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Diagnostic work report

The field work occurred on 1st of June 2023 near Hudiksvall, Hälsingland. Contact person is Nils Frank, Skogskonsulent, Skogsstyrelsen. Local media people from SVT NYHETER joined to the field and made a short video about the diagnostic work on the site (https://www.svt.se/nyheter/lokalt/gavleborg/skador-pa-trad-i-nordanstig-forbryllar-experter-forsta-gangen-jag-ser-det--jebbuo?fbclid=IwAR2DCiBMuNEsp5qpUoq4gdaSS_PN489Xz_EOXFuRm_wwskQtEbtppZF2b5Y) (Fig. 1).

Fig. 1. Diagnostic team work (Iryna Matsiakh, analyst Forest Damage Center, Nils Frank and media group)

The first site visited had some damaged 16-18-year-old lodgepole pine *Pinus contorta* trees (Fig.2). Not all trees were symptomatic but while walking in the stand it was possible to see that the crowns of some trees were more transparent, showing loss of needles, and branch dieback (Fig.2).

Fig. 2. General view of symptomatic *Pinus contorta* trees

The symptomatic trees were inspected carefully, in particular needles, branches, stems and roots. One tree was cut for closer inspection of the crown symptoms and collecting samples for further analyses.

The symptoms of the foliage were found on the current needle growth. Some tips of the needles showed brown-red-yellow discoloration and appeared dried while other needles were detected as half chlorotic/discoloured having their tips pale brown or orange/yellow (Fig.3). These symptoms were found often on several branches, or in some cases the annual growth of needles were symptomatic and spread throughout the tree.

Fig. 3. Needle discoloration on *Pinus contorta*

Five trees were found with shoot or branch dieback symptoms (Fig. 4). Infected shoots/branches were detected in the lower or middle part of the trees. It was seen that shoots started growing in the spring but remained stunted in growth. Symptoms included the browning of needles on new shoots as the needles grew from the fascicle sheath (Fig.4). Infected shoots with brown needles and cones remained attached to the shoots but were dead (Fig. 4).

Fig. 4. Symptoms of shoot and branch dieback on *Pinus contorta*

Some of the infected shoots were dissected in the field to observe wood discoloration (Fig. 5) and six of these infected shoots/branches were taken to the laboratory for further analyses.

Fig. 5. Dissected shoots in the field showing wood discoloration

The second site visited was located near Strömsbruk, Hälsingland. The site was planted with *Pinus contorta* and thinning occurred in 2022. Generally, the trees were healthier than on the previous site but similarly had some sporadic foliage discoloration (Fig. 6a). The cut trees lying on the ground from the thinning were actively colonized by secondary bark beetle. The exposed galleries indicated *Pityogenes* species and two dead beetles were collected for further analyses and species identification (Fig. 6b).

Fig. 6a. *Pinus contorta* stand in near Strömsbruk and collected samples for diagnostic work

Fig.6b. Exposed *Pityogenes sp* bark beetle gallery, and two dead beetle images taken for identification.

- **Laboratory work (Hudiksvall material)**

The laboratory analyses were conducted 6.06- 3.07.2023 (sequencing analyses 26.07-5.08) and included classical fungal isolation technique, molecular identification of obtained fungal isolates and real-time quantitative PCR (qPCR) based on TaqMan chemistry to test the presence of *Diplodia* species in collected pine tissues. In total 122 fungal isolates were obtained from symptomatic needles, wood discoloured tissues, fruiting bodies on the shoots and cones. Sixty-two fungal isolates proceeded for DNA sequencing.

Fungi associated with needle symptoms. Needles with symptoms were inspected under a microscope (Nikon SMZ645) (Fig. 7). Discoloured patches with tan- to yellow-colored bands and/or spots were found under close observation. This part of infected needles was used for fungal isolation.

These discoloured parts at the edge of healthy looking tissue were cut in small pieces and placed on nutrient media to isolate fungi. Of the 62 isolates that were sent for DNA sequencing, 20 were obtained from collected infected needles (Hudiksvall site), 10 were obtained from black round fruiting bodies on collected shoots and 4 - from symptomatic needles of lodgepole pine (Strömsbruk site) (Fig. 7).

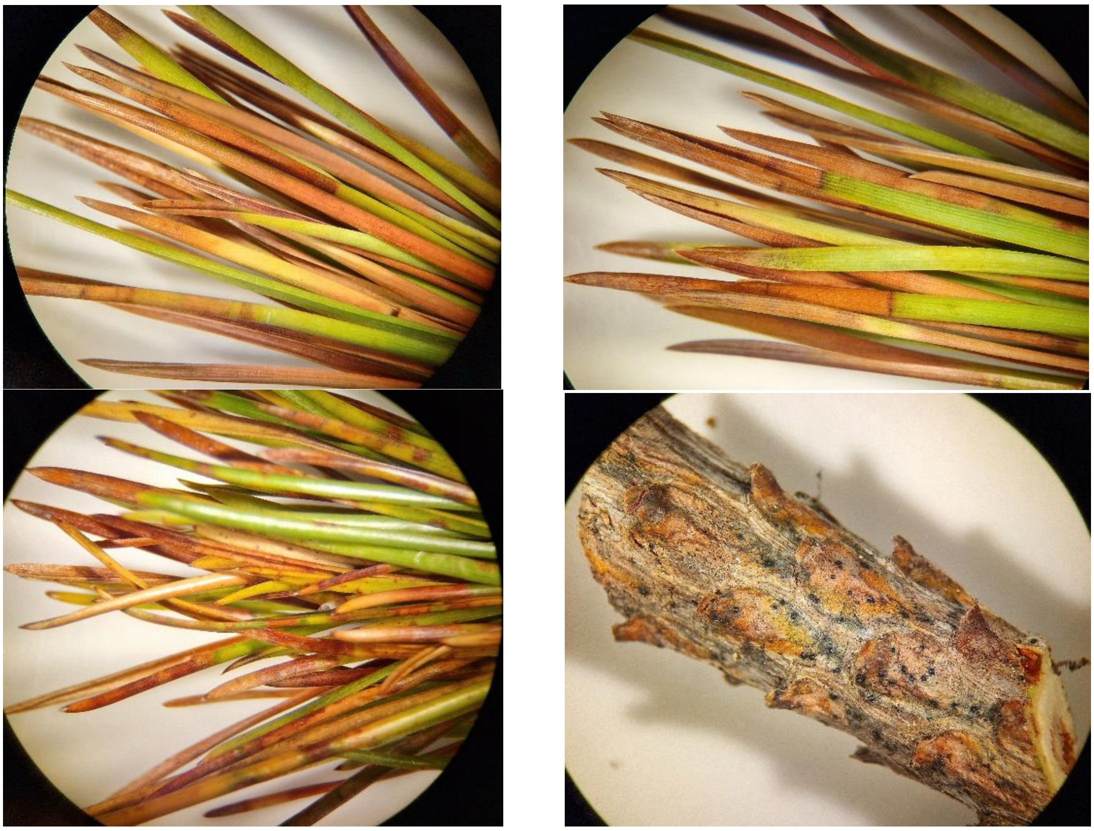


Fig. 7. *Pinus contorta* needle discoloration with tan- to yellow-coloured bands/spots and round black fruiting bodies on collected shoots under microscope

Fungal identification. Ten isolates from symptomatic needles of lodgepole pine and fruiting bodies on shoots were identified as *Sydowia polyspora* (Annex 1), a pathogen frequently associated with current season needle necrosis (CSNN) on conifer species, particularly *Abies* spp. and *Pinus* sp., across Europe and North America (Talgø et al., 2010; Tinivella et al., 2014; Silva et al., 2020). *Sydowia polyspora* is a widespread and extremely polyphagous fungus. Symptoms consist of tan to yellow-colored bands and/or spots, which turn reddish brown during the summer (Fig 4). Entire needles may become necrotic and are cast.
Sydowia polyspora is typically reported as a needle endophyte (Carroll and Carroll 1978), as a secondary pathogen in damaged tissues (Smerlis 1970), or as a minor foliar pathogen (Funk 1985). The fungus might also be present in seeds (Cleary et al., 2019).

Sydowia polyspora (asexual form *Sclerophoma pityophila*) occurred frequently at lodgepole pine provenance trials in northern Sweden (Karlman, 1986). It was recorded on plants previously damaged by voles, moose, insects, unfavorable weather conditions, and possibly other fungi. It was occasionally found as a secondary pathogen but is usually a completely harmless saprophyte. In the provenance trial at Moskosel, the fungus was mainly recorded on weather-damaged plants and following infection by *Gremmeniella abietina* (Karlman, 1986).
Since last year, *Sydowia polyspora* was also reported causing more pronounced and problematic foliage damage on *Pinus contorta* particularly on the visited site (Nils Frank, personal communication).

According to the current research, under certain stress factors (either biotic and/or abiotic), *S. polyspora* can become pathogenic (Busby et al. 2016) and it can enhance disease expression when co-inhabiting needles with other pathogens such as *Dothistroma septosporum* (Ridout & Newcombe 2015).

Two isolates of *Lophodermium pinastri* and 10 isolates of *Lophodermium conigenum* were isolated from symptomatic needles (Annex 1). This is a known problem in Sweden since 1990s, particularly in nurseries (Stenström and Ihrmark, 2005). *Lophodermium* needle cast disease infects the current season's foliage, which is often killed before the next growing season. Infected needles may develop yellow spots in late summer to early autumn before finally becoming yellow to brown (Fig. 4). The symptoms first appear at the needle tips and will slowly progress to the needle base. Dead needles may not abscise normally and can linger in the canopy into the next growing season. The spore-bearing structures (apothecia) can be readily visible on diseased needles (Stenström and Arvidsson, 2001). Spore dispersal by *L. pinastri* starts one to a few months earlier than *L. conigenum* (Minter and Millar, 1980a). The fungal cultivation of both species takes up to several weeks before mycelial cultures show typical characteristics that enable identification (Minter 1981). Since *L. pinastri* is one of the fungi most frequently found in pine needles, while *L. seditiosum* is not always present (Patejuk et al. 2021; Lazarevic and Menkis 2020), it is suspected that *L. pinastri* occupies the same infection sites and thus partially prevents colonisation by *L. seditiosum*.
Fruiting bodies of *L. pinastri* are mainly found on needles that have died after senescing, whereas fruiting bodies of *L. conigenum* are predominantly found on healthy needles that have died prematurely, for example, as a consequence of snow break or previous years of needle cast (Minter & Millar, 1980).
The occurrence of *L. pinastri* increases with the age of the needle (Behnke-Borowczyk et al. 2019). Nowadays, a distinction is made between three different groups of *L. pinastri* (Reignoux et al. 2014), which are pathogenic to varying degrees.

Two isolates associated with symptomatic needle were identified as *Rhizosphaera kalkhoffii*, the causal agent of Rhizosphaera needle cast (Annex 1). Rhizosphaera needle cast is a disease more commonly associated with spruce but recently described associated with pine damage causing yellowish areas/bands on the hosts' needles that subsequently leads to the development of more extensive lesions and/or necrotic areas (Monteiro et al., 2022).
Other common hosts for *R. kalkhoffii* are Austrian and eastern white pines as well as Douglas-fir, balsam fir and western hemlock. The pathogenicity of *Rhizosphaera* sp. on pines still needs to be confirmed but it is suggested that it has the potential to be one of the active players in the symptomatology initially associated to red band and brown spot needle blight diseases (Monteiro et al., 2022). Recently, *R. kalkhoffii* was reported as a pitch canker pathogen causing severe dieback and bark cracking on *Pinus sylvestris* in China, where trees showed profuse resin flow from infected branches, cones, and trunks (You et al., 2013). The fungus is listed as a quarantine pathogen in China (You et al., 2013).
To our knowledge, it has never been detected on lodgepole pine and it is difficult explain how the fungus appeared on the site and if it can be pathogenic to lodgepole pine. It could have been brought with spruce trees to the forest in the past, but the origin is unclear. More research to prove its pathogenicity and host association is needed.

One isolate was identified as *Preussia intermedia* (Annex 1) - a fungal endophyte that has an important ecological role in decomposing and recycling nutrients from animal dung. It is rarely found in Sweden (*Preussia intermedia* - Artfakta från SLU Artdatabanken).

Fungi associated with fruiting bodies on the lodgepole pine cones. Eight cones collected together with shoots were inspected under the microscope (Fig. 8). Small black structures were found which erupted through the surface of the cones and developed on the second-year and older cones.

healthy looking cones (for comparison)

Fig. 8. *Pinus contorta* cones with small black fruiting bodies on the surface of the cones (healthy looking cones showed for comparison)

It was suspected to have *Sphaeropsis sapinea* (Diplodia tip blight pathogen of conifers) pathogen but no Diplodia isolates were obtained.
Six isolates were identified as *Sydowia polyspora*, which also produces small, black, spherical fruiting bodies, and protrude irregularly from any colonized plant tissue.

One isolate was identified as *Godronia fuliginosa* – a rare brown discomycete in the family Helotiaceae (Videskål - Artfakta från SLU Artdatabanken).

Fungi associated with tip branches/shoots dieback. Six collected branches/shoots showing tip dieback were dissected in the laboratory and wood discoloration was inspected under the microscope (Fig. 9). Pale reddish brown discoloration with drops of resin was observed inside the infected shoots. These tissues were used for plating on petri dishes with nutrient media for fungal isolation.

Fig. 9. Dissected branches/shoots of *Pinus contorta* with tip dieback showing discolored wood tissues inside of shoots

Seventeen isolates were sequenced and 7 of them were identified as *Sydowia polyspora.* It is known that *S. polyspora* is able to colonise all organs of the tree including shoots and wood, but not the roots. When wood is affected, it is usually grey (Dubach et al. 2022). The fungus can be often found in association with pathogenic fungi and also with insect pests and symptoms of different species can appear together.
There are studies demonstrating that *S. polyspora* is able to colonize stem phloem and pine needles of *Pinus yunnanensis* and thus may cause secondary damage to this host after bark beetle attack (Pan et al., 2018). Recently, it was reported that *S. polyspora* caused tip dieback, needles with tan- to yellow-coloured lesions and shoot death in stands of Stone pine in Portugal (Silva et al., 2020).
Some scientists suggest that fungus can be more aggressive under certain climatic conditions or other biotic factors (Talgø et al., 2010; Ridout and Newcombe, 2018).
Sydowia polyspora most likely is involved within a disease complex, but on its own, it is not clear how damaging it is.

There were also some interesting fungal findings related to wood discoloration of lodgepole pine (Annex 1). Two isolates were identified as *Sarea resinae*. It is one of the rarely found *Sarea* species, a small, non-lichenized, inoperculate discomycete fungi in the family

Zythiaceae. *Sarea* species are found growing on the resin of conifers in the Cupressaceae and Pinaceae in the northern hemisphere. The species is recorded in Sweden. Two isolates were identified as *Pragmopora pini* species – a newly described species the genus *Pragmopora* and not reported in Sweden yet (Artfakta från SLU Artdatabanken).

For detecting the presence of the Diplodia tip blight pathogen (*Sphaeropsis sapinea)* in collected lodgepole pine material, four DNA samples extracted from discolored wood were amplified using real-time quantitative PCR (qPCR). *Sphaeropsis sapinea* was detected in one of the shoots with tip dieback symptoms (Fig. 10).

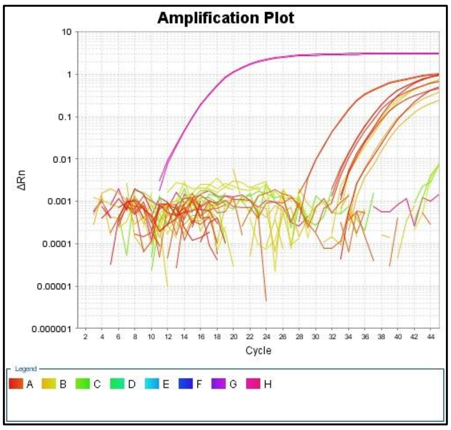


Fig.10. Positive amplified samples woody tissue DNA (curves) detecting *Sphaeropsis sapinea* (one samples is Hudiksvall, the rest are from Frövi, Sörmland-Örebros distrikt).

Despite having only one positive Diplodia DNA sample, it was confirmed that *S. sapinea* is present on the lodgepole pine site. Typical disease symptoms of Diplodia tip blight were observed in the field and they can be summarized and demonstrated in a picture portfolio (Fig.11). Symptoms include the browning of needles on new shoots as the needles grow from the fascicle sheath (Fig. 11a). One of the first indications that a shoot is infected is the oozing of small drops of resin from the shoot buds as growth begins in early spring. Infected buds stop growing and do not reach normal size. New buds may grow, but these too will become infected. As the disease continues, whole branches may be killed but the needles remain attached. While collecting infected shoots, they were covered with drops of resin (Fig. 11b-c). *Diplodia* persists in the black fruiting structures in dead shoot tips and infected cones that remain on the tree (Fig. 11d-e). Other symptoms are resinous cankers on the main stem and branches (branch and bole canker) (Fig. 11f), misshapen tops and dieback (Fig. 11j) and sapwood staining (Fig. 11h). *Sphaeropsis sapinea* can also cause death of cones, seedling blight, damping off and collar rot of seedlings, and root diseases, all of which may lead to the death of the entire tree (Blumenstein et al. 2021). Seeds also seem to be a carrier of the pathogen (Cleary et al. 2019).

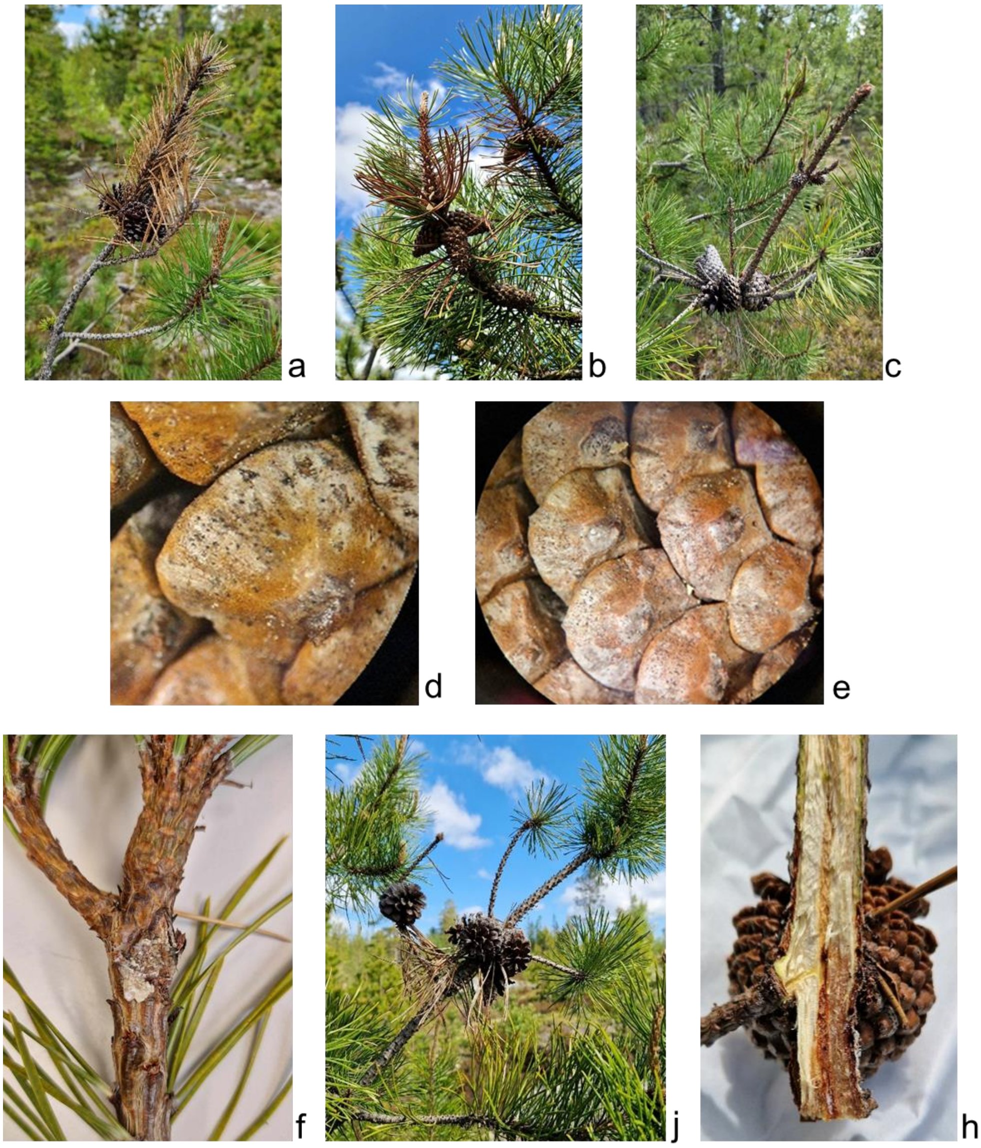


Fig. 11. Diplodia tip blight symptoms on longepole pine

The fungus can live asymptomatically as an endophyte in its host tree (Stanosz et al., 1997; Terhonen et al., 2021), and can transform from a latent to an opportunistic pathogen (Swart and Wingfield, 1991) or/and saprotroph. Stress-inducing factors, such as drought, hail, extreme temperatures, or mechanical wounding may trigger development of *S. sapinea* to become pathogenic, leading to sudden disease outbreaks (Blumenstein et al., 2021b; Stanosz et al., 2001). Trees may be infected without showing symptoms until they are affected by drought, hail or other stressors.

There is some evidence that Diplodia tip blight on its way to the north (Brodde et al., 2019). It has been recently reported in Estonia (Hanso and Drekhan 2009; Brodde et al., 2019) and in Sweden (Oliva et al., 2013). The first outbreak in Sweden was discovered in 2016 between Odensala and Märsta on *Pinus sylvestris*. A 20-year-old plantation appeared to be severely damaged where trees had lost completely all current year's shoots, and some of them were dead. The incidence was high, with the majority of trees affected, in most cases having lost their main leader shoot. A closer look in the stems revealed that most trees were either bifurcated or displayed bushy crowns, suggesting that they had probably suffered S. *sapinea* attacks in the past (Brodde et al., 2019). According to the conclusions of Brodde et al. (2019), warm May and June temperatures were associated with higher damage and low tree growth, while cold and rainy conditions seemed to favor growth and deter disease development.

Pinus contorta is on the host list of Diplodia tip blight. It was reported in Nebraska, USA (Stanosz et al., 1996; Stanosz and Kimbler, 1997), in New Zealand (Gadril, 2005) and in Canada (Ginns, 1986). This is the first record of Diplodia tip blight on lodgepole pine in Sweden.

Refections from this investigation. Despite observing Diplodia fruiting bodies on the scales on the surface of cones, it became problematic to obtain Diplodia cultures as *Sydowia polyspora* grew fast and inhibited other fungi (Fig 12). This process will be repeated using other type of media and other antibiotics that are used for Diplodia tip blight. More research should be done to obtain Diplodia tip blight cultures and confirm its pathogenicity towards lodgepole pine.

Fig. 12. Fast growth of *Sydowia polyspora* on nutrient media

In addition, DNA samples extracted from discoloured woody tissues were amplified using Loop-mediated isothermal amplification (LAMP) assay with *Gremmeniella abietina* primers (ongoing research project to develop rapid assay) to target *Gremmeniella abietina* DNA in samples. All samples were negative (it might be an issue with primers). Negative results don't mean that *G. abietina* is not present on the site. Its symptoms can be confused with Diplodia tip blight.

- **Laboratory work (Strömsbruk material)**

Symptomatic needles were also inspected under the microscope (Nikon SMZ645) (Fig. 13). Discoloured patches with tan- to yellow-coloured bands or/and spots were found under close observation. This part of the infected needles was used for fungal isolation.

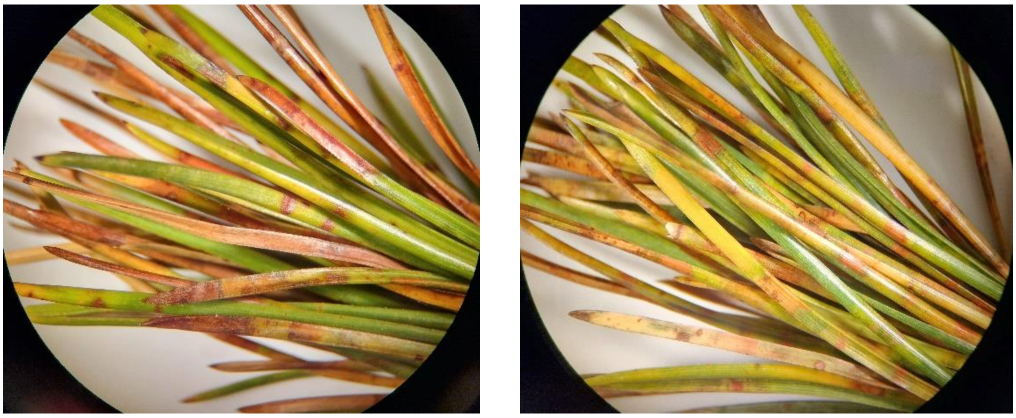


Fig. 13. Needles discoloration of longepole pine from Strömsbruk

Four fungal isolates were sequenced for identification. Two of them were identified as *Sydowia polyspora*, one of them was *Lophodermium conigenum* and one was *Fusarium tricinctum.*

- **Laboratory work (*Pinus sylvestris* branches with needles material obtained from Nils Frank in the field)**

Several branches of *Pinus sylvestris* with symptomatic needles were obtained for laboratory analyses. The sample had been collected some days before being received at the lab. Material was inspected under the microscope and some needles were plated on nutrient media for fungal isolation.
Close observation revealed that needles were covered with small fruiting bodies of *Phacidium infestans* (snow blight pathogen) (Fig. 14).

Fig. 14. Snow blight infection on *Pinus sylvestris* needles

Phacidium infestans is well-known pathogen of lodgepole pine in high-altitude stands in northern Sweden (Karlman, 1986). In the Baltic countries, the fungus can survive the winters with light and short-term snow cover, developing on poor forest site types where the lowest branches of Scots pine trees have green needles close to the ground. Only during repeated occurrence rich in snow winters the disease becomes problematic, both nurseries and young plantations (or naturally regenerating areas) can have considerable damage (Hanso, 2000). The fungus is widespread in Sweden (*Gremmenia infestans* - Artfakta från SLU Artdatabanken).

Four fungal isolates were sequenced for identification. One of them was identified as *Hormonema* (*Rhizosphaera merioides*), another was identified as *Rhizosphaera minteri* and two remained unidentified species. Both confirmed species are new pine associated needle cast pathogens and could have been brought from the nursery to plantation.

- **Entomological analyses**

The presence of pine resin-gall moth Hartsgallvecklare (*Retinia resinella*) was found on both visited sites. The exit holes of moths were found on lodgepole pine shoots in Hudiksvall and evidence of a freshly emerging moth in Strömsbruk (Fig. 15) was captured.

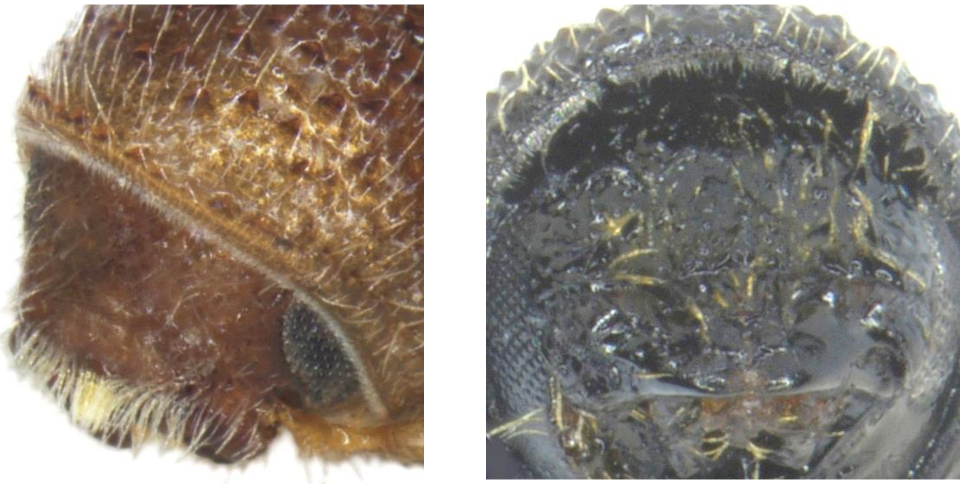
Fig. 15. Hartsgallvecklare on lodgepole pine

Retinia resinella is a pine moth, and depending on climatic zone, adults are generally found from May to June. The larva are known to live in the shoots of *Pinus sylvestris* where it causes a resin gall to develop. Development takes two years. This insect is widespread in Sweden (Hartsgallvecklare - Artfakta från SLU Artdatabanken).

The microscopy photo documentation and identification of collected beetles (Fig. 16) was done by entomologist James Connell with photos authorship (Connell/BFW, Austria).

Fig 16. Two dead bark beetles Ex *Pinus contorta,* Hudiksvall, Sweden June 2023 *Pityogenes bidentatus* f. 2,3mm

There are five *Pityogenes spp* in Sweden (Pfeffer, 1995). The samples are females, in two species, females have distinctly indented foreheads, and as per samples, three do not. Two of these three species have three small but distinct spaced spines on the declivity (sloping hind section of the wing covers), and only one female has a pair of indistinct warts on the upper declivity. The samples found had an appearance of 4-5 grains with setae emerging from them on the wing covers prior to the start of declivity. Through a detailed searching of the Austrian forest research center (BFW) bark beetle collection in August 2023, a match indicative towards *P. bidentatus* could be found, and comparison material is now presented (Fig. 17).

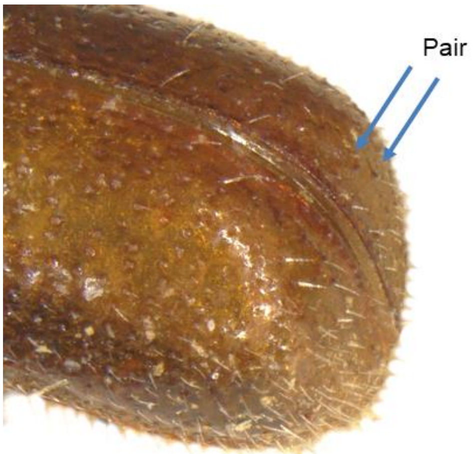


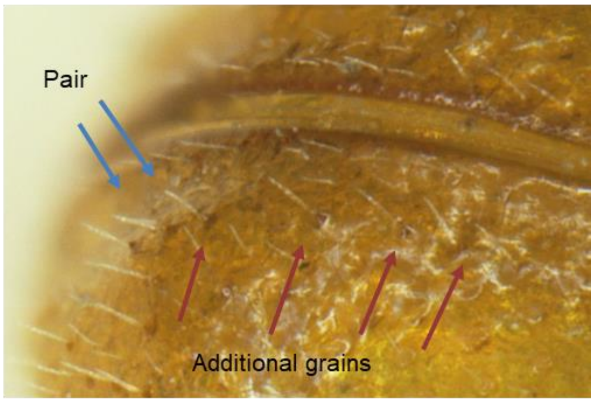
Specimen from BFW collection

Ex *Pinus contorta,* Hudiksvall*,* Sweden June 2023

Fig 17. Comparison material and a match indicative towards *P. bidentatus* collected in Sweden

Pityogenes bidentatus f, forehead with small triangle form, sometimes shiny, not indented (the yellow dotted area of triangular form is indicative only not absolute) (Fig. 18).





Both specimens from BFW collection

Fig 18. *Pityogenes bidentatus* f, features

Pityogenes bidentatus f, is described as having a pair of very indistinct wart like grains on the declivity (Peffer, 1995). However not mentioned but found present on a collection specimen too, there apparently can be similar sized grains before the pair on the declivity with setae emerging from them. Through magnification and tilting of adult images these are relatively easy to be found. The omission of such detail in descriptions is not uncommon (Fig. 19).

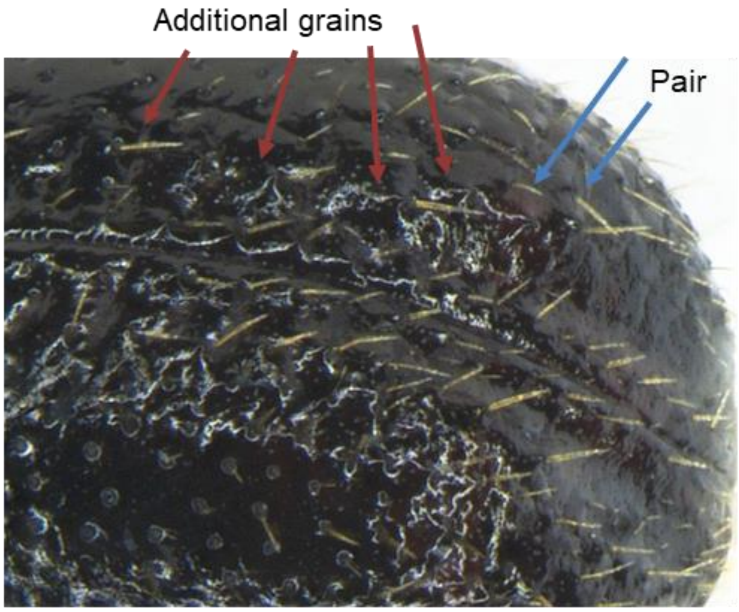


Fig 19. *Pityogenes bidentatus* grains on the declivity

In personal communication between James Connell & Nils Frank, despite *Pityogenes bidentatus* being polyphagia on pines (Pfeffer, 1995), it has not yet been recorded on *Pinus contorta* in Sweden, so the obtained specimens will come back to Sweden in September for final clarification.

Conclusions

Damage of *Pinus contorta* at visited sites in Hudiksvall and Strömsbruk include the complex of needle cast and tip blight pathogens.
As the pathogenicity of *Sydowia polyspora* has not as of yet been fully clarified, there are no reliable control measures. It is also unclear whether the removal of diseased parts of the tree right down to healthy tissue (i.e. pruning) would be beneficial.
As a preventive measure, improving the general health of the tree, i.e. through adequate watering and fertilization, could prevent damage as the fungus benefits from low tree vitality. In infected stands, phytosanitary measures such as the removal of badly infected trees (degree of damage >60 % of the needle mass) can be implemented to prevent secondary infections. The infection risk can be reduced by regularly cutting the dense surrounding young growth within stands to improve air humidity around the desired crop trees. Regular thinning measures can reduce competition for water and nutrients among the vegetation and thus the susceptibility of the trees too.

The new record of Diplodia tip blight and new host for the pathogen was found in this diagnostic work. More research will be conducted with sampled material to support this finding and a first report publication will be submitted for publication.

The extremely low number of observed resin galls from pine resin-gall moth Hartsgallvecklare (*Retinia resinella*) would conclude it is low risk to crop health. The confirmation of *Pityogenes bidentatus* as a secondary bark beetle species on the cut *Pinus contorta* material would be interesting, as it could then be added to the Swedish list of potential bark beetles found on lodgepole pine.

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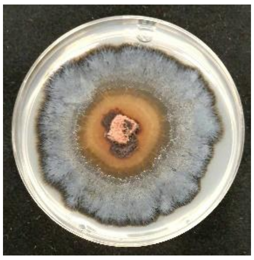
Annex 1. Collection of fungal isolates obtained from *Pinus contorta* infected material in Hälsingland district


Sydowia polyspora


Sydowia polyspora

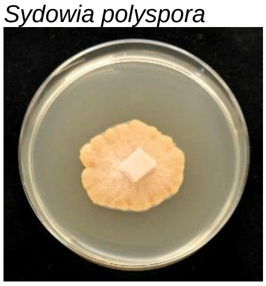

Sydowia polyspora


Sydowia polyspora


Sydowia polyspora


Sydowia polyspora


Lophodermium pinastri


Lophodermium conigenum


Lophodermium conigenum


Lophodermium conigenum


Lophodermium conigenum


Lophodermium conigenum


Rhizosphaera kalkhoffii


Rhizosphaera kalkhoffii


Preussia intermedia


Godronia fuliginosa


Pragmopora pini