Neofusicoccum arbuti: Survey of a latent endophytic pathogen reveals widespread infection, broad host range, and a hidden threat.

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Abstract

*Arbutus menziesii* is an iconic tree species of the Pacific Northwest that has been in decline for the past 40 years. *Neofusicoccum arbuti*, a latent endophytic fungal pathogen in the Botryosphaeriaceae family, has been implicated as the primary cause of disease in *A. menziesii*, causing wart-like cankers on the stem and branches when the host is stressed. *Neofusicoccum arbuti* is suspected of causing the symptoms of decline in *A. menziesii* in Lighthouse Park in West Vancouver, BC. Little is known about the host range of *N. arbuti*, which has only been reported on *A. menziesii*, *Vaccinium corymbosum*, and once from *Cytisus scoparius*. A survey of Lighthouse Park was carried out to determine the cause and prevalence of cankers on *A. menziesii* in Lighthouse Park and to identify additional hosts of *N. arbuti*.

*Neofusicoccum arbuti* was the fungus most commonly associated with cankers on *A. menziesii*. The pathogen was isolated from 87% of cankers sampled. Cankers were prevalent throughout the park, with at least 75% of arbutus trees having one or more cankers at the majority of sites.

Furthermore, the host range of *N. arbuti* is much broader than previously thought. Seven non-arbutus hosts, spanning four taxonomic orders were identified, including *Amelanchier alnifolia*, *Cytisus scoparius*, *Gaultheria shallon*, *Ilex aquifolium*, *Rosa sp.*,*Sorbus sitchensis*, and *Spiraea douglasii*. These hosts could act as a reservoir providing additional inoculum or may be infected by spores produced on arbutus. The impact of these additional hosts is unknown.

**Key Words:** *Neofusicoccum arbuti*, *Arbutus menziesii*, arbutus, madrone, Botryosphaeriaceae, arbutus decline, Lighthouse Park, host range
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**Introduction**

*Arbutus menziesii*

*Arbutus menziesii* Pursh, referred to as Pacific madrone, madrona, or simply arbutus, has been experiencing a decline in parts of its range for the last 40 years (Davison 1972, Bressette 1995, Farr *et al.* 2005). While declining arbutus was originally noticed in an urban context, diseased trees have been reported in natural forests throughout the range of the tree species (Elliott 1999, Bressette 1995)

Arbutus is a broad-leaved evergreen tree with white, urn-shaped flowers, orange-red berries and characteristic bark. The red-orange, paper-like bark peels off to reveal pistachio-green juvenile bark. The leaves are dark green, leathery, and hairless, with a smooth margin and light-green underside. *Arbutus menziesii* can grow as tall as 30 m, though it rarely reaches such heights. A shade-intolerant, drought-tolerant species, in the Northwest part of its distribution arbutus grows in the open on dry, rocky slopes, seldom far from the ocean shore (Alaback *et al.* 2004). The range of arbutus extends along the Pacific coast of North America from southwest British Columbia to southern California (McDonald and Tappeiner 1990).

Arbutus trees hold ecological and cultural value. The berries of arbutus are an important food source for wildlife and the trees are used by cavity nesting bird species (Gurung *et al.* 1999). Arbutus is an important component of several different forest types throughout its range, from the Coastal Douglas-fir biogeographic zone to the coast redwood-tanoak forests of California (McDonald and Tappeiner 1990). While not a commercial timber species, arbutus is a prized ornamental and its sinuous, richly colored wood lends itself to artisan woodworking (McDonald and Tappeiner 1990). Arbutus is
also an important cultural species for the Saanich and Straits Salish tribes (Turner and Hebda 1990, Alaback et al. 2004)

A range of fungal pathogens, including a dozen foliar pathogens, causing defoliation, wood decay, root rots and cankers affect arbutus (Elliott 1999, Hunt et al. 1992). According to Elliott (1999), for the most part, foliar pathogens are thought to not pose a major threat to the long-term survival of arbutus, unless defoliation occurs over several successive years. Recent papers have implicated Neofusicoccum arbuti (D.F. Farr & M. Elliott) Crous, Slippers & A.J.L. Phillips as the leading cause of cankers and disease in the Northwest portion of the Arbutus menziesii range (Farr et al. 2005, Elliott et al. 2002). It has been theorized that a number of additional factors have contributed to the success of the pathogen, including the removal of fire from the ecosystem, climate change and the Pacific Decadal Oscillation (Elliott 1999, 2005; Bressette 1995).

The Decline

Declining arbutus trees were first reported in Washington’s Puget Sound in 1972 (Davison 1972). A particularly severe drought in the summer of 1967 followed by a very cold winter in 1969 was thought to have made conditions ripe for the establishment of a fungal pathogen identified by Davison as Hendersonula toruloidea.

In 1989, Hendersonula toruloidea was erroneously renamed Nattrassia mangiferae (associated with fruit trees and nut trees in sub-tropical and tropical locations) (Nattrass 1933, Sutton and Dyko 1989, Elliott et al. 2002). The erroneous classification was recognized in 2003 and the canker pathogen affecting arbutus was attributed to an as yet, undescribed species that had been misidentified as Nattrassia mangiferae (Elliott and
Edmonds 2003, Farr et al. 2005,). In 2005, this canker causing pathogen of arbutus was formally described as *Fusicoccum arbuti*, and placed into the genus *Botryosphaeria* (Botryosphaeriaceae family) (Farr et al. 2005). In 2006, the taxonomy of the Botryosphaeriaceae underwent major revisions and it was determined that *Fusicoccum arbuti* was actually in a distinct genus with morphology closely resembling but differing from that of species in the *Fusicoccum* genus. This newly defined genus, which includes several other former *Fusicoccum* species, is called *Neofusicoccum*, thus *F. arbuti* became *Neofusicoccum arbuti* (Crous et al. 2006).

**Neofusicoccum arbuti Description**

Species in the fungal family Botyosphaeriaceae are commonly endophytic and many are latent pathogens of woody shrubs and trees (Slippers and Wingfield 2007, DeWet et al. 2008). Latent endophytic pathogens may live undetected in a host without expressing any disease or damage until stressful conditions weaken the host. When the host is stressed, the latent endophytic pathogen takes the opportunity to express itself and in the case of the majority of pathogenic species in the Botryosphaeriaceae, cankers are formed on the main stem and/or branches (Slippers and Wingfield 2007).

*Neofusicoccum arbuti*, like other members of the Botryosphaeriaceae, is a latent endophytic pathogen. When an infected arbutus tree is stressed, *N. arbuti* causes the development of cankers on the main stem (Elliott 2005). Cankers formed by *N. arbuti* may appear open with a sooty appearance or partially callused over (Elliott 2005, Davison 1972). Open cankers are usually sunken, often with a raised and convoluted margin (Figure 1) (Elliott 2005, Davison 1972). If the tree is healthy enough it may callus
over the canker completely, but if the tree is very stressed many cankers can form, girdling small stems or branches and leading to water stress and dieback in the crown (Elliott 1999, Elliott 2005). Arbutus that have been subjected to heat damage or sudden exposure to sunlight (i.e. after logging) are more prone to infection by *N. arbuti* (Davison 1972, Bressette 1995). Older arbutus trees grown in direct sunlight with thick, persistent bark tend to have fewer infections than thin-barked, shade-grown trees (Bressette 1995).

It has also been theorized that cankers formed by *N. arbuti* act as inoculation points for secondary pathogens, such as *Fusicoccum aesculi*, which cause further dieback in the crown as trees become increasingly water-stressed (Elliott 1999). After 50% dieback in the crown, the trees enter a decline spiral, which can lead to mortality (Elliott *et al.* 2002).

In culture, Koch’s postulates, and pathogenicity trials, it has been shown that the minimum growing temperature for *N. arbuti* is 15°C and the ideal growing temperature is 20-25°C (Davison 1972, Elliott 1999, Espinoza *et al.* 2009). Koch’s postulates have also shown that canker development is greatly increased in drought stressed trees (Elliott 1999). While *N. arbuti* can enter a host through injuries on the stem and branches, wounds are not necessary for infection at temperatures near 25°C (Davison 1972). Water activity (a_w) has also been shown to affect the growth rate of *N. arbuti* in culture with optimal growth at a_w = 0.99 and dramatic reduction in growth when a_w is less than 0.98 (Latorre *et al.* 2012).
Host Range

If a pathogen is to be successfully prevented, controlled, or quarantined, it is important to understand its host range (Dinoor and Eshed 1984, Gilbert and Webb 2007). If the host range is unknown, there is the potential for human facilitated distribution of the pathogen to ecologically sensitive areas or to agricultural crops (Dinoor and Eshed 1984). Host ranges in the Botryosphaeriaceae and the genus *Neofusicoccum* vary from specific to cosmopolitan (Slippers and Wingfield 2007). For example, *Neofusicoccum parvum* has a circumpolar distribution and has been isolated from 90 host species, while at the other end of the spectrum, *Neofusicoccum ribis*, is found only on *Ribes* sp. in North America (Sakalidis *et al.* 2013).

Very little is known about the host range of *Neofusicoccum arbuti*. Until recently, *N. arbuti* had only been isolated from *Arbutus menziesii* in the United States (Washington, Oregon, and California) and Canada (British Columbia) and once from *Cytisus scoparius* (scotch broom, location not reported) (Farr *et al.* 2005, Elliott 2005). In 2009, *N. arbuti* was isolated from several cultivars of the commercial blueberry species *Vaccinium corymbosum* in Río Negro, Chile (Espinoza *et al.* 2009). Similar to symptoms on infected arbutus, *N. arbuti* was found to be causing cankers and dieback in *V. corymbosum*, and was restricted to areas of Chile with cool wet conditions (Espinoza *et al.* 2009). Reports of *N. arbuti* on *V. corymbosum* and *C. scoparius* suggest that the host range of *N. arbuti* may be even broader than these three species. If *N. arbuti* is found on a commercially important species in the native environment of arbutus, it may become an important pathogen in the future with the advent of climate change (Sturrock *et al.* 2011).
Thus, the identification of additional hosts would be beneficial for the management of *N. arbuti*.

One of the best places to see arbutus trees near Vancouver is Lighthouse Park, an area with historical and ecological significance. The park is about 75 hectares (185 acres) in size and is one of the few remaining examples of Coastal Douglas-fir old growth in the Vancouver area (Coates and Mondor 1978). It has been the location of the Point Atkinson Lighthouse since 1874 and in WWII was outfitted with heavy artillery as a strategic defense point (Catherine Berris Associates Inc. 2004). Today, Lighthouse Park is a popular public park maintained by District of West Vancouver. In recent years, declining arbutus trees with wart-like cankers on their stems have been reported in the park (Pynn 2006). Our preliminary sampling of the park indicated disease symptoms consistent with *N. arbuti* infections in arbutus and one culture isolated from arbutus tissues was identified as *N. arbuti*. To determine the prevalence of cankers in the park, and to look for additional hosts of *Neofusicoccum arbuti*, a survey was carried out in Lighthouse Park in September-October of 2012.

There were three broad objectives of this study. The first objective was to confirm that *Neofusicoccum arbuti* is the primary fungus causing cankers and disease in arbutus in Lighthouse Park, and determine the prevalence of cankers in the park. The second objective was to identify any additional non-arbutus hosts of *N. arbuti* within the native ecosystem of arbutus. The final objective was to see if any genetic diversity was present in *N. arbuti* within the park, or if isolates differed genetically from those collected in previous studies.
Methods

Survey

To determine the prevalence of arbutus cankers in Lighthouse Park, the park was surveyed over the course of eight days in late August and early September of 2012. First, the distribution and location of arbutus was mapped. Using a map of the trail system, every trail in the park was walked at least once and any area near the trail that had a group of four or more arbutus trees was marked using a Garmin Etrex Venture HC GPS device. In total, 30 sites were identified (Figure 2).

At each site a variety of factors were noted. A rough estimate of the number of trees was recorded; trees were counted twice and the two values were averaged. Since arbutus stands are typically maintained by fires and have evolved to regenerate from a basal starch ball, it is common to see several stems growing very close together, which are actually one tree (Elliott et al. 2002, McDonald and Tappeiner 1990). If it was obvious that several trees were actually a cluster sprouting from a root burl, they were counted as one tree.

The main metric used in determining the extent of the pathogen in the park, the prevalence of cankers, was recorded at each site. To determine the prevalence of cankers at each site, five or so trees were counted and the proportion of those that had at least one canker was noted. From this fraction, the percentage of trees on the site with at least one canker was estimated and the site was rated on a scale of 1-4. Where:

1 = 0-25% of trees have at least one canker
2 = 26-50% of trees have at least one canker
3 = 51-75%...
4 = 76-100%...
There was some difficulty in distinguishing a canker from a knot or an abscised branch. Erring on the side of caution, questionable cankers were not counted. The coarseness of the disease classes should account for any under or over-estimations of disease.

Since one of the main means of inoculation by *N. arbuti* is through wounds (Davison 1972, Elliott *et al.* 2002), human inflicted disturbance was also recorded for each site. Human disturbance was considered to be carvings and trampled roots. The rating system for disturbance was qualitative and subjective (none, some, common). Sites with “some” human disturbance might have one or two trees with trampled roots or carving on their trunk, sites with “common” disturbance would have several trees with moderate to heavy damage from trampling or carving. Ease of sampling was also noted for each site, based on accessibility, hazard level, and height of cankers on trees.

Finally, to narrow the focus of potential non-arbutus hosts that we would be sampling, vegetation was recorded for each site. The vegetation survey focused on woody plants, shrubs, and trees since these are the most common hosts of pathogens in the Botryosphaeriaceae (DeWet *et al.* 2008). The plants that occurred most frequently in association with arbutus were selected for sampling.

**Sampling**

Collection of canker material and non-arbutus vegetation was carried out in late September and early October of 2012. In total, 10 sites were picked for sampling based on distribution throughout the park, inclusion of a “healthy” site and a “very diseased” site, number of trees, and ease of sampling (Figure 2). At eight sites, samples were taken
from a single canker on three separate arbutus trees and from three non-arbutus hosts. Much broader sampling was employed at one “healthy” site and one “very diseased” site. At these two sites up to nine (eight at the healthy site, nine at the very diseased) samples were taken from non-arbutus hosts and from six arbutus trees. At the very diseased site, arbutus tree samples were tissues from cankers. At the healthy site, arbutus tree samples were tissues from one canker, one raised bump that did not match descriptions of *N. arbuti* cankers, and tissues from four healthy stems.

In accordance with the canker sampling methods in Elliott (1999), a hammer and chisel were used to take chips from the canker margin; the raised edge of the canker where the healthy looking tissue meets the diseased. Between each canker the chisel was sterilized with ethanol. To sample non-arbutus vegetation, stem or branch cuttings were taken using ethanol-sterilized pruners. If cankers were seen on the stems or branches, those stems/branches were collected.

At eight sites, stem sections were taken from three species: *Amelanchier alnifolia* Nutt. and *Gaultheria shallon* Pursh., plus another less frequent plant; either, *Cytisus scoparius* (L.) Link, *Ilex aquifolium* L., *Spiraea douglasii* Hook., or *Vaccinium parvifolium* Sm.. At the two sites with broad sampling, *Paxistima myrsinites* (Pursh) Raf., *Arctostaphylos uva-ursi* (L.) Spreng., *Rosa* sp., *Prunus* sp., *Rubus discolor* Weihe & Nees, *Sorbus sitchensis* M. Roem, were also collected.

**Cultures and Processing**

Samples were surface sterilized in bleach, ethanol, and rinsed in several baths of autoclaved, deionized water as described in Taylor *et al.* (2009). Each sample was cut
into three or four pieces and plated on 2% MEA as in Farr et al. (2005). Tools were sterilized between each sample. Cultures were then allowed to grow approximately one week before sub-cultures were made. (Farr et al. 2005)

From each primary isolate, up to three sub-cultures were made from distinct fungal outgrowths. These were, in turn, isolated a second time under the fume hood. Further sub-isolations and transfers were made if cultures appeared mixed or contaminated.

Cultures were sorted based on morphology, growth characteristics, and host. Species in the Botryosphaeriaceae tend to grow relatively fast and have a fluffy cotton-like appearance due to their aerial mycelium. As cultures age, the center become darkly pigmented and appears grey, indigo-grey, or black from the reverse side of the petri dish (Slippers and Wingfield 2007). Neofusicoccum arbuti follows this trend in culture morphology (Farr et al. 2005, Espinoza et al. 2009). Cultures were grouped into four categories: Botryosphaeriaceae-like (bot-like) cultures from arbutus, bot-like from non-arbutus hosts, non-bot-like from arbutus, and non-bot-like from non-arbutus hosts. Once pure cultures were obtained they were scraped and placed in Eppendorph Flex-Tubes® and stored at -80° C.

DNA Extraction and PCR

DNA was extracted from all cultures except for non-bot-like cultures from non-arbutus hosts. While still frozen, small pieces of mycelium were placed in 1.5 μl tubes containing two tungsten beads. Samples were then placed in a tissue lyser at maximum speed for three minutes. Tungsten beads were removed and 1 μl of sterile, deionized
water was added to each tube. The mycelium solution was then vortexed and placed in a water bath set at 96°C for 5 minutes. Reusable locks were placed on tubes to prevent them from popping open in the water bath. The boiled mycelium solution was used directly for PCR.

Because the identities of the fungi were unknown and variable, a “touchdown” PCR protocol was used to amplify the internal transcribed spacer (ITS) gene using the standard ITS1F and ITS4R primers. The ITS gene region has been identified as an ideal molecular barcode for identification of fungi because it is generally species specific, present in large quantities in each cell, and can be amplified using universal primers (Gardes et al. 1991). A touchdown PCR cycles through multiple annealing temperatures. In this case annealing temperatures ranged from 52°C to 57°C. PCR was carried out in a BIORAD T100 thermal cycler and an Applied Biosystems 2720 thermal cycler with the following protocol: denaturation at 95°C for three minutes, followed by a second denaturation step (95°C for 30 seconds), a variable annealing step that started at 57°C for 30 seconds and decreased by 1°C each cycle, and an elongation step (72°C for one minute). Steps two though four were repeated five times. This was followed by 34 cycles of denaturation (95°C for 30 sec), annealing (55°C for 30 sec), and elongation (72°C for one minute). The protocol finished with a 10 minute elongation at 72°C.

Sequences were aligned and manually edited using Geneious Pro version 6.0.5 created by Biomatters (available from http://www.geneious.com); a phylogenetic analysis program. A BLAST (Basic Local Alignment Search Tool) search in the NCBI (National Center for Biotechnology Information) database was used to identify species, and the species with the highest pairwise identity match was noted.
To look at the genetic diversity of *N. arbuti*, ITS gene sequences were compared between all isolates in Lighthouse Park, and with isolates from throughout the range of arbutus. In the ITS region, the only known diversity is a single nucleotide polymorphism (SNP) identified by Elliott (2005), located at the 196th base pair (beginning of the 5.8s region of the rDNA). This SNP consists of an adenine in the isolates from Washington, southern California, and Sierra Nevada mountains; and a guanine in the central Oregon and northern California isolates (Figure 4) (Elliott 2005).

Using TCS version 1.13, a haplotype network was constructed for the aligned ITSrDNA region (Figure 3)(Clement *et al.* 2000). The program determines the haplotypes present based on all input DNA sequences and calculates the frequency of each haplotype in the sample. Haplotype frequency is then used to estimate out-group probabilities, which are proxies for the age of each haplotype (Castelloe and Templeton 1994, Donnelly and Tavare 1986, Sakalidis *et al.* 2011). The program then creates an absolute distance matrix, which is used to estimate phylogenetic networks. A probability of parsimony is used to create phylogenetic networks, using 0.95 as the threshold probability (Templeton *et al.* 1992, Sakalidis *et al.* 2011).

**Results**

**Survey Results**

All of the 30 sites identified in the park had cankers on arbutus. While there was some variability in the prevalence of cankers from site to site, the majority of sites were in health class four (Figure 2). In total, 19 sites were in health class four, eight sites were in class three, two sites in class two, and one site in class one. Approximately 76% to
100% of the trees had at least one canker at the majority of the sites surveyed. Only site one could be considered a “healthy” site (less than 25% of the trees had at least one canker). Human disturbance was higher in places with benches for seating and at heavily visited viewpoints. Trees that had visible signs of human disturbance tended to have the worst cankers. Cankers were also observed on *Gaultheria shallon*, from which *N. arbuti* was isolated.

**Primary Pathogen in Arbutus Cankers**

*Neofusicoccum arbuti* was the dominant pathogen isolated from cankers on arbutus (Table 1). *Neofusicoccum arbuti* was isolated from arbutus at every site sampled and was found in 27 out of 31 (87%) of cankers sampled. *Neofusicoccum arbuti* was also isolated once from the smooth un-cankered stem of an apparently healthy arbutus at the “healthy” site. Seven cankers produced non-bot like cultures. Of these, four isolates were unable to be identified because their DNA did not amplify during PCR. Also, three of the cankers that produced non-bot-like cultures also produced cultures identified as *N. arbuti*.

**Additional Hosts**

All Botryosphaeriaceae-like cultures isolated from non-arbutus hosts were identified as *N. arbuti* except for one; a fungus identified via BLAST search as *Botryosphaeria stvensii* (100% pairwise identity match), which was isolated from *Amelanchier alnifolia*. In total, *N. arbuti* was isolated from seven of the twelve non-arbutus hosts sampled (Table 2). New hosts identified include *Amelanchier alnifolia*, *Gaultheria shallon, Ilex aquifolium, Rosa sp.*, *Sorbus sitchensis*, and *Spiraea douglasii*. 
Cytisus scoparius was also confirmed as a host. Also, dead shoots of Gaultheria shallon with cankers (and some without) were noticed during the collection of samples, N. arbuti was isolated from some of these cankers.

**Non-Botryosphaeriaceae-Like Cultures From Arbutus**

In addition to N. arbuti, there was a total of seven other species of fungi isolated from arbutus (Table 1). Three of the non-bot-like fungi came from the stems of non-cankered trees; one culture was identified as Cytospora sp. (97.6% pairwise identity match), another was identified as Cytospora austromontana (99% match), and the third was identified as Leptosphaerulina chartarum (100% match).

A different non-bot fungus came from a raised bump, or “blister”. This blister did not match descriptions of N. arbuti cankers and the culture isolated from the blister was identified as Diaporthe viticola (98.9% match).

Four non-bot fungi were isolated from three cankers. One of these cankers was a rather old, cracked lesion with a burnt appearance, from which two fungi were isolated and identified. One was identified as Cytospora sp. (97% match) and the other was Absidia glauca (97.7% match). The other two non-bot fungi came from typical cankers. Hypocrea rufa (100% match) was isolated from one, though it should be noted that N. arbuti was also isolated from the same canker. The other canker produced a culture that was identified as Candida zeylanoides (100% match).
**Genetic Diversity of *Neofusicoccum arbuti***

All isolates of *N. arbuti* had identical sequences in the ITS region. At the region of the SNP identified in Elliott (2005), the Lighthouse Park isolates all matched the Washington/Southern California/Sierra Nevada mountains haplotype, and were grouped with those isolates in the haplotype network (Figures 3). Based on haplotype frequencies the haplotype network shows that isolates with an adenine at the SNP are probably ancestral to those with a guanine.

**Discussion**

The results of this study show that *Neofusicoccum arbuti* is the primary fungal pathogen associated with cankers in *Arbutus menziesii* at Lighthouse Park in West Vancouver, BC. *Neofusicoccum arbuti* was isolated from arbutus at all 10 sites sampled and 87% of cankers sampled. Cankers are quite common in the park and 19 of the 30 sites surveyed (63%) were in health class 4, indicating that at least 76% of the trees at those sites had at least one canker. Furthermore, the host range of *N. arbuti* is much wider than previously thought. The pathogen was isolated from seven non-arbutus species in four different orders, including indigenous and introduced species. All isolates of *N. arbuti* were genetically identical in the ITS region and shared the same sequence as the Washington/Southern California/Sierra Nevada haplotype identified in Elliott (2005). *Neofusicoccum arbuti* is very prevalent throughout Lighthouse Park, causing cankers on *Arbutus menziesii* and has a much broader host range than previously thought.
Primary Pathogen Isolated From Cankers

The result of *N. arbuti* being the most frequently isolated fungus from cankers is not surprising and confirms the results of previous canker surveys on arbutus (Elliott 1999, Elliott *et al.* 2002). In Elliott *et al.* 2002, *N. arbuti* was isolated from the margin of cankers 90% of the time. As distance from the canker margin increased, the frequency of *N. arbuti* decreased but was still the most frequent pathogen isolated even 8 cm from the canker margin. In the same study, *Neofusicoccum arbuti* was also the most frequently isolated fungus from both new and old cankers on stems and branches (Elliott *et al.* 2002).

One caveat that should be taken into consideration when reviewing the results of this survey is that *Neofusicoccum arbuti* and most species in the Botryosphaeriaceae are very fast growing (Farr *et al.* 2005, Slippers and Wingfield 2007). In surveys of endophytes, species in the Botryosphaeriaceae family are frequently the first species to emerge and grow in culture (Slippers and Wingfield 2007). While primary isolations were checked multiple times for other cultures, it is possible that some very slow growing species were missed. Despite this, the frequency of isolations of *N. arbuti*, both in this study and others, plus Koch’s postulates carried out in previous studies, strongly support that *N. arbuti* is the dominant pathogen causing cankers in arbutus (Elliott 1999, Elliott *et al.* 2002). Furthermore, disease symptoms observed in the park, including canker morphology and crown dieback match descriptions of disease and damage caused by *N. arbuti* (Elliott 1999, Farr *et al.* 2005). Therefore, the results of this study show that *N. arbuti* is the dominant pathogen causing cankers in arbutus in Lighthouse Park.
**Additional Hosts of *Neofusicoccum arbuti***

It remains to be seen whether *N. arbuti* can cause disease on non-arbutus species. It might be noted, however, that there were dead shoots of salal observed during sampling, some of which had cankers that produced *N. arbuti* cultures. In the future, pathogenicity trials need to be carried out on newly identified hosts of *N. arbuti* to confirm that they are indeed hosts and to ascertain the degree to which the pathogen causes disease and damage, and under what conditions it expresses itself on those plants.

In total, *Neofusicoccum arbuti* was isolated from seven non-arbutus hosts, both native and introduced, from four different taxonomic orders: the Rosales, Ericales, Fabales, and the Aquifoliales. The new hosts are *Gaultheria shallon*, *Amelanchier alnifolia*, *Spiraea douglasii*, *Sorbus sitchensis*, *Rosa* sp. (either *R. gymnocarpa* or *R. nutkana*), *Cytisus scoparius* (confirmed), and *Ilex aquifolium* (Table 2). This has increased the known host range of *N. arbuti* from two hosts to nine hosts.

*Gaultheria shallon* (salal) is an indigenous evergreen shrub with dark-green leathery leaves and bell shaped flowers in the Ericaceae family (Order Ericales) (Alaback *et al.* 2004). Salal was one of the most common species at arbutus sites in the survey of Lighthouse Park and may act as source of inoculum for *N. arbuti*. In parts of the Northwest there is a thriving market for the production of salal as a filler plant for flower arrangements (Wills and Lipsey 1999). In BC, an estimated 12-15,000 people are employed by the salal industry and on the Southern tip of Vancouver Island alone, annual revenues have been estimated at $6-10 million (Wills and Lipsey 1999, Hobby *et al.* 2010). While salal is primarily exported to Europe, there are numerous alternatives for background filler and so the market is somewhat delicate. Since reduced quality may lead
to lower European demand (Hobbs et al. 2010), *N. arbuti* may become an important pathogen to look out for in the future.

*Amelanchier alnifolia* (Saskatoon, pacific service berry), *Spiraea douglasii* (hardhack, steeple bush), *Sorbus sitchensis* (Sitka mountain-ash), and *Rosa* sp. (rose) are all indigenous shrubs in the Rosaceae family (order Rosales) (Alaback et al. 2004). *Spiraea douglasii* is useful in restoration projects for stabilizing stream banks, and can compete well with exotic canary reed grass (Darris and Gonzalves 2009). The sweet, nutty-flavored pomes of *A. alnifolia*, which resemble blueberries, are gaining popularity as a horticultural crop in the prairie provinces of Canada due to their antioxidant properties (St. Pierre 1992). Healthy appearing *Amelanchier alnifolia* specimens supporting endophytic populations of *N. arbuti* could act as vectors for the transport of the pathogen to new environments. These four species, which are the first identified hosts of *N. arbuti* in the order Rosales, likely act as additional sources of inoculum for *N. arbuti* in areas where arbutus are in decline. Future pathogenicity test will reveal what conditions are needed for *N. arbuti* cankers to develop on these species.

*Cytisus scoparius* (Scotch broom), is a widespread shrub in the Fabaceae family (order Fabales), often seen growing along roadsides and in disturbed soils (Alaback et al. 2004). Scotch broom is considered a problem species due to its capacity to outcompete native species (Alaback et al. 2004). A single isolation of *N. arbuti* from *Cytisus scoparius* in an area with a large source of inoculum was reported in Elliott (2005), a PhD thesis, but it was not included it in the formal description of the pathogen (Farr et al. 2005). The results from this survey confirm Elliott’s report of *Cytisus scoparius* as a host.
An introduced, broad-leaved evergreen in the Aquifoliaceae (order Aquifoliales) *Ilex aquifolium* (English holly) grows as a tree or shrub and is native to Great Britain and Europe with a large distribution, extending as far south as Northern Africa and Southwest Asia (Peterken and Loyd 1967, Jones and Reichard 2009). English holly is a common ornamental and a frequent garden escapee, dispersed primarily by frugivorous birds (Zika 2010).

*Cytisus scoparius* and *Ilex aquifolium* are especially interesting as hosts of *N. arbuti* because they are both introduced species (Dennis 1980, Weber 2003, Jones and Reichard 2009). These non-indigenous hosts from two different taxonomic orders, both of which are different from the order of the known hosts for *N. arbuti* (Ericales), are interesting because they demonstrate a flexible host affinity in *N. arbuti*. Also, since both non-natives are fairly widespread they might increase inoculum and facilitate an increase in the distribution of the pathogen.

Given that *N. arbuti* was isolated from 7 out of 12 non-arbutus species sampled, it’s likely that further hosts of *N. arbuti* remain undiscovered. A broad host range may suggest that the species has the capacity to be very pathogenic. A review of studies on the Botryosphaeriaceae family notes that the species that are most frequently isolated and cause the most damage are often those which have the broadest host range and widest geographic distribution (Slippers and Wingfield 2007). As an example, *Neofusicoccum parvum* has been proven to cause disease on a wide variety of commercial timber and agricultural species (Sakalidis et al. 2013).

The cosmopolitan distribution of *N. parvum* is thought to be the result of global trade of plant material (Sakalidis et al. 2013). Due the endophytic nature of
Neofusicoccum spp. and other species within the Botryosphariaceae, successful recognition of infected plant material and adequate quarantine measures are nearly impossible (Slippers and Wingfield 2007 and Sakalidis et al. 2013). Given that N. arbuti has already been isolated from blueberries in Chile, and is apparently found in other commercial plant species such as salal and saskatoon, the probability that the range of N. arbuti will be broadened through international trade is very high.

If it is introduced to another region, climate will likely play a role in where N. arbuti can establish populations. For example, in Chile, N. arbuti was only reported in regions with cool, wet weather and was not isolated in the warm, dry, central region of the country (Espinoza et al. 2009). This climate-limiting trend of Botryosphaeriaceae has been observed in other instances and is thought to be just as important as, if not more than, host range in determining distribution since Botryosphaeriaceae species often lack host specificity (Slippers and Wingfield 2007). As an example, it has been suggested that climate was the key factor determining where Botryosphaeriaceae species could colonize Vitis vinifera (grape) in parts of Spain, Mexico, and Australia (Urbez-Torres et al. 2006, 2008, Pitt et al. 2010, Sakalidis et al. 2013).

Non-Botryosphaeriaceae-Like Fungi Isolated from Arbutus

In addition to N. arbuti, seven other fungal species were isolated from arbutus. Half of the non-bot fungi were isolated from trees that appeared healthy or had non-typical cankers (raised bumps or blisters, which may have been cankers that were healed over by the tree or had not yet broken the bark surface) (Table 1). Non-bot-like fungi isolated from the healthy stems and non-typical canker included Cytospora sp.,
Cytospora austromontana, Diaporthe sp., and Leptosphaerulina chartarum. Non-bot-like fungi isolated from cankers included Cytospora sp., Absidia glauca, Trichoderma viride and Candida zeylanoides.

Cytospora spp. are latent endophytes that have been known to cause cankers and dieback and are also secondary invaders found in association with cankers caused by other pathogens, such as Botryosphaeria dothidea (Adams et al. 2005, Betucci et al. 1999, Sinclair and Lyon 2005). Cytospora spp. are found in over 85 hosts species, primarily woody trees and shrubs, and are capable of causing serious damage in fruit crops and orchards (Sinclair and Lyon 2005, Adams et al. 2005). In this study, it is possible that the Cytospora spp. isolated were acting as endophytes in the healthy trees they were isolated from and as a secondary pathogen in the older canker.

Cytospora austromontana G.C. Adams & M.J. Wingf. has only been isolated from dead cankered branches of Euclyptus pauciflora in New South Wales, Australia (Adams et al. 2005). Since it has only been previously reported in a single, remote region in the Southern Hemisphere the Cytospora sp. identified in this study may actually be a species closely related to C. austromontana.

Another culture isolated from arbutus was identified as Diaporthe sp. The genus Diaporthe, which has anamorphs in the genus Phomopsis, is comprised of hundreds of species which range in pathogenicity from the highly aggressive to opportunistic saprophytes found exclusively in necrotic plant tissues (Sinclair and Lyon 2005). The Diaporthe sp. was isolated from a bump or blister, not from a typical canker. It is not known if it caused the formation of the bump or if it was living as saprophyte in a canker underneath that had healed over.
Leptosphaerulina chartarum, the teleomorph of Pithomyces chartarum, is a primarily saprophytic, occasionally pathogenic fungus in the Pleosporaceae family that has a very wide host range and distribution (Kodsueb et al. 2006, Toth et al. 2007). It has been isolated from over 186 plant species around the world, including Africa, Asia, Europe, North America and South America, though not from Arbutus menziesii (Farr and Rossman 2013 a).

Trichoderma viride (Teleomorph=Hypocrea rufa) is one of the most widely isolated and reported species of fungi and is commonly used as a biocontrol agent (Hermosa et al. 2000). However, Jaklitsh et al. (2006) showed that the majority of species in the Trichoderma genus are indistinguishable in the ITS 1 and ITS 2 region and thus it is likely that many of the reports of T. viride are actually misidentifications. Since we only looked at the ITS region in our own study, it is very possible that the species isolated from arbutus was also a misidentified Trichoderma sp., especially since it was identified using a BLAST search on NCBI and not by culture or spore morphology.

Trichoderma spp. were also isolated from a number of cankers in Elliott (1999) and were more frequent in older cankers than new (Elliott 1999, Elliott et al. 2002). Seedlings inoculated with Trichoderma alone did not express any symptoms of disease (Elliott 1999, Elliott et al. 2002).

Candida is a group of yeast fungi associated with humans and other animals, causing disease (candidiasis) in primarily immunodeficient hosts (Coutinho 2009). Candida zeylanoides has been occasionally, albeit rarely, isolated as a human pathogen causing onychomycosis (nail fungus) (Crozier 1993), fungaemia (a kind of fungal blood infection) (Levenson et al. 1991), and arthritis (Bisbe et al. 1987). Considering that
Candida spp. are primarily associated with animals, it is likely that this was a culture contamination. Furthermore, early photos of the culture appeared very much like *N. arbuti* in morphology.

Species in the genus *Absidia* are ubiquitous in soil, and are common airborne contaminants (Donnison *et al.* 2000, Cvetnic and Pepeljnjak 1997). In immune-compromised humans, *Absidia* spp. have been known to cause lung disease and other health issues (Ribes *et al.* 2000). *Absidia glauca* is a common model organism used in a range of molecular and mycological studies involving zygomycetes (Kellner *et al.* 1993, Kayser and Wöstemeyer 1991, Wöstemeyer et al 1987,). Given that *Absidia* species are frequently found in soil and air and have not been reported on *Arbutus menziesii* (Farr and Rossman 2013b), it is possible that the *Absidia* isolate in this study was a culture contaminant.

**Health of Arbutus in Lighthouse Park**

Several factors may be involved in the decline of arbutus in Lighthouse Park. Firstly, the natural disturbance regime of Lighthouse Park has been significantly altered. Fires, however infrequent they may naturally occur, have been entirely removed from the ecosystem (Catherine Berris Associates Inc. 2004). While this leads to a beautiful and complex climax Coastal Douglas-fir forest, it also means a higher degree of competition for shade-intolerant species like arbutus in some areas of the park (Elliott 1999, Bressette 1995). Without fire, arbutus is shaded out and more susceptible to insects and pathogens such as *N. arbuti* (Elliott 2005, Bressette 1995). In this way, the decline of arbutus can be seen as a natural successional process in the absence of disturbance (Castello *et al.* 1995).
For arbutus growing on open rocky sites, an alternative theory is that human disturbance, through peeling off bark, carving, and trampling roots, has increased points of inoculation for pathogens and induced stress in arbutus (Davison 1972, Elliott 1999, Bressette 1995). Many of the open growing arbutus in the park are found at viewpoints along the shore. More trees with human disturbance were noticed at these areas, and those with heavy abuse (carving was most noticeable) tend to have high levels of disease and *N. arbuti* cankers. However, since human disturbance was usually restricted to a few trees at a site, it is unlikely that human-inflicted wounds caused by carving or trampled roots are the main points of inoculation for *N. arbuti* populations in Lighthouse Park.

Furthermore, Lighthouse Park is home to a large population of arbutus, as well as the additional hosts identified in this survey, which means the source of inoculum is high. Incidence of plant pathogens is positively correlated with the density of suitable host plants (Burdon and Chilvers 1982). The non-arbutus hosts identified in this study are prevalent throughout the park, meaning that host density is much higher than when considering arbutus alone.

The results of the survey may also paint a more dire picture of health in the park than reality. While the prevalence of cankers seems very high, the health rating classes were very broad and estimates of canker prevalence were rough; calculated from five trees per site. Also, only one canker needed to be present on a tree in order to be counted which does not mean necessary death for the tree (Elliott 1999). Furthermore, while many trees in the park did indeed have cankers, many of the trees appeared to be in otherwise good health despite some dieback in the crown. *Neofusicoccum arbuti* is suspected to be an indigenous pathogen (Elliott 2005). If it is indeed an indigenous
pathogen, arbutus should be able to tolerate low levels of disease, however if the trees become increasingly drought stressed, pathogenicity trials have shown that the situation may become much worse (Elliott 1999, Ma et al. 2001).

Genetic Diversity

It is interesting that isolates of *N. arbuti* from Lighthouse Park match the Washington/Southern California/Sierra Nevada mountains haplotype identified by Elliott (2005) (Figures 3 and 4). This hints at some diversity within the species, however it is hard to speculate from just one gene region. To determine if there is any genetic diversity within the *N. arbuti* population of Lighthouse Park or between the Lighthouse Park population and other *N. arbuti* populations, other gene regions besides ITS, such as EF 1-α, β-tubulin, and RPB2 should be sequenced (Slippers and Wingfield 2007, Sakalidis et al. 2011). This may reveal genetic differences between *N. arbuti* isolated from arbutus and non-arbutus hosts or clarify whether *N. arbuti* is indigenous or introduced.

The Future of Arbutus in Lighthouse Park and Non-Arbutus Hosts

Given that *N. arbuti* and other latent endophytic pathogens in the Botryosphaeriaceae are only expressed when the host is weakened by suboptimal growing conditions, increasing drought frequency and intensity from climate change may increase the potential for wide scale decline of arbutus and an increase in the disease on non-arbutus hosts. Studies have shown that drought-stress creates prime conditions for the expression of pathogens in the Botryosphaeriaceae (Desprez-Loustau et al. 2009, Ma
et al. 2001). Koch’s postulates and pathogenicity trials of *N. arbuti* on seedlings have also shown dramatic increase in canker intensity under drought conditions (Elliott 1999).

Climate change predictions for the Northwest region vary from model to model, though on average a warming trend is predicted with a change of +1.1°C in average annual temperature by the 2020s, +1.8°C by the 2040s and +3°C by the 2080s (Mote and Salathe 2010). Predicted changes in precipitation are less pronounced with an average of +1 to +2% change (Mote and Salathe 2010). The modest predicted change in precipitation may be misleading. Some models predict that seasons will become more pronounced, that is, wetter autumns and winters and drier summers (Mote and Salathe 2010). In the case of warmer drier summers, canker pathogens in the Botryosphaeriaceae, such as *Botryosphaeria dothidea*, are predicted to increase in severity (Sturrock et al. 2011). *Botryosphaeria dothidea*, like *N. arbuti*, has been shown to be especially damaging in drought stressed hosts (Ma et al. 2001). Thus, it’s not difficult to imagine an increase in cankers caused by *N. arbuti*, on any of the hosts, in the case of increasingly warm, dry summers.

For individual trees, which may be valuable or culturally important, certain fungicide regimes may be effective in reducing canker size and preventing new infections (Elliott and Edmonds 2008). The best treatments, however, are arboricultural methods such as reduction or removal of sources of inoculum (i.e. heavily diseased trees), proper pruning methods that encourage healing, and management of competing vegetation (Elliott 2005). Unfortunately, since the host range of *N. arbuti* extends to indigenous and ubiquitous species other than arbutus, efforts to reduce inoculum levels in a natural forest context may be in vain.
Future Research Directions

Now that *N. arbuti* has been isolated from these seven non-arbutus species, it is imperative to understand the role that *N. arbuti* plays in the health of the new species and whether they might play a role in the spread of the disease. In the future, Koch’s postulates and pathogenicity trials should be carried out for each of the newly identified host species. Pathogenicity trials should focus on temperature and precipitation and should mimic climatic regimes predicted by climate change models. This will clarify the potential for disease and decline in native ecosystems resulting from *N. arbuti* in stressed hosts. It would also be interesting to see if *N. arbuti* isolates from non-arbutus hosts have the same pathogenicity when used to inoculate arbutus and vice-versa. Also, as mentioned above, several gene regions should be used to make any concrete assessments of genetic diversity among isolates and populations of *N. arbuti*.

Acknowledgments

I extend my sincere gratitude to Dr. Monique Sakalidis and Dr. Richard Hamelin, for their expert guidance and advice throughout this project. Also, thanks to Dr. Allan Carroll, Dr. Sarah Gergel, Avneet Brar, Padmini Herath, Stéphanie Beauseigle, Angie Dale, and the rest of the Hamelin Lab for their assistance in the lab and words of encouragement. Thanks to Dan Henegar, Manager of Parks Arboriculture and Horticulture for the District of West Vancouver for permission to sample in Lighthouse Park. Finally, thanks to my amazing Mom, for lending a sympathetic ear to my many worries.
References


Appendix

Figure 1: An arbutus stem with *Neofusicoccum arbuti* cankers

Figure 2: Arbutus sites identified in survey of Lighthouse Park, prevalence of cankers, and human disturbance. Each letter corresponds to one site. Brackets denote sites picked for canker/vegetation sampling. Letters stand for level of human disturbance: N= None, S= Some, C= Common. Sites are color coded by prevalence of cankers (disease class) where: green represents a ‘1’ (0-25% of trees have at least one canker); yellow = ‘2’ (26-50% of trees have at least one canker); orange = ‘3’ (51-75%); and red = ‘4’ (76-100%)
Figure 3: *Neofusicoccum arbuti* haplotype network.
Created using *N. arbuti* isolate ITS sequences from Elliott 2005 (OR, WA, Northern and Southern CA, Sierra Nevada Mountains) and sequences from Lighthouse Park isolates collected in this study.

–ATAAAGAAGCTTT— OR/ Northern CA Haplotype
–ATAAACTAACTT— WA/ Southern CA/ Sierra Nevada Mountains Haplotype
–ATAAACTAAACTT— Lighthouse Park Isolates

^196th Nucleotide of ITS Region

Figure 4: Single nucleotide polymorphism (SNP) in the 196th base pair of the internal transcribed spacer (ITS) region identified in Elliot (2005) differentiating two haplotypes of *Neofusicoccum arbuti*. One haplotype (with a guanine) exists in Oregon and Northern California, while the other haplotype (with an adenine) exists in Washington, Southern California and the Sierra Nevada Mountains. All Lighthouse park isolates are identical in the ITS region and match the WA/Southern CA/ Sierra Nevada haplotype.
Table 1: Fungi isolated from cankers and stems of arbutus.

<table>
<thead>
<tr>
<th>Site</th>
<th>Canker/Tree:</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>(NTC*) Cytospora sp. 97%, Absidia glauca 100%</td>
<td>(AHT) Neofusicoccum arbuti</td>
<td>(AHT) Cytospora sp. 97.8%</td>
<td>(AHT) NBL</td>
<td>(NTC**) Diaporthe sp. 98%</td>
<td>(AHT) Leptosphaerulina chartarum 100%</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>(T) N. arbuti (+1 NBL) (T) Candida Zeylanoides 100%</td>
<td>(T) N. arbuti (+1 BL)</td>
<td>(T) N. arbuti</td>
<td>(T) N. arbuti</td>
<td>(T) N. arbuti</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>(T) N. arbuti, Trichoderma viride 100%</td>
<td>(T) N. arbuti</td>
<td>(T) N. arbuti</td>
<td>(AHT) Cytospora austromontana 99%</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>(T) N. arbuti</td>
<td>(T) N. arbuti</td>
<td>(T) N. arbuti</td>
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<td></td>
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</tr>
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<td>5</td>
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<td>(T) N. arbuti</td>
<td>(T) N. arbuti</td>
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</table>

Non-bot fungi are reported with BLAST pairwise identity match percentage. All *Neofusicoccum arbuti* isolates had a 100% pairwise identity match to *N. arbuti* in NCBI database. Initialisms: (AHT): Apparently Healthy Tree; (BL): Bot-like culture, DNA did not amplify in PCR; (NBL): Non-bot-like culture, DNA did not amplify in PCR; (NTC*): Non-typical canker, very old, appearing canker, burned looking; (NTC**): Non-typical canker, bump or blister; (T): Typical canker
Table 2: Species sampled for endophytic populations of *Neofusicoccum arbuti*

<table>
<thead>
<tr>
<th>Potential Host</th>
<th>Common name</th>
<th>Order, Family</th>
<th>Botryosphaeriaceae-like culture produces?</th>
<th><em>Neofusicoccum arbuti</em> isolated?</th>
</tr>
</thead>
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<td><em>Amelanchier alnifolia</em> Nutt.</td>
<td>Saskatoon, Service Berry</td>
<td>Rosales, Rosaceae</td>
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<td>Yes</td>
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<td><em>Arbutus menziesii</em> Pursh.</td>
<td>Madrone, Arbutus</td>
<td>Ericales, Ericaceae</td>
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<td><em>Arctostaphylos uva-ursi</em> (L.) Spreng.</td>
<td>Kinnikinnick, Bearberry</td>
<td>Ericales, Ericaceae</td>
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<td><em>Cytisus scoparius</em> (L.)Link</td>
<td>Scotch broom</td>
<td>Fabales, Fabaceae</td>
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<td>Yes</td>
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<td><em>Gaultheria shallon</em> Pursh</td>
<td>Salal</td>
<td>Ericales, Ericaceae</td>
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<td><em>Ilex aquifolium</em> L.</td>
<td>Holly</td>
<td>Aquifoliales, Aquifoliaceae</td>
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<td><em>Paxistima mysinites</em> (Pursh) Raf.</td>
<td>Falsebox</td>
<td>Celastrales, Celastraceae</td>
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<td>-</td>
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<td>Rose</td>
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<td>Himalayan Blackberry</td>
<td>Rosales, Rosaceae</td>
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<td><em>Spiraea douglasii</em> Hook.</td>
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<td><em>Vaccinium parvifolium</em> Sm.</td>
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