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REVIEW ARTICLE

Recent insights into the pandemic disease butternut canker caused by the invasive pathogen *Ophiognomonia clavigignenti-juglandacearum*

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Summary

In the 25 years following the initial 1967 report of the disease, butternut canker was able to quickly spread throughout the entire range of butternut (*Juglans cinerea*) in North America, from Minnesota in the upper Midwest to Tennessee in the south and Quebec in the north-east. The speed of this dispersal is notable as butternut trees do not make up a significant proportion of any single forest type. Instead, they are usually found sparingly in most mixed hardwood forests. In this review, we synthesize the current knowledge of the invasion process of the butternut canker pathogen, *Ophiognomonia clavigignenti-juglandacearum*, an invasive fungal pathogen, that as its emergence has spread across North America and is now found wherever butternut naturally occurs. Taxonomic studies have determined that the fungus belongs in the genus *Ophiognomonia*, which includes a number of saprophytes, endophytes and pathogens of members of the Fagales, rather than the genus *Sirococcus,* which includes several important pine pathogens. The ability of fungus to be dispersed by rain splash, transported on and in beetle vectors, transmitted by infected seed and successfully colonized several species of *Juglans* and *Carya* have all likely contributed to the rapid increase in abundance and severity of disease and tree mortality in the invaded forest ecosystems. Recent genomic and population genetic analyses have determined that there were at least three emergence events. A less virulent strain of the fungus likely has been present in the north eastern United States for over a century, but it was the emergence of a more virulent strain of the fungus in Minnesota and Wisconsin in the 1960s that resulted in range-wide mortality and pushed butternut to be listed as an endangered species in Canada and a number of states in the United States.

1 Introduction

In many cases involving invasive forest pathogens, there is an extended latent period between infection and symptom development and this delay often results in diseased trees being overlooked. It is, therefore, quite difficult to determine whether a new pathogen was introduced, if subsequent introductions have occurred, or how far a pathogen has spread since its original introduction. In the case, when a source population cannot be identified, it is also possible that an emergent pathogen was derived from an avirulent strain, such as an endopyhyte, on the same host, or has made a host jump from a plant species that was either introduced into the new host's range or has migrated into the new host's range due to climate change. A good example of this situation is the case of the butternut canker pathogen, *Ophiognomonia clavigignenti-juglandacearum* (Nair, Kostichka, & Kuntz) Broders & Boland (Fig. 1), which has caused extensive damage to butternut populations in North America. In recent years, the tree has been decimated by this fungal pathogen, with reports indicating declines of as much as 70–90% in some regions (Anderson and LaMadeleine 1978). In Canada, the impact of butternut canker has led to butternut being classified as an endangered Species (Neilson et al. 2003). A recovery strategy has been developed to support the conservation and recovery of this species (Poisson and Ursic 2013).

2 Current taxonomy

The first report of butternut canker was in Wisconsin in 1967 (Renlund 1971), and in 1979, the fungus responsible for the disease, *Sirococcus clavigignenti-juglandacearum* (*Sc-j*), was described for the first time (Nair et al. 1979). A later multigene phylogenetic study determined that, in fact, the fungus is a member of the genus *Ophiognomonia* and not *Sirococcus*, and it was subsequently reclassified as *Ophiognomonia clavigignenti-juglandacearum* (*Oc-j*) (Broders and Boland 2011). This finding is of particular relevance as species of *Ophiognomonia* are most frequently recovered from members of the *Fagales* including the *Betulaceae, Fagaceae* and *Juglandaceae,* but some species have also been reported from other plant families (Sogonov et al. 2008). Classification into *Ophiognomonia* would seem ecologically appropriate for *Oc-j*, which was recovered from *J. ailantifolia* var. *cordiformis, J. cinerea* and *J. nigra* (Ostry 1997; Ostry et al. 1997; Broders and Boland 2011), as opposed to the ecology of other members of *Sirococcus*, which are primarily pathogens of coniferous hosts (Rossman et al. 2008). The symptoms of *Oc-j* on *J. nigra* are typical of other species of the *Gnomoniaceae*, which occur most commonly on fallen or still attached overwintered leaves including petioles and herbaceous stems. While a teleomorph for the butternut canker fungus has not been observed, most members of the genus produce perithecia on the leaves of their host. It is possible that *Oc-j* is able to sexually reproduce, but both mating types may not be present in North America or the fungus may reproduce sexually very rarely and so a teleomorph has yet to be observed.

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Fig. 1. Symptoms of butternut canker on: (a, b) trunk and (d) stem of butternut trees caused by the (c) fungus *Ophiognomonia clavigignenti-juglandacearum*.

3 Long-distance dispersal of *Oc-j*

The rate at which *Oc-j* spread across North America remains an area of uncertainty and debate. In the 25 years following the initial 1967 report of the disease (Renlund 1971), *Oc-j* quickly spread throughout the entire range of butternut in North America (Neilson et al. 2003; Ostry and Woeste 2004) (Fig. 2). At the northern range of butternut in Canada, butternut canker was first detected in Québec in 1990 (Innes and Rainville 1996), in Ontario in 1991 (Davis et al. 1992) and in New Brunswick in 1997 (Harrison et al. 1998). This dispersal is notable because butternut trees occur as a minor component of deciduous and mixed forests where they are usually found as scattered individuals or in small groups. It would be easy to assume that due to their sporadic distribution, many butternuts would have escaped infection but, unfortunately, that is not the case. The rate of spread of this epidemic over the entire range of the host was faster than other lethal diseases of Carolinian hardwood species. For example, chestnut blight was first reported in North America in 1904 (Murrill 1906) in the Bronx Zoo in New York, but it was not until the late 1940s that it spread to the limits of the natural range of American chestnut (Gravatt 1949; Anagnostakis 1987). Similarly, Dutch elm disease was first introduced into North America in the late 1920s and, over the next 50 years, made slow but steady progress in devastating the American elm (Gibbs 1978).

Numerous studies have examined the dispersal mechanisms of the butternut canker pathogen. Tisserat and Kuntz (1983a) examined the dispersal of *Oc-j* conidia during rainfall events and found that conidia were readily transported down the trunk in rainwater stemflow. Additionally, airborne conidia, resulting from raindrop impaction, were detected up to 40 m away from diseased trees while it was raining. Although the authors speculated on the possibility of airborne spores being dispersed farther than 1 km in distance, the asexual conidia alone were not able to account for the pathogen's rapid migrations. This is evidenced by a second study from the same authors (Tisserat and Kuntz 1983b), which noted that the viability of the airborne conidia was greatly affected by temperature, vapour pressure deficit and ultraviolet light. The conidia of *Oc-j* are produced within pycnidia and are unlikely to travel more than 10 km under the most ideal conditions (Tisserat and Kuntz 1983b). Conversely, thick-walled sexual spores are known to be able to travel great distances but, to date, have not been observed for *Oc-j*.

Research into possible insect vectors of *Oc-j* has identified multiple insect species that are capable of spreading the pathogen. Species frequently found to carry *Oc-j* conidia include *Astylopsis macula*, *Acoptus suturalis*, *Eubulus parochus*,

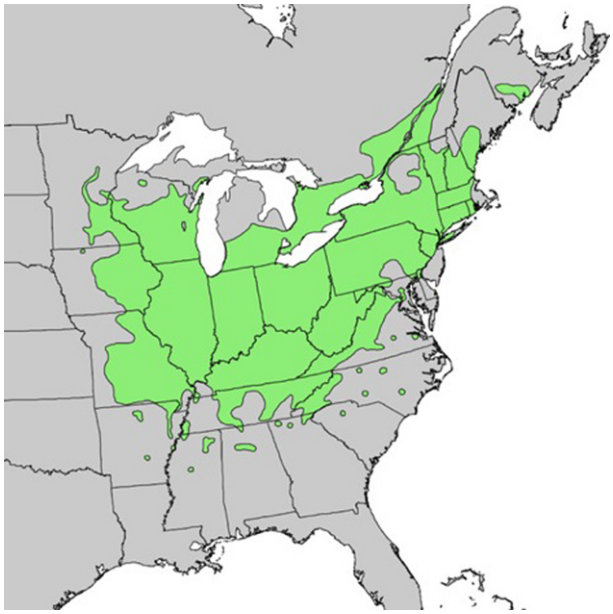

Fig. 2. Geographic distribution of butternut (*Juglans cinerea* L.).

Glischrochilus sanguinolentus and *Hyperplatys maculate* (Halik and Bergdahl 2002, 2006; Stewart et al. 2004). The exact role these insects play is still not well understood nor is the degree of their impact. However, various interactions between these insects and the fungus resulting in the acquisition and transport of conidia have been observed. Examples include feeding on bark as well as the fungus itself, females ovipositing and larvae overwintering underneath the bark of dead or dying branches, and movement between dead, infected branches on the ground and dead branches within the tree canopy (Halik and Bergdahl 2002, 2006; Stewart et al. 2004). Additionally, viable spores of *Oc-j* have been isolated from faecal pellets of *E. parochus* (Halik and Bergdahl 2006).

Alternate hosts of butternut canker may play a critical role in the dispersal of the pathogen. For instance, while mortality only results in *J. cinerea*, Ostry and Moore (2007) found that, in greenhouse inoculations, the pathogen was able to colonize several genera of hardwoods, including *Juglans, Carya, Quecus, Castenea* and *Prunus*, and several important commercial Persian walnut (*J. regia* L.) cultivars that were moderately to highly susceptible. Species of Asian origin such as Japanese walnut (*Juglans ailantifolia*) and heartnut (*Juglans ailantifolia* var. *cordiformis*) displayed a measure of resistance to the pathogen, however (Ostry and Moore 2007), providing a possible source of genetic variation for the development of resistant cultivars. Additionally, several species in the genus *Carya* (family *Juglandaceae*) such as pecan (*Carya illinoinensis*) and shagbark hickory (*Carya ovata*) are susceptible to the pathogen (Ostry and Woeste 2004). As previously mentioned, butternut is distributed sporadically as scattered individuals or small groups throughout its range and it is often found in stands containing individuals of these alternate host species. As such, it is possible that wind, rain and insect dispersal mechanisms may interact with a larger host base and expedite the dispersal of *Oc-j* conidia. Unfortunately, spread of the pathogen via alternate hosts and infected seed has not been investigated outside of greenhouse and nursery settings, even though these variables (climate, vector, host range) may provide valuable information on the long-distance dispersal of this pathogen. To better assist epidemiological studies, a PCR-based diagnostics assay was developed which was proven to detect the presence of the pathogen on different types of *J. cinerea* plant tissue (i.e. terminal buds, stem cankers, trunk cankers, leaf lesions, seed pericarp and cotyledons). The same assay was used to detect the presence of the pathogen on other hosts, including members of the genera *Juglans* and *Carya* (Broders and Boland 2010). In addition to detecting the pathogen on alternate hosts, the authors also indicated a potentially important role of butternut seed in disease transmission.

Ophiognomonia clavigignenti-juglandacearum is capable of infecting the pericarp of butternut and black walnut seeds (Innes and Rainville 1996), and this may represent an important avenue for long-range transmission as squirrels frequently gather and disperse seeds. Seeds may also be moved by hydrology in rivers, streams and other waterways. During the course of developing a molecular diagnostic assay for detection of the fungus in the pericarp, it was observed that the fungus was also able to infect the cotyledon of the seed (Fig. 3). In a sample of 55 butternut seeds from a single tree, *Oc-j* was successfully isolated from 14 of the cotyledons. This raised several interesting questions. How were these cotyledons becoming infected? Does *Oc-j* have an endophytic phase or can it cause a latent infection? In 2010, three trees in southern Ontario, including the tree that produced the infected seed in the previous year, were monitored starting at flowering for early signs of infection. *Ophiognomonia clavigignenti-juglandacearum* was successfully recovered from flowers and young nutlets on all three trees. Unfortunately, mature seeds could not be tested, as each of the three trees experienced a significant seed abortion event in July of that year. Whether *Oc-j* was responsible for the seed abortion event is still undetermined. Further monitoring and controlled inoculations will be needed to determine the impact of flower infection by *Oc-j*

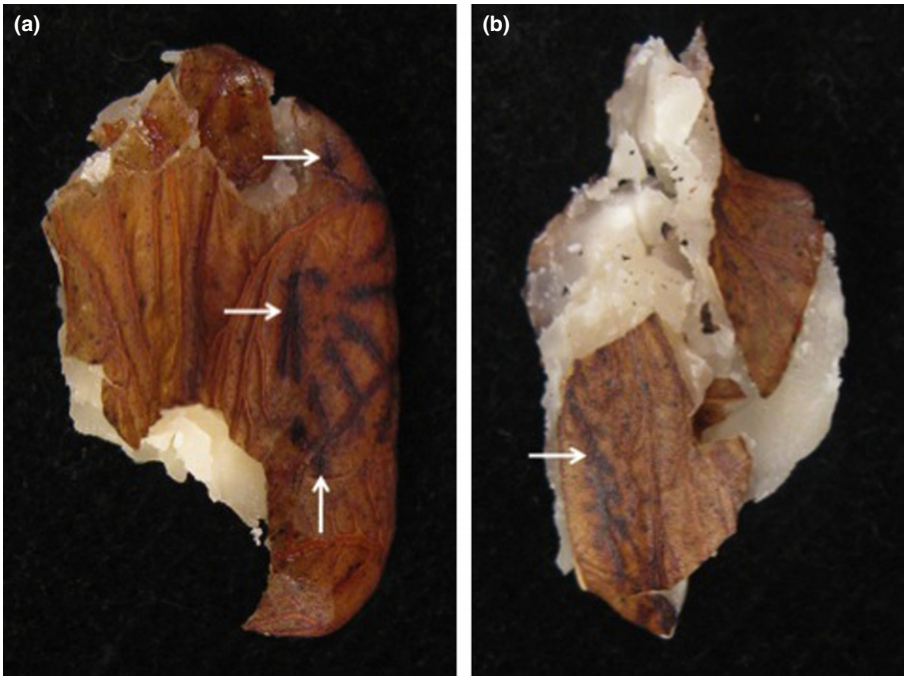

Fig. 3. Symptom of *Oc-j* infection on cotyledon tissue of butternut seeds from two seed sources (A & B). Arrows indicate areas of infection from which the fungus was successfully isolated.

on spontaneous seed abortion of butternut. Nevertheless, the potential importance of seed infection as a mechanism for pathogen dispersal and its potential impact on current butternut recovery programs must be considered.

4 How long has *Oc-j* been in North America?

Given the potential spore dispersal mechanisms (wind, rain, insects, alternate hosts and seed borne) described previously (Tisserat and Kuntz 1983a,b; Innes and Rainville 1996; Katovich and Ostry 1998; Halik and Bergdahl 2002), the rapid spread of *Oc-j* from Minnesota to New Brunswick in the east and Tennessee to the south-east in <30 years, is remarkable in comparison with other historic forest tree epidemics such as chestnut blight or Dutch elm disease. It is likely, therefore, that *Oc-j* was present for a number of years prior to its formal discovery and initial description. Early reports of butternut decline date back to 1923 and asserted the weak, secondary pathogen *Melanconium oblongum* Berk. (perfect state: *Melanconis juglandis* Ellis & Everhart Graves) as the pathogen responsible for diseased butternuts (Graves 1923). In fact, Graves states that 'Almost without exception the mature butternut trees in the region surveyed (New York and Connecticut) were in a moribund condition, sometimes only a few of the smaller branches being dead, while in extreme cases the entire tree had succumbed' (Graves 1919). The author go on to state that the disease is widespread, having been reported in Connecticut, Maryland, Pennsylvania and New York. However, we now know that *Melanconis juglandis* has a close association with *Oc-j* and can be found colonizing dead branch and trunk tissue following infection of *Oc-j,* and is more readily isolated from this tissue and grows faster in culture than *Oc-j*. Furthermore, Graves was not able to sufficiently replicate disease symptoms when butternut trees were inoculated in the greenhouse with *M. juglandis*. These facts indicate that butternut trees may have been mistakenly diagnosed and that *Oc-j* was likely more prevalent than was previously thought. This perspective is supported by work done by Broders et al. (2012), which demonstrated that the *Oc-j* populations in the north-eastern United States were more diverse than populations in Ontario, Minnesota and Wisconsin, indicating that the pathogen had been resident in the region for a longer period of time.

5 Emergence of *Oc-j*

For an invasive fungal pathogen to succeed in a new environment, it must evolve in response to the availability of new hosts. Upon introduction, the pathogen may primarily infect a single host species. But, as the pathogen begins to spread through the range of the new host, and the new host becomes more rare due to mortality, the pathogen may begin to shift onto other hosts through evolutionary processes. Such a case may be occurring with the butternut-*Oc-j* pathosystem, as the fungus has caused mortality rates of 70–90% in butternut trees in North American forests (Ostry and Woeste 2004). Several recent studies have documented the potential host range of *Oc-j* through inoculation experiments with other species of *Juglans* and *Carya* (Ostry and Moore 2007), as well as the isolation of the fungus from natural lesions or cankers on the closely related species *Juglans ailantifolia* and *J. nigra* (Innes and Rainville 1996; Broders and Boland 2010). These observations may indicate that pathogen infectivity may change over time through frequency-dependent selection (e.g. the Red

Queen Hypothesis) as the primary host becomes more rare and other species become more common. We might expect an increase in the frequency of pathogen genotypes most able to infect and reproduce on the dominant host species, leading to increased disease incidence or severity. As butternut has become more rare, several genotypes of *Oc-j* may have been able to expand their host range and, therefore, gained the ability to infect other members of the genus *Juglans,* including *J. nigra* and *J. ailantifolia*. In a preliminary study, we demonstrated that isolates of *Oc-j* recovered from *J. ailantifolia* and *J. nigra* were significantly more virulent on butternut than isolates recovered from butternut itself (Fig. 4). This indicates that through host expansion, *Oc-j* has increased virulence on its primary host butternut. An alternative conclusion may be that only the most virulent strains infecting butternut are capable of making a host jump. Future research is in progress to test these two competing hypotheses.

6 Genomics and population genetics of *Oc-j*

The emergence of new pathogens of animals, plants and humans has increased instep with the rise in global trade and travel resulting in a number of new host–pathogen interactions. With current sequencing technologies, many of these new or emerging pathogens (e.g. mostly of animals, including humans) have gone from virtually unknown to systems with abundant genomic and population genetic resources in a matter of years (Prospero et al. 2007; Goss et al. 2009; Broders et al. 2011, 2012; Stukenbrock et al. 2012; Rosenblum et al. 2013). In the case of plant pathogens, attention has focused primarily on developing genomic data for pathogens of agricultural crops with limited emphasis on the importance of developing genomic tools and data for pathogens of forest species or other native plants, with the exception of those that have potential as biofuels crops (i.e. poplar and switchgrass). This was the case for *Oc-j* only 4 years ago. At that time, there were only a handful of ITS sequences for *Oc-j* in GenBank and the fungus was classified as a member of the genus *Sirococcus*. A phylogenetic analysis of multiple isolates of *Oc-j* clearly demonstrated that the organism belonged in the genus *Ophiognomonia*; however, the phylogenetic study was completed with only five genes and clear phenotypic differences indicated that the organism was not a single clone spread across North America. To access this diversity, we were one of the first groups to pool-sequence multiple strains of a non-model fungus where a reference genome was not previously available (Broders et al. 2011). While a *de novo* assembly could not be completed, a wealth of genomic data was available to mine for markers to be used in population genetics. This led to the development of a series of SNP markers that were later used to complete a population genetic analysis of *Oc-j* in the United States and Canada that demonstrated there are at least three distinct clonal lineages of the fungus in North America that likely represent independent introduction events (Broders et al. 2012). In fact, there may have been many independent introductions, but whether by selection or random evolutionary process, these lineages persisted and expanded while others did not. The reclassification of *Oc-j* into the genus *Ophiognomonia* followed by the development of SNP markers and subsequent population analysis completely changed our understanding of the taxonomic placement and evolutionary relationship of this organism, how it was introduced and subsequently spread through North America, how long it has been present in North America and how rapidly this type of information can be generated for a non-model organism.

7 Ecology and evolution of *Oc-j*

In the past, many epidemics caused by invasive pathogens were assumed to be initiated by a single introduction event. However, several recent studies have found that this is likely not the case and that, in most invasion events, multiple introductions have occurred (Ciosi et al. 2008; Delmotte et al. 2008; Goss et al. 2011; Keller et al. 2012). While it has been

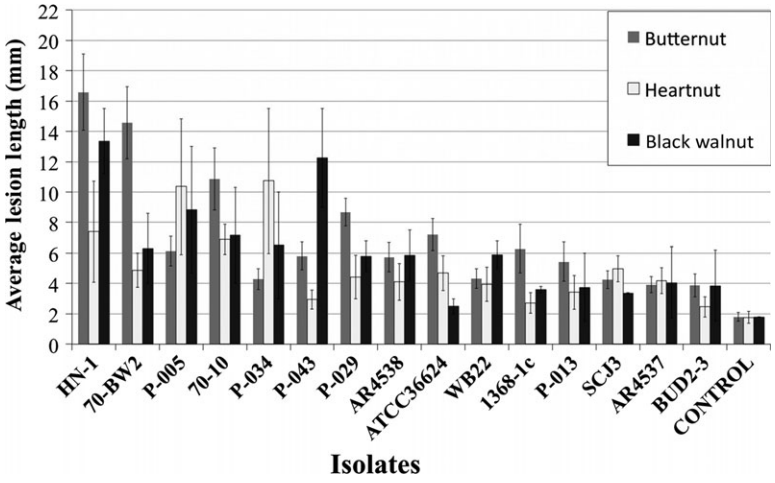

Fig. 4. Virulence of isolates of *Oc-j* on butternut, black walnut and heartnut. Isolates HN-1 and 70-BW2 were recovered from heartnut and black walnut, respectively. Remaining isolates were recovered from butternut including the type culture ATCC36624.

documented that at least three distinct clonal lineages of *Oc-j* in North America (Broders et al. 2012), the origin of *Oc-j* is still unknown. The most likely scenario is that *Oc-j* was introduced on a foreign plant species, such as the closely related Japanese walnut (*Juglans ailantifolia*), which is a close relative of butternut and has been known to hybridize with butternut. While *Oc-j* does not kill Japanese walnut, the fungus has been isolated from leaves, flowers, seeds and branch cankers on the tree (Broders and Boland 2010). The above situations are based on recent studies indicating the importance of live plant imports as major pathways for insect and pathogen invasions into the United States (Liebhold et al. 2012), as well as a recent study that identified *Oc-j* living as an endophyte on *Acer truncatum* in China (Sun et al. 2011). The isolates recovered by Sun et al. (2011) were obtained by the author (KB) and, while it does not appear to be the same species as the fungus causing butternut canker, it is to date the closest known relative of *Oc-j* (Fig. 5). In addition, recent phylogenetic studies have shown that several *Ophiognomonia* species closely related to *Oc-j* commonly occur on *Alnus* species in North America (Broders and Boland 2011; Walker et al. 2012) that are frequently found in similar forest habitats as *J. cinerea* (Fig. 5). Finally, the author recently recovered *Oc-j* from leaf tissue of *J. cathayensis*, an Asian species, located in the Arnold Arboretum in Boston, MA, as well as a number of isolates from Japanese walnut × butternut (*J* × *bixby*) hybrids frequently found in urban areas on the east coast and as planted trees throughout butternuts range. Phylogenetic inference clearly indicates that species in *Ophiognomonia* evolved through host jumps followed by adaptive specialization on plants belonging to multiple genera in the Fagales (Walker et al. 2012, 2014). In most instances, *Ophiognomonia* species only infect members of a single genus, including seven species that are limited to members of the Juglandaceae. Future work will focus on sequencing the genome of several closely related members of *Ophiognomonia*, including pathogens and endophytes, to evaluate the evolution of virulence and host specialization in this genus.

In summary, our research to date on the *Oc-j* populations in North America has provided new insight into the taxonomy, ecology, genetic diversity and evolution of *Oc-j* and support a model of multiple recent introduction or emergence events of *Oc-j* clonal lineages in North America. These findings also provide an example of how multiple introduction events were

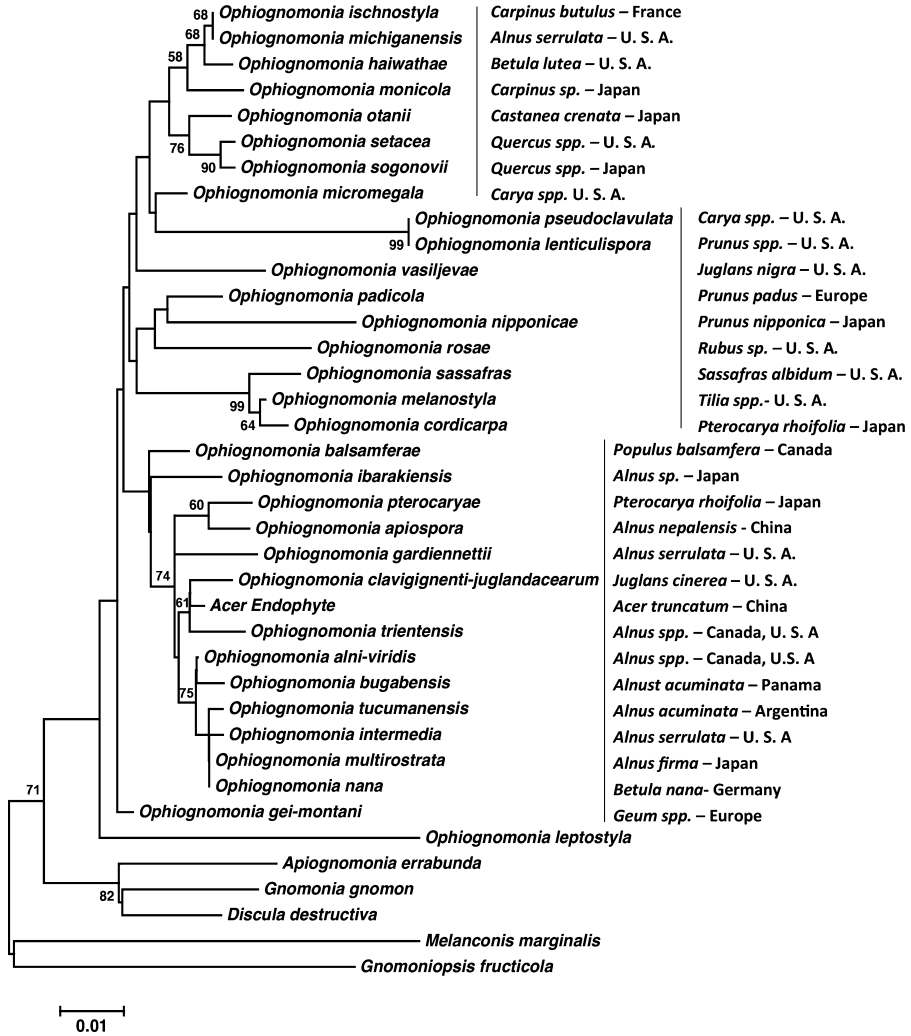

Fig. 5. Maximum likelihood tree based on the analysis of the ITS gene of 33 members of *Ophiognomonia.* Species names are followed by host and country of origin. Posterior probability values <60% are not shown.

probably required for a pathogen to become resident in a new environment. In addition, once a new pathogen has established in a location on a novel host, there is still potential for a more virulent strain of the pathogen to emerge through a variety of evolutionary processes, such as through a host jump. Therefore, quarantine procedures may need to take into consideration not just the fact that the invasive pathogen has become established but the likelihood of a more virulent strain being introduced and causing greater mortality than has already occurred. Further genotyping and population genomic studies are needed to provide a more complete picture of the host range, geographic range and interactions among the clonal lineages. Future research will attempt to determine the evolutionary processes and adaption that allow fungal pathogens to establish and then expand into a novel environment. A major aim of this future work is to identify genes under positive or purifying selection during speciation and adaptation to a new environment or host during an invasion event.

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