THESIS

HOST ASSOCIATION AND EVIDENCE OF GEOGRAPHIC ISOLATION OF THE GENUS *ONNIA* IN NORTH AMERICA AND THE DEVELOPMENT OF A MOLECULAR TOOL FOR FIELD DETECTION

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ABSTRACT

HOST ASSOCIATION AND EVIDENCE OF GEOGRAPHIC ISOLATION OF THE GENUS *ONNIA* IN NORTH AMERICA AND THE DEVELOPMENT OF A MOLECULAR TOOL FOR FIELD DETECTION

Tomentosus root rot, a disease found in coniferous forests throughout the northern hemisphere, is caused by *Onnia* spp. This disease causes growth reduction, root decay, and weakens trees, which increases the likelihood of mechanical failure. The genus has a wide geographic distribution, and this distribution, alongside a substantial reduction in growth of infected trees, makes Tomentosus root rot a highly impactful disease for timber operations. However, management of this disease is difficult because these saprophytic fungal pathogens can persist in stumps and other decaying organic matter making it impossible to eradicate. The aboveground symptoms caused by *Onnia* spp. are common for most root diseases, which makes diagnosis challenging. This difficulty in identification and persistence in a stand brings challenges to managing this disease for both large-scale operations and recreational sites such as campgrounds. Due to recent taxonomic changes, the geographic distributions and host ranges of several North American *Onnia* spp. is not well understood. A clear understanding of host association and species distribution is important for developing management strategies. Quick and accurate identification of these pathogens would enable early detection and a proactive response for management. Therefore, this research had three objectives to better characterize these pathogens and develop tools for the detection and identification of *Onnia* spp. in North America: 1) provide an overview of the existing literature regarding the host associations,

geographic distributions, and molecular tools available, 2) conduct surveys and use DNA-based identification techniques to describe the host associations and geographic distributions of North American *Onnia* spp., and 3) design and validate a rapid field-based molecular detection assay for North American *Onnia* spp.

Chapter one is a literature review that consolidates and summarizes previous research and knowledge on the genus. Historical accounts of Tomentosus root rot are obfuscated by numerous changes in genus and species classifications and the debates that followed. As such, making confident inferences on the distribution, host association, and management strategies are difficult. However, what is clear is that historically, two closely related species that both produce a white pocket rot and similar basidiocarps were recognized in North America. These were referred to as *O. tomentosa* (= *Inonotus tomentosus*) and *O. circinata* (= *I. circinatus*). A defining feature that was used to distinguish these species was the hymenial setae, which were either hooked (*O. circinata*) or straight (*O. tomentosa*). However, investigations using molecular tools showed that the species that exhibited hooked hymenial setae comprised two distinct species, *O. subtriquetra* and *O. leporina*. The fact that *O. subtriquetra* and *O. leporina* were considered the same species has resulted in a misunderstanding of their actual geographic distributions. There has also been some doubt cast on the distribution and host association of *O. tomentosa.* This is because the main distinguishing feature between *O. tomentosa* and the other two species is that it has straight hymenial setae. This feature requires the use of a microscope for identification and there is some concern that previous reports of *O. tomentosa* were based solely on the existence of basidiocarps without further investigation of the morphology of the hymenial setae.

In Chapter two, 58 isolates of *Onnia* spp. collected during surveys, along with those borrowed from herbaria, were identified using DNA sequencing. We found evidence of three

species of *Onnia* spp. in North America: *O. tomentosa*, *O. subtriquetra,* and *O. leporina*. *O. subtriquetra* appears to be primarily a pine specialist found from as far west as California to the Carolinas. We confirmed *O. tomentosa* as a widespread generalist across North America and discovered evidence that the species can be separated into three lineages, there is no evidence of recombination across the lineages. However, a neighbor-net phylogenetic network showed some evidence of reticulation among *O. tomentosa* lineages, suggesting that there is a soft barrier that prevents random recombination among lineages, but some gene flow may be present. Unfortunately, few inferences could be made regarding *O. leporina*, as only a single specimen, from Quebec, Canada was available. Chapter two also describes the development and validation of a set of primers for a loop mediated isothermal amplification (LAMP) assay. This molecular assay requires little training, equipment, and reaction time, making it a valuable tool for land managers or forest pathologists who need an inexpensive and rapid technique for diagnosing infected trees. The efficacy of the LAMP was determined by testing it against 30 North American *Onnia* isolates as well as other co-occurring fungi (e.g., *Armillaria* spp.*, Coltricia* spp.*, Porodaedalea* spp.*, Phellinus* spp.*, Fomitopsis* spp.*, Heterobasidion* spp.*,* and *Coniophora puteana*). The limit of detection for this molecular test was 170 pg of DNA for *O. tomentosa* isolates, 83 pg for *O. subtriquetra*, and 121 pg for *O. leporina*. We demonstrated the field applicability of this assay and found that temperature fluctuations of premixed reagents increased the likelihood of false positives. This molecular tool will allow for reliable detection and identification of *Onnia* spp. in North America even when white pocket rot is the only indication of infection, which will aid in the diagnosis and management of Tomentosus root rot.

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CHAPTER 1: LITERATURE REVIEW

1.1 *Onnia* spp.

1.1.1 Tomentosus Root Rot

Tomentosus root rot is a major disease in North American conifer forests (Hunt & Unger, 1994; Lockman & Kearns, 2016; Woods, 2003). It is also known as red root rot (Scharpf, 1993; Worrall & Nakasone, 2009). The causal organisms for this disease are basidiomycetes in the genus *Onnia* (Larsson et al., 2006). This genus is distributed globally throughout temperate, boreal, and sub-boreal forests and primarily affects conifer species (Ji et al., 2017; Zhao et al., 2022). These fungal pathogens are characterized by a white pocket rot that primarily degrades lignin and hemicellulose in the roots and butt of trees (Blanchette, 1984). Their orangish basidiocarps (known as false velvet top) are found on either the forest floor or directly on their hosts (Scharpf, 1993). Infections originate in small roots, damaged root collars or wounds, or root contacts with an infected root, and progress through the heart wood of root systems (Lewis et al., 1992). Decay is preceded by a red staining, known as incipient red decay, and as an infection progresses the decay spreads radially from the heart wood into the sapwood and cambial region (Lewis et al., 1992; Lewis, 1997; Myren & Patton, 1971). Once the vascular system is infected, transportation of sugars and water is reduced, which then in turn leads to symptoms such as chlorotic leaves, needle loss, thinning crowns, and occasionally whole-tree mortality. These above-ground symptoms are common to root disease and often do not occur until the root system is heavily colonized (Lewis et al., 1992). Trees often succumb to windthrow

due to the presence of advanced decay in the roots and butts. Windthrown trees are often still green and may be completely asymptomatic prior to mechanical failure.

The prevalence of Tomentosus root rot in a stand is important information for land managers. It is known for reducing the growth of infected trees (Lewis, 1997; Lewis et al., 2005; Woods & Watts, 2019). Studies on the effects of thinning as a management method have produced conflicting results and should be investigated further (Morrison et al., 2022; Whitney, 1993). It has been observed to persist in stumps for upwards of 30 years, which can act as an inoculum source for root-to-root infections (Lewis & Hansen, 1991a, 1991b; Myren & Patton, 1971). The length of time it can persist in stumps and the lack of diagnostic above ground symptoms make it difficult to manage (Lewis, 1997; Lockman & Kearns, 2016). For managers of recreational sites, the effect of wood decay is not just un-merchantable wood, but increased risk to visitors (Johnson, 1981; Mortimer & Kane, 2004). Over the years, regions have developed hazard tree assessments to determine the risk of tree failure in a repeatable way (Blodgett et al., 2021; Johnson, 1981). These methods are typically based on a visual assessment, which makes accurate identification of Tomentosus root rot critical.

1.1.2 Distribution in North America

Historically, North American forest pathologists have recognized two species as the causal agents of Tomentosus root rot and red root rot, *O. tomentosa* and *O. circinata*. These distinctions were based on morphological characteristics such as the shape of the hymenial setae, with *O. tomentosa* exhibiting straight setae and *O. circinata* exhibiting hooked, or curved, setae (Haddow, 1941; Whitney & Bohaychuk, 1976, 1977; Whitney & Fleming, 2005). Current

evidence suggests that there are at least three distinct species in North America— *O. tomentosa* (formerly *Inonotus tomentosus, Coltricia tomentosa, Polyporus tomentosus*) (Karst, 1882), *O. subtriquetra* (formerly *O. circinata*, and *I. circinatus*) (Xiao-Hong, 2017), and *O. leporina* (formerly *O. circinata*, *I. circinatus*, and *I. leporinus*) (Germain et al., 2002; Jahn, 1978; Ji et al., 2017; Zhao et al., 2022). *O. tomentosa* and *O. leporina* both have global distributions in the northern hemisphere, while *O. subtriquetra* has only been reported in North America (Ji et al., 2017; Lee et al., 2024). While genetic evidence supports the occurrence of *O. leporina* in Canada, its prevalence remains unknown, with only one reported instance documented using molecular evidence (Germain et al., 2002).

The recent description of *O. subtriquetra* and the documented presence of *O. leporina* in Canada suggest that historical studies of North American samples of *O. circinata* may be confounded because what was once considered a single species is now known to represent at least two distinct species (Germain et al., 2002; Ji et al., 2017). Therefore, it is important to interpret historical reports of *Onnia* spp. cautiously, as identification methods often relied on characteristics such as the presence of decay and host association (Lockman & Kearns, 2016; Woods & Watts, 2019). For example, both *O. leporina* and *O. tomentosa* cause white pocket rot in spruce (*Picea* spp.). Thus, any identification of *Onnia* spp. in North America based solely on the presence of white pocket rot in spruce, without any other supporting evidence, could have been either *O. leporina* or *O. tomentosa* (Niemelä & Kotiranta, 1983; Whitney & Bohaychuk, 1976). Although *Onnia* spp. are commonly thought to exist primarily in boreal and sub-boreal biomes, there are reports of *Inonotus tomentosus* and *I. circinatus* (found only on pine) in southern Mexico (Cibrian Tovar, 2007).

1.1.3 Phylogenetic relationships

Members of *Onnia* have been classified in various genera over the past 100 years, and their taxonomic journey can be traced through 3-4 different classifications before settling into their current designation. As such, following the history of their taxonomic names can be difficult (Haddow, 1941; Niemelä & Kotiranta, 1983). The designation of *Onnia* is well supported, with two genetic analyses of the Hymenochaetales providing evidence that these species form a monophyletic clade (Larsson et al., 2006; Wagner & Fischer, 2002).

The two most comprehensive molecular examinations of *Onnia* were conducted by Zhao et al. (2022) and Ji et al. (2017). These studies provide a clearer understanding of the evolutionary history and phylogenetic relationships among *Onnia* spp. and collectively describe five additional species within *Onnia*. Not all these new species are strongly supported; one of them, *O. himalayana*— had relatively low bootstrap support (60%) in the maximum likelihood phylogeny and with Bayesian inference (BI) posterior probability less than 0.8 (Zhao et al., 2022). Zhao et al. (2022) also includes a molecular clock analysis of *Onnia* spp. From this analysis, the authors hypothesize that *Onnia* spp. originated in Europe before dispersing eastward into Asia and subsequently into North America (Zhao et al., 2022).

1.1.4 Host association

Taxonomic revisions and the reliance on observational studies have made forming definitive conclusions regarding host association difficult. However, an adequate understanding of these species and their hosts can be gained by summarizing past studies. Current literature states that *Onnia* spp. are composed primarily of gymnosperm specialists (Ji et al., 2017; Palla et al., 2023). Most described *Onnia* species are reported as infecting *Pinus*, with two gymnosperm

generalists being *O. tomentosa* and *O. leporina* (Ji et al., 2017; Palla et al., 2023). There are reports of *Onnia* being found in association with angiosperms, but these reports did not include DNA-based identification and are typically set aside in studies of this genus (Zhao et al., 2022). Few experimental or molecular studies exist examining the host associations of these species.

Observational reports and experimental studies that do exist find that *O. tomentosa* is found to be primarily on spruce with the ability to infect a wide range of conifer trees. This is supported by (Whitney & Bohaychuk, 1976), which showed both *Inonotus tomentosus* (*O. tomentosa*) and *Inonotus circinatus* (*O. leporina/O. subtriquetra*) were pathogenic to 11 conifer species (*Pinus ponderosa*, *Pinus contorta*, *Pinus sylvestris*, *Pinus strobus*, *Picea glauca*, *Picea mariana*, *Picea abies*, *Picea pungens*, *Picea rubens*, *Larix laricina*, and *Pseudotsuga menziesii*). In pathogenicity trials, isolates of the species with straight setae (*O. tomentosa*) were more virulent than those from the species with hooked setae (*O. leporina* or *O. subtriquetra*). While pathogenicity tests indicate that some genets (individuals) of *O. tomentosa* can infect multiple host species (Whitney & Bohaychuk, 1976), there has not been rigorous testing of host association using isolates collected from a wide geographic distribution. No substantial work has been done to determine whether *O. tomentosa* is a generalist white rot pathogen with a wide host range or if it is composed of multiple host specific clades.

Onnia subtriquetra has only been reported infecting pines (*Pinus* spp.) (Ji et al., 2017; Lee et al., 2024). This aligns with literature that reports that the majority of species within *Onnia* are pine specialists (Palla et al., 2023). To date, no pathogenicity or virulence study has been conducted with *O. subtriquetra*. *Onnia leporina* is typically found in association with spruce (Hakala et al., 2004; Niemelä & Kotiranta, 1983). While it may have a broad host range like *O. tomentosa*, it is documented to be less virulent (Whitney & Bohaychuk, 1976, 1977). It is

important to note that these two studies were completed prior to the division of *O. circinata* into *O. subtriquetra* and *O. leporina.* The isolations were made from a spruce-dominated forest, which indicates that the *Inonotus circinatus* isolates used were likely *O. leporina* and not *O. subtriquetra*. That said, the virulence and host range of *O. leporina* should be investigated further.

1.2 Signs and symptoms

1.2.1 Basidiocarps

All species within the genus *Onnia* form a basidiocarp (Ji et al., 2017). In general, the basidiocarps produced by this genus are described as having a yellowish-orange cap with an off-white underside. The location of fruiting bodies appears to be dependent on the species, with *O. tomentosa* often stipitate, growing from the forest floor, and commonly found in association with spruce trees. *Onnia subtriquetra* is commonly astipitate or laterally sub-stipitate and most often directly attached to the host substrate. However, it can also be found on the forest floor in stipitate form (Lee et al., 2024). *Onnia leporina* is also usually astipitate or laterally sub-stipitate and directly attached to its host (Niemelä & Kotiranta, 1983). Hymenial setae exist within the pores of *Onnia* spp. basidiocarps (Ji et al., 2017). The exact purpose of hymenial setae is unknown, but it is hypothesized to be an insect deterrent (Larsson et al., 2006). When studied, basidiocarps were found not to fruit regularly, nor did they always appear in the same location, but fruiting was associated more often with dead trees rather than living infected hosts (Whitney & Fleming, 2005). However, it is not uncommon for fruiting bodies to be the only above-ground feature of Tomentosus root rot, as trees often do not show symptoms until the advanced stages of infection (Lewis et al., 1992). When basidiocarps are not available, forest pathologists typically

rely on isolations of *Onnia* spp. from infected wood tissue to diagnose disease. When added to the isolation medium, the fungicide benomyl has been shown to successfully deter ascomycetes while allowing hymenomycetes to grow (Worrall, 1991). *Onnia* spp. isolates in a stationary liquid malt extract broth has been shown to produce enough mycelium for protein extraction after 4 weeks of growth (Hunt & Ekramoddoullah, 1996). Cultures have been described as having a range of colors from light brown to dark brown or being cream-colored (Whitney & Bohaychuk, 1977).

When dried and properly stored, the basidiocarps can persist for decades. This makes them a valuable resource for researchers. When herbarium specimens are available, a researcher is not limited to what they can collect within a season, but they have access to the collections made across many seasons and geographic locations. However, extracting DNA from herbarium specimens can be very difficult (Aras & Duman, 2006.; Cubero et al., 1999; Redchenko et al., 2012). Herbarium specimens are often treated with pesticides that prevent the degradation of the macroscopic features of the fruiting bodies but, unfortunately, these applications can degrade the DNA (Särkinen et al., 2012), resulting in problems with down-stream applications such as PCR. DNA fragmentation is common when working with herbarium samples (Bradshaw et al., 2023; Redchenko et al., 2012; Särkinen et al., 2012). The likelihood of DNA fragmentation increases over time which can limit the utility of older specimens. If conventional PCR is the goal of extractions, amplification of small regions (< 300 bp) is recommended when trying to amplify loci from herbarium samples (Särkinen et al., 2012). The addition of bovine serum albumin (BSA) during PCR can improve amplification of DNA from older herbarium specimens (Särkinen et al., 2012).

1.2.2 Host symptoms and White Pocket Rot

Due to the nature of root rot, infected trees often exhibit few above-ground symptoms and those produced are not diagnostic because they are the same as those produced by other root disease pathogens (Lewis et al., 1992; Reich et al., 2013). Above-ground symptoms include foliar chlorosis, needle loss, thinning crowns, a reduced or dead leader, and stress cone production. These generic symptoms lead to a common scenario where infection is noticed only after tree failure or harvest. A common characteristic used to diagnose Tomentosus root rot is the presence of white pocket rot or white pitted decay resulting in a honeycomb-like pattern (Lewis & Hansen, 1991a; Woods, 2003; Woods & Watts, 2019). Diagnosing disease caused by *Onnia* based solely on decay characteristics could result in misdiagnosis. Fungi in the genus *Porodaedalea*, a sister genus to *Onnia*, also cause white pocket rot with similar characteristics. These fungi also appear brownish-orange in culture (Hunt, 1997; Hunt & Ekramoddoullah, 1996). *Porodaedalea* spp. are stem-rotting fungi whereas *Onnia* spp. typically cause butt and root rot. However, both infections have been reported to form decay columns from the root collar up to 4.5 feet (the standard measurement for diameter at breast height (DBH)) (Hunt, 1997; Hunt & Ekramoddoullah, 1996).

To further highlight the saprophytic nature of *Onnia* spp., Finnish isolates of *O. leporina* were able to successfully decay blocks of dried spruce wood (Hakala et al., 2004), and *O. tomentosa* has been shown to exhibit vegetative growth on multiple media types, such as thatch products, agricultural waste, and sterilized wood (Martin, 1980). It appears that the species studied within this genus are selective de-lignifiers, but this might not be the case for all *Onnia* spp. (Blanchette, 1984; Hakala et al., 2004). A Finnish study found a large difference between

two isolates of *O. leporina* when looking at percent loss of lignin over percent loss of cellulose components from decay caused by polypores (Hakala et al., 2004).

1.3 Disease Cycle

1.3.1 Establishment and spread

Onnia spp. spread via airborne basidiospores and vegetatively through root-to-root contact (Lewis et al., 1992). Initial infection occurs primarily in the heartwood and then spreads outward into the sapwood (Lewis et al., 1992). As an infection progresses, the likelihood of root-to-root transmission increases. Uninfected trees whose roots are touching infected roots are at risk of infection (Lewis et al., 1992; Lewis & Hansen, 1991a). Researchers have noted a slow spread outward from initial infections. A study of 12 stump pairs showed a spread rate of 20 cm/yr (Hunt & Peet, 1997). This study, and others from this time, used the existence of white pocket rot as a diagnostic feature. *Onnia* spp. have been shown to act saprophytically in dead stumps, suggesting that dead infected trees and stumps may function as long-term sources of inoculum (Lewis & Hansen, 1991a).

The exact mechanism of how a tree becomes infected by basidiospore is not well studied, though it has been hypothesized that it infects either through a wound or through small roots (Lewis et al., 1992; Lewis & Hansen, 1991b; Myren & Patton, 1971). A study that investigated the population genetics of 180 *O. tomentosa* basidiocarps reported evidence of multiple genets in a stand. The distribution of these genets suggests that the establishment of new infections via basidiospore dispersal is more common than previously thought (Germain et al., 2009). A separate study also presented evidence that concurred with these results. Following a stem mapping study, Woods and Watts (2019) noted that there were multiple instances where the

clumps of trees infected with white pocket rot was too large given the radial spread rates recorded in previous studies (Hunt & Peet, 1997), suggesting that either the radial spread rates need to be updated, or that there were multiple basidiospore infections within close proximity. A study of vegetative compatibility among isolates from a single stand revealed the presence of more distinct genotypes than would be expected if spread was predominately vegetative (Lewis & Hansen, 1991b). These studies suggest that the role(s) and mechanisms of infections established via basidiospores should be investigated further.

1.4 Molecular Identification

1.4.1 Molecular tools for Onnia spp. identification

Analyses of genetic variation in the internal transcriber spacer unit (ITS) and large ribosomal subunit (LSU) regions have been shown to successfully discriminate species within *Onnia* (Ji et al., 2017). A set of species-specific primers was developed based on a 21 bp insertion on the ITS locus that is unique to *O. tomentosa* (Germain et al., 2002). This primer set was developed when *O. tomentosa* was classified in the genus *Inonotus,* so some of the fungal species that were tested as presumed congeners were not as closely related as the researchers thought. The efficacy of this primer set needs to be revisited considering the newly described species of *Onnia*. A second genus specific primer set was also developed for the ITS locus, and like the other primer set, this marker was designed and tested prior to the confirmation of new *Onnia* spp. and needs validation for other species (Gonthier et al., 2015).

1.4.2 Loop Mediated Isothermal Amplification

A loop mediated isothermal amplification (LAMP) assay is a molecular tool that allows for convenient amplification of template DNA at a single constant temperature with minimal equipment and sample preparation (Nagamine et al., 2002; Notomi et al., 2000). When designed correctly, it can be a highly specific and sensitive assay that can be performed with a simple heat block and pipette. These primers allow for the creation of amplicons that are different shapes than the original template DNA and these amplicons can act as both template and primer (Panno et al., 2020). This allows for a higher rate of amplification than typical polymerase chain reaction (Panno et al., 2020). The reaction also creates an excess of (Mg^{2+}) which increases the overall pH of the solution (Wong et al., 2018). If a pH indicator is added to the solution, then it becomes a colorimetric assay and visual detection of amplification is possible. This technique is useful for detecting a target organism in the field with few tools.

Specificity and sensitivity must be evaluated when developing a species-specific molecular marker. Specificity refers to the likelihood of a false positive (type one error). A test that is highly specific has a very low likelihood of reporting a false positive. We determine the specificity of a test by testing the assay in question with a wide range of non-target organisms. Sensitivity is used when describing the likelihood of getting a false negative (type two error). The sensitivity of a molecular test is determined by testing progressively smaller amounts of target DNA until a false negative occurs. It is also important to test this assay against many different target individuals to confirm that a false negative is unlikely.

Since the LAMP assay's inception, there has been a growing body of literature to aid in the development of primers. The ideal GC content for a specific primer is between 40% and 60% (Panno et al., 2020). While the original creation of a LAMP assay includes only four primers, it

is very common for researchers to include two “loop” primers (Nagamine et al., 2002), with four thymine bases in the loop primer to aid in amplification (Lamas et al., 2023). While the implementation of a LAMP assay seems straightforward, the actual development and validation of an assay can be fraught with roadblocks and difficulties. Due to its rapid and sensitive nature, false positives caused by contamination are a common issue when developing LAMP primers (Panno et al., 2020). However, the utility a functional test provides is well worth the effort put into the development process. Due to its ease of use and minimal processing time, a LAMP assay is an ideal tool for land managers. A LAMP assay allows for a reliable form of identification even when diagnostic features are unavailable. This is especially important for early detection and proactive management of root disease such as *Onnia*. In recreation areas, the availability of tools such as LAMP will allow land managers to detect and identify *Onnia* in standing trees so they can be treated proactively.

1.5 Research Objectives

While many forest diseases have reliable diagnostic features that allow them to be quickly identified, infections caused by *Onnia* spp. do not. Although basidiocarps of this genus can indicate the presence of the pathogen, these are not always reliably produced, and it is often difficult to determine which trees are infected unless the fruiting bodies are attached directly to the host. With the newly recorded presence of three distinct species in North America, questions arise regarding the host ranges and geographic distributions of each species. Therefore, the goals of this study were to: 1) investigate the phylogenetic relationships between samples of North American *Onnia* spp., 2) evaluate their host ranges and geographic distributions, and 3) develop a rapid field-based molecular detection assay to detect *Onnia*.

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CHAPTER 2: HOST ASSOCIATION AND EVIDENCE OF GEOGRAPHIC ISOLATION OF THE GENUS *ONNIA* IN NORTH AMERICA AND THE DEVELOPMENT OF A MOLECULAR TOOL FOR FIELD DETECTION

2.1 INTRODUCTION

In forested areas that are managed as recreation sites, managers must balance site aesthetics, tree health, and visitor safety simultaneously with few financial or educational resources (Blodgett et al., 2021; Johnson, 1981; Li et al., 2022). With an estimated 219 million visits to national forests in 2019 and roughly $10 billion spent in nearby economies (Warren, 2020), the stakes can be high for these land managers. Unlike common logging operations, a tree failure can have serious repercussions, as visitors' lives and property are at risk and affected parties are likely to seek compensation if damage occurs due to a tree failure (Johnson, 1981; Mortimer & Kane, 2004). As an attempt to mitigate risk to visitors, hazard tree evaluations have been developed specifically for established recreation sites such as campgrounds and picnic areas (Blodgett et al., 2021; Johnson, 1981). These visual evaluations allow land managers to better assess hazardous locations within a site so they can respond proactively with management actions (Johnson, 1981).

Wood rotting fungi grow deep within the tree, degrading xylem and heartwood tissues. The internal nature of decay makes identification difficult without destructive sampling. This problem is exacerbated with root decay fungi. Management of these fungi often proves difficult because of a lack of diagnostic above ground symptoms and persistence of decay in a stand. As

the number of trees infected with root decay fungi increases, so does the risk of tree failure. An accurate identification of wood decay pathogens when evaluating for hazard trees in a site is vital to developing vegetation management plans and proactive risk mitigation strategies.

Tomentosus root rot (also known as red root and butt rot) is caused by fungi in the genus *Onnia*, which cause white pocket rot primarily in conifers. *Onnia* spp. are found globally in temperate and boreal forests of the Northern Hemisphere and infections can be tedious to identify due to a lack of diagnostic features when fruiting bodies are not available (Ji et al., 2017; Lewis, 1997; Lockman & Kearns, 2016). The most common symptom found in association with this disease is a white pocket rot that preferentially decays the heart wood during initial infections but progressively spreads outward into the sapwood (Lewis, 1997; Lewis et al., 1992; Myren & Patton, 1971). Infections are thought to begin in small roots, damaged wood near the root collar, or through root contact with the roots of infected trees or stumps (Myren & Patton, 1971). Decay columns caused by *Onnia* spp. expand from the roots up into the root crown and lower bole of the tree. As the decay advances in the roots, the structural integrity of the tree diminishes, and the risk of mechanical failure increases. Unfortunately, white rot is not unique to *Onnia* spp. Several other fungal genera, including *Porodaedalea* spp. cause white pocket rot of the stem and during advanced infections can colonize the root collar and/or roots (Hunt, 1997; Hunt & Ekramoddoullah, 1996). This makes identifying Tomentosus root rot based solely on the presence of white pocket rot imprecise. The management strategies for decay fungi that reside primarily in the stem are different from managing fungi that exist in roots. Being able to quickly distinguish *Onnia* from other white pocket rot fungi is important for landowners.

Previous molecular investigations of *Onnia* have focused on a global perspective of the genus, which have included only a few representative isolates from any specific region (Ji et al.,

2017; Zhao et al., 2022). Also, a limited number of molecular based studies have been conducted on the diversity of North American samples of *Onnia* spp. Currently, it is hypothesized that three species of *Onnia* spp. exist in North America *O. tomentosa* (formerly *Inonotus tomentosus, Coltricia tomentosa, Polyporus tomentosus*) (Karst, 1882), *O. subtriquetra* (formerly *O. circinata*, and *I. circinatus*) (Xiao-Hong, 2017), and *O. leporina* (formerly *O. circinata*, *I. circinatus*, and *I. leporinus*) (Germain et al., 2002; Jahn, 1978; Ji et al., 2017; Zhao et al., 2022). Due to recent taxonomic changes and reliance on morphological features for identification, each species' distribution in North America and host association is unclear (Ginns, 2017; Lockman & Kearns, 2016). Because molecular backed evidence of host association is lacking, landowners must make decisions based on historical studies that relied on morphological features for species identification. A molecular based analysis of North American samples of *Onnia* spp. would allow for a more accurate assessment of geographic distributions and host associations, which have previously been characterized primarily based on morphological features.

Many tools, such as PCR primers and DNA extraction techniques, have been developed for the identification of wood rotting fungi (Gonthier et al., 2015). However, these tools often require a considerable amount of laboratory training, specialized equipment, and time. There exists a need for a fast and reliable molecular method for the detection of *Onnia* spp. in North America that requires minimal training and equipment. Therefore, to-aid in the development of management tools and strategies for *Onnia* spp., our objectives are to investigate host associations and geographic distribution of North American *Onnia* species, and to develop a field based molecular marker to detect North American *Onnia* spp. The recent division of *O. circinata* into *O. leporina* and *O. subtriquetra* led us to question whether *O. tomentosa* is also composed of cryptic species. Our aim is to determine if *O. tomentosa* is composed of a single or multiple

genetic lineages or species and if multiple, we expect that these lineages will separated be based on host associations.

2.2 METHODS

2.2.1 Samples

Samples of *Onnia* spp. were acquired from field surveys and from donations or loans from forest pathologists and from two herbaria, the Center for Forest Mycology Research (CFMR), USDA Forest Service, Northern Research Station, Madison, WI, U.S.A, and the Laurentian Forestry Center of Natural Resources, Canadian Forest Service Station, Sainte-Foy, Québec, Canada. Genera other than *Onnia* were also collected as non-target species to test the specificity of the newly developed molecular assay. Non-target species were collected based on their phylogenetic relationship to *Onnia* spp., morphological similarity to *Onnia* spp., co-occurrence with *Onnia* spp., and general abundance in North America.

Using past Forest Service site reports, areas with known instances of *Onnia* spp. basidiocarps and white pocket rot were used to select areas for surveys (Lockman & Kearns, 2016). Field samples of *Onnia* spp., including basidiocarps and wood cores were collected from symptomatic and surrounding susceptible host trees. An increment borer was used to extract cores from each cardinal direction at the root collar. cored (Haglof Increment core, Madison, Mississippi, USA). Increment borers were cleaned with 90% ethanol between trees to minimize the risk of cross-contamination. Wood cores were placed in sterile tubes and stored in a cooler with ice packs for up to 14 days.

Wood cores with white pocket rot, red staining due to incipient decay, or other indications of wood rotting fungi were plated on selective agar media in petri dishes for fungal isolation. From each symptomatic tree core, areas at the edge of the infected tissue were sampled, whereas for cores with no symptoms of infection, wood samples were taken from the heart wood (Lewis et al., 1992). Sampled wood cores were cut into five pieces approximately 1x1 mm^2 in size and then cut in half (ca. 10 pieces). Five pieces were directly plated onto 2% malt agar (2% Malt extract powder, HIMEDIA, Kennet Square, PA, U.S.A) amended with 10mg benomyl and 10 mL streptomycin (BSMA) and the other five were surface sterilized by soaking for 30 s in 90% ethanol, followed by soaking for 30 s in 10% bleach, and rinsing twice with sterile water (Worrall 1991). The wood pieces were then briefly dried with a sterile paper towel and plated onto 2% BSMA. Fungal growth originating from the wood pieces was then isolated onto 2% malt agar (MA) using the hyphal-tipping method.

2.2.2 DNA extraction and PCR

Five agar plugs from actively growing cultures were placed in 75ml of 2% malt broth that was left stationary at room temperature and in the dark (Hunt & Ekramoddoullah, 1996). Once hyphal growth from the agar plug was approximately 2.5 cm in diameter, mycelia were filtered under vacuum using Whatman #4 filter papers (Whatman™) and DNA was extracted using OPS SYNERGY 2.0 Plant DNA extraction Kits (OPS Diagnostic, Lebanon, NJ, U.S.A). Sample homogenization was performed using a FastPrep-24 (MP Biomedicals, Santa Ana, CA, U.S.A) with a setting of 5.5 for 30 sec for 3 rounds. Dried fruiting bodies were ground with mortar and pestle in liquid nitrogen prior to kit extraction. Extractions were completed based on the

manufacturer's protocol except that the extraction buffer amount was increased to 0.75mL for extracting fruiting bodies. For herbarium samples that failed to amplify, a modified CTAB extraction method was used (Cubero et al., 1999; Kozhar et al., 2023). Field-based DNA extractions from wood were achieved using a 200 µL of 5% Chelex 100 (BioRad; Walsh et al, 2018) and 2% polyvinylpyrrolidone (PVP) for every 0.005g of wood. Samples were heated at 98ºC for 30 minutes and cooled for 5 min. Wood cores used in the development of the LAMP assay were also extracted in a lab setting with the modified CTAB method (Cubero et al., 1999; Kozhar et al., 2023).

Samples were confirmed as *Onnia* spp., or other closely related species for the development of the molecular assay by sequencing PCR amplicons of the internal transcriber spacer region using primers ITS1f (5′-CCT GGT CAT TTA GAG GAA GTA A-3′; Gardes & Bruns, 1993) and ITS4 (5′-TCC TCC GCT TAT TGA TAT GC-3′; White et al., 1990). *Onnia* spp. samples were additionally sequenced using the RNA polymerase II 2nd largest subunit (*rpb2*) primers fRPB2-5F (5′-GAY GAY MGW GAT CAY TTY GG-3′; Matheny, 2005) and fRPB2-7cR (5′-CCC ATR GCT TGY TTR CCC AT-3′; Liu et al., 1999), translation elongation factor alpha 1 (*tef1a*) primers EF1-983F (5′-GCY CCY GGH CAY CGT CAY TTY AT-3′; Matheny, 2007) and EF1-1567R (5′-ACH GTR CCR ATA CCA CCS ATC TT-3′; Rehner & Buckley, 2005), and the large subunit of nuclear ribosomal RNA gene (LSU) primers LR0R (5′-ACC CGC TGA ACT TAA GC-3′; Zhao et al., 2022) and LR7 (5′-TAC TAC CAC CAA GAT CT-3′; Vilgalys & Hester, 1990).Amplification was visualized using a 2% agarose gel with electrophoresis at 60V for one hour. Prior to Sanger sequencing, excess primers and dNTPs in the PCR products were degraded using EXOSAP-IT (Thermo Fisher Scientific, Waltham, MA, U.S.A), using the recommended thermocycler program of 37°C for 15 minutes and then 80°C for 15 minutes. When a PCR

product of an herbarium sample contained two amplicons, bands were cut out and then the amplicons were extracted using a Zymoclean™ Gel DNA Recovery Kit (Zymo Research). Purified PCR amplicons were sequenced at Eurofins Genomics (https://eurofinsgenomics.com/en/home/). Species were identified by using the ITS sequences to search for similar sequences in National Center for Biotechnology Information (NCBI) GenBank with the basic local alignment search tool (BLAST) (https://www.ncbi.nlm.nih.gov/).

2.2.3 Phylogenetic analyses

All sequence data were initially edited, aligned and analyzed in Geneious Prime® 2024.0.5 (). Forward and reverse sequences from *Onnia* spp. and non-target species were aligned using the MAFFT algorithm with default settings (Katoh & Standley, 2013) and modified manually using IUPAC codes where heterozygous sites occurred. A North American isolate of *Porodaedalea pini* was used as an outgroup as in previous studies of *Onnia* spp. (Ji et al., 2017a; Zhao et al., 2022). Substitution models for each locus were determined using the Bayesian information criterion and symmetry tests performed using IQ-TREE2 (Kalyaanamoorthy et al., 2017; Minh et al., 2020; Naser-Khdour et al., 2019).

Alignments were formatted using the program Beauti prior to Bayesian phylogeny construction in BEAST2 (Bouckaert et al., 2019). The phylogenetic analyses were completed in BEAST using all four loci to produce a single linked phylogeny, whereas the clock and site models for each locus were unlinked (Bouckaert et al., 2019). Two reference sequences of *O. tomentosa*, one sample from North America (Dai23683) and one sample from China (Dai22935), were used in the partitioned Bayesian phylogeny. One hundred million chains were used, and the

first twenty percent of the trees were discarded as burn in. The trace files were assessed using the MCMC Trace Analysis Tool to ensure convergence (Rambaut et al., 2018). Trees were consolidated and posterior probabilities were produced using the program Tree Annotator (Bouckaert et al., 2019) and visualized using FigTree (http://tree.bio.ed.ac.uk/software/figtree/). Separate Bayesian phylogenies were also produced with an expanded dataset to include reference sequences and samples that were not sequenced at all four loci. For these phylogenies, 10 million chains were sufficient for convergence. A concatenated dataset of all four loci was used in the construction of a maximum likelihood phylogeny (ML) with 200 bootstraps using IQ-TREE2 (Kalyaanamoorthy et al., 2017; Minh et al., 2020; Naser-Khdour et al., 2019). Support values were considered statistically significant when they exceeded 0.70 for posterior probability in the Bayesian phylogenies and 50% bootstrap support for maximum likelihood phylogenies.

O. tomentosa samples were placed into lineages based on their location on the Bayesian phylogeny. To assess the influence of geographic location and host association on lineage formation, each samples characterized as belonging to one of four regions, Northeast, Northwest, Southeast, and Southwest. Region was determined by relation to the approximate geographic center of North America (44.422, -100.351). Lineage dissimilarity was assessed by host genus and region using Bray-Curtis distance with statistical significance calculated through a PERMANOVA using the vegan package in R (Oksanen et al., 2020).

The hypothesis of recombination within species was tested with the program Splitstree2 (Huson & Bryant, 2006). P-distances between samples were calculated using a concatenated dataset of all four loci and a neighbor-net phylogenetic network was constructed based on these distances (Bryant & Huson, 2023; Hamming, 1950). Using SplitsTree2, a principal coordinate analysis (PCoA) based on the calculated P-distances was produced for the concatenated data set.

Implemented in Splits trees, we calculated the pairwise homoplasy index (Φ_w) separately for *O. tomentosa* and *O. subtriquetra* sequences (Bruen et al., 2006). This measurement infers the likelihood of recombination within a set of samples. A test statistic of $\Phi_w < .05$ was considered to be statistically significant evidence of recombination. This was calculated by determining the number of convergent mutations needed for two sites to be the same.

2.2.4 Primer development

The molecular marker was developed as a loop mediated isothermal reaction (LAMP) assay. This assay consists of four required primers and two supplemental loop primers that aid in amplification (Nagamine et al., 2002; Notomi et al., 2000). The ITS, LSU, *tef1a*, and *rpb2* were assessed for primer viability using an alignment of sequences produced using MAFFT within Geneious Prime. Loci were determined to be viable for primer design if they had low variation within *Onnia* spp. Reference sequences of *Onnia* spp., downloaded from GenBank, were also used for determining the usability of each locus for a LAMP assay. The *tef1a* locus was determined to be suitable for LAMP assay primer development because of its low variation within the genus *Onnia* and its high variation when compared to non- target species. Using *Onnia tomentosa* isolate C15, a 448 bp long region was imputed into Primer explorer V5 (http://primerexplorer.jp/e/index.html) to produce prospective primer sets. The *tef1a* region was categorized as adenine and thymine rich, so the "AT rich" setting was used. To assist with amplification, four thymine's were added as a linker between Fc1 primer and F2 primer and between the Bc1 and B2 primers (Lamas et al., 2023). The 5' stability ΔG threshold was changed from the default −3°C to −4°C (Kozhar et al., 2023).

2.2.5 LAMP assay conditions and validation

To allow for visual identification of amplification, NEB WarmStart Colorimetric LAMP 2X Master Mix (New England Biolabs) was used (12.5 µL per sample) for all reactions. Primer concentrations were as follows: F3 and B3 primers (0.2 µM each), LoopF and LoopB primers (0.4 µM each), FIP and BIP primers (1.6 µM each). Template DNA (1 µL) or wood core extractions (2 µL) were added to the reaction tube containing primer mixture (2.5 µL) and molecular grade H_2O (9 µL) for a total reaction volume of 25 µL. Reactions were placed in a MyBlock mini dry bath (Benchmark Scientific, Sayreville, NJ) set to 65°C for 35 min, and then samples were assessed for color change. Amplification was determined when a sample changed color from pink (negative) to yellow (positive).

To validate our newly designed molecular marker (LAMP assay), DNA extracted from wood cores (see above) were amplified using a genus specific primer set ITS3 (5′-CTT GGT CAT TTA GAG GAA GTA A-3′; White et al., 1990) and ONR2 (5′-AAG TCC CTT TTC CTT TCA GT-3′; Gonthier et al., 2015). However, the efficacy of this primer set for *O. subtriquetra* needed to be confirmed due to the recent description of this species. Therefore, six samples of *O. subtriquetra* and one isolate of *Porodaedalea* spp. were amplified. Thermocycler programs followed the methods of Zhao et al. (2022) for all primer sets except ONR2 and ITS3 which followed (Gonthier et al., 2015). Unless otherwise specified, PCR reagent mix consisted of 0.4µM of forward primer, 0.4 µM of reverse primer, 12.5µL of GoTaq Green, 9.5µL of molecular grade H20, and 10ng of template DNA with a total reaction volume of 25µL. Amplification of the samples were verified with electrophoresis, samples were cleaned with EXOSAP-IT, and sequenced at Eurofins Genomics.

2.2.6 Specificity and sensitivity

Specificity was determined by testing the LAMP assay against 30 samples of *Onnia* spp., comprising 23 of *O. tomentosa,* six of *O. subtriquetra,* and one of *O. leporina.* These samples were chosen for their genetic variation as well as their geographic diversity. Multiple tissue types were tested as well. Additionally, 12 non-target samples were tested against the LAMP assay (Table 2.3). To determine the sensitivity of the LAMP assay, the DNA concentration of one sample each of *O. tomentosa, O. subtriquetra,* and *O. leporina* were measured using a Qubit 4MT with a dsDNA HS Assay Kit (Invitrogen, Thermo Fisher Scientific, Waltham, MA, U.S.A) in triplicate and the average of the three measurements was used. A series of two- fold dilutions were made until the final concentration was ≤ 10 pg/µL. A LAMP reaction was run at each dilution using 1 µL of DNA. Each test had two technical replicates run on separate days.

2.2.7 Sample diversity and field applications

Multiple types of samples were used to test the versatility of the *Onnia* LAMP assay, including herbarium samples, infected wood samples, and DNA extracted from pure cultures (Table 2.3). To test the viability of the LAMP assay in the field, two field collection trips occurred to areas surrounding Durango, CO and Cabin City, MT. Wood cores were sampled from symptomatic and nearby asymptomatic trees. DNA was extracted from wood cores with a 5% Chelex solution amended with 2% PVP within 12 hours of collection. Additional wood samples were stored on ice and, once in the lab, were extracted with a modified CTAB protocol (Cubero et al., 1999; Kozhar et al., 2023). Initially, samples were confirmed to be *Onnia* spp. through the amplification of ITS3 and ONR2 (Gonthier et al., 2015) as well as the amplification and sequencing of the ITS using primers ITS1F and ITS4 to confirm sample species (White et al.

1990). In order to determine if a wood sample without *Onnia* spp. DNA negatively impacted the LAMP assay, we applied 1µL of crude wood core extract to our sensitivity assay of *O. tomentosa.* Previous studies and reports have shown that a LAMP assay is able to amplify crude DNA extract from wood (Atkinson et al., 2017; Hamilton et al., 2020; Kozhar et al., 2023). The wood core used to test the effects was from an asymptomatic tree in an area with no indication of root diseases.

2.3 RESULTS

2.3.1 Sample collection

In total, 58 samples of *Onnia* spp. were sequenced at all four loci and used in phylogenetic analyses. Eighty-one fruiting bodies and 40 cultures were loaned from herbaria and culture collections. Twenty-two basidiocarps were collected and 21 cultures were isolated from infected wood or fresh basidiocarps. Sequencing at all 4 loci revealed a total of 47 North American samples of *O. tomentosa*, ten samples of *O. subtriquetra* and one isolate of *O. leporina*. The morphology of hymenial setae was observed for all fruiting bodies, and all *O. tomentosa* fruiting bodies had straight setae and all *O. subtriquetra* had hooked setae (Supplemental Figure 2.1**)**. *O. tomentosa* samples were found in association with *Picea glauca*, *Picea engelmannii*, *Picea lutzii*, *Picea mariana*, *Pinus rigida*, *Pinus monticola*, *Pinus strobus*, *Abies bifolia*, *Abies balsamea*, *and Larix lyallii* (Supplemental Figure 2.1). With 76% of samples being found in association with *Picea* spp.**)**. All *O. subtriquetra* samples were found in association with *Pinus muricata*, *Pinus contorta* var. *contorta*, *Pinus contorta* var. *latifolia*, *Pinus elliottii*, *Pinus echinata*, and *Pinus virginiana* except for a single isolate found on the forest floor near a *Quercus* sp. (Supplemental Figure 2.1**)**.

The sole *O. leporina* sample was found in association with *Picea glauca*, and there were no fruiting bodies of *O. leporina* to observe setae morphology.

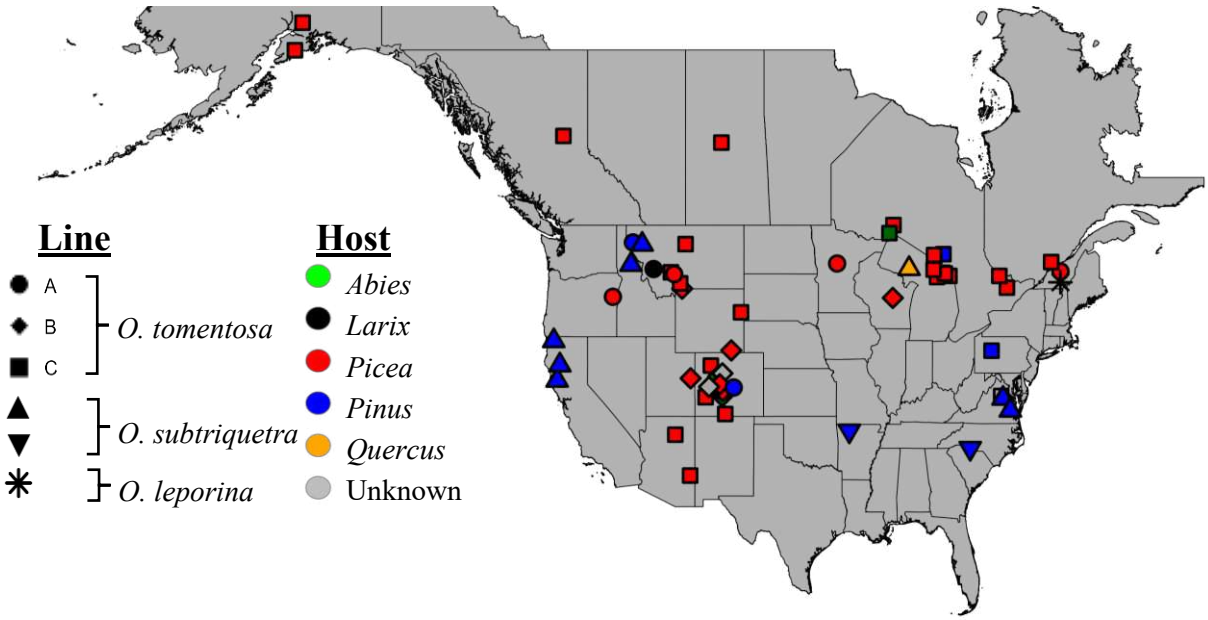

Figure 2.1. Map of location where samples were collected or originated in North America. Isolate lineages, determined by phylogenetic analyses, are indicated through shape and host

2.3.2 Phylogenetic analysis

The four loci used in this study across our samples were conserved with 81-95% of sites being constant across *Onnia* spp. (Table 2.1). The LSU was the most conserved locus with 1,327 sites of 1,391 being invariant across all species of *Onnia* observed. The ITS had the greatest variation among samples with 81% of sites being invariant. The model tests produced by IQTREE2 provided the same base substitution model for the *tef1a*, LSU, and the *rpb2*, (Table 2.1). The statistical tests for homogeneity and stationarity provided evidence that the common assumptions made for phylogenetic analyses were met for each locus (Table 2.1). These tests showed that nucleotide frequency (homogeneity) and substitution rate (stationarity) for each

locus remain constant over time. All four loci were used in phylogenetic analyses. Both the partitioned Bayesian and concatenated ML phylogenies had few nodes considered as statistically significant (PP>0.7, BB>50%). However, both the ML and Bayesian phylogenies separated *O. tomentosa, O. subtriquetra,* and *O. leporina* into well supported clades (Figure 2.2 and Supplemental Figure 2.1). Both the ML and Bayesian phylogenies separated the reference sequence from China (*O. tomentosa* Dai22935) from the North American *O. tomentosa* samples with significant support (PP>0.7, BB>50%) (Figure 2.2 and Supplemental Figure 2.1), showing that the *O. tomentosa* sample from China was distinct from the North American samples of *O. tomentosa.* The ML and Bayesian phylogenies both produced three clades within the larger O. *tomentosa* clade. One isolate from Montana, T166, was an exception to the similarities between Bayesian and ML phylogenies. This sample was basal to all North American samples of *O. tomentosa.* This isolate formed its own well-supported clade distinct from the remaining North American isolates (Supplemental Figure 2.1).

Table 2.1. Models of evolution used for Bayesian analyses.

					Symmetry test		
Loci	**Model**	**Sites**	**Informative**	**Invariant**	**SymPval[A]**	**MarPval[B]**	**IntPval[C]**
tef1	Tne+I	587	35	483	0.14	0.08	0.45
ITS	HKY+F+G4	714	41	584	0.27	0.10	0.71
LSU	TN+F+I	1391	19	1327	0.18	0.18	0.26
rpb2	Tne+G4	1083	69	928	0.35	0.73	0.15

[A]P-value test of symmetry

[B]P-value for test of marginal symmetry

[C]P-value for test of internal symmetry

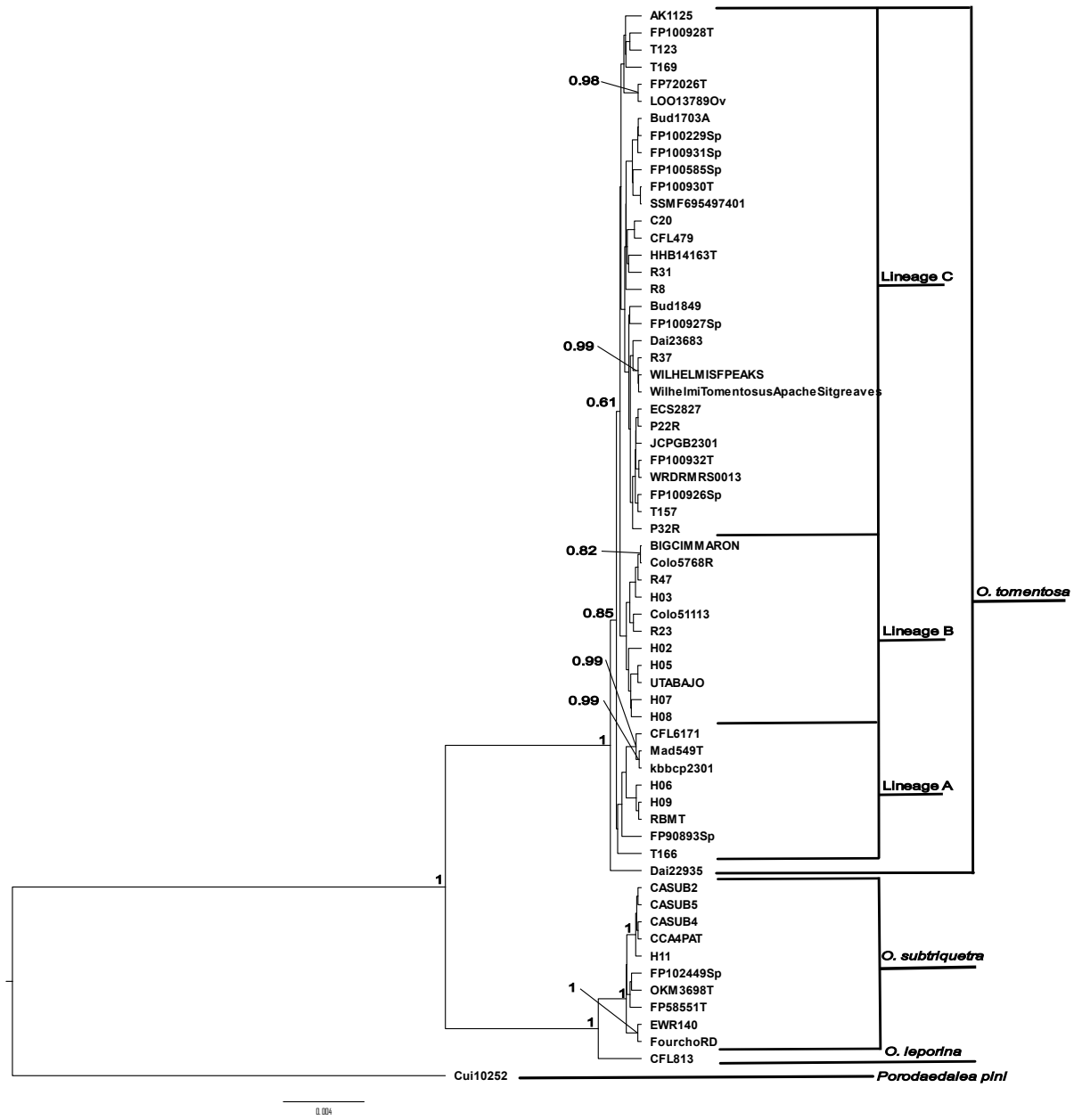

Figure 2.2 Consensus phylogeny with Bayesian analyses based on a partitioned dataset of the ITS, LSU, *tef1*, and *rpb2*. Node support is highlighted above each node as posterior probability (PP).

Not all samples collected were used in the four-locus partitioned dataset. Separate phylogenies were generated for each locus independently with the available and relevant *Onnia* spp. sequences downloaded from GenBank (Supplemental Figures 2.5). Little congruence was observed among the Bayesian phylogenies produced with the single loci compared to the larger partitioned dataset produced in BEAST2 (Supplemental Figures 2-5). The ITS and *tef1a* both separated the three North American species. The LSU did not separate the three species with a high degree of support (Supplemental Figure 2.4). The *rpb2* successfully discriminated *O. tomentosa* from *O. leporina* and *O. subtriquetra* but did not separate *O. subtriquetra* from *O. leporina* with statistically significant support (Supplemental Figure 2.5).

2.3.3 Neighbor-net Phylogenetic Network

When all three species were present in the neighbor-net phylogenetic network of the concatenated four-locus dataset, a clear differentiation among *Onnia* species was observed (Figure 2.3), with the greatest difference between *O. tomentosa* and the other two species of *Onnia* (*O. leporina* and *O. subtriquetra*). In addition, *O. tomentosa* separated into three distinct clades (Figure 2.5). These three clades share similarity with the three lineages produced by the Bayesian and ML phylogenies (Figure 2.5). All samples in lineage A (Figure 2.2) except for four isolates from Montana on *Picea engelmannnii*, T166 and the addition of T169, T157, and T123, formed a group in the neighbor-net phylogenetic network (Supplemental Figure 2.6). Lineage B (Figure 2.2) also formed a distinct group with the addition of R21 (Supplemental Figure 2.6). Lineage C (Figure 2.2) loosely groups together, with samples joining Lineage A and B (Supplemental Figure 2.6). A visual analysis of the phylogenetic network of *O. tomentosa* and *O.*

subtriquetra showed reticulation between samples, suggesting a history of recombination (Figure 2.5 and 2.6).

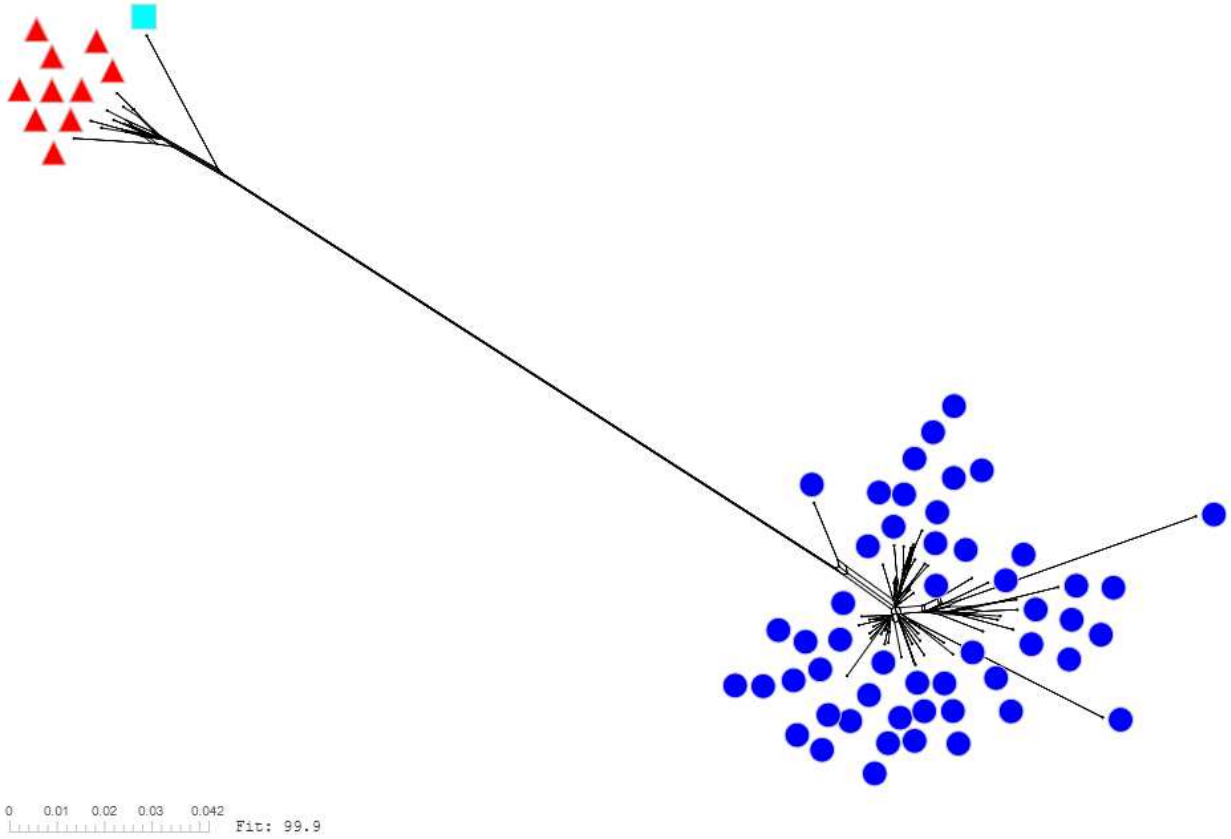

Figure 2.3 Neighbor-net phylogenetic network of the concatenated dataset for *O. tomentosa* (in blue), *O. subtriquetra* (in red), and *O. leporina* (in Cyan).

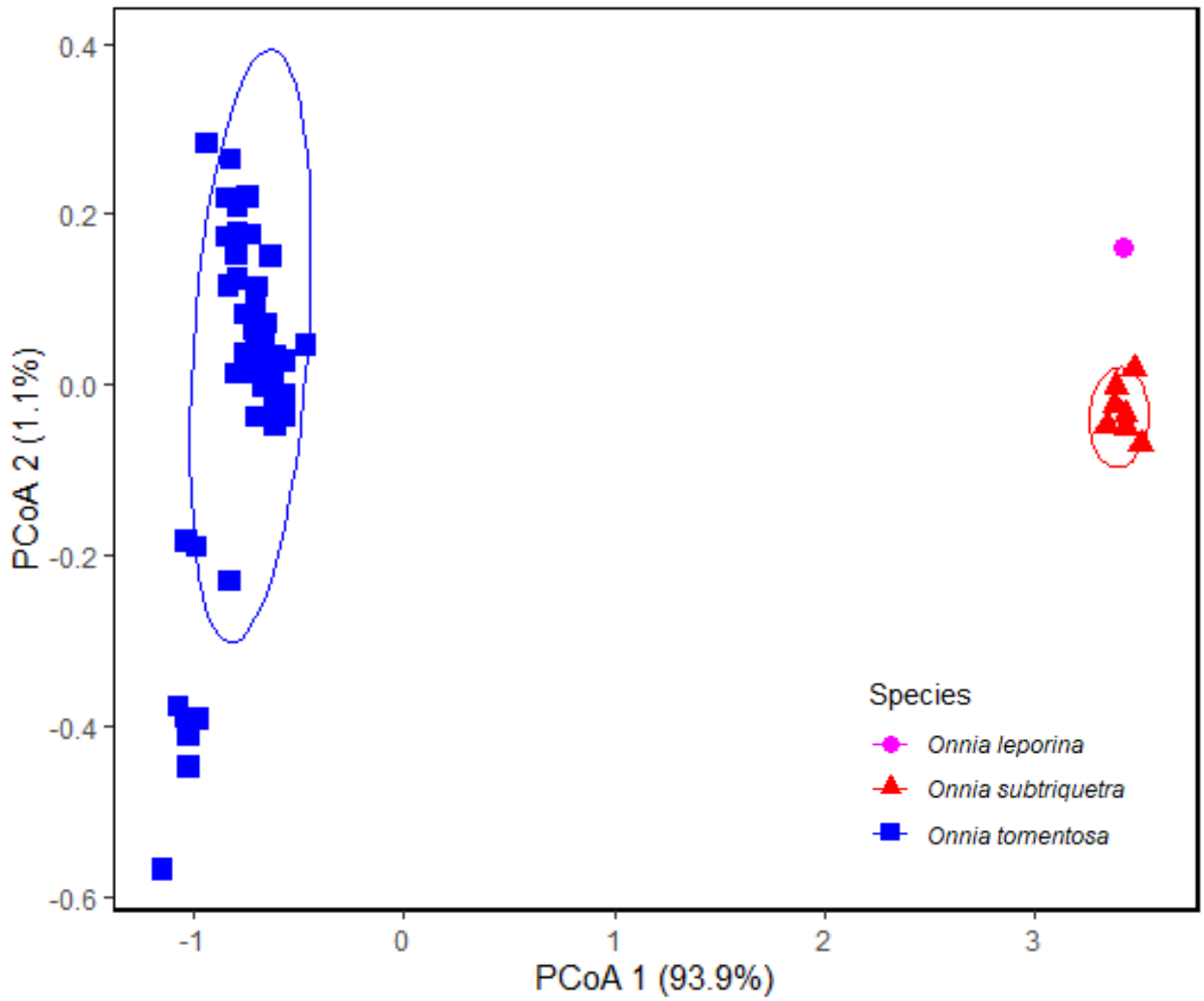

Figure 2.4 A principal coordinate analysis (PCoA) of P distances (a measurement of diversity between samples) for the concatenated dataset of all *Onnia* species. Isolate species is indicated by color.

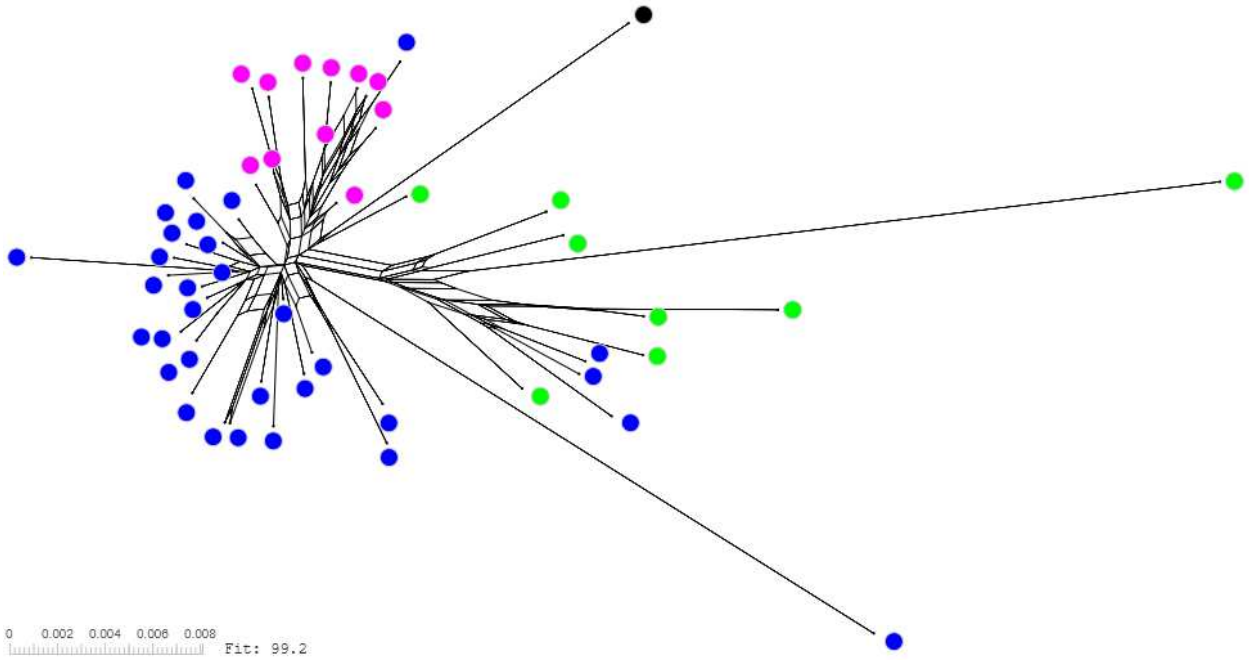

Figure 2.5 A near neighbor splits tree of the concatenated dataset for *O. tomentosa.* Nodes are colored by lineage as designed by the phylogenic analyses: Clade A (green), clade B (fuchsia), Clade C (blue), and the Chinese reference sequence (black).

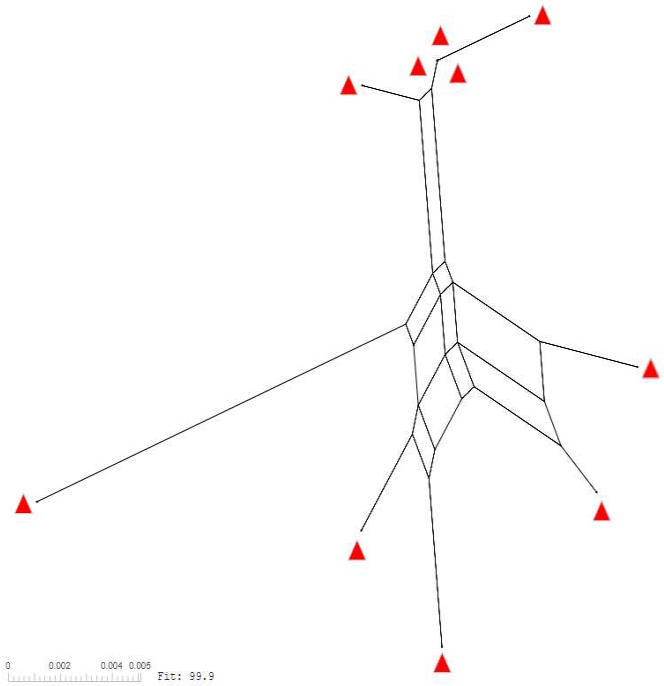

Figure 2.6 A near neighbor splits tree of the concatenated dataset for *O. subtriquetra*.

2.3.4 Host Association, Geographic association, and Recombination.

A visual assessment of the geographic distribution of the lineages produced by BEAST and ML analyses showed some geographic separation (Figure 2.1). For example, clade B is found predominately in the west, and clade A is found more in the North. No significance was observed in the PERMANOVA when examining if North American samples of *O. tomentosa* were separated into clades based on host association (R^2 = 0.353, F = 0.956, P =0.471), but geography was a significant predictor of clade formation (R^2 = 0.582, F =3.714, P =0.012). With a Φ_w of 0.91, we did not find statistically significant evidence to support recombination between all samples of *O. tomentosa*. We did find statistically significant evidence, ($\Phi_w = 0.008$), to support recombination among *O. subtriquetra* samples.

2.3.5 Primer Development

The *tef1a* was a suitable site for LAMP primer development due to low intraspecific variation of *O. tomentosa* and *O. subtriquetra* (Table 2.2). Multiple primer sets were designed and tested against representative samples of both *O. tomentosa* and *O. subtriquetra.* Due to the lack of available samples, only a single isolate of *O. leporina* was used in tests to determine the efficacy of the primer set. An analysis of the final primer set showed the primers to be on an area of high nucleotide variation when comparing *Onnia* spp. to off target species and low nucleotide variation when compared to *Onnia* spp. sequences. A four base-pair indel can be found in *O. tomentosa* samples that is not present in *O. subtriquetra* or *O. leporina*. This insertion is on the forward loop primer (Figure 2.9).

Table 2.2 Newly designed loop mediated isothermal reaction (LAMP) primers with thymine linkers in lowercase, and PCR primers used to amplify loci.

LAMP Primer ID	Sequence	
F3_Set_M2	CGT CAA CAA AAT GGA TAC CAC	
B3_Set_M2	AAC ATG TTG TCA CCG TGC	
FIP_Set_M2	AAC GAT CCT CGG ACC ACT GAttttCAA GGT TCG CTT GCA TTG	
BIP_Set_M2	TCATTGAGATTGTCAAGGAAACGTCttttCCAGAAATCGGGACGAAAG	
LF_Set_M2	CTTTGTGCTTTTTTGAAACACGACC	
LB_Set_M2	GTTACAACCCGAAGGCCGTG	
PCR Primer ID	**Sequence**	**Reference**
ITS1f	5′-CCT GGT CAT TTA GAG GAA GTA A-3′	Gardes & Bruns, 1993
ITS4	5′-TCC TCC GCT TAT TGATAT GC-3′	White et al., 1990
ITS3	5′-CTT GGT CAT TTA GAG GAA GTA A-3′	White et al., 1990
ONR2	5′-AAG TCC CTT TTC CTT TCA GT-3′	Gonthier et al., 2015
LR0R	5′-ACC CGC TGA ACT TAA GC-3′	Zhao et al., 2022
LR7	5′-TAC TAC CAC CAA GAT CT-3′	Vilgalys & Hester, 1990
EF1-1567R	5′-ACH GTR CCR ATA CCA CCS ATC TT-3′	Rehner & Buckley, 2005

EF1-983F	5′-GCY CCY GGH CAY CGT CAY TTY AT-3′	Brandon Matheny et al., 2007
fRPB2-5F	5′-GAY GAY MGW GAT CAY TTY GG-3′	Matheny, 2005
fRPB2-7cR	5′-CCC ATR GCT TGY TTR CCC AT-3′	Liu et al., 1999

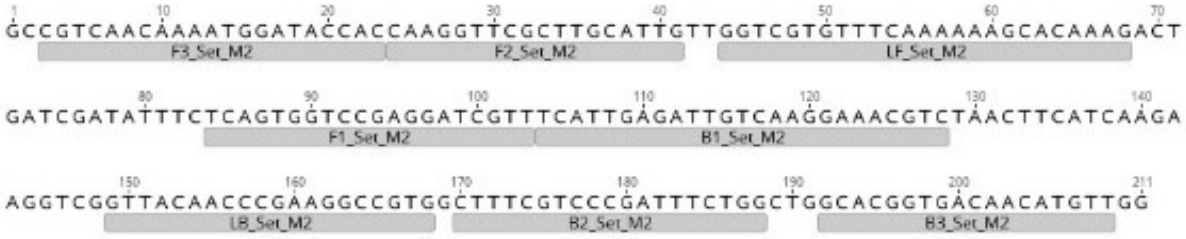

Figure 2.9 Location of loop mediated isothermal reaction (LAMP) primers on the *tef1a.*

2.3.6 Specificity and Sensitivity

The LAMP assay was effective in detecting *Onnia* from across North America (Table 2.3). The LAMP assay was able to reliably detect North American *Onnia* spp. samples at a sensitivity of 170 pg for *O. tomentosa*, 83 pg for *O. subtriquetra*, and 121 pg for *O. leporina* (Figure 2.10). We showed that the genus specific primer set developed by Gonthier et al. (2015) was able to successfully detect six samples of *O. subtriquetra,* which allowed us to use it as a lab-based tool to validate our LAMP results. An analysis of the ITS region and the primer set developed by Gonthier et al. (2015) showed that the primers are in a well conserved region for *Onnia* spp.

Table 2.3 *Onnia* spp. samples and non-target samples used to determine the efficacy of the loop mediated isothermal reaction (LAMP) assay designed.

Sample ID	Host Genus	Genus	Species	Origin	Sample Type	LAMP RESULTS
T123	*Picea engelmannnii*	*Onnia*	*tomentosa*	MT	Wood core	+
T169	*Picea engelmannnii*	*Onnia*	*tomentosa*	MT	Wood core	+
T157	*Picea engelmannnii*	*Onnia*	*tomentosa*	MT	Wood core	+
NICH-1	*Picea sp.*	*Onnia*	*tomentosa*	BC	Wood core	+
NICH-2	*Picea sp.*	*Onnia*	*tomentosa*	BC	Wood core	+
NICH-3	*Picea sp.*	*Onnia*	*tomentosa*	BC	Wood core	+
H10 (Apache-Sitgreaves)	*Picea engelmannnii*	*Onnia*	*tomentosa*	AZ	Dried FB	+
H11(Cabin City)	*Pinus contorta* var. *latifolia*	*Onnia*	*subtriquetra*	MT	Dried FB	+
CFL-813	*Picea glauca*	*Onnia*	*leporina*	BC	DNA	+
FP-58551-T	*Pinus* sp.	*Onnia*	*subtriquetra*	VA	DNA	+
FP-102449-Sp	*Quercus* sp.	*Onnia*	*subtriquetra*	MI	DNA	+
EWR-140	*Pinus elliottii*	*Onnia*	*subtriquetra*	SC	DNA	+
CASUB5	*Pinus muricata*	*Onnia*	*subtriquetra*	CA	DNA	+
OKM-3698-T	*Pinus virginiana*	*Onnia*	*subtriquetra*	MD	DNA	+
ECS-2826	Unknown	*Onnia*	*tomentosa*	ON	DNA	+
McWilliams Bear Creek	*Picea engelmannnii*	*Onnia*	*tomentosa*	OR	DNA	+
JC-PGB-23-01	*Picea engelmannii x glauca*	*Onnia*	*tomentosa*	BC	DNA	+
R8	Unknown	*Onnia*	*tomentosa*	CO	DNA	+
WRD-RMRS-0013f	*Picea glauca*	*Onnia*	*tomentosa*	SD	DNA	+
Mad-549-T	*Picea mariana*	*Onnia*	*tomentosa*	MN	DNA	+
LOO-13789-Ov	*Pinus rigida*	*Onnia*	*tomentosa*	PA	DNA	+
FP-72026-T	Unknown	*Onnia*	*tomentosa*	VA	DNA	+
RBMT	*Larix lyallii*	*Onnia*	*tomentosa*	MT	DNA	+
CFL6171	*Picea glauca*	*Onnia*	*tomentosa*	BC	DNA	+

H02	*Picea engelmannii*	*Onnia*	*tomentosa*	CO	DNA	+
R37	Picea engelmannii	*Onnia*	*tomentosa*	CO	DNA	+
UTABAJO	*Picea engelmannii*	*Onnia*	*tomentosa*	UT	DNA	+
WilhelmiSFpeaks	*Picea engelmannii*	*Onnia*	*tomentosa*	AZ	DNA	+
FP100926Sp	*Picea glauca*	*Onnia*	*tomentosa*	ON	DNA	+
La Sal Horse Creek Tomentosus 1	*Picea engelmannii*	*Onnia*	*tomentosa*	UT	DNA	+
ST-1		*Armillaria*	*solidipes*		DNA	-
ST-8		*Armillaria*	*gemina*		DNA	-
RM-092922		*Coltricia*	ssp.	AK	DNA	-
RM-060617		*Coltricia*	ssp.	AK	DNA	-
JSA-IDA-23-02		*Porodaedalea*	ssp.	ID	DNA	-
H100		*Porodaedalea*	ssp.	CO	DNA	-
H101		*Phellinus*	*Hartigii*	CO	DNA	-
FRIM-617		*Phellinus*	*Noxious*	CO	DNA	-
H95		*Fomitopsis*	*orchacea*	CO	DNA	-
FP-JW-GUN		*Fomitopsis*	*pinicola*	CO	DNA	-
GR-CNP-20-01		*Heterobasidion*	ssp.		DNA	-
CP-JW-GUN		*conoiphora*	*puteana*	CO	DNA	-

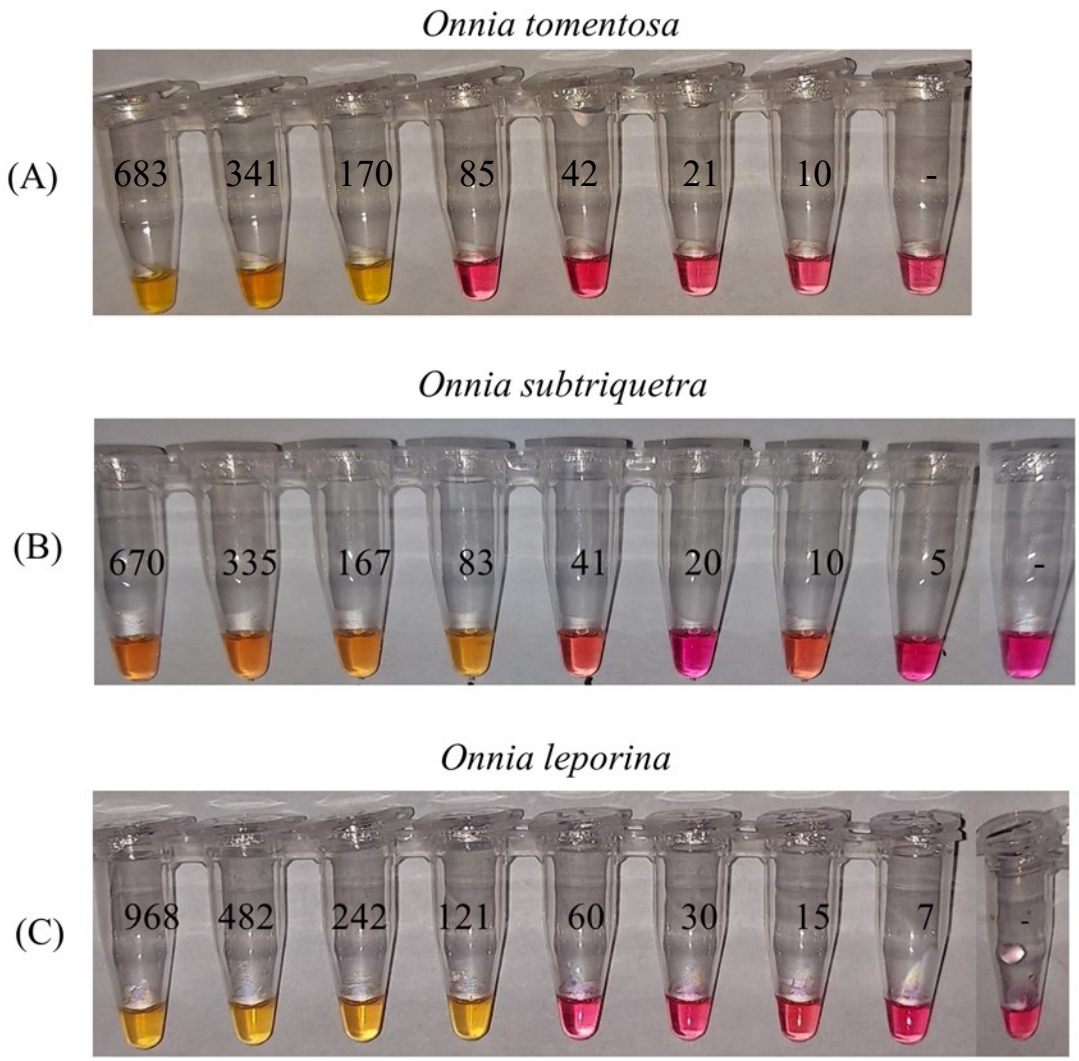

Figure 2.10 Results of a two-fold dilution assay (in picograms) for each of the three North American species of *Onnia* spp. (A = *O. tomentosa,* B = *O. subtriquetra*, C = *O. leporina*). Yellow tubes indicate a positive result, and red tubes indicate a negative result. This is the limit of detection of LAMP assay. The PCR tube furthest to the right of each dilution assay is the negative control.

2.3.7 Sample diversity and field applications

The LAMP assay was shown to be an effective test for common sample types including crude wood core extract, fruiting body, and kit extracted DNA (Table 2.3). The addition of *Picea* sp. wood extract did not inhibit the assay. The molecular tool developed here did not react when tested against fungi that commonly co-occur with *Onnia* spp. (Table 2.3). The reagents were able to successfully discriminate against target and non-target DNA after remaining at 4°C for 24 hours, though there is minor color change if kept at 4°C for an extended amount of time. During one trip, premixed reagents were exposed to fluctuating temperatures for 48 hours. Premixed reagents were regularly above 4°C and as a result, false positives occurred. We discovered that past 24 hours, there is a possibility of excess primer dimer formation and false positives. Crude DNA extracts containing *Onnia* spp. were successfully detected using the LAMP assay when processed using PVP and Chelex (Table 2.3). Samples that were extracted and tested positive using the LAMP assay, were also shown to contain *Onnia* spp. DNA using common molecular tools (PCR).

2.4 DISCUSSION

2.4.1 Onnia spp. in North America

We observed three distinct species of *Onnia* spp. within North America and our study sheds light on the host association of *O. subtriquetra* and the broad host range of *O. tomentosa*. We found evidence that *O. tomentosa* does not form distinct lineages based on host association, but rather lineages may be influenced by geographic region. While the data presented here provides an updated distribution of *O. tomentosa* and *O. subtriquetra* in North America, the

distribution of *O. leporina* remains unclear. Even though we sampled a wide geographic area of the United States, only one sample was identified as *O. leporina*, suggesting that this species may be distributed primarily in the boreal forests of Canada. The LAMP assay is a robust tool for investigating *Onnia* spp. in North America and may be useful in other regions of the world. The data and tools presented in this research provide a solid foundation for understanding the host specialization and geographic range of *O. tomentosa* and *O. subtriquetra* and provides support for future investigations of North American instances of *Onnia* spp.

The phylogenetic analysis and neighbor-net phylogenetic network supported the separation of the three *Onnia* species, with *O. leporina* and *O. subtriquetra* being more closely related to each other than to *O. tomentosa*, concurring with the global analyses of Zhao et al. (2022). This research provided evidence to support the existence of three *Onnia* species in North America which was first hypothesized by Ji et al. (2017). In our study, *O. tomentosa* was found across North America, which challenges the implication that North American *O. tomentosa* primarily occurs in boreal and sub boreal forests of the continent, as suggested by previous studies (Lewis et al., 1992, 2004; Woods & Watts, 2019). Likely, these previous results were premature because many of the studies focused on only Canadian isolates rather than including a wider distribution of *O. tomentosa* within different forest types. While *Onnia* spp. is a major damage agent in the boreal forests of North America, it is also an important organism in conifer forests throughout North America. The distribution of *O. tomentosa* presented here aligns with historical accounts of the species which has been documented throughout conifer forests in North America (Gilbertson & Ryvarden, 1986).

Our research supports the hypothesis that *O. subtriquetra* is a distinct North American species from *O. leporina* (Ji et al., 2017; Zhao et al., 2022). None of our collections of *O.*

subtriquetra were from Canadian provinces or Alaska, yet this does not preclude the presence of the species outside of the contiguous United States. It is hypothesized *O. subtriquetra* may have been introduced to North American via Asia (Ji et al., 2017), therefore further sampling within Alaska and Canada is warranted. Of the ten samples identified as *O. subtriquetra* via DNA sequencing, nine were found in association with *Pinus* spp. and one was found in association with *Quercus*. This is surprising due to the prevailing hypothesis that *Onnia* spp. are gymnosperm specialists (Ji et al., 2017; Palla et al., 2023). This specific *O. subtriquetra* isolate was a loaned culture and the only collection notes on host association were "*Quercus*, ground under living". *O. subtriquetra* is known to grow on the forest floor near infected trees, so it is not unreasonable to assume that the fruiting body was on a *Pinus* sp. root near the *Quercus* sp. (Lee et al., 2024). However, no studies have examined the pathogenicity of *O. subtriquetra,* so an infection of *Quercus* is not impossible. Inoculation studies would be required to determine whether *O. subtriquetra* is capable of colonizing oaks.

We were only able to retrieve a single isolate of *O. leporina*, and we were not able to demonstrate the distribution of *O. leporina* or make any inference on host association. As such, there is a need for future research to better characterize the distribution and host range of *O. leporina.* Regarding historical accounts of the now defunct *O. circinata* (*Inonotus circinatus, Polyporus tomentosus* var. *circinatus*), the taxonomic classification of *O. circinata* was based solely on the existence of hooked hymenial setae, a morphological feature shared by both *O. leporina* and *O. subtriquetra*. As a result of this, literature regarding *O. circinata* represents data for two different species. Based on our current results, we believe that historical accounts of *O. circinata* in association with *Pinus* spp. are likely to be *O. subtriquetra,* and that collections from *Picea* spp. are likely *O. leporina* based Scandinavian reports that *O. leporina* is found in

association with *Picea* (Niemelä & Kotiranta, 1983). Without further genetic evidence, previous accounts of *O. circinata* should be interpreted cautiously.

Both Bayesian and maximum likelihood phylogenetic analyses separated North American *O. tomentosa* samples into two well supported lineages, and a third lineage exists albeit with lower phylogenetic support. The neighbor net phylogenetic network showed evidence of reticulation among the three *O. tomentosa* lineages. The lineages produced by the neighbor net phylogenetic network and the phylogenetic analyses shared similar sample composition. The evidence of reticulation among the three lineages suggest that recombination may be occurring, however these results were not supported with the pairwise homoplasy index with *O. tomentosa* samples, which indicated a lack of recombination. This suggests that there is a barrier preventing recombination between the lineages. The exact mechanism for this barrier is unknown, but the results of the PERMANOVA suggested that geography may play a role. However, the sample map showed that samples from each of the lineages were near each other, i.e., in Colorado, and did not have clear geographic boundaries. It may be that each lineage is adapted to specific site characteristics, which in turn prevents reproduction among lineages, but because many of the samples used in the analysis were loaned, and did not have precise GPS points associated with them, we were unable to adequately investigate this hypothesis. Another hypothesis that would explain the genetic variation between lineages is that there were three separate introductions of *O. tomentosa* into North America. A previous study hypothesized that *O. tomentosa* was introduced into North America from Asia through the Bering Land Bridge, thus limiting gene flow to off chance wind events once submerged (Zhao et al. 2022). This may explain the PERMANOVA results of geographic region as a significant predictor. A lineage that has only recently been introduced to the North American continent would have a limited distribution

when compared to the lineage that was from the first introduction. We currently do not consider these lineages to be speciating because 1) this study was primarily based on genetic evidence and did not consider variation in morphological traits (or lack thereof), 2) There was little topological congruence among phylogenies of single locus analyses, and 3) the neighbor net phylogenetic network showed evidence of reticulation among the three clades. The PERMANOVA tests also supported the hypothesis that North American instances of *O. tomentosa* are generalist pathogens, rejecting the idea that *O. tomentosa* samples separate into distinct lineages based on host association. Our results are similar to studies of *Porodaedalea,* the genus most closely related to *Onnia* (Brazee & Lindner, 2013). Brazee and Lindner (2013) showed that North American isolates of *Porodaedalea* spp. separated into geographic lineages rather than by host. Future studies examining genetic diversity of North American isolates of *O. tomentosa* should focus on environmental preferences or host virulence.

Interestingly, all North American samples of *O. tomentosa* separated from the reference sequences collected from China. An investigation of *O. tomentosa* on a global scale is warranted to better characterize the genetic diversity of the species. Previous literature has documented that *O. tomentosa* separates into well supported clades (Ji et al., 2017; Zhao et al., 2022). However, the phylogenies produced in these studies had small sample sizes of *O. tomentosa* and the isolates did not represent a wide geographic range. Further analyses should include large sample sizes from both Eurasia and North America to better understand the genetic diversity within *O. tomentosa.*

2.4.2 The LAMP assay

The LAMP assay we developed is an effective tool for the detection of North American samples of *O. tomentosa* and *O. subtriquetra.* Since we were only able to collect one isolate of *O. leporina,* this species was not thoroughly tested. The locus that was targeted by the LAMP primers had low intraspecific variation with the *O. leporina* sequence compared to the other *Onnia spp.*, suggesting that this molecular test will likely work with other samples of *O. leporina.* This could be validated by testing a geographically and genetically diverse set of *O. leporina* isolates. It is important to note that this primer set was only tested against North American samples of *Onnia* spp. While the primer set was developed to detect all *Onnia* spp., the effectiveness of this test for all *Onnia* spp. should be confirmed.

While testing this assay, we discovered that the likelihood of a false positive increases in field conditions. While it is possible to use these primers in the field, there are important caveats to keep in mind. This assay is sensitive to temperature fluctuations. Keeping reagents on ice is effective for a single-day trip, but leaving premixed reagents overnight in a cooler increases the risk of temperature fluctuations resulting in the formation of structures like primer dimers. It is also important to understand that the mechanism for color change is pH dependent. Therefore, using dry ice (solid CO2) to keep these reagents cool will result in abrupt color changes (false positive).. This test is very sensitive and if a researcher is using it in the field with *Onnia* basidiocarps present then the test may be at risk of contamination and false positives from basidiospores. The range of dissemination of *Onnia* spp. basidiospores has yet to be investigated. Therefore, any outdoor use of this assay should consider the risk of aerial basidiospore contamination. If this assay is used indoors, it is recommended to make sure that the freezer can

reliably be set below 4°C and that the researchers have a clear understanding of potential sources of contamination.

2.4.3 Management implications

The research presented here provides tools for land managers and researchers that enable early detection of *Onnia* spp. in standing trees, and the development of management strategies to proactively mitigate the impacts of Tomentosus root rot. The evidence provided herein that North American lineages of *O. tomentosa* are generalists will allow land managers to better develop vegetation management plans. Previously, land managers and researchers were unsure of the host range of *O. tomentosa.* This was due to multiple name changes and high morphological similarity with other species of *Onnia*. This study has provided molecular data that updates and validates the established host range for *O. tomentosa.* For example, a land manager aiming to reduce visitor risk at a recreational site that contains *O. tomentosa* may choose to transition their stand composition to hardwoods, or avoid planting *Picea* spp., *Pinus* spp*., Abies* spp. and *Larix* spp. Further, the prevalence of *O. subtriquetra* on pine indicates a host preference; This is important information for determining the species to use for replanting a site. However, due to the *O. subtriquetra* isolate associated with *Quercus*, further research is needed. If a fruiting body and host tree are available, our research supports the ability to make a species identification without genetic evidence. If setae are straight, then the fruiting is *O. tomentosa.* If setae are hooked and found in association with *Pinus*, then the fruiting is likely *O. subtriquetra,* and a fruiting is from *O. leporina* if the setae are hooked, and it is found in association with species other than *Pinus.* The LAMP assay developed here will give land managers an economical tool to detect *Onnia* spp. The availability of this tool will allow for more accurate hazard tree assessments and more effective vegetation management planning, in turn reducing risk and

liability. Researchers will also benefit from this tool because identification no longer will require more costly and time-consuming analyses to confirm a diagnosis. Our research also provides a rule of thumb to follow when reading historical studies of the organism *O. circinata* and its many synonyms. When collecting samples, five isolations of white pocket rot were identified as *Porodaedalea* spp. This potential for misidentification is not a new problem (Hunt, 1997). It is important to be able to differentiate these two pathogens, because both *Onnia* spp. and *Porodaedalea* spp. can infect the roots and butts of trees, but the two species are managed differently. When white pocket rot is the only available symptom and no signs are present, genetic evidence is needed to confirm any identification of *Onnia* spp. in North America. This research also solidifies the idea that the existence of white pocket rot is not enough evidence to suggest *Onnia* infection in North American forests and campgrounds.

2.4.4 Concluding remarks

This study provides a greater understanding the host specialization and geographic range of *O. tomentosa* and *O. subtriquetra*, and with the development of the field-based LAMP marker, the work done here will greatly benefit future landowners in developing downstream management strategies. This research demonstrated that *O. tomentosa* acts a generalist pathogen in North America and can be separated into three lineages. This research also supports the hypothesis that *O. leporina* and *O. subtriquetra* are separate species in North America, which aligns with previous research. Overall, this research provides a useful basis for understanding and researching *Onnia* spp. in North America in the future.

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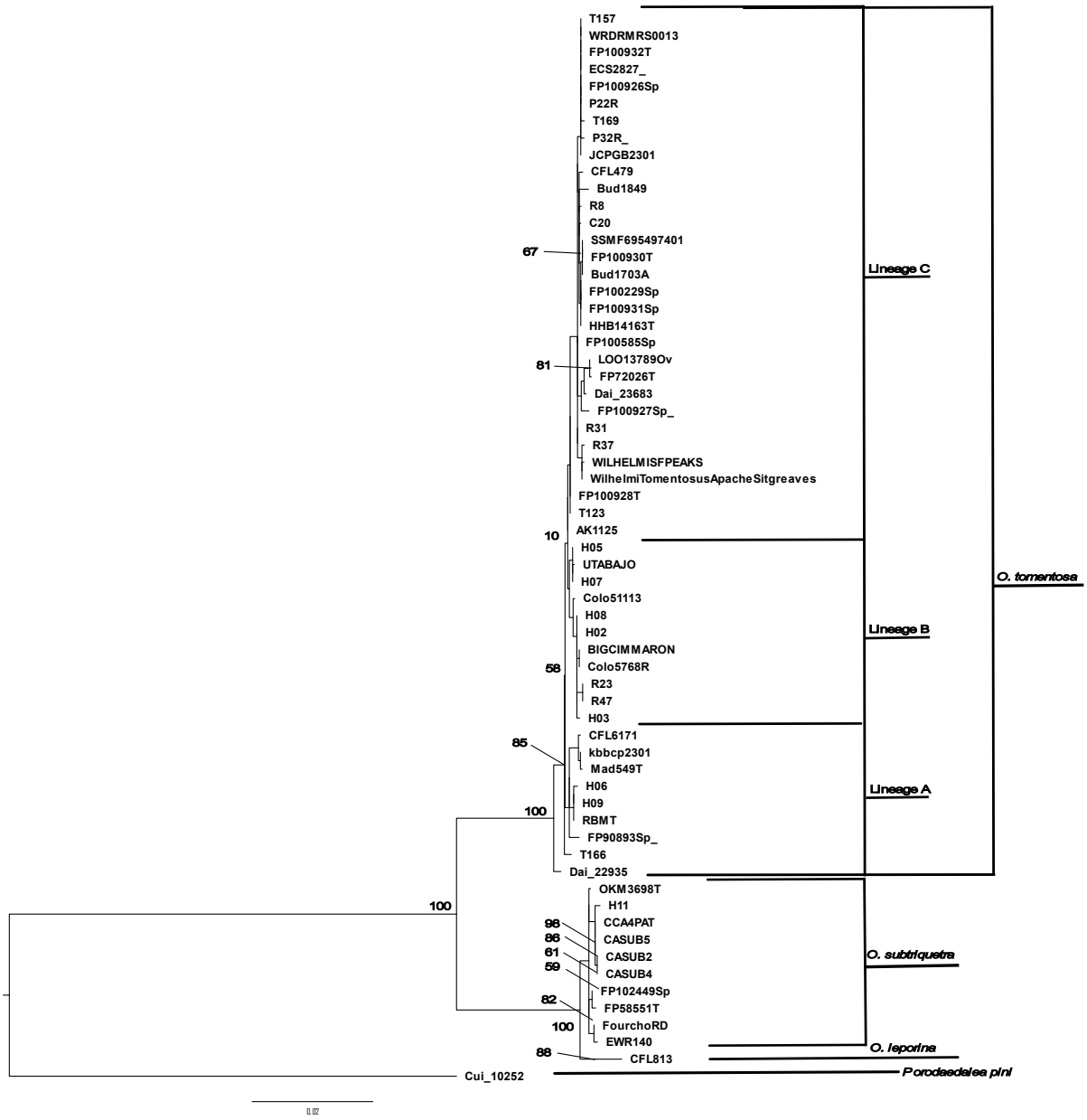
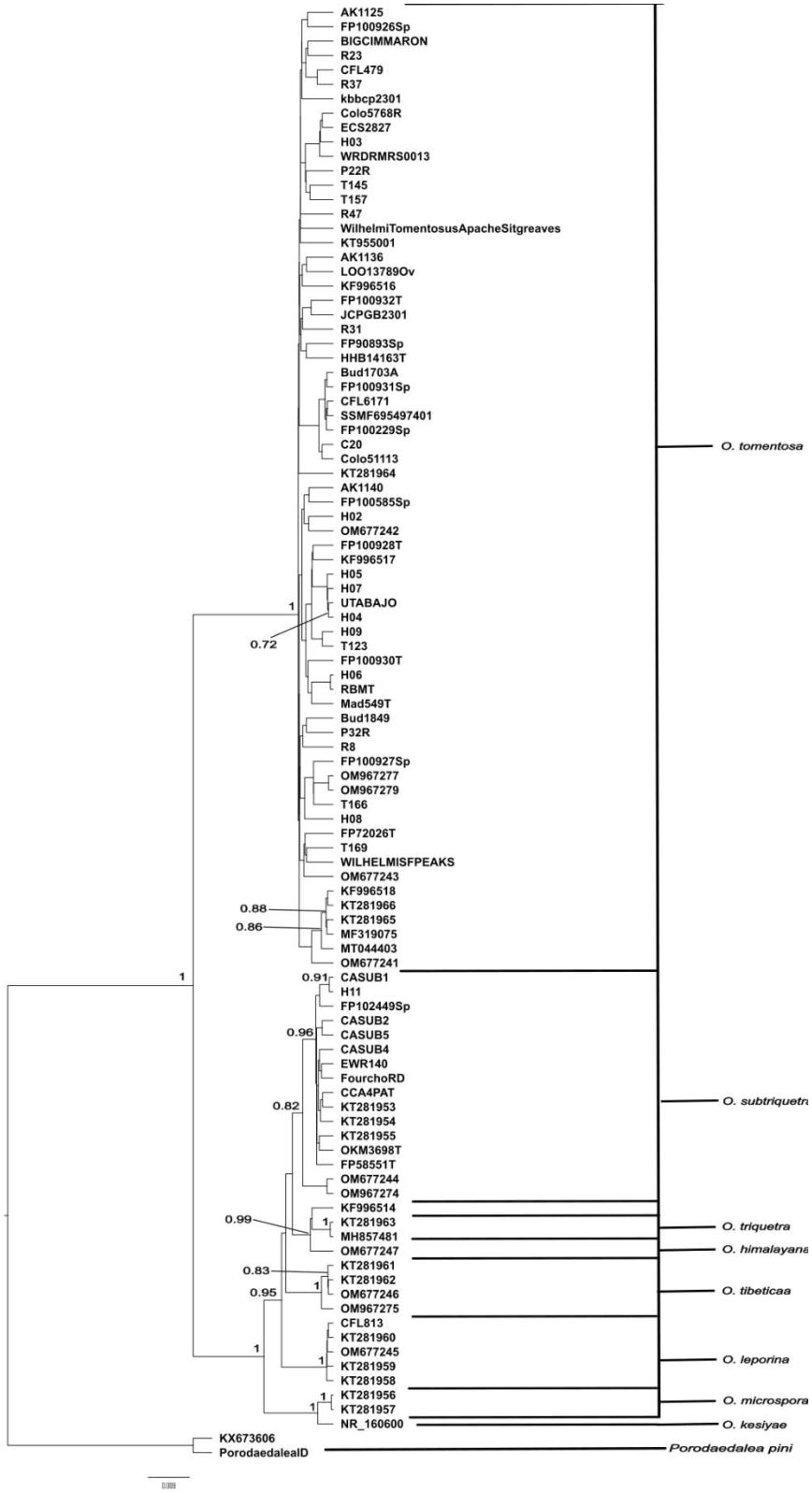
APPENDIX

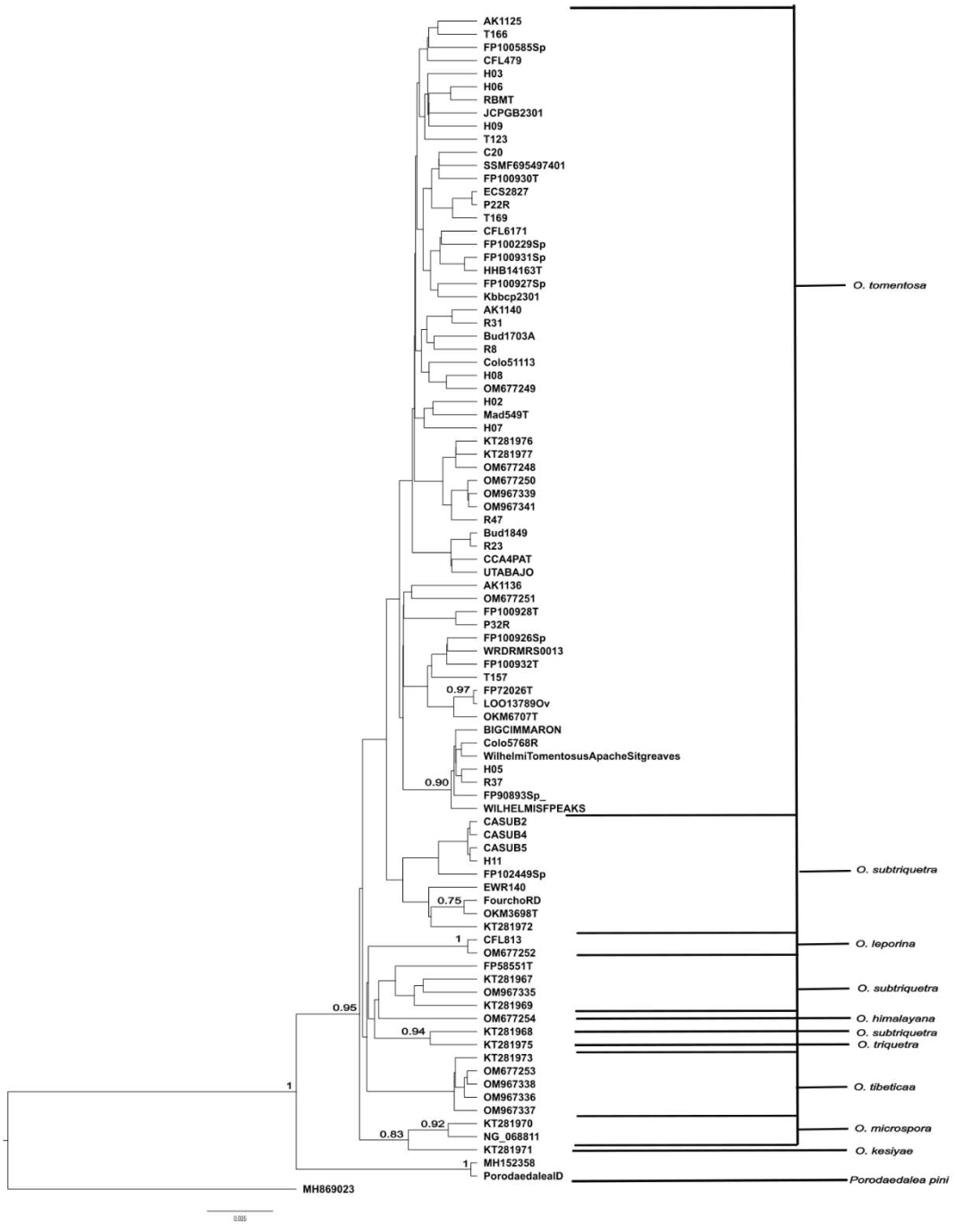


Supplemental Figure 2.1 Maximum likelihood phylogenetic tree based on a concatenated dataset of the ITS, LSU, *tef1a*, and *rpb2*. Node support is highlighted above each node as bootstrap support (BS).

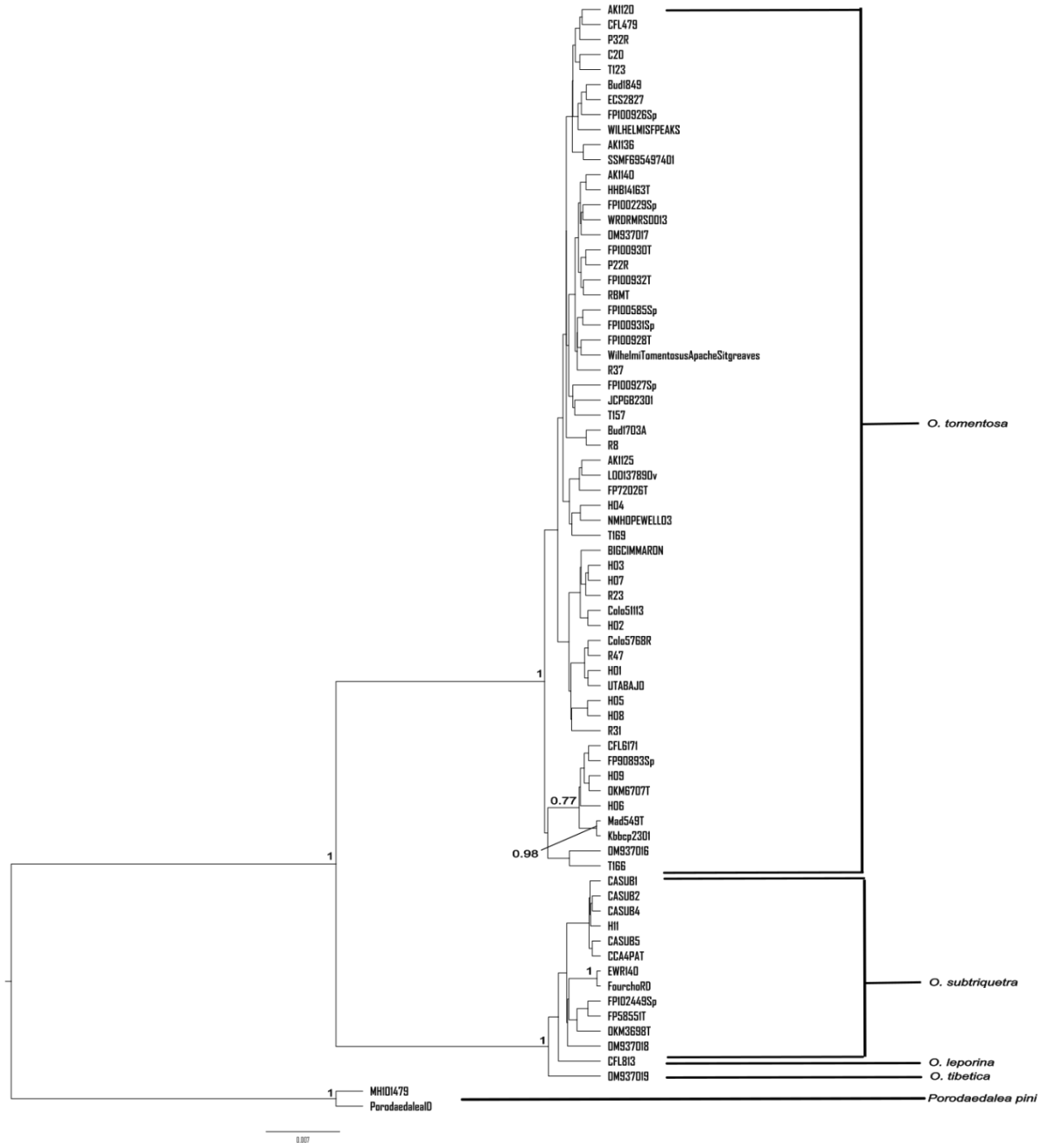
Supplemental Figure 2.2 Cladogram of Bayesian phylogenetic tree based on the ***tef1a***. Node support is highlighted above each node as posterior probability (PP).



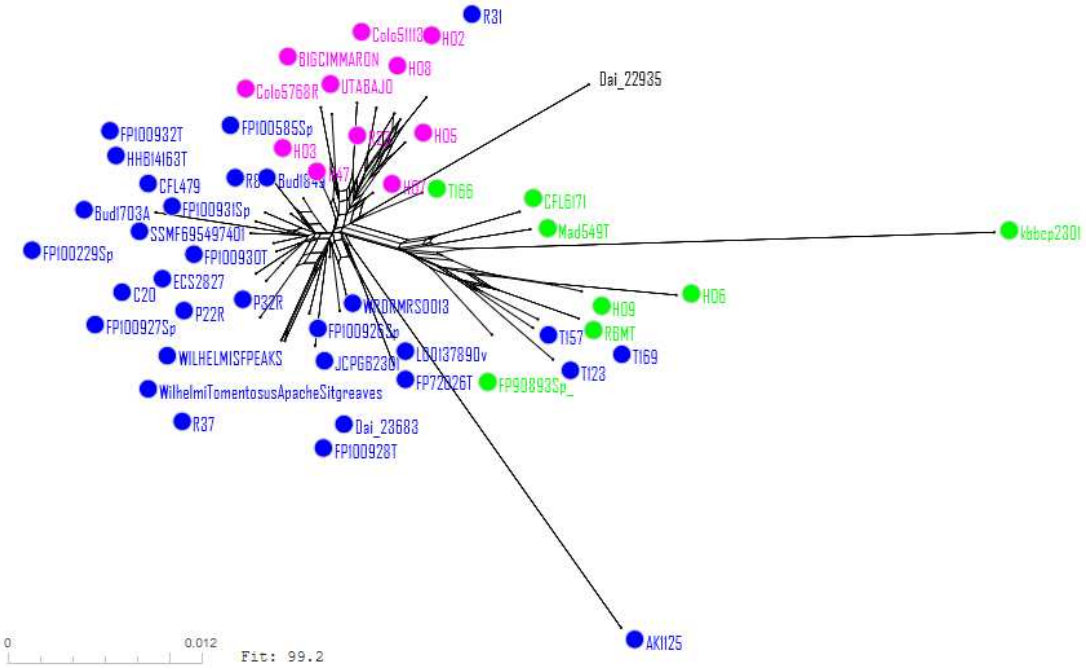
Supplemental Figure 2.3 Cladogram of Bayesian phylogenetic tree based on the ITS. Node support is highlighted above each node as posterior probability (PP).



Supplemental Figure 2.4 Cladogram of Bayesian phylogenetic tree based on the LSU. Node support is highlighted above each node as posterior probability (PP).



Supplemental Figure 2.5 Cladogram of Bayesian phylogenetic tree based on the *rpb2*. Node support is highlighted above each node as posterior probability (PP).



Supplemental Figure 2.6 neighbor-net phylogenetic network of concatenated data set for *O. tomentosa.* Nodes colored by lineage produced by phylogenies: Clade A is in green, clade B is in fuchsia, Clade C is in blue, and the reference sequence from China (*O. tomentosa* isolate Dai22935) in black.

Supplemental Figure 2.1 Table of samples used in phylogenies. Sample species, host, state/province, hymenial setae, sample type, and source provided.

Sample ID	Host Genus	Species	State/Province	Country	Source	Sample Type	Setae	ITS	nLSU	rpb2	*tef1α*
Bud1703A	*Picea glauca*	*O. tomentosa*	ON	Canada	CFMR[1]	Culture	-	x	x	x	x
Colo51113	*Picea engelmannii*	*O. tomentosa*	CO	USA	CFMR[1]	Culture	-	x	x	x	x
Colo5768R	*Picea engelmannii*	*O. tomentosa*	CO	USA	CFMR[1]	Culture	-	x	x	x	x
LOO13789Ov	*Pinus rigida*	*O. tomentosa*	PA	USA	CFMR[1]	Culture	-	x	x	x	x
Mad549T	*Picea mariana*	*O. tomentosa*	MN	USA	CFMR[1]	Culture	-	x	x	x	x
P22R	*Picea glauca*	*O. tomentosa*	ON	Canada	CFMR[1]	Culture	-	x	x	x	x
P32R	*Picea glauca*	*O. tomentosa*	ON	Canada	CFMR[1]	Culture	-	x	x	x	x
HHB14163T	*Picea lutzii*	*O. tomentosa*	AK	USA	CFMR[1]	Culture	-	x	x	x	x
FP100926Sp	*Picea glauca*	*O. tomentosa*	ON	Canada	CFMR[1]	Fruiting body/culture	S[7]	x	x	x	x
FP100927Sp	*Picea glauca*	*O. tomentosa*	ON	Canada	CFMR[1]	Fruiting body/culture	S[7]	x	x	x	x
FP100928T	*Picea glauca*	*O. tomentosa*	ON	Canada	CFMR[1]	Fruiting body/culture	S[7]	x	x	x	x
FP100929Sp	*Picea glauca*	*O. tomentosa*	ON	Canada	CFMR[1]	Fruiting body/culture	S[7]	x	x	x	x
FP100931Sp	*Picea glauca*	*O. tomentosa*	ON	Canada	CFMR[1]	Fruiting body/culture	S[7]	x	x	x	x
FP100932T	*Picea glauca*	*O. tomentosa*	ON	Canada	CFMR[1]	Culture	-	x	x	x	x
FP90893Sp	*Picea engelmannii*	*O. tomentosa*	CO	USA	CFMR[1]	Fruiting body/culture	S[7]	x	x	x	x
FP72026T	Unknown	*O. tomentosa*	VA	USA	CFMR[1]	Fruiting body/culture	S[7]	x	x	x	x
FP100229Sp	*Picea engelmannii*	*O. tomentosa*	CO	USA	CFMR[1]	Fruiting body/culture	S[7]	x	x	x	x
FP100585Sp	*Picea glauca*	*O. tomentosa*	SK	Canada	CFMR[1]	Fruiting body	S[7]	x	x	x	x
SSMF695497401	*Pinus strobus*	*O. tomentosa*	ON	Canada	CFMR[1]	Fruiting body/culture	S[7]	x	x	x	x
FP100930T	*Picea glauca*	*O. tomentosa*	ON	Canada	CFMR[1]	Fruiting body/culture	S[7]	x	x	x	x
H08	*Picea engelmannii*	*O. tomentosa*	MT	USA	CSU[2]/USFS[5]	Fruiting Body	S[7]	x	x	x	x
T169	*Picea engelmannii*	*O. tomentosa*	MT	USA	CSU[2]/USFS[5]	Culture	-	x	x	x	x
WRDRMRS0013f	*Picea glauca*	*O. tomentosa*	SD	USA	USFS[6]	Culture	-	x	x	x	x
WilhelmiApacheSitgreaves	*Picea engelmannii*	*O. tomentosa*	AZ	USA	USFS[6]	Fruiting body/culture	S[7]	x	x	x	x
WilhelmiSFpeaks	*Picea engelmannii*	*O. tomentosa*	AZ	USA	USFS[6]	Culture	-	x	x	x	x
T123	*Picea engelmannii*	*O. tomentosa*	MT	USA	CSU[2]/USFS[5]	Culture	-	x	x	x	x
T157	*Picea engelmannii*	*O. tomentosa*	MT	USA	CSU[2]/USFS[6]	Culture	-	x	x	x	x
T166	*Picea engelmannii*	*O. tomentosa*	MT	USA	CSU[2]/USFS[6]	Culture	-	x	x	x	x
H06	*Picea engelmannii*	*O. tomentosa*	OR	USA	USFS[6]	Fruiting body	S[7]	x	x	x	x
H07	*Picea engelmannii*	*O. tomentosa*	UT	USA	USFS[6]	Fruiting body	S[7]	x	x	x	x

H02	*Picea engelmannii*	*O. tomentosa*	CO	USA	USFS[6]	Fruiting Body	S[7]	x	x	x	x
H03	UNKNOWN	*O. tomentosa*	CO	USA	USFS[6]	Fruiting Body	S[7]	x	x	x	x
H05	*Abies bifolia*	*O. tomentosa*	CO	USA	USFS[6]	Fruiting Body	S[7]	x	x	x	x
R31	*Picea engelmannii*	*O. tomentosa*	CO	USA	USFS[6]	Culture	-	x	x	x	x
R37	*Picea engelmannii*	*O. tomentosa*	CO	USA	USFS[6]	Culture	-	x	x	x	x
R47	*Picea engelmannii*	*O. tomentosa*	CO	USA	USFS[6]	Culture	-	x	x	x	x
R8	*Picea engelmannii*	*O. tomentosa*	CO	USA	USFS[6]	Culture	-	x	x	x	x
UTABAJO	*Picea engelmannii*	*O. tomentosa*	UT	USA	USFS[6]	Fruiting Body	S[7]	x	x	x	x
AK1125	*Picea mariana*	*O. tomentosa*	AK	USA	USFS[6]	Fruiting Body	S[7]	x	x	x	x
RBMT	*Larix lyallii*	*O. tomentosa*	MT	USA	UMN[4]/USFS[6]	Culture	-	x	x	x	x
JCPGB2301	*Picea engelmannii* x *glauca*	*O. tomentosa*	BC	Canada	UNBC	Fruiting body	S[7]	x	x	x	x
BIGCIMMARON	Unknown	*O. tomentosa*	CO	USA	USFS	Fruiting body	S[7]	x	x	x	x
CFL6171	*Picea glauca*	*O. tomentosa*	QC	Canada	LFC[3]	Culture	-	x	x	x	x
CFL479	*Picea glauca*	*O. tomentosa*	QC	Canada	LFC[3]	Culture	-	x	x	x	x
kbbcp2301	*Pinus* sp.	*O. tomentosa*	CO	USA	USFS[6]	Culture	-	x	x	x	x
H09	*Pinus monticola*	*O. tomentosa*	MT	USA	USFS[6]	Fruiting body	S[7]	x	x	x	x
Bud1848	*Abies balsamea*	*O. tomentosa*	ON	Canada	CFMR	Culture	-	x	x	x	x
OKM6707T	Unknown	*O. tomentosa*	MT	USA	CFMR	Fruiting body/culture	S[7]	-	x	x	x
AK1136	*Picea mariana*	*O. tomentosa*	AK	USA	USFS[6]	Fruiting Body	S[7]	x	x	x	-
AK1140	*Picea mariana*	*O. tomentosa*	AK	USA	USFS[6]	Fruiting Body	S[7]	x	x	x	-
H04	*Abies bifolia*	*O. tomentosa*	CO	USA	USFS[6]	Fruiting body	S[7]	x	-	x	x
T145	*Picea engelmannii*	*O. tomentosa*	MT	USA	CSU[2]/USFS[6]	Culture	-	x	-	-	x
R22b	Unknown	*O. tomentosa*	CO	USA	USFS[6]	Culture	-	-	-	-	x
H01	*Picea* sp.	*O. tomentosa*	CO	USA	USFS[6]	Fruiting body	S[7]	-	-	-	x
AK1120	*Picea mariana*	*O. tomentosa*	AK	USA	USFS[6]	Fruiting Body	S[7]	-	-	x	-
NMHOPEWELLLAKE	*Picea engelmannii*	*O. tomentosa*	NM	USA	USFS[6]	Culture	-	-	-	x	-
CFL813	*Picea glauca*	*O. leporina*	QC	Canada	LFC[3]	Culture	-	x	x	x	x
CASUB5	*Pinus muricata*	*O. subtriquetra*	CA	USA	USFS[6]	Fruiting body	H[8]	x	x	x	x
CASUB2	*Pinus contorta* var. *contorta*	*O. subtriquetra*	CA	USA	USFS[6]	Fruiting body	H[8]	x	x	x	x
CASUB4	*Pinus muricata*	*O. subtriquetra*	CA	USA	USFS[6]	Fruiting body	H[8]	x	x	x	x
H11	*Pinus contorta* var. *latifolia*	*O. subtriquetra*	MT	USA	USFS[6]	Fruiting body	H[8]	x	x	x	x
CCA4PAT	*Pinus contorta* var. *latifolia*	*O. subtriquetra*	MT	USA	USFS[6]	Culture	-	x	x	x	x
FP58551T	*Pinus* sp.	*O. subtriquetra*	VA	USA	CFMR[1]	Fruiting body	H[8]	x	x	x	x
OKM3698T	*Pinus virginiana*	*O. subtriquetra*	MD	USA	CFMR[1]	Fruiting body	H[8]	x	x	x	x
EWR140	*Pinus elliottii*	*O. subtriquetra*	SC	USA	CFMR[1]	Fruiting body	H[8]	x	x	x	x
FourchoRD	*Pinus echinata*	*O. subtriquetra*	AR	USA	CFMR[1]	Culture	-	x	x	x	x

FP102449Sp	*Quercus* sp.	*O. subtriquetra*	MI	USA	CFMR[1]	Culture	-	x	x	x	x
CASUB1	*Pinus contorta* var. *contorta*	*O. subtriquetra*	CA	USA	USFS[6]	Fruiting body	H[8]	x	-	x	x

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[6]United States Department of Agriculture, Forest Service

[7]Straight hymenial setae

[8]Hooked hymenial setae

Supplemental Figure 2.2 Table of reference sequences used in phylogenies. GenBank Accession numbers provided.

Isolate ID	Species	Country	ITS	LSU rDNA	rpb2	*tef1α*
Dai 18415	*Onnia kesiyae*	Vietnam	NR_160600	NG_068811	–	OM800827
Dai 13501	*Onnia leporina*	China	KT281958	–	–	–
Dai 20866	*Onnia leporina*	China	OM677245	OM677252	–	OM800829
JV0609/15	*Onnia leporina*	Czechia	KT281959	–	–	–
JV1207/2	*Onnia leporina*	Czechia	KT281960	KT281972	–	–
Phaeo1	*Onnia leporina*	Italy	KF996514	–	–	–
Dai 11886	*Onnia microspora*	China	KT281956	KT281970	–	–
Dai 11897	*Onnia microspora*	China	KT281957	KT281971	–	–
Dai 22620	*Onnia himalayana*	China	OM677247	OM677254	–	–
Dai 23686	*Onnia subtriquetra*	United States	OM677244	OM677251	OM937018	OM800828
Dai 23687	*Onnia subtriquetra*	United States	OM967274	OM967335	–	–
MB2	*Onnia subtriquetra*	United States	KT281955	KT281969	–	–
JV0410/12J	*Onnia subtriquetra*	United States	KT281954	KT281968	–	–
JV0109/D6J	*Onnia subtriquetra*	United States	KT281953	KT281967	–	–

Cui 12254	*Onnia tibetica*	China	KT281961	KT281973	–	–
Dai 23621	*Onnia tibetica*	China	OM967275	OM967336	–	–
Dai 23622	*Onnia tibetica*	China	–	OM967337	–	–
Dai 23642	*Onnia tibetica*	China	OM677246	OM677253	OM937019	OM800830
Dai 23643	*Onnia tibetica*	China	OM967276	OM967338	–	–
CBS 278.55	*Onnia triquetra*	Germany	MH857481	MH869023	–	–
JV1410/3	*Onnia triquetra*	Czechia	KT281963	KT281975	–	–
Dai 14806B	*Onnia tomentosa*	China	KT281965	KT281976	–	–
Dai 18900	*Onnia tomentosa*	China	OM677241	OM677248	OM937015	OM800824
Dai 22935	*Onnia tomentosa*	China	OM677242	OM677249	OM937016	OM800825
Dai 23682	*Onnia tomentosa*	United States	OM967277	OM967339	–	–
Dai 23683	*Onnia tomentosa*	United States	OM677243	OM677250	OM937017	OM800826
Dai 23685	*Onnia tomentosa*	United States	OM967279	OM967341	–	–
Vampola 2010	*Onnia tomentosa*	Czechia	KT281966	KT281977	–	–
FP-100585-5p	*Onnia tomentosa*	Canada	KF996516	–	–	–
OT-Slu	*Onnia tomentosa*	Sweden	KF996518	–	–	–
T. Niemela 9079	*Onnia tomentosa*	Finland	MF319075	MF319006	–	–
SFC20170810-01	*Onnia tomentosa*	Russia	MT044403	–	–	–
Cui 9986	*Onnia tomentosa*	China	KT281964	–	–	–
HHB-18573	*Onnia tomentosa*	United States	KT955001	–	–	–
LOO-13789-Q	*Onnia tomentosa*	United States	KF996517	–	–	–
Cui 10252	*Porodaedalea chinensis*	China	KX673606	MH152358	MH101479	MG585301