Diplodia sapinea found on Scots pine in Finland

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Abstract

Diplodia sapinea is an important pathogen of *Pinus* spp. in many parts of the the world's temperate and sub-tropical zones. In Central Europe, this fungus has been known since its first description in the 19th century. But it was only in 2007 that the fungus was identified using DNA-markers in Northern Europe (in Estonia). Viljo Kujala reported observing *D. sapinea* on logging debris of Norway spruce in Finland in 1945, but we have not been able to verify the identifications from his herbarium samples. In 2004, we collected cones under asymptomatic Scots pine (*Pinus sylvestris*) trees at one location near the southern coast of Finland but failed to isolate *D. sapinea* from the samples. In 2015 and 2016, we collected fallen Scots pine cones in 15 Scots pine stands in various parts of Finland and the fungus was found at five locations in southwestern Finland including the location investigated in 2004. Frequency of cones with pycnidia of *D. sapinea* varied from 1 to 12 % in the infested stands. No symptoms of Diplodia tip blight or resinous cankers were detected in trees in the stands where the cones were collected. The results suggest a recent introduction after 2004 of the *D. sapinea* in Finland.

Key words: Climate, latent infection, fungi, forest pathology, conifer.

Introduction

Diplodia sapinea (Fr.) Fuckel (earlier *Diplodia pinea* and *Sphaeropsis sapinea*) is a pathogen of *Pinus* spp. that causes tip blight, resinous cankers on main stems and branches, dieback, misshapen tops, death of cones, seedling blight, sapwood staining, and sometimes root disease in seedlings (Sinclair & Lyon, 2005). Especially in managed *Pinus radiata* and *Pinus patula* plantations of the southern hemisphere, *D. sapinea* has caused large-scale dieback (Chou, 1976; Swart & Wingfield, 1991; Zwolinski, Swart, & Wingfield, 1995). In Europe the most susceptible native pine species are Austrian pine (*P. nigra*), mountain pine (*P. mugo*) and Scots pine (*P. sylvestris*) (Vujanovic, Arnaud, & Neumann, 2000; Iturritxa et al. 2013; Fabre, Piou & Desprez-Loustau, 2011). In France and Germany, Diplodia tip blight and canker damage on Scots pine has increased in incidence and importance in recent decades (Blaschke & Cech, 2007; Fabre, Piou & Desprez-Loustau, 2011). This has also been true on *P. mugo* and *P. nigra* in southern Europe (Maresi et al. 2002; Georgieva & Hlebarska, 2016).

Diplodia sapinea can persist for long periods of time as asymptomatic latent infections in pine hosts (Stanosz et al. 1997; Flowers et al. 2001; Bihon et al. 2011). Drought and other sources of stress trigger pathogen proliferation and development of disease outbreaks (Stanosz et al. 2001; Blaschke & Cech, 2007; Chhin & O'Brien, 2015). Also, hail damage has frequently been mentioned to result in serious disease outbreaks caused by *D. sapinea* on pines and the effect is particularly pronounced in pine stands stressed by drought (Swart & Wingfield, 1991). Infections of pines by *D. sapinea* have also been observed in association with insect damage in the absence of physiological stress (Haddow & Newman 1942; Swart, Wingfield & Knox-Davies, 1987a).

Diplodia sapinea is among the most common and widely distributed pathogens of pines worldwide. Consequently, it is interesting that, until recently, it has not caused disease outbreaks in northern Europe where the susceptible Scots pine is one of the dominant tree species. The pathogen was originally described in the 19th century as *Sphaeria sapinea* by Fries in Sweden (Fries, 1823) and as *Sphaeria pinea* by Desmazieres (1842) in France. It has, therefore, been known in Europe for over 170 years. However, DNA-based taxonomy has revealed high diversity within the *Botryosphaeriaceae* with many morphologically similar species (Phillips et al. 2013) and it is questionable whether the initial species descriptions correspond to what we now regard as *D. sapinea*. The first observations of *D. sapinea* in northern Europe (including Fennoscandia, the Baltic countries and North-East northwest Russia), verified by DNA-sequence based methodology, were from Estonia in 2007 and subsequently from north-western Russia and Sweden in 2013

(Hanso & Drenkhan 2009; Oliva, Boberg & Stenlid, 2013; Adamson et al. 2015). In Estonia, the fungus was initially found only on cones of the non-native Austrian pine but in 2010 it was also collected on needles. The fungus spread throughout Estonia within a few years and was detected in 2012 at one location on cones, needles and twigs of Scots pine (Adamson et al. 2015). The Russian record on native Scots pine in 2013 was made 110 km from the south-eastern border of Finland (Adamson et al. 2015). In Sweden *D. sapinea* was first detected in 2013 (Oliva, Boberg & Stenlid, 2013) and in 2016 a Diplodia tip blight outbreak occurred in a Scots pine stand in central Sweden (latitude and climate corresponding to that of the south coast of Finland) and in another stand in 2017 in southern Sweden (https://www.skogsstyrelsen.se/nyhetslista/nytt-fall-av-diplodia-pa-tallbestand/).

Environmental conditions could previously have prevented *D. sapinea* from becoming established in northern Europe because low winter temperatures are known to correlate negatively with its occurrence in France (Fabre, Piou & Desprez-Loustau, 2011). This view is supported by observations of the temperature response of this fungus *in vitro*. Both the maximal and optimal temperature for growth and minimal threshold temperature of *D. sapinea* in artificial growth media are high; 40 °C, 28 °C and 4 °C, respectively (Keen & Smits, 1989; Milijašević 2006). By comparison, *Gremmeniella abietina*, a pathogen occupying a very similar niche in boreal pine forests (i.e. infecting needles and shoots of Scots pine), occurs in the most northern Scots pine forests of Europe and is able to cause necrosis in Scots pine seedlings at -4 °C (Petäistö, 1993). Ettlinger (1945) recorded growth at -3 °C, highest growth at 19 °C and a thermal death point at 25-28°C for two isolates of this species on agar media. Hence, *D. sapinea* appears to be adapted to considerably higher temperatures than *G. abietina*, which is also consistent with the distribution range of the fungus in warmer regions.

The recent increases in observations of *D. sapinea* in northern Europe coincides with considerable climate warming. In Finland, the warming has been more rapid during the last four decades than ever since temperature recordings were initiated in the country in 1847. During 1980 – 2010 mean annual air temperature rose by 1 °C (Mikkonen et al. 2015). The temperature increase has been highest during November – January and winters have consequently become milder.

Viljo Kujala (1950) reported observations of *S. sapinea* in Finland. He found the fungus on Norway spruce bark and deposited several samples in between 1945-1949 in the herbarium of the Finnish Forest Research Institute. In order to assess the possibility that this fungus was present in Finland

in the 1950's, we sourced and examined the herbarium specimens collected by Kujala. An informal survey of *P. sylvestris* cones in Finland in 2004 failed to result in any isolations of *D. sapinea* at that time. However, in 2015, pycnidia of *S. sapinea* were found on *P. sylvestris* cones in Helsinki. These contradictory results prompted the present study to determine the current distribution of *S. sapinea* in Finland.

Materials and Methods

Herbarium specimens

Viljo Kujala noted pycnidia occurring on two samples of Norway spruce bark (no. 406 dated 17.04.1947, from Sippola, Ruotila, Finland and no. 409 dated 4.11.1945 from Tuusula, Ruotsinkylä, Finland) to *Diplodia pinea* (Kujala 1950) and deposited the samples in the herbarium of the Finnish Forest Research Institute (presently included in the collections of the Finnish Museum of Natural History, LUOMUS). We examined these specimens and found abundant immersed dark brown globose pycnidia similar in appearance. Single pycnidia were aseptically detached with forceps under a microscope and placed into Eppendorf tubes (3-5 pycnidia/tube) for DNA-PCR of the Internal Transcribed Spacer (ITS). Additionally, pycnidia were placed on microscope slides, crushed with a scalpel blade in a droplet of water for microscopic examination (Table 1).

Three methods were used in an attempt to use PCR to amplify DNA of the ITS region using the primer pairs: ITS1/ITS4, ITS5/ITS4 and ITS7A/ITS4 (Gardes and Bruns 1993; White et al. 1990; Bertini et al. 1999). These included: (i) disrupting the pycnidia in quartz sand with a glass rod and thereafter extracting DNA with phenol + chloroform (modified from Vainio, Korhonen & Hantula, 1998, as described by Müller, Valjakka & Hantula (2007). Biotools polymerase (Biotools B&M Labs, Madrid, Spain) was used following to manufacturer's instructions, (ii) DNA extraction by powdering the pycnidia with a glass rod in liquid nitrogen and thereafter dissolving the powder in an extraction buffer containing CTAB followed by heating and extraction in chloroform + isoamyl alcohol as described by Cubero et al. (1999). In separate reactions alternatively Biotools or Dream Taq polymerase (Thermo Fisher Scientific, Waltham, MA USA) was used following the manufacturer's instructions and (iii) rupturing pycnidia at room temperature in Eppendorf tubes containing quartz sand using a glass rod. The sample was then subjected to direct PCR using the Phire Plant Direct PCR kit (Thermo Fisher Scientific, Waltham, MA USA) following the instructions of the manufacturer.

Table 1. Conidia and pycnidia dimensions of Finnish specimens compared to previously reported.

Species or specimen	Geographical origin	Conidia dimensions		Number of septa	Pycnidium width (µm)	Reference
		length (µm)	width (µm)			
D. pinea A	USA	36 - 39	12 - 13	0 - 1		Palmer et al., 1987
D. pinea B (=*D. scrobiculata*)	USA	33 - 34	11 - 12	0 - 3		Palmer et al., 1987
D. pinea		30 - 45	10 - 16	0 - 1	≤ 250	Hansen and Lewis, 1997
D. pinea	Estonia	31 - 45	14 - 17	0 - 1		Hanso and Drenkhan, 2009
D. sapinea	Sweden	30 - 37	12 - 15	0 (-3)		Oliva et al., 2013
D. sapinea	The Netherlands and South Africa	30.5 - 52.5	12.5 - 20	0 (-3)	300 -500	Phillips et al., 2013
Norway spruce bark sampled in 1947-1949 [1)]	Finland	38 - 48	19 - 26	1		Kujala, 1950
Norway spruce bark sampled in 1947-1949 [1)]	Finland	40 - 53	19 - 21	0 - 1	400 - 800	This study
Pycnidia on Scots pine cones in 2015/16	Finland	33 - 45	15 - 18	0	200 - 250	This study

[1)] collected by Viljo Kujala

All PCR reactions were done with undiluted and diluted (1:10 and 1:50) extracts. Two *Diplodia sapinea* strains provided by Dr. Paolo Gonthier (SS 1.2 A.I.A from Torino, Italy and S81 from Trento, Italy) were used as positive controls in all PCR runs. Double distilled water was used as negative template control. The PCR protocol included: 5 minutes at 95 °C and thereafter 25 cycles including a start with 30 seconds at 95 °C, 30 seconds at 57 °C and 1 minute at 72 °C. The reaction was terminated with 5 minutes at 72 °C and thereafter cooling to +4 °C. Amplification success was checked by electrophoresis in gels containing 1.0% agarose in TAE buffer and the amplification products were visualized by ethidium bromide staining under UV light as described in Müller, Valjakka & Hantula (2007). Best results were obtained with the primers ITS7A and ITS4 and the PCR products were subjected to sequencing in both directions with this primer pair at Macrogen Inc., Korea (http://www.macrogen.com).

Field inventory

Scots pine plots were surveyed for presence of *D. sapinea* by collecting cones on the ground under mature Scots pines and by inspecting these for presence of *D. sapinea* pycnidia. Cones were collected in an area of ca. 30 ha of the Keräkankare ridge in October 2004 (site 3 in Table 2) and during October 2015 – September 2016 from fifteen mature Scots pine stands (Table 2 and Fig. 1). In each stand 50-110 cones were collected from the ground. Only fresh cones that appeared to have fallen during that calendar year, were collected. The cones were examined using a dissecting microscope and the number of cones with pycnidia resembling those of *D. sapinea* were counted. Identification was confirmed by detaching single pycnidia and examining conidial morphology under a microscope. In the case of the 2004 collections, isolations were made from the pith tissue of all the cones on 2% Malt Extract Agar (MEA: 20 g malt extract, 20 g agar 1000^{-1} ml water).

Table 2. Investigated Scots pine stands and frequency of cones with pycnidia of D. sapinea.

Site number	Municipality	Locality	Coordinates		Date of sampling	Number of cones collected	Stand age	Symptoms of Diplodia tip blight in pine shoots	Frequency of infected cones (%)
			N	E					
1	Helsinki	Kallahdenniemi	60° 11,4'	25° 08,5'	18.10.2015	100	mature	no	3
2	Helsinki	Linnanmäki	60° 11,4'	24° 56,3'	11.10.2015	100	overmature	no	5
2	Helsinki	Linnanmäki	60° 11,4'	24° 56,3'	9.8.2016	100	overmature	no	12
3	Lohja	Keräkankare	60° 29,0'	23° 55,6'	20.10.2015	100	40-50 years	no	2
4	Kemiönsaari	Helgeboda	60° 11,2'	22° 33,4'	20.9.2016	100	mature	no	4
5	Eurajoki	Lutta	61°07,9'	21° 46,1'	14.7.2016	110	mature	no	0
6	Eurajoki	Lehtimäki	61° 11,5'	21° 43,6'	14.7.2016	110	mature	no[1]	0
7	Pori	Metsäkulma	61° 31,7'	21° 38,7'	14.7.2016	110	mature	no	1
8	Nokia	Peltokankaanvuori	61° 30,0'	23° 12,6'	14.7.2016	110	mature	no	0
9	Joutsa	Koivula	61° 45,1'	26° 08,0'	28.7.2016	110	mature	no	0
10	Joutsa	Kaurasaari	61° 43,4'	26° 16,6'	19.5.2016	56	mature	no	0
11	Taipalsaari	Sammaljärvi	61° 14,3'	28° 04,2'	17.8.2016	110	mature	no	0
12	Savonlinna	Punkaharju	61° 46,5'	29° 20,4'	9.6.2016	100	overmature	no	0
13	Kauhava	Laakso	63° 13,3'	23° 11.0'	21.6.2016	50	40-50 years	no[2]	0
14	Kontiomäki	Sipola	64° 26,0'	28° 09,4'	19.8.2016	100	mature	no	0
15	Inari	Inkavaara	68° 59,1'	26° 59,7'	20.8.2016	110	mature	no	0

[1] some shoot beetle damages

[2] some Heterobasidion butt rot damages

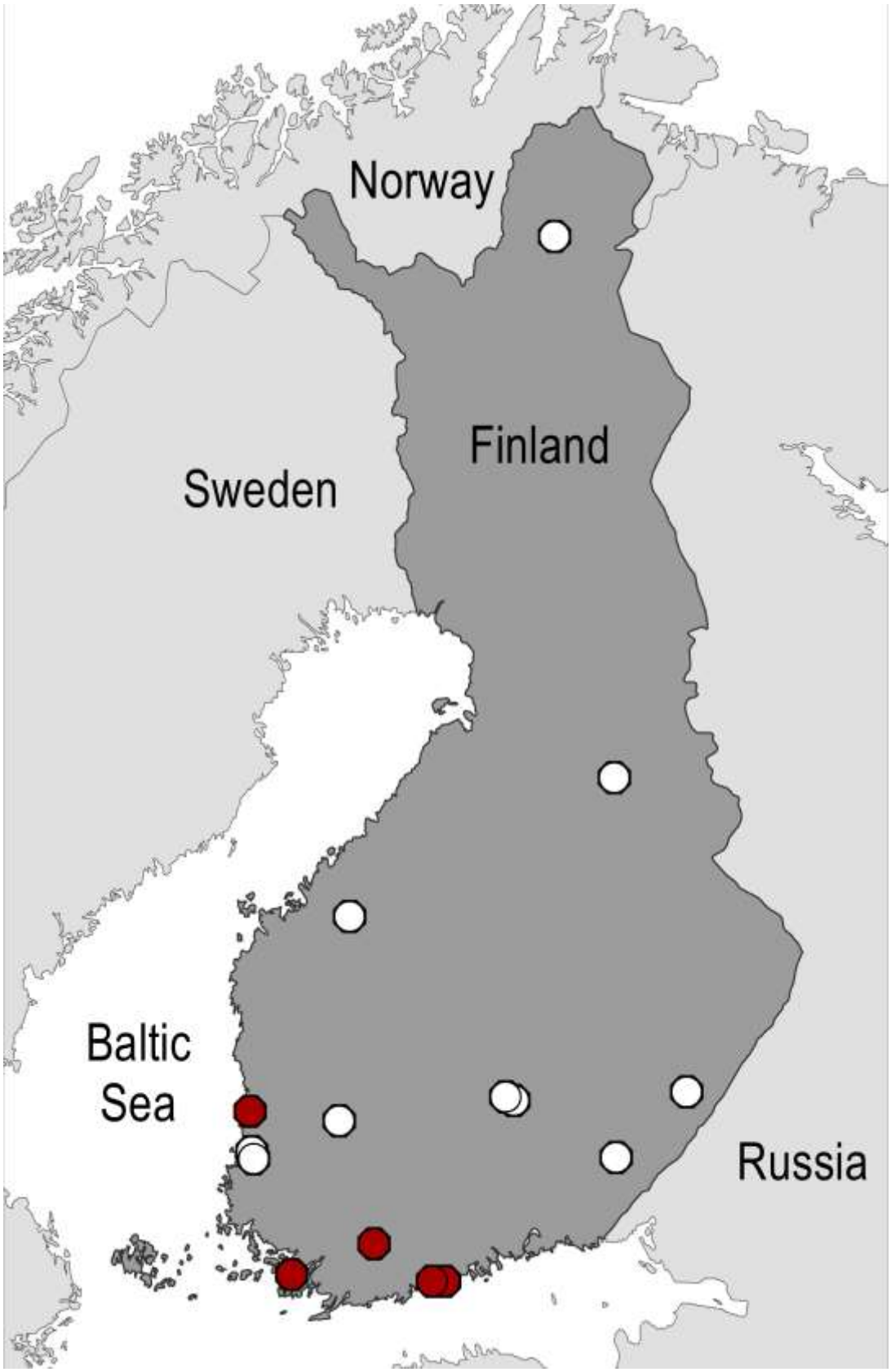

Figure 1. Collection sites of fallen Scots pine cones in 2015-2016. *D. sapinea* was found on Scots pine cones from five sites, indicated by red symbols. White symbols indicate sites were *D. sapinea* was not found. Numbering refers to that used in Table 2.

Fungal isolation and identification

When the conidia matched the shape, colour and dimensions described for *D. sapinea* by Phillips et al. (2013), they were removed from the microscope slide by rinsing them with 1 ml of sterile water and onto water agar plates. The plates were incubated at room temperature for 5 to 20 hours after which several germinating single conidia were lifted using a modified Pasteur pipette and transferred to fresh MEA in a Petri dish. Up to five single conidium isolates per stand (each from a different cone) were transferred to fresh malt extract agar plates covered with a cellophane membrane (Surface Specialities; Wigton, UK). After an incubation period of five days at 20 °C, hyphal mass from culture edges was removed and stored at -20 °C until DNA extraction. DNA-PCR of these samples was done using the first method described above. The primer pair ITS5/ITS4 was used for PCR. Amplification products were subjected to sequencing in both directions with the primers ITS5/ITS4 at Macrogen Inc., Korea. Global sequence alignments were constructed using Geneious R10 version 10.0.8 (Biomatters Ltd.) and MAFFT alignment algorithms. Sequence comparisons were conducted using NCBI Blast.

Results

The pycnidia abundantly present on Kujala's bark samples were larger (diam. 400 – 800 µm) than those found on cones freshly collected in pine stands in this study or described earlier for *D. sapinea* (Table 1). Also, conidia from Kujala's bark samples were longer (40 – 53 µm) and wider (19 – 21 µm) compared to conidia measured recently from cones in Estonia, Sweden and this study (30 – 45 and 12 – 18 µm, respectively). The sequences obtained from pycnidia on Kujala's bark samples also failed to match with sequences of *D. sapinea* in GenBank (National Center For Biotechnology Information, Bethesda, MD). PCR-products were obtained from the herbaria samples only using the Phire Plant Direct PCR kit (Method 3) and using the primer pair ITS7A/ITS4. The best of these sequences, a 244 bp long sequence (from sample 409; GenBank accession number MH260637) was obtained and this sequence was 95 % similar to *Aplosporella sumachi, Phaeobotryosphaeria porosa Sphaeropsis citrigena and S. visci* in GenBank (all equally similar to our sequence) but no match to any species at ≥ 98 %.

Field collected cones in 2004 had no pycnidia on their bracts and *D. sapinea* was also not isolated from them. In the more recent (2015-2016) field inventory, *D. sapinea* was found on Scots pine cones in five stands. Identification of pycnidia and conidia on the cone scales was based on morphology of pycnidia and conidia as well as on colony appearance of isolates on malt extract agar. The isolates produced on malt extract agar were fluffy, white to grey mycelia and stained the

agar dark greenish to grey. The morphological characteristics observed corresponded to those described by Phillips et al. (2013). The pycnidia were immersed, nearly globose, dark brown or black and 200 – 250 µm in diameter (Table 1). The conidia were thick-walled, ellipsoid, oblong, aseptate, brown in colour, 33 – 45 µm long and 15 – 18 µm wide (Table 1). The conidial dimensions fell within the range observed in several previous studies (Table 1).

Identification of *S. sapinea* for the recently collected isolates was verified by ITS sequences of ten isolates originating from stands 1, 2, 3, 4 and 7. All ten sequences (444 – 515 bp) were identical to each other and identical to numerous sequences in GenBank assigned to *D. sapinea, D. pinea* or *Sphaeropsis sapinea*. They were also identical to those obtained recently in Estonia, Latvia and north-western Russia. The sequences were deposited in GenBank under the accession numbers: Helsinki, Linnanmäki: MH260627, MH260628, MH260629, MH260632, MH260633; Helsinki, Kallahdenniemi: MH260630; Lohja, Keräkankare: MH260631; Kemiönsaari, Helgeboda: MH260634, MH260635; Pori, Metsäkulma: MH260636.

All five Scots pine stands infested by *D. sapinea* were close to the south-west coast of Finland (Fig. 1). The percentage of cones with pycnidia was low: 1 – 12 % (Table 2). Most of the investigated stands appeared to be healthy, but one suffered from shoot beetle damage and in one stand several trees had fallen due to butt rot. None of the 15 investigated stands showed any symptoms typical for diseases caused by *D. sapinea*.

Discussion

The results of this study provide good evidence that the pine pathogen *D. sapinea* is a recent colonist of *P. sylvestris* in Finland. There was no evidence to suggest that it is causing disease problems at present. However, its apparently new area of occurrence and presence on a *Pinus* spp. known to be susceptible to infection, might suggest that in times of stress, it could become a problem. This would be consistent with similar situations elsewhere in the world.

. Both morphological and DNA sequence comparisons have led us to conclude that it is unlikely that the fungus described by Kujala (1950) is *D. sapinea* or the related pine-infecting fungus *D. scrobiculata* (De Wet et al 2003). Consequently, our observation of *D. sapinea* on Scots pine cones in Helsinki in 2015 appears to be the first true record of this pathogen in Finland. Given that a previous survey in 2004 failed to produce any evidence of the pathogen and the subsequent

detection in 2015-2016 of a limited south-western distribution support the view that the pathogen is a relatively recent invader of Finland.

The frequencies of detection on cones in this study (1 – 12 %) are relatively low compared to those of 50 – 100 % frequently recorded in France on *P. nigra* and *P. sylvestris* stands (Fabre, Piou & Desprez-Loustau, 2011). In Latvia and Estonia detection frequencies of 31 % and 12 % have been reported on the same pine species (Adamson et al. 2015). Abundance of inoculum on cones is of primary importance for disease development in pines (Palmer, McRoberts & Nicholls, 1988; Fabre, Piou & Desprez-Loustau, 2011). Though conidial abundance is required for disease progression, conducive environmental conditions and often stress to trees are also required for disease development (Palmer, McRoberts & Nicholls, 1988; Fecci et al. 2002; Munck & Stanosz, 2010). Winter temperatures correlate positively with the disease severity (Fabre, Piou & Desprez-Loustau, 2011), and especially winters have become milder but also summers have become warmer during the last decades in Finland (Mikkonen et al. 2015; Ruosteenoja et al. 2016). The current distribution of *D. sapinea* only in southwestern Finland may also reflect the importance of mild climate because winters are mildest in that area of this country where the average daily temperature of the coldest month is -9 °C and of the warmest month +16 °C.

The disease history (i.e. disease outbrakes in southern Europe for over 100 years; Luchi et al. 2014) in Europe suggests that recent climate warming could be an explanation for the current spread of *D. sapinea* to northern Europe. In contrast, *D. sapinea* causes shoot blight and top kill on pines in north central United States and even in Ontario, Canada, (Myren & Davis 1989; Ostry et al. 2012) where winters are colder (but summers are considerably hotter) than in southern Finland. Hence, the explanation for the recent spread of *D. sapinea* to northern Europe remains open to debate. Many studies have hypothesised that the distribution of Diplodia shoot blight is climate restricted because direct effects on the pathogen and/or indirect effects through host susceptibility (Fabre, Piou & Desprez-Loustau, 2011; Bosso et al. 2017; Wyka et al. 2018). One justification for this assumption is the fact that *D. sapinea* has been present in Europe for a long time yet has not spread to all areas of this region despite the presence of susceptible pine species.

Diplodia sapinea has been observed on Scots pine at the northern coast of Estonia in 2012 and in north-western Russia, near St Petersburg (110 km from the Finnish border) in 2013 (Adamson et al. 2015). In Central Sweden, the first observation of the pathogen was in 2013 (Oliva, Boberg & Stenlid, 2013). Consequently, *D. sapinea* could have spread to the southern coast of Finland over

the Baltic Sea from Sweden or Estonia or along forested landscapes from north-western Russia into south-eastern Finland.

D. sapinea can survive in a latent state in plant tissues for long periods of time (Bihon et al. 2011, 2012). As is true for other members of the *Botryospaheriaceae* (Marsberg et al., 2017), it is well suited to be accidentally introduced into new environments in infected plant material such as seedlings. Conidia can be disseminated by rain-splash and wind-driven rain (Hansen & Lewis, 1997; Swart, Wingfield & Knox-Davies, 1987b) although their relatively large size might argue against their being blown over long distances such as the Finnish Gulf. It is also possible that the fungus could have been transported to Finland with insects. In this regard, at least one insect, the pine engraver beetle *Ips pini*, has been shown to carry *D. sapinea* to logs of *P. nigra* (Whitehill, Lehman & Bonelle, 2007). Several other insect species have also been found to co-occur with *D. sapinea* in pine cones and have been proposed to act as vectors of the fungus (Feci et al. 2002; Haddow & Newman, 1942; Luchi et al. 2012; Drenkhan et al. 2017). Hence, we conclude that the introduction of *D. sapinea* into Finland may have occurred via one or a number of several different modes.

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