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ORIGINAL ARTICLE

Low disease incidence and cone bagging in *Picea abies* are associated with low genotypic diversity in *Thekopsora areolata*

Ke Zhang[1] | Berit Samils[1] | Juha Kaitera[2] | Åke Olson[1]

[1]Department of Forest Mycology and Plant Pathology, Swedish University of Agricultural Sciences, Uppsala, Sweden

[2]Natural Resources Institute Finland, Oulu, Finland

Correspondence
Ke Zhang, Department of Forest Mycology and Plant Pathology, Swedish University of Agricultural Sciences, Almas Allé 5, 75651, Uppsala, Sweden.
Email: ke.zhang@slu.se

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Abstract

Thekopsora areolata infects pistillate cones of *Picea* spp. with monokaryotic basidiospores in the spring. Receptive monokaryotic hyphae in the cones are fertilized by monokaryotic spermatia in the summer, and dikaryotic aecia are produced in cones in late summer. Infected cones produce no fertile seeds, meaning the disease causes large reductions in seed production. To understand the seasonal variation of *T. areolata* genotypic diversity, 548 aecia from 55 infected cones were sampled from multiple seed orchards in 2015, 2019 and 2020. Cone bagging experiments were performed during two seasons to investigate the sexual reproduction of *T. areolata*. In addition to the published simple-sequence repeat (SSR) markers, we developed 10 new polymorphic SSR markers to improve the resolution of population genetic analysis. Aecia were genotyped with 18 SSR markers in total. In 2015, when disease incidence was high in the seed orchards, the *T. areolata* populations had high genotypic diversity ($H = 4.69$). In 2019 and 2020, when disease incidence was low, the *T. areolata* populations had lower genotypic diversity ($H = 3.88$ and 3.85) and several cones were dominated by a single multilocus genotype. The genotypic diversity of *T. areolata* in a recently established seed orchard was exceptionally low ($H = 2.01$). Seven bagged cones that were infected produced either aecial primordia or aecia with lower diversity than exposed cones. The results indicate that cross-fertilization is important for sexual reproduction and aecia formation of *T. areolata*, and genotypic diversity of *T. areolata* increased with higher disease prevalence.

KEYWORDS
heterothallism, Norway spruce, population genetics, rust fungi, spermatia

1 | INTRODUCTION

Norway spruce (*Picea abies*) produces abundant female flowers and seeds in mast years. The interval between mast years is usually about 7 years, and the frequency is influenced by weather (Nussbaumer et al., 2016). The supply of genetically improved high-quality seeds produced in seed orchards is often in shortage due to this irregular flowering (Lundströmer et al., 2020). Furthermore, insects and fungal diseases can decrease the seed yield significantly in a mast year, increasing the imbalance between the supply and demand of seeds. Cherry spruce rust is a rust disease that associates with the primary host, Norway spruce, and the alternate host, mainly bird

cherry (*Prunus padus*) in Scandinavia (Kaitera et al., 2014, 2017, 2019; Kaitera, Aarnio, Ylioja,et al., 2021; Kaitera, Kauppila et al., 2021; Zhang et al., 2021). The typical symptoms of infected spruce cones are reddish-brown to dark brown aecia that fully cover both sides of the scales (Kaitera et al., 2009a). Therefore, seeds in infected cones are either missing or poorly developed (Kaitera & Tillman-Sutela, 2014). This disease can cause a 10-fold reduction in seed germination (Kaitera & Tillman-Sutela, 2014) and as high as 89% of seed yield loss (Kaitera, 2013).

The causal agent of cherry spruce rust is the rust fungus *Thekopsora areolata*. This pathogen is widely distributed in Europe and Asia, but absent in North and South America, Africa and Oceania (CABI, 2022). *T. areolata* is heteroecious; both Norway spruce and bird cherry are required to complete its macrocyclic life cycle (Kaitera et al., 2017, 2019). During its 2-year life cycle, *T. areolata* produces five types of spores (Littlefield & Heath, 2012; Olive, 1943). Teliospores are the overwintering structures in bird cherry leaf litter. In the early stage, they are dikaryotic, having two haploid nuclei in each cell. During the germination of teliospores in the spring, karyogamy and meiosis occur, after which haploid monokaryotic basidiospores are produced and then spread under optimal weather conditions to infect pistillate cones (Kuprevich & Transchel, 1957; Zhang et al., 2022). From late spring to early summer, *T. areolata* hyphae colonize the developing cones and produce spermogonia with haploid monokaryotic receptive hyphae and spermatia. The mating of receptive hyphae and spermatia occurs in mid-summer, after which aecia with dikaryotic aeciospores are produced in the cones in mid- to late summer (Kaitera et al., 2009a; Kaitera, Aarnio, Karhu, et al., 2021). After overwintering, aeciospores are released to infect the bird cherry, where uredinia with dikaryotic urediniospores are produced from late spring to late summer (Kaitera, Aarnio, Karhu, et al., 2021), and teliospores are produced in autumn.

Maintaining the mechanism of sexual reproduction has a cost to the pathogen and two individuals with different mating-type genes are required (Lee et al., 2010); however, it is considered advantageous to plant pathogens, because the mating of two individuals creates new combinations of genetic alleles that help adaption to new environments (Drenth et al., 2019). In rust fungi, including *T. areolata*, sexual reproduction is achieved by the transfer of spermatia to receptive hyphae, which requires insect vectors or rain. In cones infected by *T. areolata*, a sugary liquid is produced by spermogonia in mid-summer (Kaitera, Aarnio, Karhu, et al., 2021; Murray, 1955). No study has addressed the relationship between this sugary liquid and the visitation by insects leading to fertilization in *T. areolata*, but this type of insect attraction has been demonstrated in other rusts (Alexopoulos et al., 1996). For example, *Puccinia arrhenatheri* infects *Berberis vulgaris* leaves, where insects can be attracted by the bright yellow colour of the diseased leaves and the sugary nectar and volatiles produced by the fungal spermogonia. Subsequently, the insect transfers spermatia in the sugary nectar between leaves and enables successful outcrossing (Naef et al., 2002).

Most studied species in Basidiomycota are heterothallic, that is, two individuals with different mating types are required for sexual reproduction (Whitehouse, 1949). In Pucciniomycotina, most known mating systems are bipolar (such as *Puccinia graminis*) or tetrapolar (such as *Melampsora lini*), although some strains are homothallic or anamorphic (Kües et al., 2011). The mating system of *T. areolata* is not well understood. Capador et al. (2018) developed eight polymorphic microsatellite/simple-sequence repeats (SSR) markers and analysed the diversity of *T. areolata* individuals from Finland, Norway and Sweden. The results suggested high gene flow, sexual reproduction and multiple infections of Norway spruce cones by *T. areolata*. However, a few homozygous individuals were found from four cones (Capador et al., 2020). This homozygosity could be explained either by self-fertilization between genetically identical spermatia and receptive hyphae or by the limited resolution of the molecular markers.

The temporal dynamic of pathogen populations often undergoes "boom and bust" cycles, especially for crop pathogens. These fluctuations are the result of variations in environmental factors, pathogen inoculum dispersal, and host susceptibility and senescence (Burdon et al., 1989; Mundt et al., 2011). The genetic and genotypic diversity of pathogen populations can be greatly affected by population fluctuation through random genetic drift (McDonald & Linde, 2002). In the case of *T. areolata*, the population fluctuation in seed orchards can be influenced by additional factors, such as the change of availability of alternate hosts and long mast year intervals in Norway spruce. Information about the variation in pathogen genotypic diversity between years with different disease prevalence would improve our understanding of the infection and reproduction processes of this pathogen and support the selection of strategies for disease control. Norway spruce seed orchards in Sweden had high cherry spruce rust disease incidence in 2015 (Olle Rosenberg, Skogforsk, olle.rosenberg@skogforsk.se, personal communication), providing material for previous studies (Capador et al., 2018, 2020). Population genetic analysis with SSR markers revealed a high genotypic diversity of *T. areolata* aecia at the levels of seed orchards, trees, cones and even scales (Capador et al., 2020). The disease incidences of cherry spruce rust in 2019 and 2020 in the studied seed orchards were lower than in the previous years (Rosenberg, personal communication). We hypothesized that during these years with low cherry spruce rust incidences, *T. areolata* populations would have lower genotypic diversity.

The previous study by Capador et al.(2020) provided evidence for multiple infection and cross-fertilization events in single cones. Accordingly, we hypothesized that bagging a cone before the production of the sugary fluid containing spermatia would lead to lower genetic diversity of *T. areolata* aecia by reducing the frequency of the mating between receptive hyphae and alien spermatia, therefore reducing the sexual reproduction frequency between cones. The results from such cone bagging would increase understanding of the biology of the rust and provide new evidence for the involvement of insects in the sexual reproduction of *T. areolata*.

In this study, we developed new SSR markers to improve the resolution in the population genetics analysis of *T. areolata*. The objectives of this research were (a) to study the variation in genotypic diversity of *T. areolata* populations in Swedish and Finnish seed orchards during years of high and low disease incidences; and (b) to

TABLE 1 List of Norway spruce samples infected with *Thekopsora areolata* that were processed for genotyping

Year	Seed orchard	Coordinates	Cone production	Disease incidence[a]	Exposed or bagged cone	No. of cones	Sampling	No. of aecia
2015	Söregarde, Sweden	56°47′ N, 16°21′ E	Mast year	High, 10%	Exposed	8	One aecium per scale, 10 scales	80
	Ålbrunna, Sweden	59°30′ N, 17°32′ E	Mast year	High, na	Exposed	5	One aecium per scale, 9 or 10 scales	48
2019	Suhola, Finland	62°15′ N, 27°42′ E	Mast year	Low, ≤1%	Exposed	6	One aecium per scale, 10 scales	60
					Bagged	4	One aecium per scale, 10 scales	40
	Ålbrunna, Sweden	59°30′ N, 17°32′ E	Mast year	Low, 3%	Exposed	8	One aecium per scale, 10 scales	80
2020	Vuolenkoski, Finland	61°05′ N, 26°08′ E	Low production	Low, ≤1%	Exposed	6	One aecium per scale, 10 scales	60
					Bagged	2	One aecium per scale, 10 scales	20
	Suhola, Finland	62°15′ N, 27°42′ E	Low production	Low, 90%[b]	Exposed	8	One aecium per scale, 10 scales	80
	Söregärde, Sweden	56°47′ N, 16°21′ E	Mast year	Low, 0%	Exposed	7	One aecium per scale, 10 scales	70
					Bagged	1	One aecium per scale, 10 scales	10

[a]The disease incidences were calculated either by the owners of the seed orchards after harvest or by the authors during the final sample collection.

[b]Due to the low flowering rate, very few new cones (about 20) were produced in Suhola 2020, and although about 90% of these cones were infected, the occurrence of disease was still very low and so we regard disease incidence as low for the purpose of this study.

investigate the effect of bagging of Norway spruce cones on the infection, fructification, and genetic diversity of *T. areolata*.

2 | MATERIALS AND METHODS

2.1 | Fieldwork and sample collection

Because of the irregular flowering of Norway spruce, it was impossible to collect samples every year from all seed orchards included in this study. The cone bagging experiments were conducted in seed orchards in Rörby, Sweden, and Suhola (sv 403), Finland, in 2019, and Söregärde, Sweden, and Vuolenkoski (sv 445), Finland, in 2020. The seed orchards were established in 1967 (Rörby), 2009 (Söregärde), 1997 (Suhola) and 2014 (Vuolenkoski). In May, 2019 and 2020, 300 to 500 pistillate Norway spruce cones were bagged with paper sterilization pouches and sealed with zip-ties in each seed orchard. Pistillate cones were bagged individually unless it was impractical to do so (if two or more cones were tightly aggregated). All the cones were bagged before the formation of *T. areolata* spermatia, and therefore, the mating of spermatia and receptive hyphae between cones during the summer was inhibited. Pistillate cones were covered in the bags until mature cones with aecia had developed. All bagged cones were harvested in early September and examined in the laboratory: four cones were infected in Suhola, Finland, in 2019; two cones were infected in Vuolenkoski, Finland, in 2020; and one cone was infected in Söregärde, Sweden, in 2020. Additional infected exposed (unbagged) cones were collected from Vuolenkoski (6) and Suhola (8 and 6), Finland, and Söregärde (7), Sweden, in 2019 and 2020 (Table 1). These bagged and exposed cones from 2019 and 2020 represented samples from seed orchards with low disease incidence. One special case was Suhola 2020, where very few cones were produced in the seed orchard (in total about 20), but among these, approximately 90% were infected, meaning that the disease incidence was high but the occurrence of disease was very low. Therefore, in this study, we regarded Suhola as having low disease incidence.

In 2015, high disease incidences of cherry spruce rust were observed in many Swedish seed orchards including Ålbrunna (established in 1982) and Söregärde (O. Rosenberg, personal communication). Scale samples and aecial DNA samples from eight cones from Söregärde and five cones from Ålbrunna collected in 2015 (Capador et al., 2020) were retrieved from −20°C storage. These samples represent aecia from seed orchards with high disease incidence. In total, samples from 55 cones were investigated in this study (Table 1).

2.2 | Single aecium sampling and DNA extraction

From each infected cone, aecia were collected as described by Capador et al. (2020): each cone was split vertically into two halves and then horizontally into five sections in each half, and one scale with aecia was selected from each section. From each scale, one aecium

was picked up carefully with a scalpel and transferred into a 1.5-ml tube. In this way, 10 evenly distributed aecia were sampled from each cone. In the 1.5-ml tube, the aecium was precleaned with 0.2% Tween 20 (Merck), then surface sterilized with 1.5% sodium hypochlorite for 1 min, 70% ethanol for 1 min and rinsed with sterilized water three times. DNA from each aecium was extracted with a NucleoSpin Plant DNA extraction kit (Macherey-Nagel) according to the manufacturer's manual. In total, 548 aecia were included in the study.

2.3 | Development of SSR markers and screening for polymorphism

A draft genome assembly of *T. areolata* isolate SB-2076-252 was obtained by next-generation sequencing (unpublished data). The original assembly consisted of 165,963 contigs with lengths from 200 to 31,680 base pairs (bp). The assembly was filtered with Geneious Prime 2019.1 (https://www.geneious.com); subsequently, 413 contigs longer than 10,000 bp were screened with BatchPrimer3 v. 1.0 (You et al., 2008) for SSR loci that were longer than 12 bp and with corresponding primer pairs that yielded a product of optimal size (100–300 bp).

The preliminary screening yielded 359 SSR loci that contained repeated motifs: 51 dinucleotide, 97 trinucleotide, 141 tetranucleotide, 41 pentanucleotide and 29 hexanucleotide loci. Thirty-three loci and corresponding primers (Table S1) were selected to test the polymorphism in standard PCR with 12 randomly selected aecial DNA samples from Sweden and Finland collected in 2019. The selection was made according to the following criteria: (a) only one SSR locus was selected from each contig, (b) primer GC content was between 40% and 60%, and (c) longer SSR loci with more repeats were favoured. Standard PCR was performed in 20 μl reaction volumes (2 μl DNA template, 0.5 μM forward primer, 0.5 μM reverse primer, 0.2 mM each dNTP, 1× PCR buffer, 0.5 mM $MgCl_2$ and 1 U DreamTaq DNA polymerase [Thermo Fisher Scientific]), under the following cycling conditions: 95°C for 5 min; 40 cycles of 95°C for 30 s, 56°C for 30 s and 72°C for 30 s; followed by a final extension of 10 min at 72°C. Amplification products were loaded on a 3% agarose gel for electrophoresis at 100 V for 2 h, then examined in a gel imaging system. SSR loci that were polymorphic among tested samples were identified based on the variation of amplicon sizes.

Of the 33 loci tested for polymorphism (Table S1), 13 loci produced detectable polymorphic amplicons on 3% agarose gel, but three of them had either weak amplicon bands or the amplicon size significantly differed from the estimated size based on NGS contigs. Therefore, 10 new SSR markers were included in the genotyping along with eight published markers from Capador et al. (2018) (Table 2).

2.4 | Genotyping

A total of 18 SSR markers were divided into six groups according to the amplicon size, and the forward primers were labelled with fluorescein amidite (FAM) or hexachloro-fluorescein (HEX) (Table 2). Standard PCR of each locus was performed individually in 15 μl reaction volumes (2 μl of DNA template, 0.5 μM forward primer, 0.5 μM reverse primer, 0.2 mM each dNTP, 1× PCR buffer and 0.75 U DreamTaq DNA polymerase [Thermo Fisher Scientific]), with cycling conditions as described above for SSR marker development. The PCR products of three markers in the same group were mixed together and then diluted 50 times for fragment length analysis (Macrogen Europe) with an ABI 3730xl DNA analyzer (Applied Biosystems) and GeneScan 400HD ROX as size standard.

Raw data were scanned with GeneMarker v. 3.0.1 (SoftGenetics) to determine the SSR allele sizes.

2.5 | Population genetics analysis

Genotypic diversity analysis and haplotype reconstruction were performed with non-clone-corrected data, while clone-corrected data was used to analyse the SSR loci statistics.

The genotyping data of 18 SSR loci of all aecium individuals were summarized in GenAlEx (Peakall & Smouse, 2006). The data were stratified and analysed according to year, seed orchard, bagged or exposed, and individual cone. The SSR loci statistics and genotypic diversity were analysed with R in Rstudio and poppr v. 2.8.0 (Kamvar et al., 2014). Data of the 478 samples from 48 exposed cones were used to compare the variation of *T. areolata* genotypic diversity between years. Data of the 70 samples from seven bagged cones and 190 samples from 19 unbagged cones from the same year and seed orchard were compared to study the effect of cone bagging. Indices of genotypic diversity included the number of observed multilocus genotypes (MLG), the number of expected multilocus genotypes based on rarefaction (eMLG), Shannon–Wiener diversity index (*H*) (Shannon, 2001), Stoddard and Taylor's index (*G*) (Stoddart & Taylor, 1988) and evenness (Grünwald et al., 2003).

The haplotypes of individuals from the same cones were inferred in Arlequin v. 3.5 (Excoffier & Lischer, 2010) with Excoffier–Laval–Balding (ELB) algorithm, where parameters used in the analysis were Dirichlet prior $\alpha = 0.01$, $\gamma = 0$, $\varepsilon = 0.1$, sampling interval = 500, number of samples = 20,000, burn-in steps = 10,000, recombination steps = 0. The numbers of *T. areolata* haplotypes in aecia from the same cone were summarized and visualized in R. The haploid diversity (HD) was represented by the average haplotype number per aecium.

2.6 | Microscopy of aecial primordia from bagged cones

In 2020, poorly developed fruiting bodies identified as aecial primordia were found in three infected cones from the cone bagging experiment in both Sweden and Finland. Scales of these cones were examined under a dissecting microscope. Individual aecial primordia were picked up from the scale with a scalpel and fixed in formaldehyde–acetic acid–ethanol (FAA) solution for 24 h. Cross-sections of the fruiting

TABLE 2 Characteristics of polymorphic simple sequence repeat (SSR) markers and primers, and their fluorescent dye labelling for determining multilocus genotypes of *Thekopsora areolata*

Marker	Primer sequence (5′–3′)		Motif	No. of repeats in *T. areolata* reference genome[a]	Amplicon size in *T. areolata* reference genome (bp)[a]	Fluorescent dye	Assigned group[b]
SSR1	F	AGATCACCAGTTTAGTGACCA	TCATCT	4	146	FAM	1
	R	AACCTAATTCTGCTGTCCTTC					
SSR24	F	CGTTGAGATGAGACTGGATTA	GA	9	154	HEX	
	R	GCGCTGTGTGTATGTAAGTATT					
SSR4	F	AAGGAGGGGGATTTTAGTAGT	TCAA	6	235	FAM	
	R	TACCACCATCTCAACCTTTTA					
SSR5	F	TTCTCATTCTCATTCTCATCC	CTTTTT	6	150	FAM	2
	R	CGTCATTAGCAAATCAAATTC					
SSR13	F	TCTCGTGTGTTCATAACTGTG	TG	9	172	HEX	
	R	AGTAAGGATGGAAATGGAGTC					
Tha9	F	AAGGCAGATGACAGTCGTGA	AC	17–21	296–300	FAM	
	R	TCCTCTGTCCAAAGCGTCTT					
SSR10	F	CAAAACAGGTGTCAAAGTGAT	CT	12	149	FAM	3
	R	CGGAGAGAGAGAAAAGAAAAA					
Tha91	F	GTCTGTGTCTCTGGTGTCGA	AG	3–8	181–191	HEX	
	R	ACCAAAGTTCCCTGATATCCC					
Tha61	F	TGGGTAATTTGGGGTGTTTGT	AC	15–17	337–341	FAM	
	R	ACAGAAGTTACTCCGCCCTT					
SSR16	F	AGGAACAAGTTGACTTGCAG	GAGAAT	5	140	FAM	4
	R	TAATCGATCAGACAACCCTTA					
SSR18	F	GAGATATGTATGCAGGCAAAG	GA	15	155	HEX	
	R	GTCTATCTGGTCTTCCCAAAC					
Tha92	F	TTCTCGGGAATGGTGTGGAA	AACAAAAT	9–10	355–363	FAM	
	R	CCCCACAAATCTTACGAGCTG					
SSR19	F	GATCACGGATCAAGTTATCAA	TCA	7	147	FAM	5
	R	GTGTTTAGGAGGTTGGTTTTT					
Tha96	F	ATCACAACGCCTGATGG	AAG	12–21	176–203	HEX	
	R	GCTCACAACATTCGCAATCC					
Tha136	F	CAAGCACAACCTTCACCACA	AG	10–40	231–261	FAM	
	R	TGGGTCATCAGCTTTACGGA					
SSR22	F	ATCCAAGTCTTTTCTTCACCT	CT	8	150	FAM	6
	R	TCGAAAAGTGTGAGAGAGTTT					
Tha105	F	GCCGATTCTCAAACCTACACC	AG	7–17	184–204	HEX	
	R	TGCTGCCAACTTTTCACGTT					
Tha137	F	AAAGGGTTTTCAGGAGGGGC	AG	7–11	225–233	FAM	
	R	CCAGTGCAAATCAAACGTCC					

Note: The markers developed in this study are in bold.

[a]No. of repeats of the motif and amplicon size in the *T. areolata* reference genome were calculated for the SSR markers developed in this study. Information about the published markers was taken from Capador et al. (2020).

[b]SSR amplicons in the same group of the same aecia sample were pooled in one tube for one fragment length analysis reaction. The signal of each SSR can be identified according to the fragment length and fluorescent label.

bodies were prepared by cryostat (CM1850; Leica) and microtome, and stained with lactophenol cotton blue. To quantify the number of nuclei in the fungal tissue, cross-sections of the aecial primordia and the hyphae around the fruiting bodies were stained with the fluorescent dye 4′,6-diamidino-2-phenylindole (DAPI) for staining DNA (Samils et al., 2011), and then examined under a fluorescence microscope.

3 | RESULTS

3.1 | Diversity of SSR markers in individual aecial samples collected in 2015, 2019 and 2020

All SSR markers included in this study were polymorphic in the entire set of 548 aecial samples, as well as in the subsets of samples collected in 2015, 2019 and 2020; the only exception was locus Tha92 in samples collected in 2019, which was monomorphic (Table 3). In total, these markers detected 123 alleles with a variation from 2 (Tha92) to 14 (Tha96) alleles at each locus. The samples from 2015 had the highest average number of alleles (5.167 alleles per locus), even though the sample size (n = 128) was smaller than the subsets from 2019 and 2020 (n = 180 and 240, respectively). However, samples from 2019 and 2020 had marginally higher expected heterozygosity (H_{exp} = 0.479 and 0.477, respectively) and alleles had more even distributions (evenness = 0.677 and 0.636).

Almost all aecial samples (518 out of 548) had at least one heterozygous locus among the 18 SSR loci. Exceptions were the three bagged cones collected in Söregärde and Vuolenkoski in 2020, from which aecial primordia instead of mature aecia were identified. These samples showed only one allele at all SSR loci, and therefore, they were excluded from the population genetics analysis.

3.2 | Variation of *T. areolata* genotypic diversity between years

SSR markers used in the study achieved high resolution that identified 276 multilocus genotypes (MLGs) from the 548 samples (Figure S1); the genotype accumulation increased linearly and later a plateau was reached. Among the 478 *T. areolata* aecia samples from 48 exposed cones, a total of 259 MLGs were identified (Table 4). Aecia that shared the same MLG were always from the same cone, whereas aecia from different cones never shared the same MLG.

A significantly higher number of MLGs was found in aecia samples collected in 2015 compared to 2019 and 2020 (MLG = 115 vs 71 and 73, respectively) despite a smaller sample size (N = 128 vs 140 and 210; Table 4). Most aecial samples (n = 106) from 2015 had a unique MLG, while two to four aecia from the remaining samples (n = 22) shared the same MLGs (Figure S2). All exposed cones from 2015 had at least seven MLGs when 9 to 10 aecia were sampled (Figure 1). Eight out of 14 exposed cones from 2019 and 2020 had only one to six MLGs when 10 aecia were sampled (Figure 1), and it was common that aecia from the same cone shared identical MLGs (Figure S2). The genotypic diversity indices calculated in this study, Shannon–Wiener index (H) and Stoddart and Taylor's index (G), were higher in 2015 than in the other years. In addition, the MLGs had a more even distribution in 2015 compared to 2019 and 2020 (Table 4).

Samples from the same seed orchard followed the general trend mentioned above: the genotypic diversity was higher in 2015 when disease incidence was generally higher than in 2019 and 2020. For example, with lower numbers of aecial samples, rust populations in 2015 in Ålbrunna and Söregärde had a much higher number of MLGs and eMLGs, and higher genotypic diversity indices than in 2019 in Ålbrunna and 2020 in Söregärde (Table 5). In Suhola, Finland, samples were collected in 2019 and 2020 when the disease incidences were low. The 2020 Suhola population had an even lower number of eMLGs and genotypic diversity than the 2019 Suhola population (Table 5).

Within the same sampling years, the diversity of populations from different seed orchards had similar levels of genotypic diversity: the diversity was the highest in 2015, lower in 2019, and the lowest in 2020. For example, Shannon–Wiener indices (H) were 3.84 and 4.14 in Ålbrunna and Söregärde in 2015, 3.13 and 3.24 in Suhola and Ålbrunna in 2019, and 2.95 and 3.17 in Suhola and Söregärde in 2020 (Table 5). The population in the youngest seed orchard, Vuolenkoski in 2020, was the least diverse (H = 2.01). Only nine MLGs were identified from 60 aecia samples from exposed cones, and the 10 aecia sampled from the same cones were always dominated by one MLG (Figure S3).

The differences in genotypic diversity between years and seed orchards were shown in the rarefaction analysis (Figure 2): the expected MLGs increased almost linearly as the sample size grew in the two 2015 populations. The rarefaction curves of the populations from 2019 and 2020 were grouped together below the 2015 curves, which indicated lower genotypic diversity. The low plateau of the rarefaction curves of 2020 Vuolenkoski suggested a very low genotypic diversity, and the levelling out in the curve indicated that most genotypes existing in the seed orchard were sampled.

3.3 | The effect of cone bagging on *T. areolata* genotypic diversity

Seven infected cones were found among about 1200 bagged cones in 2019 in Suhola, 2020 in Söregärde and 2020 in Vuolenkoski. In general, aecia from bagged cones had lower numbers of MLGs (Figure 1): one to three MLGs were usually identified from the bagged cones, except for one bagged cone in Suhola 2019, which had six MLGs. In total, 17 MLGs were identified from 70 samples from seven bagged cones (Figure 3). Among exposed cones from the same years and seed orchards, four to nine MLGs could be usually identified from the 10 aecia, except for cones from 2020 in Vuolenkoski, where one to two MLGs were identified in each cone (Figure 1). In total, 74 MLGs were identified from 190 samples from 19 exposed cones from the same seed orchards and years. The four bagged cones in Suhola 2019 had on average 3.5 MLGs, while the exposed cones had on average 5.3 MLGs.

In the four bagged cones from Suhola 2019, fully developed aecia with multiple heterozygous MLGs coexisted in the same cone. However, all fruiting bodies (N = 30) from the three bagged cones collected from Söregärde and Vuolenkoski in 2020 were poorly developed and identified as aecial primordia. All the aecial primordia samples from these bagged cones had only one allele at all SSR loci,

TABLE 3 Summary of allelic results for each simple-sequence repeat (SSR) locus in samples of *Thekopsora areolata* aecia collected in 2015, 2019 and 2020, and in the total population

	2015			2019			2020			Total		
SSR	Allele no.	H_{exp}[a]	Evenness	Allele no.	H_{exp}	Evenness	Allele no.	H_{exp}	Evenness	Allele no.	H_{exp}	Evenness
SSR1	6	0.571	0.591	4	0.612	0.746	5	0.583	0.649	7	0.601	0.566
SSR24	3	0.497	0.869	3	0.496	0.821	4	0.551	0.793	4	0.540	0.848
SSR4	9	0.671	0.714	6	0.746	0.793	6	0.696	0.766	11	0.705	0.702
SSR5	4	0.586	0.819	6	0.600	0.759	4	0.567	0.791	7	0.607	0.808
SSR13	6	0.652	0.660	8	0.751	0.730	6	0.699	0.736	9	0.755	0.710
Tha9	3	0.250	0.588	3	0.406	0.722	3	0.305	0.660	3	0.318	0.650
SSR10	7	0.738	0.760	5	0.576	0.673	7	0.758	0.793	8	0.779	0.802
Tha91	6	0.618	0.721	5	0.645	0.722	4	0.575	0.699	7	0.620	0.652
Tha61	3	0.183	0.478	3	0.091	0.401	6	0.296	0.431	8	0.190	0.365
SSR16	3	0.274	0.637	3	0.367	0.624	3	0.362	0.648	3	0.328	0.622
SSR18	6	0.622	0.746	5	0.723	0.836	6	0.415	0.477	6	0.636	0.685
Tha92	2	0.162	0.552	1	na	na	2	0.076	0.454	2	0.091	0.472
SSR19	3	0.309	0.615	3	0.525	0.861	3	0.463	0.682	3	0.449	0.712
Tha96	11	0.582	0.513	10	0.648	0.574	6	0.668	0.738	14	0.632	0.549
Tha136	7	0.276	0.405	5	0.242	0.415	5	0.244	0.438	10	0.257	0.368
SSR22	3	0.187	0.524	3	0.362	0.696	5	0.479	0.548	5	0.330	0.510
Tha105	9	0.839	0.813	9	0.785	0.749	9	0.827	0.828	12	0.856	0.843
Tha137	2	0.009	0.308	2	0.035	0.387	2	0.013	0.328	2	0.018	0.345
Mean	5.167	0.446	0.629	4.667	0.479	0.677	4.778	0.477	0.636	6.722	0.484	0.623

Note: The analysis was performed with clone-corrected data from 548 samples.

[a]Expected heterozygosity.

TABLE 4 Population diversity of *Thekopsora areolata* at the level of collection year

Year (disease severity)	*N*[a]	MLG[b]	eMLG[c]	*SE*[c]	*H*[d]	*G*[e]	Evenness
2015 (high)	128	115	115.0	0.00	4.69	99.9	0.915
2019 (low)	140	71	66.6	1.59	3.88	32.6	0.668
2020 (low)	210	73	54.7	2.92	3.85	34.7	0.733
Total	478	259	93.7	4.32	5.16	112.2	0.644

Note: The analysis was performed on samples from exposed cones.

[a]Number of samples.

[b]Number of multilocus genotypes.

[c]Expected number of MLGs and standard error at the smallest sample size (128) based on rarefaction.

[d]Shannon–Wiener index.

[e]Stoddart and Taylor's index.

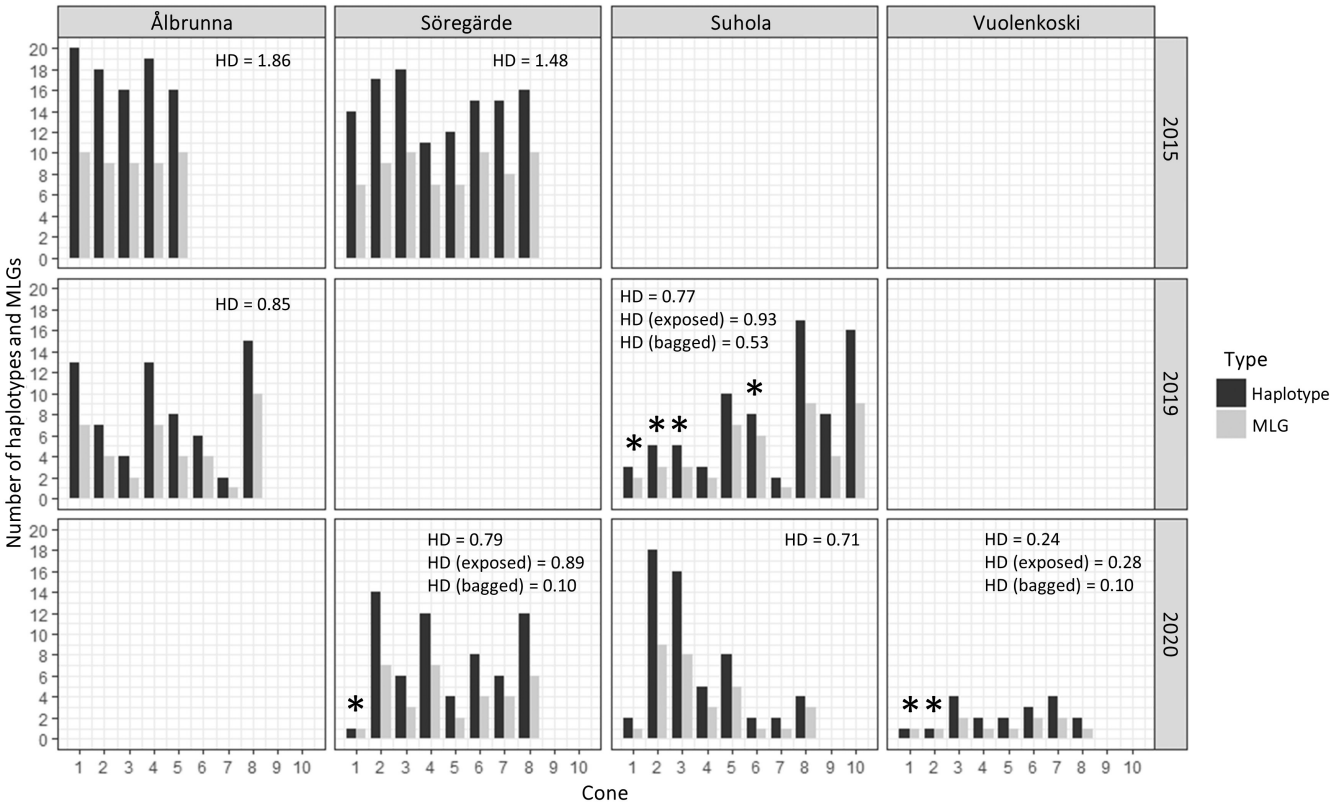

FIGURE 1 Number of multilocus genotypes (MLGs) of *Thekopsora areolata* aecia and inferred number of haplotypes of *T. areolata* in each infected cone of Norway spruce collected from four seed orchards in 2015, 2019 and 2020. The haploid diversity (HD) is represented by average haplotype number per aecium. Bagged cones are labelled with asterisks. The three bagged cones in 2020 only produced haploid aecial primordia

and all samples from the same cone belonged to the same haploid MLG (Figures 1 and 3).

3.4 | Haplotype inference

The inferred number of haplotypes included both the haplotypes of basidiospores that infected the cones and the haplotypes of the spermatia that mated with the receptive hyphae (Figure 1).

3.4.1 | Variation between years

High numbers of haplotypes (HD of 1.86 and 1.48) were inferred from each exposed cone in Swedish seed orchards in 2015 (Figure 1), when there was high disease incidence. This indicated that all cones were infected by multiple basidiospores and/or received multiple spermatia. In 2019 and 2020, with low disease incidence, the number of haplotypes in exposed cones varied. For example, four exposed cones in Suhola 2019 and 2020 had as many as 16–18 haplotypes

TABLE 5 Population diversity of *Thekopsora areolata* at the level of collection year and Norway spruce seed orchard

Year	Seed orchard	N[a]	MLG[b]	eMLG[c]	SE[c]	H[d]	G[e]	Evenness
2015	Ålbrunna	48	47	47.00	0.000	3.84	46.08	0.988
	Söregärde	80	68	43.07	1.581	4.14	56.14	0.889
2019	Suhola	60	32	27.41	1.485	3.13	15.79	0.673
	Ålbrunna	80	39	26.80	2.093	3.24	17.11	0.658
2020	Suhola	80	31	22.21	1.943	2.95	13.39	0.682
	Söregärde	70	33	25.93	1.782	3.17	17.88	0.737
	Vuolenkoski	60	9	8.73	0.476	2.01	6.95	0.918
Total		478	259	41.64	2.252	5.16	112.22	0.644

Note: The analysis is based on samples from exposed cones.

[a]Number of samples.

[b]Number of multilocus genotypes.

[c]Expected number of MLGs and standard error at the smallest sample size (48) based on rarefaction.

[d]Shannon–Wiener index.

[e]Stoddart and Taylor's index.

(Figure 1, Table S2). However, eight exposed cones from 2019 and 2020 were dominated by only one MLG and two haplotypes (Figure 1, Table S2). On average, samples from 2019 and 2020 had lower haplotype diversity (HD<1) than those of 2015 (HD = 1.86 and 1.48).

3.4.2 | Variation between exposed and bagged cones

The number of haplotypes per cone, seed orchard and year varied from 2 to 20 in exposed cones and from 1 to 8 in bagged cones (Figure 1, Table S2). The haplotype diversity of samples from four infected bagged cones from Suhola in 2019 (HD = 0.53) was lower than that of samples from the exposed cones (HD = 0.93). Three to eight haplotypes were inferred from these bagged cones (Figure 1, Table S2), which indicated that multiple basidiospores with different haplotypes had already infected the pistillate cones before the bagging. Only one haplotype was inferred from each of the bagged cones from 2020, which was supported by the observation that only aecial primordia were found in these cones.

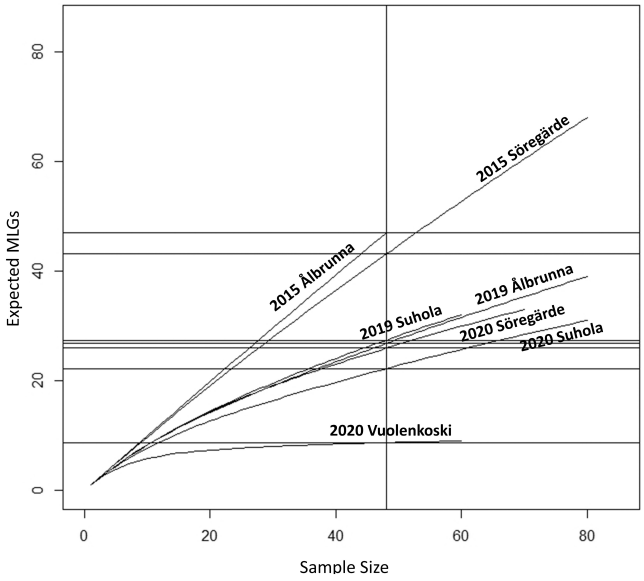

FIGURE 2 Rarefaction curve of *Thekopsora areolata* aecia collected from four seed orchards in 2015, 2019 and 2020. Vertical line indicates minimum sample size, horizontal lines indicate expected number of multilocus genotypes (MLGs) with the minimum sample size

3.5 | Microscopy of aecial primordia in infected bagged cones

In infected cones of *T. areolata* with mature aecia, the aecia that developed on the inner/adaxial side of the scale were hemispherical or polygonal, reddish-brown to dark brown, and with a smooth surface (Figure 4a). The three cones in bags collected in 2020 only developed aecial primordia, and aeciospores were not observed. These early-stage structures were hemispherical or polygonal, 0.8–1.2 mm in diameter, 80–100 μm high, disk-shaped, white (Figure 4b), and sometimes light brown because of the coverage of Norway spruce seed wings (Figure 4c,d). Aecial primordia were surrounded by white mycelia (Figure 4b,d). Cross-sections of the aecial primordia showed a dense layer of fungal basal cells growing closely to the cone scale epidermis. Chains of swollen fertile cells were produced above the basal cells and covered by a gelatinous matrix (Figure 4e–h). After DAPI staining of aecial primordia, only one nucleus could be found in each fungal hyphal cell (Figure 4i–l).

4 | DISCUSSION

In this study, 10 new polymorphic SSR markers were developed for *T. areolata* based on its genome sequence. Most of the SSR markers

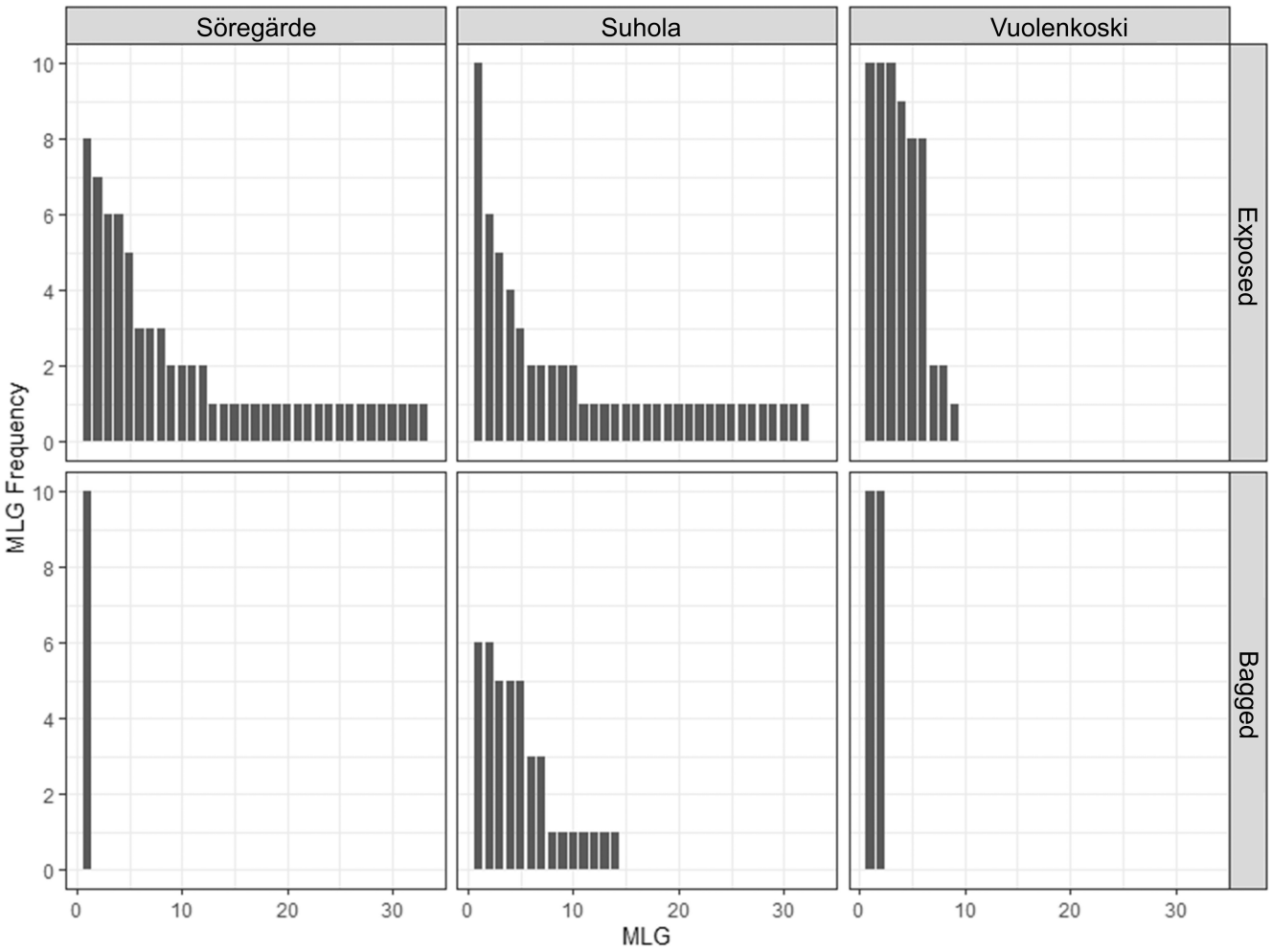

FIGURE 3 Multilocus genotype (MLG) frequency of *Thekopsora areolata* aecia in exposed and bagged cones from Norway spruce seed orchards in Suhola in 2019, Söregärde in 2020 and Vuolenkoski in 2020. Each number on the *x* axis indicates one MLG

were highly polymorphic, with 2–14 alleles per marker identified in the studied populations. The genotype accumulation curve suggested a high resolution of MLGs: 276 MLGs were identified from the 548 samples; the genotype accumulation showed a linear increase for the first few loci, and later a plateau was reached. In a previous study (Capador et al., 2018) using eight polymorphic SSR markers, one MLG was found from different cones from three seed orchards, and another four samples were homozygous at all tested loci (Capador et al., 2020). However, in the present study, individual aecia that shared the same MLG were always from the same cone, that is, no MLGs were found twice from different cones. The four samples that were homozygous for the eight SSR markers in the previous study, were shown to be heterozygous at two to five loci when tested with the new expanded set of SSR markers (data not shown).

Results from aecia samples investigated in this study agree with the previous study by Capador et al. (2020) that the Ålbrunna 2015 population had high genotypic diversity and multiple basidiospore infection in the same cone. The Söregärde 2015 population, which was not included in the previous study, showed a similar level of diversity as well as multiple basidiospore infections. The *T. areolata* populations had low genotypic diversity in 2019 and even lower diversity in 2020 in all seed orchards in this study. In both years, cherry spruce rust incidence in seed orchards was usually low and infected cones were scarce to find as samples. In Finland, the low disease incidence in 2019 was due to a low *Prunus* infection rate on overwintered leaves and poor flowering (Kaitera, Aarnio, Karhu, et al., 2021). In 2020, the weather conditions were unfavourable for the spread of rust, and the flowering of *P. abies* was also poor (authors' personal observations). The lower disease incidence and low genotypic diversity of aecia is most probably a result of infection by a limited number of basidiospores with very few haplotypes. However, there is still a possibility that cones could be infected by many basidiospores but the basidiospores shared fewer haplotypes, such as occurred in Suhola 2020: the amount of cones harvested from the seed orchard was extremely low despite a high infection rate, and *T. areolata* diversity was low. This could be explained by population bottlenecks: according to the life cycle of *T. areolata*, if any biotic or abiotic factor caused a lower infection rate by basidiospores on Norway spruce cones in 2018 or by aeciospores on *Prunus* in 2019, fewer alleles and haplotypes would be inherited by basidiospores in 2020. Thorough monitoring of *T. areolata* haplotype diversity and infection rate on Norway spruce and *Prunus* will help to investigate their association.

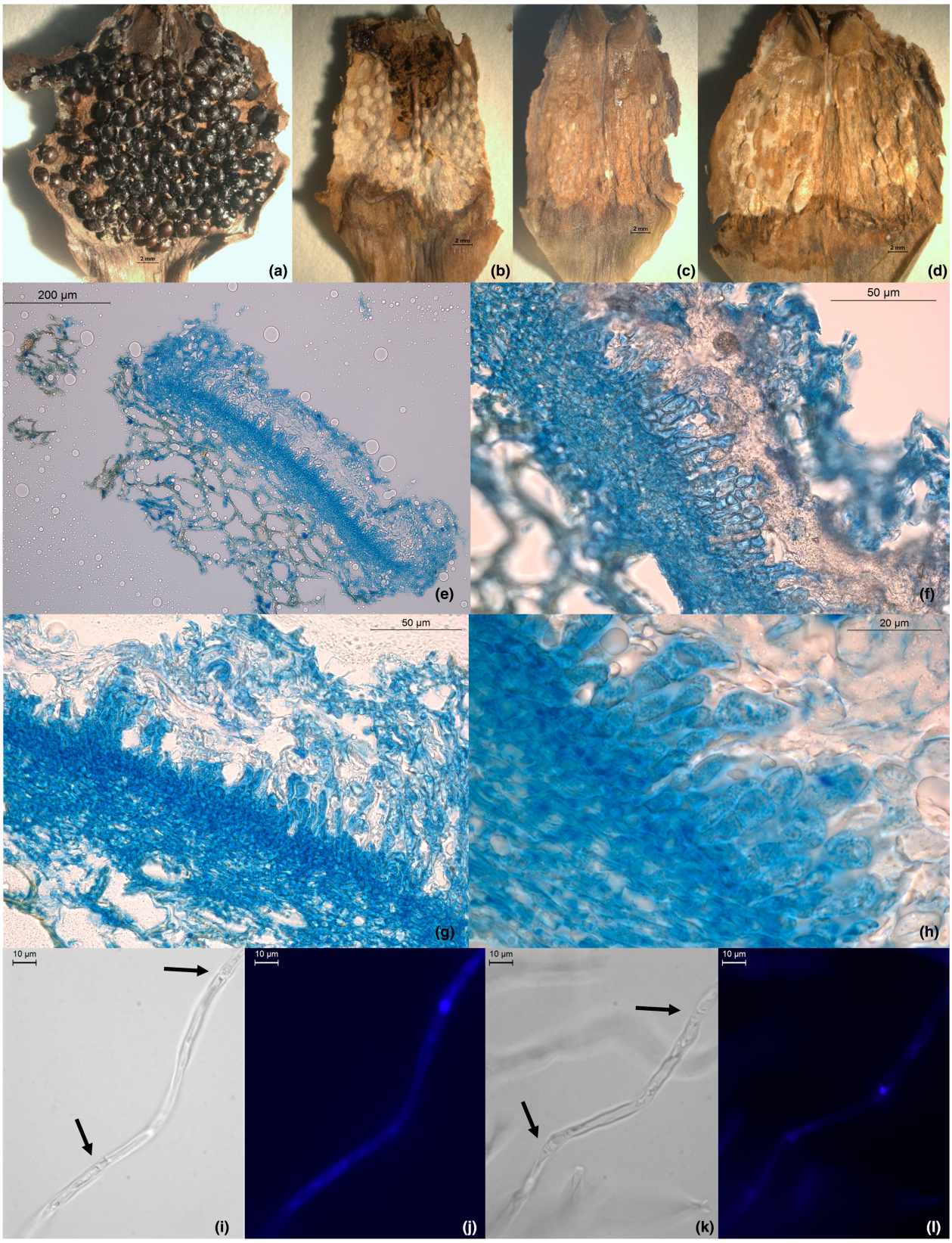

FIGURE 4 Microscopy of cone scales of Norway spruce infected with *Thekopsora areolata* showing normal and abnormal fruiting bodies. (a) Typical infected scale with mature hemispherical aecia. (b–d) Infected scales with abnormal flat fruiting bodies surrounded by white hyphae, from cones collected in Sweden (b) and Finland (c,d), bar = 2 mm. (e–h) Cross-section of abnormal fruiting bodies, stained with lactophenol cotton blue. (i–l), 4′,6-diamidino-2-phenylindole (DAPI) staining of hyphae from aecial primordial structures in bright field and fluorescence, showing only one nucleus in each fungal cell (arrows indicate septa) [Colour figure can be viewed at wileyonlinelibrary.com]

The extremely low genotypic diversity of the Vuolenkoski 2020 population is unique compared to the other studied seed orchards. Only two to four haplotypes were identified for each exposed cone, indicating that each cone was only infected by basidiospores of one or two haplotypes and fertilized with spermatia of one or two haplotypes. One explanation could be the young age of the seed orchard that created a shortage of genetic material and a lack of genetic variation. This was supported by the low observed number of old infected cones in the seed orchard indicating that previous rust epidemics were rare in Vuolenkoski before 2020 (J. Kaitera, unpublished observations). In macrocyclic rust fungi, sexual reproduction is initiated by the plasmogamy of spermatia and receptive hyphae in the aecial host, and sexual recombination of genes occurs during karyogamy and meiosis in teliospores in the telial host (Figueroa et al., 2020; Rodriguez-Algaba et al., 2014). Accordingly, the mating of *T. areolata* occurs in Norway spruce, and the recombination of gene alleles occurs in bird cherry. The Vuolenkoski seed orchard was established in 2014, and due to its young age and long interval of flowering of spruce, only one cycle of mating and recombination of the fungus might have been completed between spruces and bird cherry surrounding the seed orchard.

Many rust fungi produce spermatia in sugary nectar to attract insects, which act as vectors to transfer spermatia, such as in *Puccinia* spp. (Leonard & Szabo, 2005; Naef et al., 2002). *T. areolata* spermatia in fluid have frequently been reported (Kaitera, Aarnio, Karhu, et al., 2021; Murray, 1955) or observed by researchers over time, but the contribution of insects in the mating process has not been confirmed experimentally. Cone bagging has previously been tested to inhibit the *T. areolata* basidiospore infection of spruce cones (Kaitera et al., 2009b). In the present study, cones were bagged before the production of spermogonia, and therefore, these cones were isolated while the other exposed cones were visited by insects. Only a limited number of bagged infected cones were collected in this study, and the genotypic diversity of aecia and aecial primordia from bagged cones was lower than that of aecia from exposed cones in the same seed orchard. In 2019, bagged cones were infected by basidiospores of multiple haplotypes: gravity or shaking of the branch may have caused dripping of spermatia nectar within the bag, enabling compatible haplotypes to mate (Figure S4, case3). In exposed cones from the same seed orchard, cones were usually infected by more basidiospore haplotypes, and subsequently mated with more spermatia haplotypes. This study was not able to prove conclusively that insect vectors contributed to the higher diversity of aecia in exposed cones because the ability of insects to carry *T. areolata* spermatia was not directly tested. However, alien spermatia increase aecia diversity in cones and basidiospore infection in isolated cones results in either no aecia or aecia with reduced diversity, confirming the significance of cross-fertilization in sexual reproduction. In 2020, only aecial primordia of one haplotype were identified from all bagged cones. This indicated that each of these cones was only infected by one basidiospore, or several basidiospores of the same haplotype. Because no spermatia of other haplotypes were available, sexual reproduction never occurred. Therefore, alien spermatia can be critical for production of aecia; whether the spread of spermatia usually occurs via insects (Naef et al., 2002) or other factors such as water splashes (Tadych et al., 2014) requires further study to determine.

T. areolata aecial primordia have never been described in detail although they have been reported earlier (Kaitera et al., 2009a). Infected cones with mature aecia are usually easy to identify in trees because the cone scales open prematurely (Kaitera et al., 2009a; Murray, 1955) and their appearance is already clearly distinguishable from healthy cones by late summer (Kaitera, Aarnio, Karhu, et al., 2021). In this study, the scales of cones with aecial primordia remained closed, similar to healthy cones, but these structures could be found by pulling up the scales of the cone. Thus, aecial primordia are cryptic and hard to locate in exposed cones in seed orchards. For this reason, population genetics statistics were not performed for bagged cones and exposed cones collected in 2020 due to the risk of biased sampling. However, aecial primordia are more likely to be discovered when the cones are isolated in bags, because, unlike exposed cones infected with a single haplotype, they are less likely to receive alien spermatia, possibly by attracting insects with spermatial fluid, which produce mature aecia after sexual reproduction.

The absence of homozygous aecia and the presence of aecial primordia in bagged cones collected in Vuolenkoski and Söregärde in 2020 suggested that *T. areolata* is a heterothallic species and self-fertilization is unlikely to happen. In Pucciniales, the mating system of *Puccinia* spp., *Melampsora* spp. and *Cronartium* spp. are better understood. All these species are heterothallic according to the spermatia transfer experiment (Craigie, 1927) and/or genetic structure of mating-type genes (Duplessis et al., 2011). Their mating processes are controlled by one locus or two unlinked loci (Kües et al., 2011). Future characterization of the gene structure of mating-type loci will provide more information about the mating system of *T. areolata*.

In the previous study by Capador et al. (2020), it was proposed that basidiospore infections were local and vegetative spreading occurred only after dikaryotization/plasmogamy because only a few dominant MLGs were found throughout the cones. In this study, some cones were found to be colonized by several MLGs in different parts of the cones, and these MLGs shared one haplotype (Figure S4, case3). Similar cases were also observed in *Cronatium pini* and *Puccinia* spp.: different MLGs that share the same haplotype were often found within one aecial cluster (Berlin et al., 2017; Samils et al., 2011). It is possible that the cones in our study were infected by several basidiospores' haplotypes in the spring, and these haplotypes were fertilized by spermatia of the same haplotype in early summer. Another possible scenario is that these cones were infected by one basidiospore, after which the monokaryotic hyphae grew vegetatively to colonize the whole cone, and hyphae with the same haplotype were fertilized by different spermatia. Strong evidence for the vegetative growth of monokaryotic hyphae before dikaryotization was that one homogeneous haplotype was found throughout the same bagged cone in 2020.

In conclusion, this research increased our understanding of the infection, colonization, and sexual reproduction of *T. areolata* by population genetics analyses based on 18 polymorphic SSR markers.

In the year with lower disease incidence, cones were infected by fewer fungal haplotypes, that is, fewer basidiospores and/or spermatia, which resulted in lower genotypic diversity of aecia. In the youngest seed orchard, cones were infected by basidiospores of very few haplotypes, probably because of low disease prevalence in the local rust population. Mature aecia in cones isolated in bags had lower diversity than aecia in exposed cones, which suggested that transfer of spermatia by vectors promotes genotypic diversity of the rust fungus. Homogenous aecial primordia of *T. areolata* that occupy the entire cones were reported, and homothallic mating of the same gametes is less likely to occur in *T. areolata*.

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DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

ORCID

Ke Zhang https://orcid.org/0000-0003-4204-1516

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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