# Root and Soil-borne Oomycetes (Heterokontophyta) and Fungi Associated with the Endangered Conifer, *Torreya taxifolia* Arn. (Taxaceae) in Georgia and Florida, USA[1]

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**Abstract**: A systematic survey was conducted to isolate and identify root and soil-borne oomycetes and fungi associated with the rare and endangered southeastern USA conifer, *Torreya taxifolia* Arn. Twenty four trees showing different degrees of decline were sampled at two different sites: Torreya State Park, Liberty County, Florida (n = 12) and US Corps of Engineers in Decatur, Georgia (n = 12). All *T. taxifolia* trees sampled showed moderate to severe levels of decline (100% decline incidence) based on criteria, such as poor development of trees, stunting and fragility. Disease severity was higher, and trees were smaller with poor development at Florida sites, showing an average height of 89 cm, and a diameter at breast height (DBH) of 5 cm compared to trees in Georgia's site (174 cm h and 10.6 cm DBH in average) In addition to decline, root necrosis and stem cankers were observed in 45.8 % of trees examined. A diverse fungal community was associated with declining trees. Twenty eight fungal genera were identified belonging to the Oomycetes, Zygomycetes, Ascomycetes, Basidiomycetes and anamorphic fungi. Anamorphic fungi, Oomycetes, and Zygomycetes were the dominant groups associated with *T. taxifolia*. *Pestalotiopsis* spp., *Fusarium* spp. and *Pythium* spp. were the most common genera. Most isolates were obtained from root tissue. Seventy four percent (74%) of isolates were from samples collected at a Georgia site. *Alternaria* spp., *Cylindrocladium* sp., *Fusarium* spp., *Phoma* sp., *Pythium* spp., *Rhizoctonia* sp., *Thielaviopsis* sp. and *Verticillium* sp. are among soil-borne oomycetes and fungal species identified. Not known *Phytophthora* spp. were isolated during the survey. Interactions of oomycetes and fungi inhabiting the soil and rhizosphere could play an important role in *T. taxifolia's* decline.

**Key Words:** *Torreya taxifolia*, endangered species, soil-borne pathogens, Oomycetes, Fungi,

*Torreya taxifolia* Arn. (family Taxaceae[4]) commonly known as Florida *torreya* or 'stinking yew', is a dioecious evergreen coniferous tree. Branches are whorled with needle-like leaves of pungent, resinous odor (USFWS 1986). Florida *Torreya* is endemic to the ravine slopes on the eastern bank of the Apalachicola River in northern Florida and in parts of Georgia. Prior to 1950's, *T. taxifolia* was estimated to be the seventh most abundant tree species within Apalachicola Bluff regions (Schwartz et al., 1995). Surveys conducted in areas

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[4] The genus *Torrey*a has also been recently placed in the Cephalotaxaceae although recent studies seem to favor a placement in a more broadly defined Taxaceae (Christenhusz et al. 2011).

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with known high tree densities suggested that *T. taxifolia* has lost at least 98.5% of its total population size since the early 1900s, causing the species to be federally listed as endangered in 1984 (USFWS 1986). Current populations are characterized by small (< 2m tall) individuals that are failing to achieve reproductive maturity. Most of the wild population persists as stump sprouts; only three seed bearing adult female trees have been reported from the wild. In addition, the plants exhibit slow growth (Spector, pers. comm.). Given the lack of seed production in the wild, and potentially a decline due to a disease, all models seem to predict eventual extinction (Schwartz and Herman 1993).

The loss of *T. taxifolia* is thought to have been a result of oomycetes and fungal pathogens during the 1950's and 1960's, and/or a combination of environmental stress and native pathogens, but studies have yet to provide an explanation for this species' decline. Broad studies addressing abiotic hypotheses, in which historical observations, as well as physiological and growth responses to several environmental factors were examined, showed little supportive evidence that *T. taxifolia* was subjected to environmental stress (Schwartz et al. 1995). Studies addressing biotic factors have focused mainly on pathogens causing needle spots, blights, and stem cankers (Table 1) (Alfieri 1984, El-Gholl 1985, USFWS 1986, Alfieri et al. 1987, Farr et al. 1989, Schwartz et al. 1996, Herman and Schwartz 1997, Mount and Smith 2010, Smith et al. 2011). Important soil-borne pathogens, such as *Phytophthora* sp., *Pythium* sp., *Rhizoctonia solani* Kühn and *Sclerotium rolfsii* Sacc. have been reported associated with *T. taxifolia*, but their role in *Torreya's* decline has not been addressed (Alfieri et al. 1984). Soil-borne oomycetes such as *Phytophthora* spp. are potentially devastating pathogens causing serious damage to many trees species in a variety of ecosystems worldwide. *Phytophthora* spp. have been implicated as one of the responsible factors in *T. taxifolia's* population decline in Georgia, but species identification and pathogenicity has not been not investigated. In Florida, *P. cinnamomi* Rands has been associated with disease outbreaks in sand pines, and has been indirectly associated with *T. taxifolia*'s root rot (Alfieri et al. 1984, Barnard et al. 1985). Unfortunately, none of these studies provided a conclusive explanation for the species' decline based on virulence of a oomycete species. Overall, very few studies have addressed root or soil-borne pathogens. Therefore, oomycetes species associated with root tissue and with other trees in its ecosystem need to be examined. Recently, a newly described species, *Phytophthora ramorum* (Werres, De Cock, and Man in't Veld), has caused widespread and sudden death of oak trees in California (Rizzo et al. 2002). In Southwestern Oregon, various *Phytophthora* spp. have been regarded as the causal agent of Tanoak (*Lithocarpus densiflorus* (Hook. and Arn.) Rehder) stem cankers (Reeser et al. 2008).

In addition to *Phytophthora* spp., another oomycetes, *Pythium* spp. are known to cause post-emergence damping-off of seedlings of coniferous trees. Various species of *Pythium* have been isolated from soil in forest nurseries in

the Southeastern United States: *P. irregulare-debaryanum, P. sylvaticum* W.A. Campb. and F.F. Hendrix, *P. spinosum* Sawada, *P. helicoides* Drechsler and *P. splendens* Hans Braun (Hendrix and Campbell 1973). These species caused reduction of root and shoot systems, due to feeder root necrosis in surviving seedlings. Plant susceptibility to feeder root necrosis varied with the species of *Pythium* examined. Pathogenicity of *Pythium* spp. has been demonstrated in pine seedlings in Japan, where *Pythium ultimum* Trow and *P. aphanidermatum* (Edson) Fitzp were the most virulent (Watanabe 1988). In South Africa, root rot of seedlings have been associated with *P. ultimum* and damping–off with *P. irregulare* Buisman (Viljoen et al. 1992). In nature, non-pathogenic *Pythium* species usually coexist with pathogenic ones (Viljoen et al. 1992).

**Table 1. Fungi previously reported associated with *Torreya* spp.**

| **Species of Fungi** | **Symptoms** | **References** |
|---|---|---|
| *Alternaria* sp. | needle spot | Alfieri et al. 1984 |
| *Botrysophaeria* sp. | needle spot | Alfieri et al. 1984, Mount and Smith 2009 |
| *Caeoma torreyae* Bonar, 1951 | rust | Farr et al. 1995 |
| *Diaporthe* sp. | associated to cankers | Mount and Smith 2010 |
| *Diplodia natalensis* Pole-Evans, 1911 | twig dieback | Alfieri et al. 1984 |
| *Fusarium* sp. | root rot associated to cankers | Alfieri et al. 1984, Mount and Smith 2010 |
| *Fusarium torreyae* Aoki, Smith, Mount, Geiser, and O'Donnell, 2013 | canker | Smith et al. 2011, Aoki et al. 2013 |
| *Fusarium lateritium* Nees, 1817 | needle spot | El-Gholl 1985, Alfieri et al. 1987 |
| *Hypoxylon* sp. | associated to cankers | Mount and Smith 2010 |
| *Janetia bonarii* (M. B. Ellis) S. Hughes, 1983 | associated to needles | Farr et al. 1995 |
| *Macrophoma* sp. | needle and stem blight | Alfieri et al. 1984 |
| *Lasiodiplodia theobromae* (Pat., 1892) Griffon and Maulb., 1909 | associated to cankers | Mount and Smith 2010 |
| *Pestalotiopsis microspora* (Speg. , 1880) G. C. Zhao and N. Li, 1995 | needle spots and stem cankers | Schwartz et al. 1996 |
| *Phomopsis* sp. | associated to cankers | Mount and Smith 2010 |
| *Phyllosticta* sp.[2] | needle spot | Alfieri et al. 1987 |

**Table 1. Fungi previously reported associated with *Torreya* spp.**

| Species of Fungi | Symptoms | References |
|---|---|---|
| *Physalospora* sp. | needle stem and twig blight | Alfieri et al. 1987 |
| *Phytophthora cinnamomi* Rands, 1922 | root rot | Alfieri et al. 1984 |
| *Pythium* sp. | root rot | Alfieri et al. 1984 |
| *Rhizoctonia solani* Kühn, 1858 | root rot | Alfieri et al. 1984 |
| *Sclerotium rolfsii* Sacc., 1911 | southern blight | Alfieri et al. 1984 |
| *Scytalidium* sp. | needle spot and necrosis | Hermann and Schwartz 1997 |
| *Sphaeropsis* sp. | needle blight | Alfieri et al. 1984 |
| *Sporidesmium fragilissimum* (Berk and M.A. Curtis,1875) M. B. Ellis, 1958 | associated needles | Farr et al. 1995 |
| *Xylocoremium flabelliforme* [3] (Schwein., 1797) J. D. Rogers, 1984 | associated to needles and stems | Alfieri et al. 1987 |

[1] Current name *Botryosphaeria rhodina* (Berk. and M. A. Curtis) Arx,, 1970
[2] *Phyllosticta* sp., imperfect stage of *Guignardia* sp.
[3] *Xylocoremium flabelliforme* is the imperfect stage of *Xylaria cubensis* (Mont.) Fr.

Worldwide, *Fusarium* spp. are frequently associated with root diseases although species differ in pathogenicity, and in host specificity (Alfieri et al. 1984, Viljoen et al. 1992, Leslie and Summerell 2006). *Fusarium* spp. chlamydospores infect the roots and ramify through the root system, affecting both cortical and vascular tissues (Agrios 2005). Young seedling infections are usually lethal. In older seedlings infection causes root rot, and death of growing tips. Bark and cortex of infected roots are usually exposed due to infection. Several *Fusarium* species have been shown pathogenic to *T. taxifolia*, by causing needle spots, i.e. *F. lateritium* Nees (El-Gholl 1985, Alfieri et al. 1987), and has been associated with root rot (Alfieri et al. 1984). More recently, a novel described species *F. torreyae* Aoki, Smith, Mount, Geiser, and O'Donnell, was demonstrated to be the causal agent of stem cankers (Smith et al. 2011). Until now, none *Fusarium* spp. has been demonstrated to cause cankers comparable to those observed in the field. In other conifer species, *F. oxysporum* Schltdl. Emend. Snyder and Hansen has been associated with seedling death and root rot (Viljoen et al. 1992).

Other soil-borne pathogens such as *Calonectria* spp. (anamorph *Cylindrocladium* spp.), *Rhizoctonia solani*, *Thielaviopsis* spp. and *Verticillium* sp. have been associated with root diseases in conifers. For example, *Rhizoctonia solani* caused damping–off and root rot of *Pinus* seedlings in forest

nurseries in South Africa (Viljoen et al. 1992). It survives in the soil by the production of sclerotia that germinates and invades seedlings' roots, stems and leaves. *Calonectria* spp. are important plant pathogens associated with forestry crops in temperate, subtropical and tropical climates (Lombard et al. 2009; Lombard et al., 2010). In forest trees, symptoms includes cutting rot, damping-off, leaf spots, shoot blight, stem cankers, and root diseases (Lombard et al., 2010). In Brazil, seven *Pinus* spp. were wound-inoculated with *C. brassicae* (Pamwar and Bohra) L. Lombard, M. J. Wingf., and Crous, resulting in mortality of all tested plants within two weeks. They concluded that the pathogen was highly virulent in established plantations of *Pinus* spp. (Hodges and May 1972). *Thielaviopsis* pp. is also a common and important soil-borne pathogen of many agricultural crops (Agrios 2005). Symptoms associated with this plant disease are damping off and root rot. Infected plants are usually stunted and chlorotic, with a characteristic black root rot. The dark colored areas are produced by chlamydospores, which are thick walled spores produced in chains in infected root tissues.

Currently, there have been no systematic studies of soil-borne pathogens associated with *T. taxifolia* and with other trees in its surrounding ecosystem. Therefore, a systematic soil survey was conducted in Florida and Georgia, emphasizing the detection of oomycetes.

## MATERIALS

### Systematic survey of soil-borne fungi

A systematic survey was conducted in which samples from roots, soil and plant litter associated with *T. taxifolia* were collected from three sites at Torreya State Park, Florida, and one site in Decatur, Georgia. Samples were collected from trees showing decline symptoms (Figure 1). A composed soil sample was taken with a stainless steel (1.5 x 14 cm) soil probe in a circle mode around each tree between the drip line and the base of the tree. Two soil samples opposite from each other were collected from each tree and placed in a plastic bag. The soil top layer (10 mm) including plant litter was collected in a separate plastic bag. The soil probe was wiped off of all organic matter (i.e. soil and plant litter) with a clean paper tissue. Plastic gloves were used and changed through sample recollection process. Using a small garden shovel, soil was removed to exposed feeder roots that showed decay (necrosis) or were colonized by mycelium. These were collected and placed in plastic bags. Equipment used (soil probe and garden shovel) was sterilized on site using 95% ethanol and rinsed with sterilized distilled water to reduce contamination between sampling sites (Inglis and Hill 2007).

A total of 24 trees were sampled, twelve from each state which is a sizable proportion of the remaining known population. Diameter at breast height (DBH in cm), height (cm) and GPS coodinates were taken for each tree. Tree health or decline (feeder root necrosis, stem cankers, mycelium development on tree base

or on cortex, dying branches and dying trees) was also recorded. In addition, disease severity was estimated based on percentage of healthy to severe decline of trees as follow: healthy trees or no decline = O, L = low symptoms ranging from 1 to 25% of decline, M = moderate symptoms ranging from 26 to 50% of decline and S = severe symptoms higher than 51% of decline.

**Isolation and identification of soil-borne pathogens associated to *T. taxifolia* and its surrounding ecosystem**

***Fungal isolation methods***

To isolate soil-borne fungi, serial dilutions of soil samples, plant tissue baits or traps, and direct pathogen isolation from host tissue (roots and plant litter surrounding tree base) were used emphasizing the detection of oomycetes such as *Phytophthora* spp. and *Pythium* spp. (Mitchell and Kannwischer-Mitchell 1992, Martin 1992). Commonly used culture media were prepared to isolate fungi and included acidified Potato Dextrose Agar (PDA), carnation leaf agar (CLA), *Phytophthora* selective media known as PARP-V8 and PARP with *T. taxifolia* needles (Jeffers and Martin 1986). The last medium contains selective antibiotics and fungicides. Isolation media were kept refrigerated.

***Soil serial dilutions***

Ten grams of soil were used for serial dilutions (i.e. $10^{-2}$, $10^{-4}$, $10^{-6}$). One hundred microliters from each dilution were transferred and spread to PARP-V8 culture media (Martin 1992). Plates were incubated at 26ºC. Colonies development was examined daily.

***Baiting bioassays***

Baiting bioassays using *Camelia* and *Torreya* leaves were used to detect soil-borne fungi and oomycetes (Ferguson and Jeffers 1999). Plates were made using fresh soil samples and plant litter surrounding tree's base. Plant litter and soil samples were placed in sterile Petri plates (60 x 15 mm) and were covered with sterile de-ionized distilled water. Three leaf baits or traps were placed per plate containing either flooded plant litter or soil sample (Ferguson and Jeffers 1999). Hyphal growth on bait leaf margins was examined after 24, 48 and 72 h at room temperature and was isolated in PARP media. Colonies were purified and kept in PARP-V8 at 4ºC in the dark.

***Direct isolation from fresh plant material***

Sections of root samples were cleaned with running tap water for half hour. Root sections were transferred directly to culture media: PDA and PARP-V8 media (Mitchell and Kannwischer-Mitchell 1992, Martin 1992). Plates were kept at room temperature for colonies' development. Colonies were purified

and identified. Also, mycelium observed under stereoscope growing on plant litter (bark and leaves) were directly transferred to culture media.

***Fungal Identification: Morphology, ELISA and DNA analysis***

Oomycetes reproductive structures such as sporangia, oogonium and antheridia, coenocitic hyphae, and hyphal swellings produced on PARP or PDA media were examined under light microscopy. Fungal reproductive structures such as conidia, conidiophores and mycelium produced on PDA media were examined under light microscopy. Reproductive structures were photographed and recorded. Taxonomic keys were used for identification (Barnett and Hunter 1998, Boerema et al. 2004, Hanlin 1990, Leslie and Summerell 2006, Gallegly and Hong 2008, van der Plaats-Niterink 1981). Other techniques based on immunology such as enzyme-linked immunosorbent assay (ELISA) and DNA analysis were used to complement traditional identification. A commercial *Phytophthora* ELISA kits (Agdia®) was used to complement oomycetes characterization. DNA analysis (i.e. PCR) of a commonly used genetic region for fungal identification such as the internal transcribed spacer (ITS) of the ribosomal DNA (rDNA) (White et al. 1990) was used to complement morphology.

Sixty isolates were selected for DNA analysis. Total genomic DNA was extracted from mycelium using the Qiagen DNeasy Plant Mini Kit (Qiagen Inc., Valencia, California) following manufacturer's instructions. DNA samples were diluted in TE buffer (10mM Tris-HCL; 1mM EDTA) to a concentration of 25 ng/µl and stored at -20°C. PCR was performed using a thermocycler (Perkin Elmer, Model 2400, Wellesley, Massachusetts) with primers ITS1 and ITS4 (White et al. 1990). PCR was performed in a total volume of 50 µL containing: 25 µl Amplitaq Gold® PCR Master Mix (Roche, New Jersey USA), 12 pmol of each primer, 17 µL of ultra-pure water (Sigma), 20 to 30 ng of DNA. PCR conditions were as follows: 94°C for 4 min, followed by 35 cycles of 94°C for 2 min, 55°C for 30 s and 72°C for 1 min, with a final extension at 72°C for 4 min (Konstantinova et al. 2002; White et al. 1990). PCR products were visualized on 1.2% agarose gel with a UV illuminator (Quantity One® 4.5 2003, Bio-Rad Laboratories, Hercules, CA). PCR products were purified by using QIA quick PCR purification Kit (Qiagen®, Valencia, California) according to the manufacturer's instructions. DNA was sequenced at the Sequencing and Genotyping Facilities, Department of Biology, University of Puerto Rico, Río Piedras Campus. DNA sequences were edited using BioEdit (version 7.1.9). Codon-based MUSCLE alignment function in Guidance® was used to align ITS rDNA (Penn et al. 2010). DNA sequences were compared with nucleotide data from fungal species of the GenBank National Center for Biotechnology Information (NCBI), using BLAST (http://www.ncbi.nlm.nih.gov/BLAST/). Identification of *Cytospora* sp., *Guignardia* spp. and *Umbellopsis* sp. isolates relayed on DNA analysis of the region described above.

Phylogenetic analysis of nucleotide sequences were conducted employing maximum likelihood analysis with the software package MEGA (Molecular Evolutionary Genetics Analysis version 5.2.1) (Tamura et al., 2011). Clade support was assessed by 2000 bootstrap replications of ITS rDNA region. Estimation of nucleotide substitution were made using Tamura Nei Model. Tree inferences were made using the nearest neighborhood interexchange (NNI) heuristic method.

## RESULTS AND DISCUSSION

### *Torreya taxifolia* trees assessment

All *T. taxifolia* trees sampled showed moderate to severe degrees of decline (100% decline incidence) based on criteria such as poor development of trees, stunting and fragility (Figure 1, Table 2). In addition, feeder root necrosis and stem cankers were observed in 45.8 % of trees examined (Table 2). Disease severity was higher and trees were smaller with poor development at Florida sites, showing an average height of 89 cm, and an DBH of 5 cm compared to trees in Georgia's site (174 cm h and 10.6 cm DBH in average) (Figure 1, Table 2). In Florida sampling sites, soil samples were damped with higher humidity, compared to the soil at the Georgia site. Overall, four trees showed mycelial growth on cortex or on tree base (Figure 1). In Florida, the main trunk of two severe affected trees had cankers at the base of the tree.

### *Fungal isolates*

A diverse fungal community was identified associated with declining trees (Figures 2 and 3). Composition of fungal community included plant pathogens, lignin and cellulose decomposers, endophytes and saprophytes (Table 3). One hundred and two fungal isolates were obtained from roots, soil and plant litter associated with *T. taxifolia*. Of these 27 isolates (26%) were obtained at Torreya State Park and 75 (74%) from Georgia. Twenty eight genera were identified belonging to the oomycetes, zygomycetes, ascomycetes, basidiomycetes and anamorphic fungi. Anamorphic fungi such as *Pestalotiopsis* and *Fusarium*; zygomycetes and oomycetes (*Pythium* spp.) were the dominant taxonomic groups (Figure 3). Most isolates were obtained from root tissue collected at the Georgia site (Table 3).

Thirteen oomycetes (13%) were isolated from bark, plant litter, roots and baits using Torreya leaf disks (Figure 4). Oomycetes colonies on PARP formed aerial mycelium. Globose intercalary hyphal swellings were abundant on culture media. Oogonia were rarely formed on culture media; but when produced, oogonia were globose surrounded by various antheridia forming a knot, typical of *P. heterothallicum* (Figure 4C). Most oomycetes were identified as *Pythium* spp. One of the isolates was obtained from Georgia sampling site using *Torreya's* needles as bait. All oomycetes except for one were isolated from roots of severe affected or dying trees at Torreya State Park. *Pythium* spp. and an

unknown oomycete were isolated from roots of dying specimens at both locations. Along with Oomycetes, *Fusarium* spp. were one of the most common fungi isolated during this survey and were also commonly isolated from roots of dying trees. Oomycetes were not effective competitors in culture media when more aggressive fungal species such as *Fusarium* spp. and *Mortierella* spp. were present, making difficult their isolation.

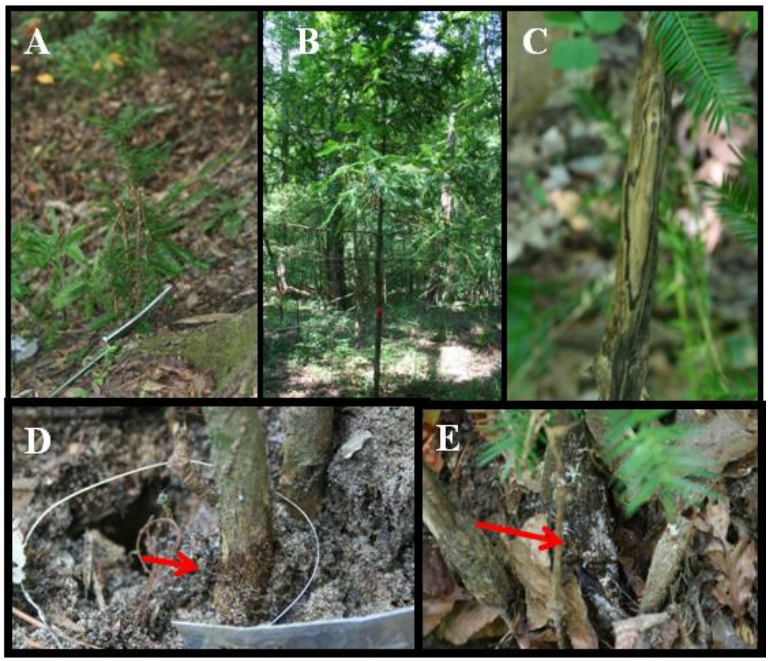



Figure 1. *Torreya taxifolia* trees from sampling sites: Poor growth and development was observed on trees at A) Torreya State Park, Florida and B) Decatur, Georgia. C. Canker symptoms observed at Torreya State Park. D. Feeder roots (red arrow) and crown base necrosis (dark circular/annular areas at base of tree). E. Mycelial mats (white areas) associated with plant litter surrounding trees.

Soil dilutions and baits using *Torreya* and *Camelia* leaves were unsuccessful in the isolation of known *Phytophthora* spp. Not known *Phytophthora* species were isolated directly from root samples, based on negative ELISA test specific for *Phytophthora* spp., even though *P. cinnamomi* had been implicated as one of the responsible factors of *T. taxifolia's* population decline (Alfieri et al. 1984, Barnard et al. 1985). DNA analysis was inconclusive in identifying oomycetes specimens. Oomycetes species may act as plant pathogens affecting roots or seedlings, and can act as saprophytes. Further studies focusing on the detailed identification of oomycetes associated with *Torreya*'s decline are urgently needed.

**Table 2.** ***Torreya taxifolia*** **trees sampled during the survey: location, identification number of individuals examined, as well as symptomatology**

| Location | Tree No. | Height (cm) | DBH (cm) | Decline[2] | Root Necrosis | Stem Canker | Disease Severity[4] |
|---|---|---|---|---|---|---|---|
| **Torreya State Park, Florida** | 1 | 116.84 | 3.81 | √ | | | M |
| | 2 | 73.66 | 0.254 | √ | √ | | S |
| | 3 | 48.26 | 1.27 | √ | √ | | S |
| | 4 | 60.96 | 3.81 | √ | | | M |
| | 5 | 48.895 | 2.54 | √ | | | S |
| | 6 | 48.26 | 3.17 | √[3] | √ | | S |
| | 7 | 27.94 | 3.81 | √ | | | S |
| | 8 | 63.5 | 2.54 | √ | | | M |
| | 9 | 320.04 | 15.24 | √ | | √ | M |
| | 10 | 152.4 | 18.415 | √ | | √ | S[5] |
| | 11 | 53.34 | 1.27 | √ | | √ | S[5] |
| | 12 | 55.88 | 7.62 | √ | | | M |
| | | **$X^1$ = 89.164** | **$X^1$ = 5.31** | **100%** | **25%** | **25%** | |

**Table 2. *Torreya taxifolia* trees sampled during the survey: location, identification number of individuals examined, as well as symptomatology (continuation)**

| Location | Tree No. | Height (cm) | DBH (cm) | Decline[2] | Root Necrosis | Stem Canker | Disease Severity[4] |
|---|---|---|---|---|---|---|---|
| **Decatur, Georgia** | 13 | 200 | 10.2 | √ | | √ | M |
| | 14 | 180 | 10 | √ | | √ | M |
| | 15 | 180 | 9 | √ | √ | √ | M |
| | 16 | 18 | 14 | √ | | | S |
| | 17 | 110 | 5 | √ | | | S[5] |
| | 18 | 140 | 10 | √ | | | M |
| | 19 | 90 | 4.5 | √ | | | M |
| | 20 | 400 | 23 | √ | | | M |
| | 21 | 330 | 16 | √ | | | M |
| | 22 | 180 | 10.5 | √ | | √ | S |
| | 23 | 140 | 11 | √ | | √ | S |
| | 24 | 120 | 4 | √ | | | M |
| | | **X = 174** | **X = 10.6** | **100%** | **8%** | **42%** | |

[1]X= average height and Diameter at Breast Height (DBH). [2]Decline included observations of feeder root necrosis, stem cankers, mycelium development on tree base or on cortex, dying branches and dying trees. [3]Necrosis was observed at tree base. [4]Disease severity was estimated based on percentage of healthy to severe decline of trees as follow: healthy trees or no decline = O, L = low symptoms ranging from 1 to 25% of decline, M = moderate symptoms ranging from 26 to 50% of decline and S = severe symptoms higher than 51% of decline. [5] Dying trees or trees with dead branches

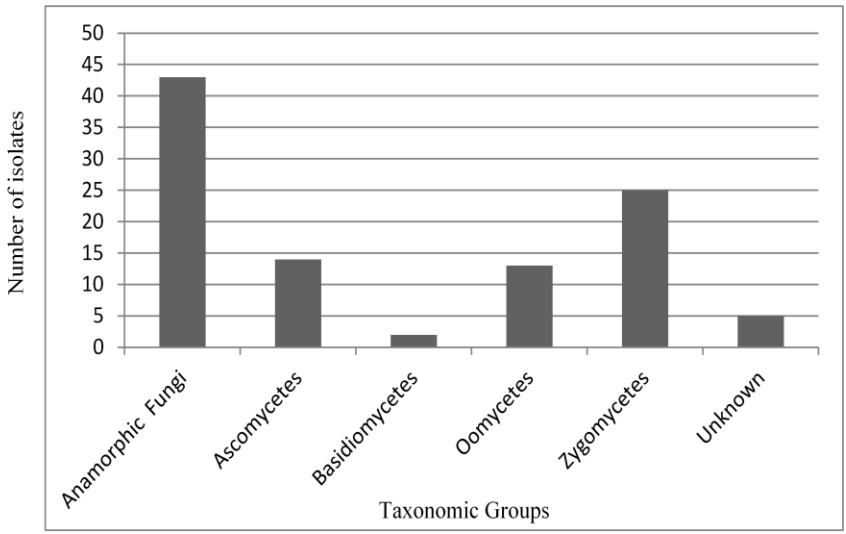



Figure 2. Taxonomic groups of microorganisms isolated from roots, soil, bark and plant litter associated to *Torreya taxifolia* in sampled sites located at Florida and Georgia, USA.

**Table 3. Fungi isolated and identified associated with *Torreya taxifolia* in this study**

| Fungal Species | Taxonomic Group | Samples and Location[1] | Habit types |
|---|---|---|---|
| *Absidia* spp. | Zygomycetes | *Torreya* leaf baits/G | saprophytes |
| *Alternaria* spp. | Ascomycetes | Roots/G | saprophytes, foliar pathogens |
| *Aspergillus flavus* | Ascomycetes | *Torreya* leaf baits, Roots /F and G | saprophytes |
| *Cephalosporium* sp. | Anamorphic Fungi | Roots/F and G | saprophytes, parasitic on fungi |
| *Chaetomium* sp. | Ascomycetes | Roots/G | saprophytes, cellulose decomposers |
| *Cladosporium* sp. | Anamorphic Fungi | *Torreya* leaf baits/G | saprophytes |
| *Cunninghamella* sp. | Zygomycetes | Roots/G | saprophytes |
| *Curvularia* sp. | Ascomycetes | Roots/F | saprophytes |
| *Cylindrocladium* sp. | Ascomycetes | Roots/G | plant pathogens |
| *Cytospora* sp. | Ascomycetes | Roots/G | plant pathogen |
| *Fusarium* spp. | Ascomycetes | Bark and Leaf Litter, Roots / F and G | saprophytes, endophytes, plant pathogens |
| *F. oxysporum* | Ascomycetes | Leaf litter, Roots/G | plant pathogens |

**Table 3. Fungi isolated and identified associated with *Torreya taxifolia* in this study**

| Fungal Species | Taxonomic Group | Samples and Location[1] | Habit types |
|---|---|---|---|
| *F. solani* | Ascomycetes | Roots/G | plant pathogens |
| *Guignardia* spp. | Ascomycetes | Roots/F and G | plant pathogens, endophytes |
| *Humicola* sp. | Anamorphic Fungi | Roots/G | saprophytes, parasitic on fungi |
| *Mortierella* spp. | Zygomycetes | Roots/F and G | saprophytes |
| *Mucor* spp. | Zygomycetes | Leaf Litter, Roots/F and G | saprophytes |
| *Nigrospora* sp. | Anamorphic Fungi | Roots/G | saprophytes |
| *Penicillium* spp. | Ascomycetes | Roots/G | saprophytes |
| *Pestalotiopsis microspora* | Anamorphic Fungi | Bark and Leaf litter, Roots/F and G | plant pathogens |
| *Phoma sp.* | Anamorphic Fungi | Roots/G | endophytes, saprophytes, plant pathogens |
| *Pythium* sp. | Oomycetes | Bark Litter, Roots, soil baits/ F and G | plant pathogens, saprophytes |
| *Pythium heterothallicum* | Oomycetes | Roots/G | plant pathogens |
| *Rhizoctonia* sp. | Basidiomycetes | Bark litter, Roots/G and F | plant pathogens |
| *Schizophyllum commune* | Basidiomycetes | Roots/G | lignin and cellulose decomposers |
| *Scytalidium* sp. | Anamorphic Fungi | Roots/G | plant pathogen |
| *Thielaviopsis* sp. | Anamorphic Fungi | Roots/G | plant pathogens |
| *Trichoderma* sp. | Anamorphic Fungi | Roots/G | saprophytes, parasitic on fungi |
| *Umbelopsis* sp. | Zygomycetes | Roots/F | endophytes, saprophytes |
| *Verticillium* sp. | Anamorphic Fungi | Leaf litter/G and F | plant pathogens, parasitic on fungi |
| *Xylaria* sp. | Ascomycetes | Leaf litter/G | decomposers |

[1] F= Torreya State Park, Florida; G = Decatur, Georgia.

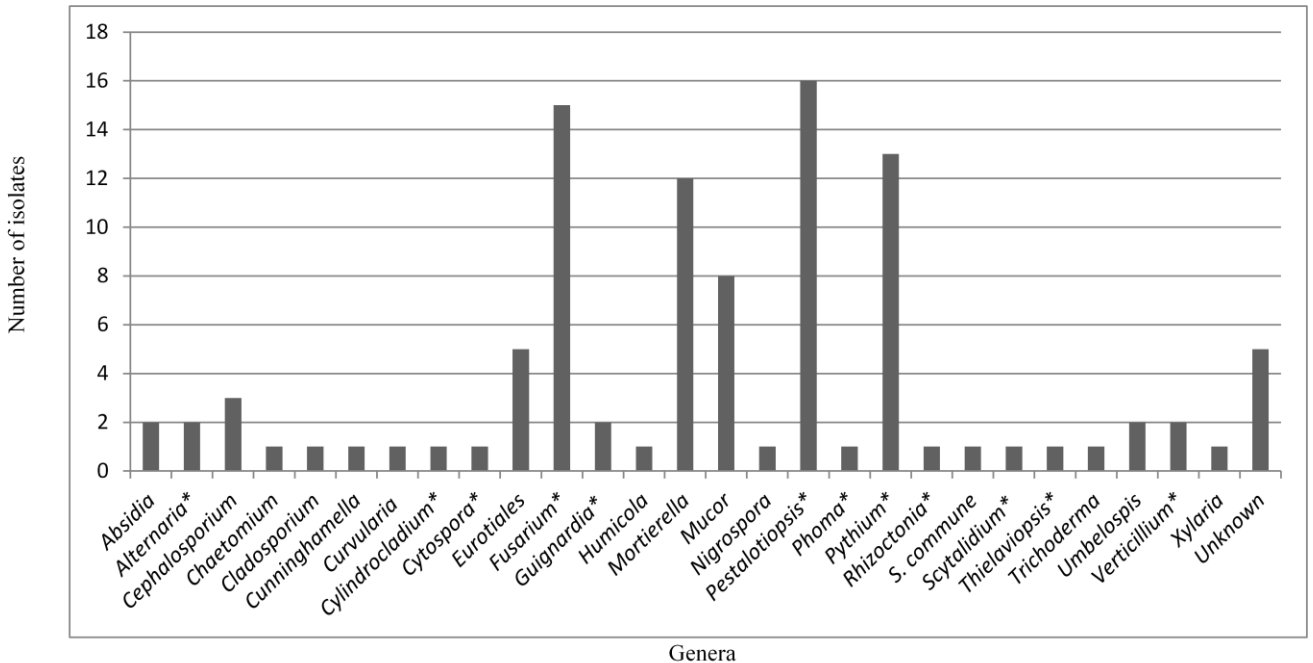



Figure 3. Oomycetes and fungal genera associated with *Torreya taxifolia* trees showing decline symptoms at sampled sites. The asterisks, *, represent plant pathogens.

Other fungi isolated using *Torreya* leaves as baits were *Absidia* sp. (Zygomycetes) and *Cladosporium* sp. (Anamorphic fungi). Fungi such as *Pestalotiopsis microspora*, *Fusarium* spp., *Rhizoctonia* spp., *Pythium* sp., in addition to an unknown Oomycetes, other than *Phytophthora* spp., were isolated directly from mycelia observed on bark associated to plant litter surrounding tree's base (Figure 4).

*Alternaria* spp., *Cylindrocladium* sp., *Fusarium* spp., *Phoma* sp., *Pythium* spp., *Rhizoctonia* sp., *Thielaviopsis* sp. and *Verticillium* sp. are among soil-borne fungal species identified (Figures 3 and 4). To our knowledge none of these soil-borne pathogens have been shown to cause disease in *T. taxifolia* roots (Alfieri 1984, El-Gholl 1985, USFWS 1986, Alfieri et al. 1987, Schwartz et al. 1996). *Pestalotiopsis* spp. was the most common genera isolated during this study, associated to bark, plant litter and necrotic roots at both locations, Florida and Georgia (Figure 3). *Fusarium torreyae*, *Pestalotiopsis microspora* and *Scytalidium* sp., have been shown to cause needle spots and stem cankers in *T. taxifolia* (Hermann and Schwartz, 1997; Schwartz et al., 1996; Smith et al. 2011). Another plant pathogen, *Phoma* spp. was found associated to *Torreya's* roots in Georgia. Some species have been associated and shown pathogenic to woody plants (Boerema et al. 2004).

In this study, various *Fusarium* species (anamorph of *Gibberella* spp.) were isolated in both locations from roots and associated to bark, plant litter and dying trees. From Georgia, *Fusarium oxysporum* was isolated from roots and plant litter. This species has been associated with seedling death and with root rot in *Pinus* and *Eucalyptus* seedling in South Africa (Viljoen et al. 1992). *Fusarium solani* was also isolated from roots of dying trees at this site (Table 3). Based on macroconidia and chlamydospores morphology, none *F. torreyae* was isolated during this study (Aoki et al. 2013). Recently this species have been shown to cause Torreya canker disease in Florida (Smith et al. 2011). DNA analysis of the ITS rDNA region showed that *Fusarium* spp. isolated during this study were closed to *F. subglutinans*, *F. oxysporum* and *Gibberella* spp. clades (Figure 5). However, amplification of other more informative genetic regions such as RNA polymerase largest subunit (RPB1 and RPB2) was not employed during this study.

Other plant pathogens identified by DNA analysis were: *Cytospora* sp., *Guignardia* spp. and *Umbelopsis* sp. (Figure 6). *Cytospora* canker has been reported in shrubs and trees in Colorado (Jacobi 2009). *Guignardia* is a genus that includes endophytes as well as important foliar pathogens (Peres et al. 2007). *Umbelopsis* spp. are zygomycetes that has been reported as endophytes of root xylem tissue of healthy conifers in the state of Washington, USA (Hoff et al. 2004).

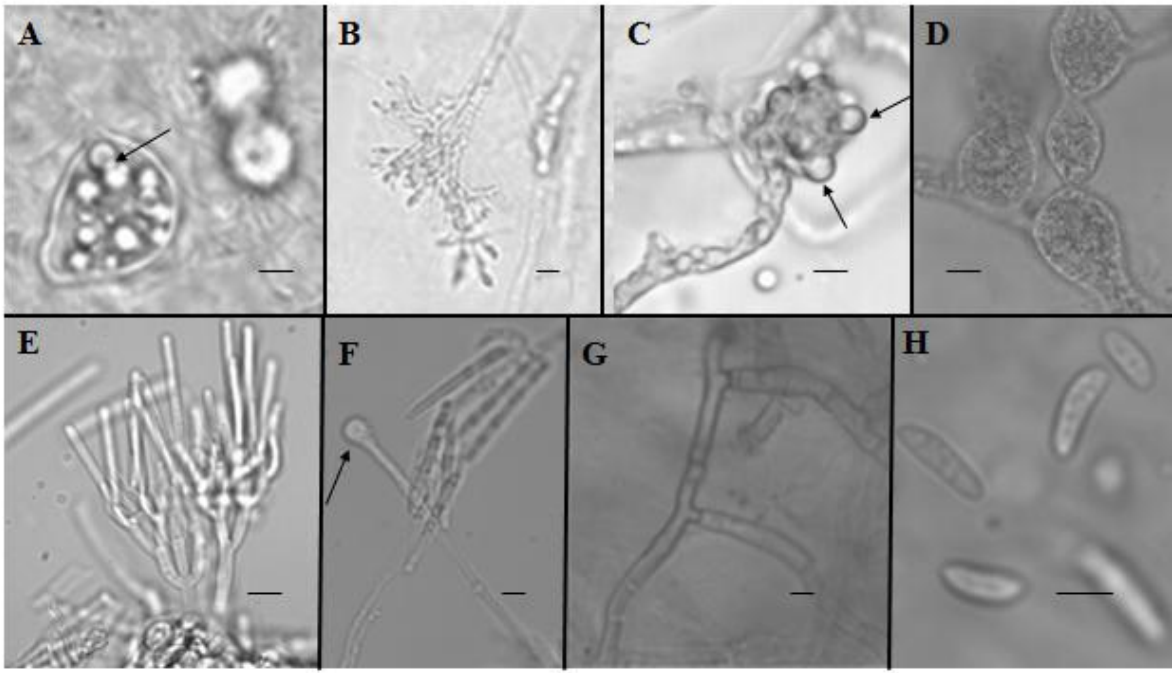



Figure 4. Oomycetes and fungal reproductive and vegetative structures. Oomycetes species from A to D: A. Sporangia with zoospores (arrow); B. Coralloid hyphae from an unknown species; C. Oogonium and antheridia (arrow) typical of *Pythium heterothalicum* isolated from roots; and D. Hyphal swellings from *Pythium* sp. Fungal species from E. to H: E. *Cylindrocladium* sp. conidia and conidiophores; F. *Cylindrocladium* sp. vesicle (arrow); G. *Rhizoctonia* sp. mycelium; and H. *Fusarium oxysporum* microconidia. Bar = 10µm.

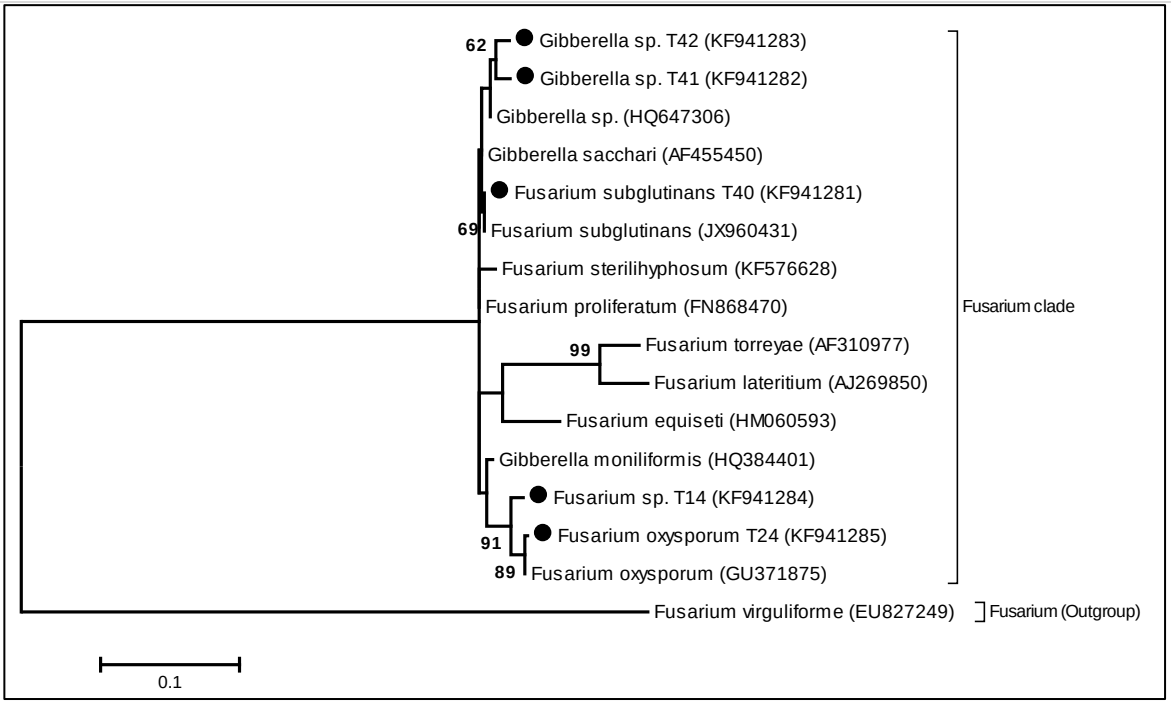
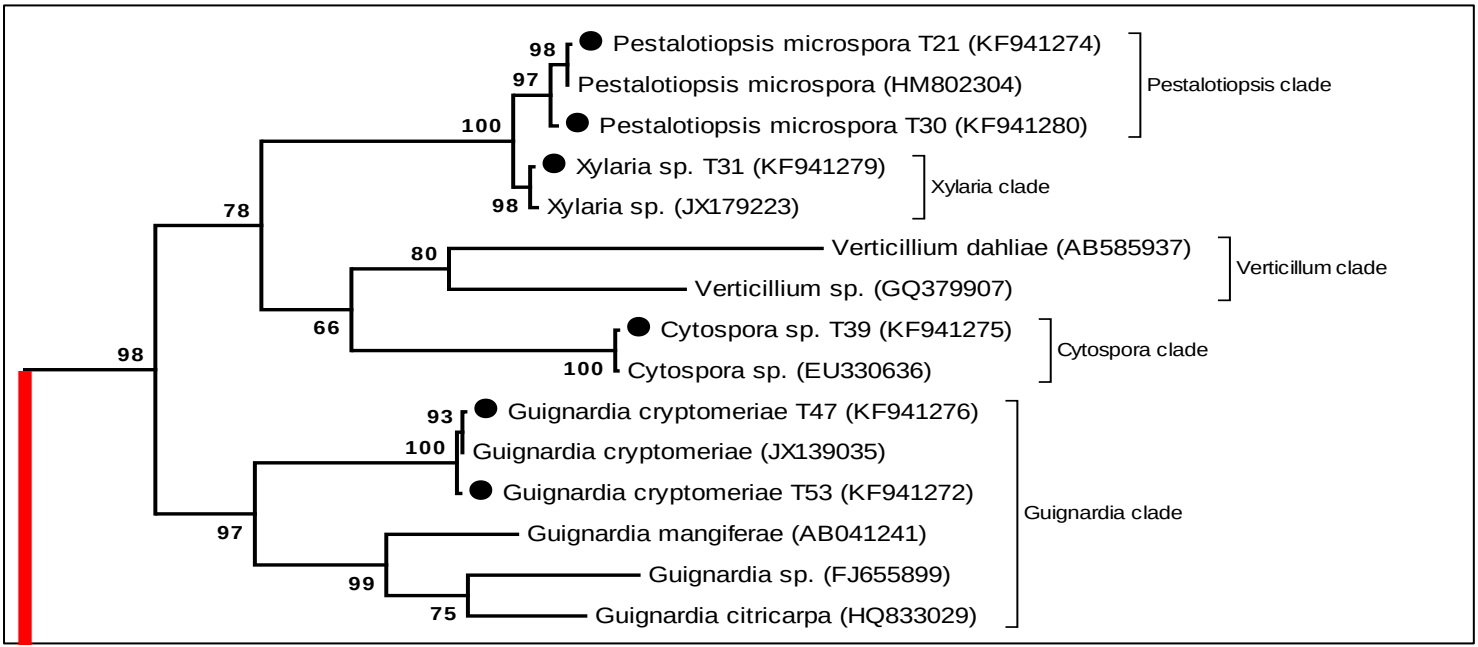



Figure 5. Phylogenetic tree inferred from the partial ITS1-5.8S-ITS2 region of *Fusarium* species isolated during this study (black dots). Sequences from known species were included for reference and comparison: *Fusarium torreyae*, *F. lateritium*, *F. equiseti*, *F. subglutinans*, F. *oxysporum* and *Gibberella* sp. GenBank accession numbers were included in parentheses.


98
97
100
98
78
80
66
100
98
93
100
97
99
75
Pestalotiopsis microspora T21 (KF941274)
Pestalotiopsis microspora (HM802304)
Pestalotiopsis microspora T30 (KF941280)
Xylaria sp. T31 (KF941279)
Xylaria sp. (JX179223)
Verticillium dahliae (AB585937)
Verticillium sp. (GQ379907)
Cytospora sp. T39 (KF941275)
Cytospora sp. (EU330636)
Guignardia cryptomeriae T47 (KF941276)
Guignardia cryptomeriae (JX139035)
Guignardia cryptomeriae T53 (KF941272)
Guignardia mangiferae (AB041241)
Guignardia sp. (FJ655899)
Guignardia citricarpa (HQ833029)
Pestalotiopsis clade
Xylaria clade
Verticillum clade
Cytospora clade
Guignardia clade

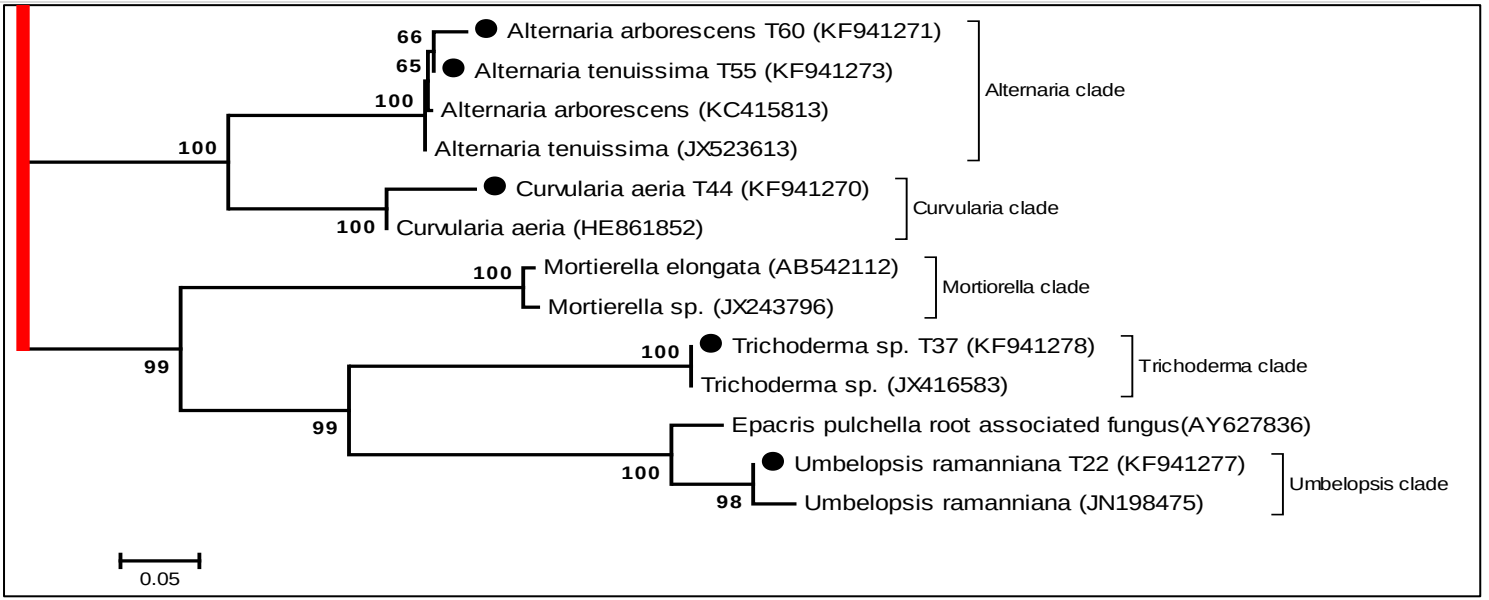



Figure 6. Phylogenetic tree inferred from the partial ITS1-5.8S-ITS2 region of different fungal species isolated during this study (black dots). Sequences from known species were included for reference and comparison: *Pestalotiopsis microspora*, Order Xylariales, *Verticillium dahliae*, *Verticillium* sp., *Cytospora* sp., *Guignardia cryptomeriae*, *G. mangiferae*, *Guignardia* sp., *Guignaridia citricarpa*, *Curvularia aeria*, *Alternaria tenuissima*, *A. arborescences*, *Verticillium* sp., *Trichoderma* sp., *Epachris pulchella* root associated fungi and *Umbelopsis ramannianna*. GenBank accession numbers were included in parentheses. The thicker vertical bar at the base of th tree indicates that the panels located on pages 219-220 are part of one phylogenetic tree.

These results suggest that fungi inhabiting the soil and rhizophere of *T. taxifolia* are diverse and some species could play an important role in its decline. Nevertheless, fungal identification is problematic due to the enormous, largely unexplored diversity and the need of accurate annotated reference DNA sequences. Research related to environmental samples revealed that the vast majority of the microbial diversity (99%) is missed by cultivation-based methods that we traditionally used (Riesenfeld et al. 2004). Concepts such as metagenomics, describes the functional and sequence-based analysis of the collective microbial genomes contained in an environmental sample (Riesenfeld et al. 2004). That is why non-culturable microbial communities which inhabit soils are define as the most complex known to science, and poorly understood despite their economic importance (Riesenfeld et al. 2004). Thus further research is needed using metagenomic technology to understand the dynamics of the microbial interactions related to *T. taxifolia* in the forest. Another very important consideration relates to the plant itself, one is the difficulty to germinate *T. taxifolia* seeds. Seedlings with healthy root systems are necessary to conduct pathogenicity tests and to complete Koch's postulates, crucial to demonstrate the role of some of the species as root pathogens. Another challenge that should be considered are the restrictions to obtained plants of a plant species that is federally listed as endangered to performed experiments.

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## ERRATUM

LoCasio III and Kudryashova. 2013.
*Life: The Excitement of Biology* 1(3):166-173

Recently, LoCasio III and Kudryashova published a paper entitled, "Preliminary survey of the butterflies and skippers (Insecta: Lepidoptera) in a wet subtropical sustainable forestry plot in Patillas, Puerto Rico" in *Life: The Excitement of Biology* 1(3):166-173. The figure captions on page 171 have several mistakes, beginning on panel L. Currently, the legend reads, "l. and m. *Pseudosphinx tetrio* (Linnaeus, 1771), photo by Shawn Hanrahan, reproduced with permission; n. and o. *Agrius cingulata* (Fabricius, 1775 photo credit George LoCascio III (l to o, Sphingidae); p. *Diaphania hylinata* (Linnaeus, 1767) Crambidae, photo credit Tom Peterson, reproduced with permission." Instead, that portion of the figure caption should read, L *Diaphania hylinata* (Linnaeus, 1767) Crambidae, photo credit Tom Peterson, reproduced with permission; M and N *Pseudosphinx tetrio* (Linnaeus, 1771) Sphingidae, N. photo by Shawn Hanrahan, reproduced with permission; O. and P. *Agrius cingulata* (Fabricius, 1775).