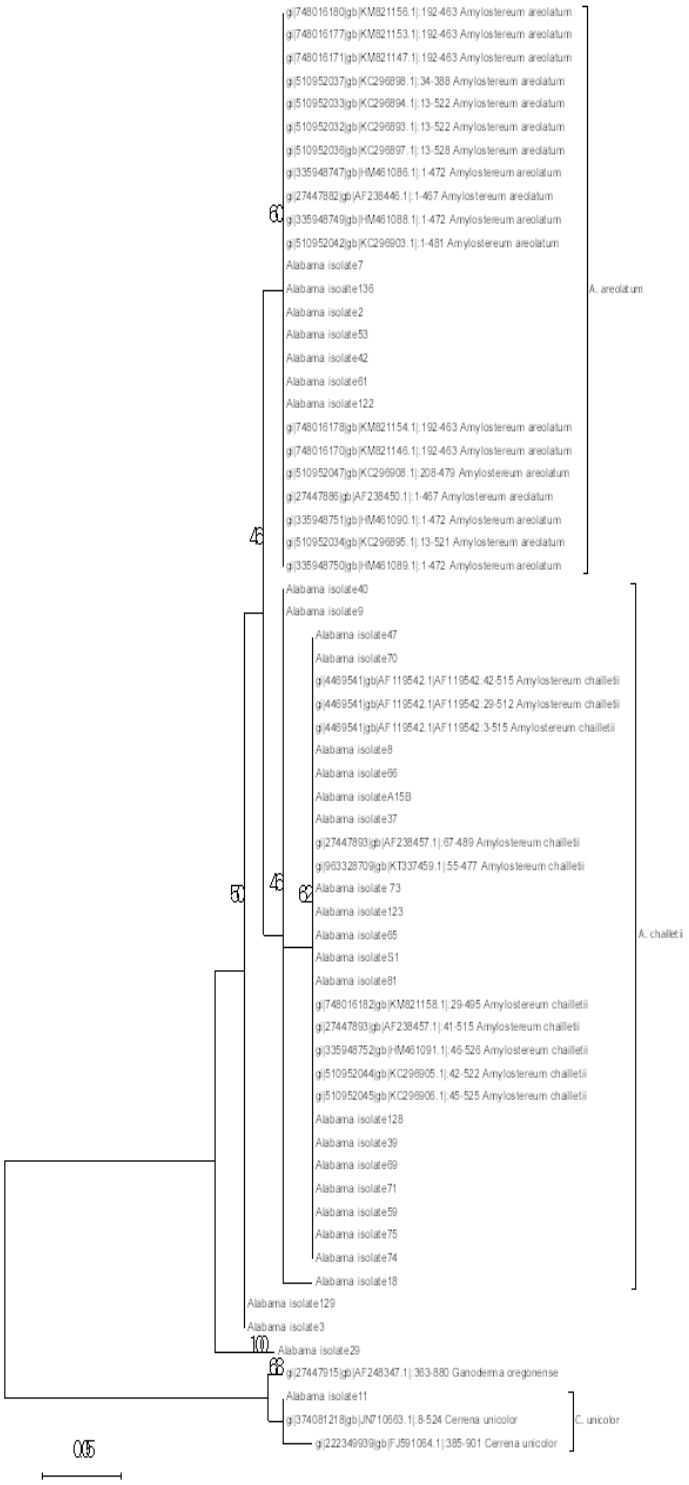

Figure 3.5. Maximum-Likelihood MS tree of 32 fungal samples isolated from woodwasps. Isolate 11 groups with *C. unicolor*. Other isolates are determined to be *A. areolatum* (n=7) and *A. chailletii* (n=21). Three isolates do not fall into a distinct species (3, 29, and 129).

Table 3.1. Species determinations of *Amylostereum* and *Deladenus* associated with woodwasp specimens that were infested with nematodes.

Specimen ID	Woodwasp species	Deladenus species	Gene region(s)	Fungal species	Gene region(s)	National Forest Locale	Date Collected
AL2	*S. nigricornis*	*?*	COI	*A .areolatum*	MS2	Oakmulgee	10/16/15
AL 11	*T. columba*	*D. siricidicola*	COI, ITS	*C. unicolor*	MS2	Oakmulgee	11/13/15
AL 23	*S. nigricornis*	*D. proximus*	COI			Tuskegee	10/30/14
AL 24	*S. nigricornis*	*D. proximus*	COI			Tuskegee	10/30/14
AL 47	*S. nigricornis*	*D. siricidicola*	COI	*A. chailletii*	MS2	Tuskegee	10/30/14
AL79	*S. nigricornis*	*D. proximus*	COI, ITS	*A.areolatum*	ITS2	Tuskegee	11/24/15
AL 81	*S. nigricornis*	*D. siricidicola*	COI			Tuskegee	12/11/14
AL 92	*S. nigricornis*	*D. siricidicola*	COI, ITS			Tuskegee	12/11/14
AL94	*S. nigricornis*	*D. proximus*	COI			Tuskegee	12/11/14

Table 3.2. Species determinations of *Amylostereum*, along with gene region sequenced.

Specimen ID	Woodwasp spp.	Fungal spp.	Gene Region(s)	National Forest	Date Collected
AL 3	*S. nigricornis*	*A. areolatum*	MS2	Oakmulgee	11/29/15
AL7	*S. nigricornis*	*A. areolatum*	MS2	Tuskegee	10/14/15
AL8	*S. nigricornis*	*A. chailletii*	MS2	Tuskegee	12/10/15
AL9	*S. nigricornis*	*A. chailletii*	MS2	Oakmulgee	11/29/15
AL18	*S. nigricornis*	*A. chailletii*	MS2	Tuskegee	10/30/14
AL29	*S. nigricornis*	*??*	MS2	Tuskegee	11/12/14
AL 30	*S. nigricornis*	*A. areolatum*	ITS2	Tuskegee	11/24/14
AL 33	*S. nigricornis*	*A .areolatum*	ITS2	Tuskegee	12/11/14
AL37	*S. nigricornis*	*A. chailletii*	MS2	Tuskegee	12/11/14
AL39	*S. nigricornis*	*A. chailletii*	MS2	Tuskegee	12/11/14
AL40	*S. nigricornis*	*A. chailletii*	MS2	Tuskegee	11/25/14
AL 41	*S. nigricornis*	*A. chailletii*	ITS2	Tuskegee	11/25/14
AL42	*S. nigricornis*	*A. areolatum*	MS2, ITS2	Tuskegee	11/12/14
AL 43	*S. nigricornis*	*A. areolatum*	ITS2	Tuskegee	11/12/14
AL 44	*S. nigricornis*	*A. areolatum*	ITS2	Tuskegee	11/12/14
AL 45	*S. nigricornis*	*A. areolatum*	ITS2	Tuskegee	11/12/14
AL 49	*S. nigricornis*	*A. areolatum*	ITS2	Tuskegee	10/30/14
AL53	*S. nigricornis*	*A. areolatum*	MS2	Tuskegee	10/30/14
AL 54	*S. nigricornis*	*A. chailletii*	ITS2	Tuskegee	10/30/14
AL59	*S. nigricornis*	*A. chailletii*	MS2	Tuskegee	11/12/14
AL 61	*S. nigricornis*	*A. areolatum*	MS2	Tuskegee	11/25/14
AL 65	*S. nigricornis*	*A. chailletii*	MS2	Tuskegee	10/30/14
AL 66	*S. nigricornis*	*A. chailletii*	MS2	Tuskegee	10/30/14
AL 68	*S. nigricornis*	*A. chailletii*	ITS2	Tuskegee	12/11/14
AL 69	*S. nigricornis*	*A. chailletii*	MS2	Tuskegee	12/11/14
AL 70	*S. nigricornis*	*A. chailletii*	MS2	Tuskegee	12/16/14
AL 71	*S. nigricornis*	*A. chailletii*	MS2	Tuskegee	12/11/14
AL 73	*S. nigricornis*	*A. chailletii*	MS2	Tuskegee	11/25/14
AL 74	*S. nigricornis*	*A. chailletii*	MS2	Tuskegee	12/11/14
AL 75	*S. nigricornis*	*A. chailletii*	MS2	Tuskegee	12/11/14
AL 77	*S. nigricornis*	*A. areolatum*	ITS4	Tuskegee	12/11/14
AL 78	*S. nigricornis*	*A. chailletii*	ITS4	Tuskegee	12/11/14
AL 79	*S. nigricornis*	*A. areolatum*	ITS2	Tuskegee	11/25/14
AL 81	*S. nigricornis*	*A. chailletii*	MS2	Tuskegee	12/11/14
AL 88	*S. nigricornis*	*A. chailletii*	ITS4	Tuskegee	10/30/14
AL 93	*S. nigricornis*	*A. chailletii*	ITS4	Tuskegee	12/11/14
AL 100	*S. nigricornis*	*A .areolatum*	ITS2	Tuskegee	12/11/14
AL 108	*S. nigricornis*	*A .areolatum*	ITS2	Tuskegee	12/11/14
AL 109	*S. nigricornis*	*A. chailletii*	ITS2	Tuskegee	12/11/14
AL 116	*S. nigricornis*	*A. areolatum*	ITS2	Tuskegee	12/11/14
AL 122	*S. nigricornis*	*A .areolatum*	MS2	Tuskegee	12/11/14
AL 123	*S. nigricornis*	*A. chailletii*	MS2	Tuskegee	12/11/14
AL 128	*S. nigricornis*	*A. chailletii*	MS2	Oakmulgee	12/9/14
AL 129	*S. nigricornis*	*A. areolatum*	MS2	Oakmulgee	12/9/14
AL 136	*S. nigricornis*	*A. areolatum*	MS2	Oakmulgee	12/9/14
AL 135	*S. nigricornis*	*A. chailletii*	ITS4	Oakmulgee	12/9/14
AL 145	*S. nigricornis*	*A .areolatum*	ITS2	Oakmulgee	12/9/14
AL 153	*S. nigricornis*	*A. chailletii*	ITS2	Oakmulgee	12/9/14
AL 156	*S. nigricornis*	*A. areolatum*	ITS2	Oakmulgee	12/9/14
AL S1	*S. nigricornis*	*A. chailletii*	MS2	MOT	10/22/15
AL 15B	*S. nigricornis*	*A. chailletii*	MS2	MOT	10/22/15