

Cherry spruce rust in the Wigry National Park and Suwałki Forest District: cone infestation and its implications

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ABSTRACT

Thekopsora areolata and *Chrysomyxa pirolata* are pathogens preferentially infesting Norway spruce cones and lead to the complete failure of spruce seed yield. Thus, the presence of seed-injuring pathogens may be a crucial factor driving close association between cherry spruce rust occurrence on trees and limited natural regeneration of the preferred host. However, a major uncertainty remains as to which pathogens are responsible for cone infection and reduced seed viability, resulting in impeded renewal of young trees. Our study aims to investigate whether *T. areolata* or *C. pirolata* presence on the Norway spruce cones was responsible for cherry spruce rust in three sites within the Wigry National Park (one site) and the Suwałki Forest District (two sites) in North-Eastern Poland. We harvested randomly five infested Norway spruce cones from each location and analysed them to verify specific pathogen occurrence. We analysed rDNA fragments such as ITS regions and 28S to precisely identify the disease causal agent on the cones and estimate the potential of genetic diversity of the analysed fungus species. *Thekopsora areolata* was identified as the main fungus promoting cherry spruce rust, suggesting that host species regeneration is constrained by this pathogen's distribution. We assumed that the apparent cherry spruce rust presence and lack of Norway spruce natural regeneration likely result from cone infection and the availability of healthy, high-quality seeds. Moreover, the occurrence of cherry spruce rust may explain the challenges associated with the deficit of seeds used for the artificial regeneration of spruce stands.

KEY WORDS

ITS, LSU, Norway spruce, *Picea abies*, *Thekopsora areolata*

INTRODUCTION

Norway spruce (*Picea abies* (L.) H. Karst) is a significant tree species in Europe, with a high biological and economic value. In southern Poland, it occurs in the

mountains, foothills and upland areas, but also outside its continuous range as a locally separated population in the northeast of the country. The Central Poland disjunction within spruce natural distribution resulted from anthropogenic pressure following intensive deforestation

in the past (Latałowa and van der Knaap 2006; Dering and Lewandowski 2009). In Polish forests, spruce contributes to more than 6% of the total tree cover (Banach et al. 2017) and is the second most important coniferous tree in forest management in Poland (Zajączkowski et al. 2023).

The changes in the tree community structure reflect an adjustment to multiple factors, like climate change, tree diseases, forest management and human activities (Dale et al. 2001; Parmesan 2006). In the past, industrial air pollution and acid rain were suggested to explain the pronounced dieback of the Norway spruce trees in the Sudetes Mountains (Jurek et al. 1983; Mazurski 1986). Nowadays, the massive decline of this species in the Beskid Mountains (Poland), the Białowieża Forest or the entire southern range in Europe by 10%–35% in 2000 and 2003 (Grodzki 2004; Bosela et al. 2011; Miśicki 2016) has been related to the increasing susceptibility to summer drought and other stresses, likely resulting in the colonization of weakened trees by the spruce bark beetle (Skrzecz and Perlińska 2018; Sierota et al. 2019).

The way seedling regeneration is related to adult tree dieback remains poorly known when considering the Norway spruce growth perspectives. According to the positive neighbourhood effect, the elimination of adult spruce individuals by the bark beetle may initiate natural regeneration processes by positively influencing the juvenile generation located in the understory and recruiting seedlings in subsequent years (Bosela et al. 2011). Such a regeneration mechanism of the Norway spruce after a bark beetle outbreak has been described in a forest complex on the Czech-German border located in two national parks, the Bavarian National Park and Šumava National Park (Schurman et al. 2018). However, similar mechanisms were not observed in protected areas of the Wigry National Park (WNP) in North-Eastern Poland, although rhizosphere fungal communities form an environment favourable for the natural regeneration of spruce (Behnke-Borowczyk et al. 2023). However, they were not associated with even scattered regeneration (Frelich and Reich 1999). Accordingly, the extent to which the reduced regenerative potential of the Norway spruce results from climate limitations or may be explained by other factors remains to be identified.

Based on the literature, one key reason for the lack of natural regeneration may be a fungal disease: cher-

ry spruce rust (Zeppenfeld et al. 2015) evoked by *Thekopsora areolata* (Fr.) Magnus (syn. *Pucciniastrum areolatum* (Fr.) G.H. Otth) and *Chrysomyxa pirolata* Wint. (Kaitera et al. 2021). Infected spruce cone scales develop reddish-brown to dark-brown spots that may completely cover the inside of all scales. Throughout its 2-year life cycle, *T. areolata* infects its primary host (i.e. Norway spruce) and its most common alternative host (i.e. *Prunus padus* L. (bird cherry)) (Kaitera et al. 2017, 2019). *Chrysomyxa pirolata* exclusively targets the cones of spruce trees. Infected cones turn brown prematurely and can be easily identified by the presence of orange-coloured aeciospores, which form between the cone scales in late summer. A light ‘dusting’ of aeciospores is often observed on vegetation beneath trees with diseased cones. *Chrysomyxa pirolata* spreads through plants such as *Moneses*, *Pyrola* and *Orthilia* (Ziller 1974). We observed symptoms on spruce cones in the WNP in 2022 for the first time (Fig. 1). Data from the WNP and surrounding Forest Districts (Polish State Forests) indicate declining availability of a sufficient number of spruce seeds (2002–2023) resulting from the withdrawn harvesting of high-quality cones. As spruce cone infection by cherry spruce rust leads to sterile seeds or low-quality seeds (Kaitera and Tillman-Sutela 2014), the cherry spruce rust epidemic affects spruce seed production (Kaitera 2013).



Figure 1. Symptoms of cherry spruce rust observed on spruce cones (July 2023; Suwałki Forest District)

Considering that generative reproduction ensures the sustainability of Norway spruce growth, harvesting good-quality seeds is the essential to their stand's stability. Especially, protection against cherry spruce

rust is problematic; it depends on many factors favouring the production of *T. areolata* basidiospores when spruce cones are open and susceptible to being infected (Zhang et al. 2022) or may result from abundant spruce seed yield occurring every 2–5 years (Wesołowski et al. 2015). However, abiotic and biotic factors disrupt the regularity of seed production. Since the 1980s, in the northeast part of Poland, only the following years were categorized as mast production years: 1980, 1981, 1992, 1993 and 1998 (Kantorowicz 2000). Besides these rare periods, seed yields were very low. *T. areolata* and *C. pirolata* induce premature opening and malformation of cones, leading to 70% of cone injury, a tenfold reduction in seed viability and general limitations in seed number caused by cherry spruce rust (Kaitera and Tillman-Sutela 2014; Almqvist and Rosenberg 2016); therefore, an enhanced decrease in natural regeneration cherry spruce rust is expected.

Analyses of the natural regeneration of the Norway spruce across the Wigry National Park (WNP) and the Suwałki Forest District are limited. Therefore, we assumed that the pathogen causing cherry spruce rust may influence seed infestation and quality, which in turn affects the regeneration potential of spruce. The aim of the research is to determine the cause of cherry spruce rust in the Wigry National Park and the Suwałki Forest District.

MATERIAL AND METHODS

Fieldwork

Cones with visible symptoms of cherry spruce rust (aecia with aeciospores on the inner (adaxial) and outer (abaxial) sides of the cone scale (Fig. 1) were collected on August 7, 2023, from spruce crowns knocked down by a strong wind blowing at night. This windfall allowed us to harvest cones without human interference in the ecosystem according to the WNP rules (August 6–7, 2023). Collection areas included division 62b (54°00'48.0"N 22°58'05.6"E) in the Płociczno forestry and division 131j (53°59'28.7"N 23°00'07.6"E) in the Pijawne forestry in the Suwałki Forest District and division 392d (53°59'57.3"N, 23°01'32.4"E) in the WNP. In all analysed stands, spruce was the main species. Five cones from three

fallen trees were collected from each forest division. All last year cones on the fallen trees show symptoms of infection (Fig. 1). We randomly selected 15 cones for molecular analysis.

Crop magnitude

Information about mast seeding in forest tree species is important for effective seed management and for the long-term tree improving programmes. In Poland, the Forest Districts are required to report on the expected magnitude of the crop, the possibilities of seed collection and seed needs. On the basis of these reports, the Forest Service makes decisions about collections of seeds (Kantorowicz 2000). Since 2023 was not a mast-year, we are unable to assess seed quality according to the rules of the National Forest.

The Wigry National Park (WNP) does not maintain crop magnitude data; therefore, we obtained our data from the Suwałki Forest District, which directly borders the WNP. Crop magnitude was judged in the autumn (2010–2023) based on the fruiting *P. abies* according to the methodology described by Kantorowicz (2000):

- 0 – No crop: No trees with cones worth collecting [0% cropping trees]
- 1 – Poor crop: Cones on only single tree fruits on forest edges and inside the stand [10% cropping trees]
- 2 – Mean crop: Cones on numerous trees at the edge and some trees inside the stand [30% cropping trees]
- 3 – Good crop (mast year): Substantial % of trees in the stand fructify [100% crop-ping trees].

Observations were carried out in excluded seed stands in Suwałki Forest District (forest divisions: 12b (54°05'61.1"N, 22°95'15.0"E), 66a (54°01'11.7"N, 22°93'99.1"), 88i (54°00'23.1"N 22°93'33.3"E), 12j (54°05'30.0"N 22°94'71.2"E). This type of assessment is not carried out in the WPN.

Seed quality assessment

In the vicinity of the Wigry National Park, symptoms of cherry spruce rust have been observed on spruce cones, potentially reducing seed viability. To assess seed quality, we obtained data from the Seed Evaluation Station in Olsztyn. The research material was sourced from seed stands in the Suwałki Forest District, specifically from divisions 12b, 66a, and 80i,

which is the same area where the crop magnitude assessment was conducted.

Seed quality was assessed during mast years and periods of lower production, but not during years of poor yield. In all years, the assessment followed the guidelines described by Załęski (2000) and met the requirements for seeds intended for trade. According to Polish regulations, specifically the Regulation of the Minister of the Environment of February 18, 2004, on the Detailed Requirements for Forest Reproductive Material, only seeds without external signs of damage from biotic agents or physical defects can be used to estimate seed quality. Briefly, the weight of 1000 seeds (i.e., 1000 seed mass) was carried out with an accuracy of 0.01 g, seed vigor and viability were assessed in the Jacobsen germination chamber, with seeds germinated on tissue paper. Vigor (germination energy) was evaluated after 10 days, while germination viability, seed viability was assessed after 21 days. Viability was determined as the percentage of germinating seeds relative to all sown seeds (Załęski, 2000). Samples of cleaned seeds i.e. seed purity and other parameters, were evaluated in three replications, each consisting of 100 seeds (Załęski, 2000).

Molecular identification of the disease agent

Material and DNA extraction

For molecular analyses, three cone samples of *P. abies* with symptoms indicating infection with the fungus *T. areolata* representing the previously mentioned three different locations (Fig. 2) were se-

lected. Cones were freeze-dried in a CoolSafe device (ScanVac, Denmark). From symptomatic scales, aeciospores of *T. areolata* were scratched with a scalpel and homogenized with quartz beads in a Magna-Lyser homogenizer (Roche, Switzerland). DNA was extracted from 40 g of fine powder samples using the Bead-Beat Micro AX Gravity kit (A&A Biotechnology, Poland). Based on the results of fluorometric measurement on a Quantus device (Promega, USA), DNA was diluted to $10 \text{ ng} \cdot \mu\text{L}^{-1}$ in ddH₂O for further analyses.

2.4.2. PCR assays

The identification of the pathogen was carried out based on the analysis of rDNA fragments such as the ITS (internal transcribed spacer) region and 28S (LSU, large subunit). PCR assays with the Pucciniales-specific primer pairs, respectively, ITS5-u/ITS4-u (Pfunder and Schürch 2001) and LRust1R/LR6 (White et al. 1990; Beenken et al. 2012), were performed in an Eppendorf EP Mastercycler (Eppendorf, Germany) with the profile: 95°C for 5 min, 35 cycles of 95°C for 1 min, 53°C for 50 sec and 72°C for 50 sec (ITS) or 80 sec (LSU), followed by 10 min at 72°C for a final extension. The final concentration of reagents in the total volume of 37.5 μL was as follows: 1 \times PCR Mix Plus (A&A Biotechnology, Poland), DNA at a concentration of 4 ng and primers at a concentration of 1.0 pM. The presence and quality of amplicons were verified by electrophoresis of 2 μL of the post-reaction mixture in tris-borate-EDTA (TBE) buffer on a 1.2% agarose gel (Pronadisa, Spain) stained with SimplySafe (EURX, Poland) and visualized using the INTAS set (Germany).

Sequencing of PCR products and data analysis

PCR amplicons were purified and sequenced in both directions by Genomed (Poland) and compared with sequences in GenBank NCBI using the BLAST search algorithm. FinchTV 1.4 (Geospiza) was used to analyse the obtained sequences. Alignments were carried out using ClustalW through the Molecular Evolutionary Genetics Analysis Version 11 (MEGAX) Toolbar (Kumar et al. 2018).

To assess genetic variability, our three ITS sequences were compared with the selected 14 *T. areolata*

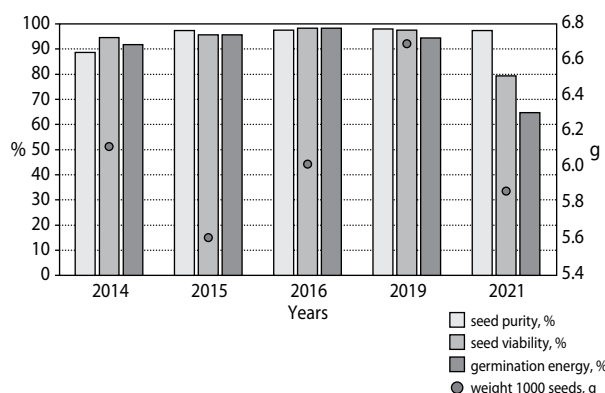


Figure 2. Results of the quality of the seeds assessed

ta sequences available in GenBank NCBI. Sequences shorter than ours by more than 100 nucleotides were not included in the analysis. To visualize the relationships between sequences, the dendrogram was constructed in MEGAX using the maximum likelihood method and Tamura 3-parameter model (Tamura 1992).

For comparative analyses of the LSU region, all four sequences available in GenBank were taken. The availability in GenBank of only four shorter sequences, overlapping with our sequences fragmentarily in different sections (the common section for all sequences was only 381 bp), made a reliable comparative analysis (dendrogram) impossible.

The sequences of isolates from three locations, that is, 62b in the Płociczno forestry and 131j in the Pijawne forestry in the Suwałki Forest District and 392d in the Wigry National Park, occurring in the three tested samples, were named Ta62Su, Ta131Su and Ta392WPN, respectively.

RESULTS

Crop magnitude

The results of the crop magnitude are included in Table 1. Norway spruce seed years occurred irregularly. In the period 2010–2023, there were two mast seeding years (2016 and 2019).

Seed quality assessment

The quality of the seeds assessed in 2014, 2015, 2016 and 2019 was very good in terms of all parameters taken into account. Its decline was noticed only in 2021 (Fig. 2). In the same year, the Seed Evaluation Station also received cones that were completely devoid of seeds.

Molecular analyses

A comparative analysis of ITS with sequences available in GenBank NCBI indicated on *T. areolata*. The three sequences, Ta62Su (710 bp), Ta392WPN (712 bp) and Ta131Su (711 bp), did not differ from each other and displayed a 100% identity with the sequences OL471665 (760 bp from *Prunus domestica* L.), OL471672 (665 bp

from *Prunus avium* L.) and OL471669 (760 bp from *P. padus* L.). They differed from other sequences by one (e.g. DQ087229 and DQ087231 [identity 99.9%]) or five (e.g. OL471667 and OL471668 [identity 98.8%]) nucleotides.

A dendrogram based on ITS region sequences indicates relationships between our sequences and GenBank NCBI isolates within a common section of 642 bp (Fig. 3).

The ITS sequences of the Ta62Su, Ta131Su and Ta392WPN isolates were deposited in GenBank NCBI under accession numbers PP309815, PP309831 and PP309832.

The comparative analysis of the LSU sequences of our isolates with sequences from GenBank NCBI confirmed, similarly to the ITS analysis, that our isolates belong to the species *T. areolata*.

Genetic diversity was found between the three analysed sequences. The Ta62Su (1,086 bp) and Ta131Su (1,089 bp) sequences differed from the Ta392WPN sequence (1,086 bp) by one nucleotide (T→A).

Table 1. Results of the assessment of Norway spruce crop magnitude in four excluded seed stands in the Suwałki Forest District registered in the Forest Seed Office

YDa	Number of seed stands		
	12b	66a	80i
2010	1	0	0
2011	1	2	1
2012	0	2	2
2013	2	0	0
2014	2	2	2
2015	1	1	2
2016	2	3	3
2017	2	0	0
2018	2	0	0
2019	3	3	3
2020	1	0	0
2021	1	1	1
2022	1	0	0
2023	1	0	0

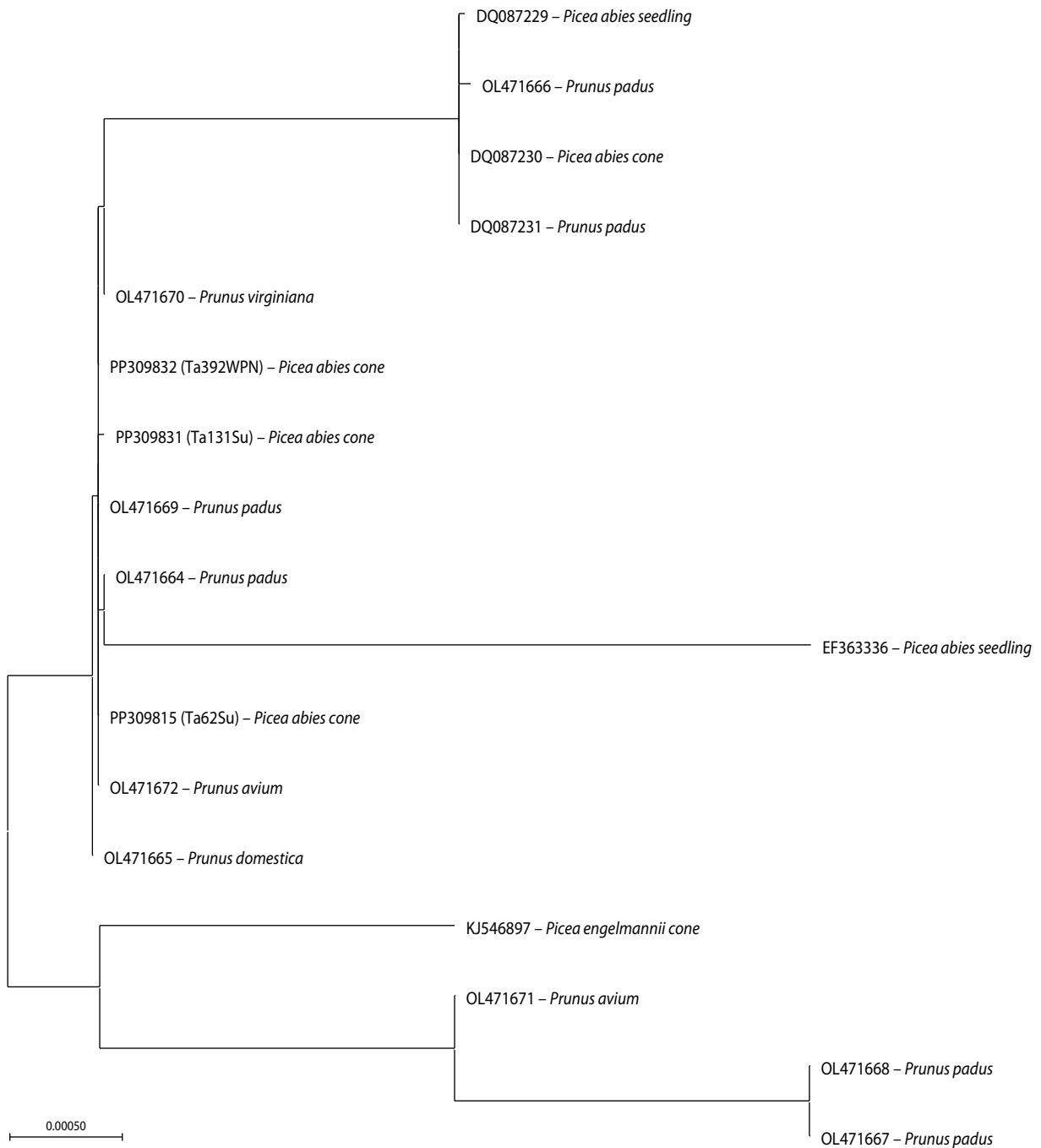


Figure 3. Dendrogram based on ITS sequences showing the relationships between the *T. areolata* isolates studied PP309815 (Ta62Su), PP309831 (Ta131Su) and PP309832 (Ta392WPN) and 14 *T. areolata* isolates from GenBank NCBI derived from different hosts

The four sequences of the 28S fragment available in GenBank were much shorter than ours and overlapped with ours at various sections. Therefore, although our sequences differed from each other, all three displayed 100% identity with AF426235 (only 520 bp). However, only two sequences, Ta62Su and Ta131Su, had 100% identity with the KJ546893 sequence (761 bp). The Ta392WPN sequence differed from KJ546893 by one nucleotide—the same level of difference between our sequences—resulting in 99.87% identity.

Our three sequences shared 99.68% of identity with KJ698629 (616 bp), differing only by one nucleotide (G→A). However, KJ546894 (763 bp) did not differ from our sequences, except for a single degenerate nucleotide (W), making it difficult to determine the level of differentiation.

The LSU sequences of the Ta62Su, Ta131Su and Ta392WPN isolates were deposited in GenBank NCBI under accession numbers PP309839, PP309852 and PP309851.

DISCUSSION

Our research demonstrated that the cause of spruce infertility in the studied area was *T. areolata*. Currently, little is known about the scale of this disease in Poland. There are also no known research results on the impact of this pathogen on spruce yield losses in Poland. The pathogen is mentioned in the Babiogórski National Park, where it was recorded as the most common species on spruce cones, next to the ubiquitous *Melanomma pulvis-pyrius* (Persoon) Fuckel (Chlebicki 2018). Its occurrence in the Białowieża National Park was reported by Majewski (1971) in the 1960s. Most reports in recent years concern Scandinavian countries, where it was considered the main cause of cone disease (Parmesan 2006; Kaitera et al. 2009; Kaitera 2013). The cherry spruce rust that persists in spruce stands intensifies the problems with the shortage of seeds necessary for the regeneration of spruce stands. The importance of this pathogen is demonstrated by the fact that, in North America, where this pathogen has not yet been detected, *T. areolata* was placed on the list of quar-

antine organisms by the United States Department of Agriculture (Farr and Rossman 2020; CABI 2022). Spruce cones with rust symptoms do not produce fertile or high-quality seeds, causing a 10-fold reduction in their germination rate (Kaitera and Tillman-Sutela 2014). Epidemics of this disease can destroy the entire seed crop (Kaitera 2013). Currently, meeting the demand for spruce seeds in other European countries (e.g. Finland or Sweden) is a challenge due to production shortages caused by the irregular flowering of spruce trees (Lundströmer et al. 2020). The Suwałki Forest District faces a similar challenge, struggling to produce the Norway spruces. Further excluded seed stands in the Suwałki Forest District are deleted from the database of the National Register of Forest Basic Material (2024). In 2023, seed stands in forest divisions 12j, 81h and 82h were excluded from the database: due to the lack of seed crops or if there was a crop of seeds, they were of poor quality. A decrease in the quality of spruce seeds and a lack of seeds in cones delivered for evaluation were also observed. This situation may be influenced by the disease we have detected.

The risk of Norway spruce extinction under climate change may be the result from low levels of phenotypic plasticity (i.e. the inability to change phenotype in response to water shortage as a major selection pressure) and from enhanced susceptibility of locally adapted ecotypes to multiple biological factors emerging with climate change (Jevšenak et al. 2021). Biotic factors related to the seed mortality caused by *T. areolata* have been frequently overlooked.

Cherry spruce rust is caused by two pathogens. *T. areolata* is characterized by more frequent occurrences in Scandinavia (up to 88%) (Kaitera and Tillman-Sutela 2014), whereas *C. pirolata* is more present in North America (Sutherland et al. 1984). The two pathogens significantly reduce seed vigour and germinability. Here, Norway spruce cones infected by *T. areolata* exhibited a lower germination potential ranging from 0% to 6%, whereas seeds from healthy cones displayed a significantly higher germination capacity (28%–48%) (Kaitera et al. 2019). *Picea mariana* (Mill.) Britton, Sterns & Poggenburg displayed a similar pattern of seed germination, which was reduced to 11% when infected by *C. pirolata*. Even non-

rusted seeds extracted from rusted cones were characterized by decreased germination (i.e. 36% in contrast to 83%–91% for seeds extracted from healthy cones). Seeds were deformed or healthy, but those obtained from infected cones were poorly developed, with an average radicle length of 2.2–6.4 mm, while healthy seed radicles were between 9.6 and 11.4 mm 7 days after germination (Kaitera et al. 2021). Considering the data and observations from the WNP and the Suwałki Forest District, further observations should be carried out to fully confirm whether the pathogen we identified is responsible for the lack of spruce seed harvest in this area.

Previous studies conducted in Finland, Sweden and Norway, yielding the same symptoms of cone disease as this study, confirmed that *T. areolata* and *C. piro-lata* were the main agents of cone disease (Kaitera et al. 2009; Kaitera 2013; Capador et al. 2020; Kaitera and Karhu 2021). These north-adapted pathogens broaden their range of activity from north to south. We presume that this adaptation to a new climate results from genetic differentiation within the *T. areolata* population. High genotypic diversity of *T. areolata* without population structure was found, suggesting a predominant sexual reproduction, random mating and a high gene flow within and between the populations in Fennoscandia (Capador et al. 2020).

The *T. areolata* isolates analysed in this study showed slight genetic differentiation within the LSU region. Their ITS sequences were identical. However, both analysed rDNA regions differed from some sequences available in GenBank NCBI. These differences suggest the possibility of genetic diversity within the *T. areolata* population. However, it is difficult to provide a reliable analysis due to the small number of sequences available in NCBI's GenBank. Low genetic diversity based on SSR markers was found by Zhang et al. (2022), but additionally, they showed that the diversity of *T. areolata* increased with higher disease prevalence. In the case of fungi pathogenic to crops or utility plants, this is a dangerous tendency because genetic diversity may be related to adaptive abilities because of generating new allele combinations (Covo 2020).

Considering the spectrum of *T. areolata* host plants, the requirement of intensive extension of the

black cherry (*Prunus serotina* Ehrh.) limits the regeneration of native plant species and reduces biodiversity. Additionally, as a potential host of spruce infertility, it may reduce the available seed base and inhibit the regeneration of spruce stands in North-Eastern Poland (Smith et al. 2009). The same solution may be applied to the other alternate hosts of *T. areolata* (i.e. *P. mahaleb* L., *P. avium* L. and *P. virginiana* L.) The general protective recommendation for the Norway spruce is to remove bird cherry from at least 400 m around Christmas tree nurseries (Talgø et al., 2020). However, Kaitera et al. (2021) suggested that this distance should be at least several kilometres to get seeds of higher quality and improve the management of naturally regenerated tree resources. However, using this method in forests is currently impossible due to the widespread occurrence of this alien invasive species (Bijak et al. 2014). In seed orchards, removing infected cones is worthwhile (Kaitera et al. 2021). This solution is challenging to apply within protected areas, such as the WNP in Poland, due to the limiting to use pesticides. Accordingly, an epidemic of this disease can destroy the entire seed crop (Kaitera 2013), hampering populations' adjustment to a new condition by negatively affecting seedlings' individual fitness and reducing genetic variability within those small populations. The irregular flowering of spruce trees increases the scale of the insufficient seed availability (e.g. Finland and Sweden) (Lundströmer et al. 2020). Therefore, special attention should be paid to preserving the genetic resources of spruce trees from North-Eastern Poland, as applied limitations heavily affect the functioning of their populations, making them vulnerable to extinction.

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