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Relationship and genetic structure among autoecious and heteroecious populations of *Cronartium pini* in northern Fennoscandia

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ABSTRACT

Epidemics of Scots pine blister rust, caused by *Cronartium pini*, have become an increasing problem in northern Finland and Sweden. The biology of the rust fungus is complex, with two different life cycle forms that cannot be morphologically distinguished, and it is unclear to what extent the two forms contribute to the epidemics. Genetic structure of fourteen populations of *C. pini* were investigated in Fennoscandia. Distinction between the two life cycle forms, a heteroecious and an autoecious one, was made by determining zygosity using microsatellite markers, and AFLP markers were developed to analyse population genetic relationships. The results showed that the two life cycle forms are clearly differentiated and occur in separate populations. Within the life cycle forms, geographic differentiation was evident, probably due to restricted gene flow as well as connection with different alternating hosts. The host-alternating form dominated in the epidemic regions in northern Fennoscandia. Implications for silvicultural practices are discussed.

1. Introduction

Epidemics of Scots pine blister rust (or resin-top disease), caused by the rust fungus *Cronartium pini* (syn. *Cronartium flaccidum*, *Peridermium pini*) have resulted in serious damage of young Scots pine (*Pinus sylvestris*) forests in northern Finland and Sweden in the last decades (Kaitera 2000; Wulff et al., 2012; Skyttä 2017). Based on the 7th Finnish NFI (National Forest Inventory), 2.55% of mature Scots pines especially in nutrient-rich and dry soils were infected in northern Finland in the early 1980s (Kaitera and Jalkanen 1995). Since then, epidemics have expanded on over 10,000 ha in private and state forests in northern Finland (Skyttä 2017). The epidemics have been severe in western Lapland and expanded southwards to Northern Ostrobothnia. Recently, fresh damage was frequent in northern Finland in 2017 (Nevalainen et al., 2018). An inventory in Sweden in 2008 showed that 130,000 ha, or 34% of young pine forests, were infected in the counties Norrbotten and Västerbotten (Wulff et al., 2012), and *C. pini* was determined as dominant cause of forest damage in the region of Norrbotten in 2019 (Normark 2019). Reasons for the severe epidemics are unknown.

Scots pine blister rust has been known in Sweden and Finland for a long time (Liro 1908; Lagerberg 1912), but usually the incidence of disease is relatively low. Severe epidemics of Scots pine blister rust have periodically caused severe losses on *Pinus* spp. throughout Europe (Diamandis and de Kam 1986; Greig 1987; Kaitera 2000). Besides northern Finland and Sweden, the Scots pine blister rust is present on Scots pine also in middle and southern parts of the countries although in most regions the disease levels are generally low (Ylikojola and Nevalainen 2006). Exceptions are the island Gotland and Åland Islands where the heteroecious form of *C. pini* has been prevalent for many years. In individual pine stands, the incidence of the autoecious form of *C. pini* may occasionally be high also in southern Finland, if the silvicultural

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sanitation practices have been neglected.

There are two forms of *C. pini* that are separated mainly by their different life cycles. The heteroecious form (syn. *C. flaccidum*) is a macrocyclic rust with five spore stages (aeciospores, urediniospores, teliospores, basidiospores and spermatia) that requires an alternate host to complete its life cycle. It alternates between pine (mainly *Pinus sylvestris* and also some other *Pinus* spp.) and herbaceous plants in 14 families within the genera of *Apocynum*, *Asclepias*, *Bartsia*, *Caiophor*a, *Castilleja*, *Delphinium*, *Euphrasia*, *Gentiana*, *Grammatocarpus*, *Hyoscyamus*, *Impatiens*, *Loasa (Nasa)*, *Melampyrum*, *Myrica*, *Nemesia*, *Nicotiana*, *Odontites*, *Paeonia, Papaver, Pedicularis, Rhinanthus, Ruellia, Saxifraga, Schizanthus, Siphonostegia, Swertia, Tropaeolum*, *Verbena*, *Veronica*, and *Vincetoxicum* (Gäumann 1959; Kaitera et al. 1999, 2012, 2015, 2018). In contrast, the autoecious form (syn. *Peridermium pini, Endocronartium pini*) completes its entire lifecycle on pine (mainly *P. sylvestris* and *P. mugo* Turra and also some other *Pinus* spp.), with aeciospores and spermatia being its only known functional spore stages (Olembo 1971; Pei and Brodie 1995; Kaitera 2003; Kaitera and Nuorteva 2008).

The life cycles of *C. pini* are complex and spans several years. The rust fungus is most easily recognized at the aecial stage on pine when lesions with orange blisters (aecia) containing aeciospores develop on a stem or branch of the tree in early summer. The lesions are perennial and can produce aecia for several years and may eventually kill the tree or branch by blocking vascular transport. In the case of the heteroecious form of *C. pini*, the aeciospores infect the alternate host plants and orange pustules (uredinia) are formed on the leaves of the plant. Urediniospores spread among host plants during the summer and the amount of spores and uredinia produced can increase excessively through multiple infection cycles. The uredinia then turn into telia and produce teliospores, from which basidiospores are formed in autumn. The wind-dispersed basidiospores will reinfect pines. On pine, the sexual stage takes place with production of spermogonia and spermatia, and the first aecia are formed 3 y or more after the basidiospore infection (Kaitera and Nuorteva 2008).

The aeciospores of the autoecious form, on the other hand, will infect other pines directly. Aecia are formed 2 y or more after infection (Kaitera 2003). The autoecious form is suggested to lack sexual reproduction and to spread clonally (Hantula et al., 2002; Samils et al., 2011). The contrasting differences in host pathogenicity between the two life cycle forms of *C. pini* have been confirmed by several infection studies where aeciospores of the heteroecious form were shown to infect alternate herbaceous host plants, but never pines (Kaitera et al., 1999; Kaitera, unpublished). In contrast, the aeciospores of the autocecious form were only able to infect pines but none of the alternate host plants (Kaitera et al., 1999; Kaitera 2003; Kaitera and Nuorteva 2008).

The aecial stages of the autoecious and heteroecious forms of *C. pini* on pine are morphologically indistinguishable (van der Kamp 1968), but at the DNA level they can be distinguished by a difference in zygosity. The autoecious form is homozygous at all loci (i.e. both alleles of genes of the dikaryotic aeciospores are always identical), while the heteroecious form shows heterozygosity (i.e. the two alleles of many genes are different) (Hantula et al., 2002; Samils et al., 2011). The two rust forms were earlier considered as two separate species (*C. flaccidum* and *P. pini*/*E. pini*; Hiratsuka 1969) but are now regarded as belonging to the same species, *C. pini* (Roskov et al., 2019), which is also suggested in recent studies where molecular markers revealed only little overall genetic differentiation between the two forms (Hantula et al., 2002; Samils et al., 2011). There are several similar pairs of closely related *Cronartium* taxa with one heteroecious and one autoecious form, e.g. *C. querquum* f. sp. *banksianae*–*C. harknessi* (syn. *Peridermium harknessi*) causing gall rust on two- and three-needled pines, and *C. comandrae*–*C. bethelii* (syn. *P. bethelii*) causing blister rust on lodgepole pine (Vogler and Bruns 1998).

To understand the progress of the current rust epidemics, it is crucial to know the distribution of the two life cycle forms and to which extent they are involved in the spread and epidemic pattern of the disease. In the case of the heteroecious form, distance between the alternate host plants and pine is a limiting factor, since basidiospores produced on telia on the alternate host plants can normally spread only short distances before infecting pine. The aeciospores and urediniospores, on the other hand, can spread up to hundreds of kilometres although both the number of aeciospores and disease rate on the alternate host are highly reduced when the distance from the aeciospore source increases from 10 m to 1000 m (Ragazzi et al., 1998). The differences in genetic composition between the heteroecious and autoecious forms on a fine scale were recently investigated (Samils et al., 2011). However, information on the general population structure was lacking in the previous study. To better understand the dispersal patterns of the Scots pine blister rust, we need information of the genetic composition of the populations of these rust forms on a larger geographic scale.

In this study, we developed AFLP markers to determine population structure and genetic diversity of *C. pini* populations in Finland and Sweden. Although AFLP markers have some limitations such as dominance and uncertain locus homology, they are advantageous since they survey the entire genome and provide high levels of allelic variability rather than selected fragments. Microsatellite markers were used to distinguish between the heteroecious and autoecious life cycle forms by analyzing the zygosity of markers. Our specific aims were to (i) examine genetic variation within and among populations, (ii) determine the distribution of the two rust forms, both within populations and on a geographical scale, and (iii) investigate whether the rust populations in the northern parts of the countries differed genetically from populations in the more southern non-epidemic regions.

2. Materials and methods

Fungal spore collection. Collection of aeciospores was made at 14 locations in Sweden and Finland in 2011, and in Ås, Sweden, in 2014 (Fig. 1). Sampling was done shortly after the rust blisters had appeared in summer (middle May to middle July, depending on the latitude of the location). At each location, 4–54 *C. pini* lesions were distinguished on stems and branches of Scots pine, and from each lesion one single spore blister (aecium) was sampled. The spore blister was picked using sterile forceps and put into a plastic tube either directly in the field, or by cutting first the whole lesion carrying several unopened blisters using branch scissors, transporting the lesion in individual separate bags into the laboratory and removing the aecia in a laminar flow cabin. To avoid spore contamination from neighbouring aecia in the field, unopened blisters were sampled when available. All collections were made in the same forest stand, except at the Swedish site Tjärby, where we merged samples from two forest stands located ca. 3 km apart because of small sample sizes. In the laboratory, aeciospores from each aecium were separated from the rest of the aecial material and preserved in small vials prior to DNA extraction.

DNA extraction. DNA was extracted from aeciospores using a CTAB procedure (Chen et al., 1993) with a modification in the spore homogenization procedure (Pei et al., 1997). For each isolate, approximately 5 mg of spores were shaken twice for 30 s at a speed of 5000 rpm in a FastPrep shaker (Precellys24-Dual, Bertin technologies) in an 1.5 ml Eppendorf tube together with three 3-mm glass beads, twenty 2-mm glass beads and 200 μl of extraction buffer (50 mM Tris-HCl pH 8.0, 150 mM Na Cl, and 100 mM EDTA).

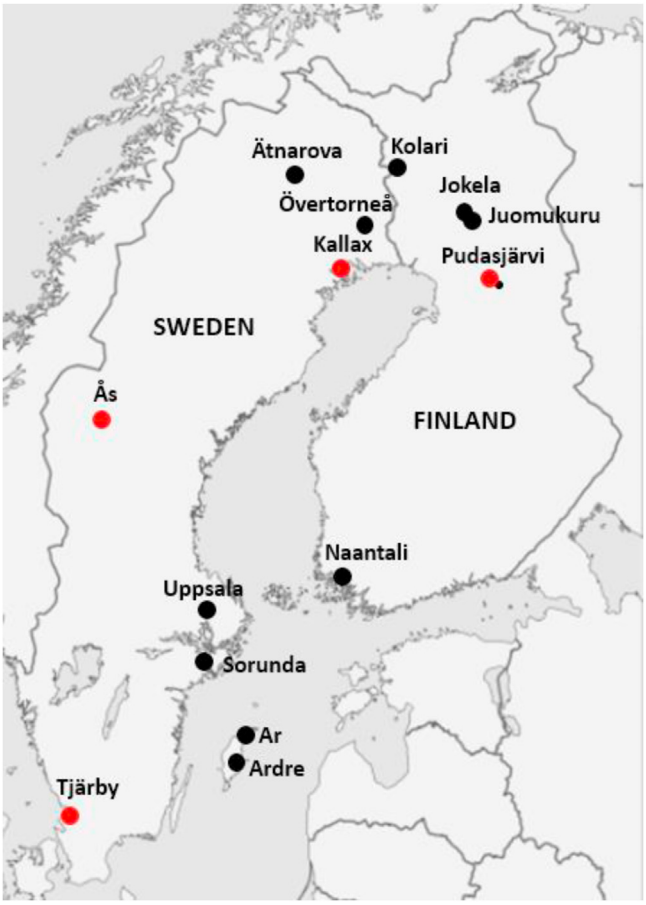

Fig. 1. Map of the sampled locations. Populations of the heteroecious form (black dots) and the autoecious form (red dots) of *Cronartium pini*.

After addition of 12 μl of 20% sodium dodecyl sulphate (SDS) the mixture was gently shaken for 1 h at room temperature, mixed with 30 μl of 5 M NaCl and 26 μl of CTAB/NaCL solution (10% CTAB in 0.7 M NaCl) and kept at 65 °C for 20 min. The mixture was extracted with chloroform/isoamylalcohol (24:1). The top aqueous phase was transferred to a clean tube and 0.6 vol (approx. 150 μl) of ice cold isopropanol was added. After 20 min of incubation at −20 °C the solution was centrifuged for 10 min at 9500×*g* to precipitate the nucleic acid. The pellet was rinsed twice with cold 70% ethanol, dried, and dissolved in 100 μl of $TE_{0.1}$ (10 mM Tris-HCl, 0.1 mM EDTA). DNA concentrations were measured with a Nanodrop spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). Before AFLP analysis, DNA was purified with E.Z.N.A. Cycle Pure Kit (PeqLab Biotechnologie GmbH, Erlangen, Germany).

Microsatellite markers. The samples were genotyped with 7 microsatellite (or microsatellite-like) markers (Pp1, Pp2, CqfSI_AAC27, CqfSI_AAC30, CqfSI_AAG13, CqfSI_AAG18, CqfSI_-GATA06) previously described by Samils et al. (2011). Approximately 1 ng μl^{-1} template DNA was added to a solution of 0.2 mM dNTP-mix, 0.025 U μl^{-1} DreamTaq Green DNA polymerase with the supplied buffer (Thermo Fisher Scientific Inc.), 0.2 μM of each forward primer either labelled with HEX or with FAM and 0.2 μM of the reverse primer and a final concentration of 2.75 mM of $MgCl_2$. PCR was performed using 2720 Thermal cycler and Veriti Thermal cycler (Applied Biosystems, Life Technologies Corporation, Carlsbad, CA, USA). An initial denaturation step at 94 °C for 5 min was followed by 35 amplification cycles of denaturation at 94 °C for 30 s, annealing at 47–62 °C for 30 s and extension at 72 °C for 30s. The thermal cycling was ended by a final extension step at 72 °C for 7 min.

Length of SSR amplicons were determined using ABI 3730XL DNA Analyzer (Uppsala Genome Center at Rudbeck laboratory, Uppsala University).

AFLP markers. AFLP reactions were performed principally as described in the protocol from the Perkin-Elmer/Applied Biosystems AFLP™ plant mapping kit for small genomes. It is based on the method of Vos et al. (1995) but uses non-radioactive fluorescent dyes to label the primers. Sequences of adaptors and primers used in this study are listed in Table 1.

Restriction of DNA and ligation of adaptors: Double-stranded adaptors were made by mixing the following in two Eppendorf tubes: 1) *Eco*RI adaptor mix (1 μl per reaction): 0.05 μl of 10 × OnePhorAll buffer (Pharmacia Biotechnology Inc.), 0.28 μl of adaptor E-ad1 at 100 ng/μl, 0.25 μl of adaptor E-ad2 at 100 ng/μl, and 0.42 μl of water. 2) *Mse*I adaptor mix (1 μl per reaction): 0.05 μl of 10 × OnePhorAll buffer, 0.52 μl of adaptor M-ad1 at 500 ng/μl, and 0.47 μl of adaptor M-ad2 at 500 ng/μl. The tubes were kept in 95 °C waterbath for 5 min, then cooled slowly to room temperature (about 20 min). A restriction-ligation mix (6.5 μl per reaction) was made combining the following: 1 × ligation buffer (Thermo Fisher Scientific Inc.), 0.05 M NaCl, 0.05 mg/ml BSA, 1 mM ATP, 0.7 μl of *Eco*RI adaptor mix, 0.7 μl of *Mse*I adaptor mix, and 0.7 μl of Enzyme master mix (1 × ligation buffer, 0.05 M NaCl, 0.05 mg/ml BSA, 1 U of *Tru1I* (*Mse*I isoschizomer; Thermo Fisher Scientific Inc.), 5 U of *Eco*RI (Thermo Fisher Scientific Inc.), and 1 U of T4 DNA ligase (Thermo Fisher Scientific Inc.). Restriction of DNA and ligation of adaptors were performed in the same reaction by mixing 3.4 μl of DNA extract (10 ng/μl), and 4.5 μl of restriction-ligation mix in an Eppendorf tube, which was incubated at room temperature overnight, or at 37 °C for two hours, and thereafter diluted with 132 μl $TE_{0.1}$.

Preselective amplification: Preselective PCR amplification was performed in a volume of 20 μl combining the following: 1 × DreamTaq Buffer 1 (ThermoFisher Scientific Inc.), 1.0 U of DreamTaq Green polymerase (Thermo Fisher Scientific Inc.), 0.2 mM of each dNTPs, 0.44 μM primer E-00, 0.4 μM primer M-00, and 4 μl of DNA solution from the restriction and ligation reaction. The thermocycle programme in the preselective PCR was 2 min at 94 °C; 32 cycles of 30 s at 94 °C, 1 min at 56 °C, 1 min at 72 °C; and finally 5 min at 72 °C. The amplification product was diluted 1:20 with $TE_{0.1}$.

Selective amplification: Selective PCR amplification was performed in a volume of 20 μl combining the following: 1 × DreamTaq Buffer, 1.0 U of DreamTaq Green polymerase, 0.2 mM of each dNTPs, 0.05 μM *Eco*RI selective primer (E-primer), 0.25 μM *Mse*I selective primer (M-primer), and 5 μl of diluted preselective amplification product. Eight different primer combinations of ECoR1 and MseI selective primers were used (Table 1). The thermocycle programme in the selective PCR was 2 min at 94 °C; 11 cycles of 30 s at 94 °C, 30 s at 65 °C (minus 0.7 °C per cycle), 2 min at 72 °C; then 23 cycles of 30 s at 94 °C, 30 s at 56 °C, 2 min at 72 °C; and finally 5 min at 72 °C. Length of AFLP amplicons were determined using ABI 3730XL DNA Analyzer (Uppsala Genome Center at Rudbeck laboratory, Uppsala University).

Data analyses. Distinction between the two forms of the Scots pine blister rust fungus, as well as measures of genotypic diversity, was based on the seven microsatellite markers. The lengths of the microsatellite amplicons were scored using the computer software GeneMarker (Softgenetics LLC, State Collage, PA, USA). The two alleles of the dikaryotic aeciospores were classified as the sizes of the amplified fragments. Samples with only one fragment were regarded as homozygous for the locus. Samples with all loci being

Table 1
Sequences of AFLP adaptors and primers used in this study.

Oligo name		Oligo sequence	Number of markers
EcoR1 adaptors	E-ad1	5′-CTC GTA GAC TGC GTA CC-3′	
	E-ad2	5′-AAT TGG TAC GCA GTC-3′	
MseI adaptors	M-ad1	5′-GAC GAT GAG TCC TGA G-3′	
	M-ad2	5′-TAC TCA GGA CTC AT-3′	
EcoR1 preselective primer	E-00	5′-AGA CTG CGT ACC AAT TC-3′	
MseI preselective primer	M-00	5′-GAT GAG TCC TGA GTA A-3′	
Selective EcoR1 and MseI primer pairs:			
1)	E-CC	5′AGA CTG CGT ACC AAT TC**C C**-3′ FAM*	5
	M-CA	5′-GAT GAG TCC TGA GTA A**CA** -3′	
2)	E-GT	5′AGA CTG CGT ACC AAT TC**G T**-3′ FAM*	6
	M-AC	5′-GAT GAG TCC TGA GTA A**AC** -3′	
3)	E-GT	5′AGA CTG CGT ACC AAT TC**G T**-3′ FAM*	5
	M-CA	5′-GAT GAG TCC TGA GTA A**CA** -3′	
4)	E-TG	5′AGA CTG CGT ACC AAT TC**T G**-3′ FAM*	6
	M-CC	5′-GAT GAG TCC TGA GTA A**CC** -3′	
5)	E-TG	5′AGA CTG CGT ACC AAT TC**T G**-3′ FAM*	9
	M-TT	5′-GAT GAG TCC TGA GTA A**TT** -3′	
6)	E-CA	5′AGA CTG CGT ACC AAT TC**C A**-3′ HEX*	7
	M-CA	5′-GAT GAG TCC TGA GTA A**CA** -3′	
7)	E-TA	5′- AGA CTG CGT ACC AAT TC**T A**-3′ HEX*	9
	M-AC	5′-GAT GAG TCC TGA GTA A**AC** -3′	
8)	E-TA	5′- AGA CTG CGT ACC AAT TC**T A**-3′ HEX*	11
	M-TT	5′-GAT GAG TCC TGA GTA A**TT** -3′	

homozygous were classified as the autoecious life cycle form, while samples with one or more heterozygous loci were classified as the heteroecious form (Samils et al., 2011).

All calculations on gene diversity and population genetic structure was performed using the AFLP data. The computer software GeneMarker (Softgenetics) was used to visualize and score the presence of amplicons of different sizes. Creating a set of markers was done manually by selecting amplicons with clearly separated size ranges and overall high signals that could be unambigously scored for all samples. The presence of an amplicon (marker allele) in a sample was denoted as 1 and absence (null allele) as 0, resulting in a binary data matrix of the different AFLP multilocus phenotypes which was used as input data in the genetic analyses described below. Clone-corrected data was used (i.e. only one individual per population of identical multilocus genotypes was retained in the data set; applicable only to the clonally reproducing populations).

The standardized index of association (rBarD) was calculated using Multilocus 1.3 b (Agapow and Burt 2001) in order to test whether populations are randomly mating or if linkage disequlibrium exist among loci. Calculation of unbiased expected heterozygosity as well as Analysis of Molecular Variance (AMOVA) and Pairwise population differentiation (PhiPT) were carried out using the software Genalex 6.502 (Peakall and Smouse, 2006, 2012). To visualize the patterns of genetic clustering of fungal individuals and populations, we performed Principal Coordinates Analysis (PCoA) based on genetic distances (GD) in GenAlEx 6.502 (Peakall and Smouse, 2006, 2012). To investigate further groupings of the fungal individuals, we used the program Structure 2.3.4 that uses a Bayesian approach to assign individuals into groups (clusters) based on genetic similarity (Pritchard et al., 2000). Structure was run by varying the number of clusters (K) from 1 to 10. The admixture model assuming no linkage between the loci and without a priori information on populations was applied. For each K, we made 10 repeated simulations with a burn-in period of 500,000 iterations of the Markov Monte Carlo Chain (MCMC) and a run length of 1,000,000 MCMC iterations. Results were compiled, and bar plots constructed, using Structure Harvester (Earl and von Holdt 2012). Microsoft Excel was used to create pie charts of cluster membership for each population.

3. Results

Genotypic diversity and distribution of the heteroecious and the autoecious forms of *C. pini*. The seven microsatellite markers could identify 2 to 33 different alleles per locus (in total 93 alleles) over the entire dataset. Only samples without missing data were used in the analyses. Out of 196 samples, 48 samples were homozygous for all seven loci and thus designated as the autoecious form, while 122 samples were heterozygous for one or more loci and thus designated as the heteroecious form (Table 2). All samples in each population were either heterozygotic or homozygotic, i.e. they belonged to either the heteroecious or autoecious form. The only exception was Pudasjärvi where all but one sample belonged to the autoecious form. The geographic distribution of the two forms are shown in Fig. 1. All heteroecious samples had unique multilocus genotypes (MLGs), while three of the autoecious populations (Kallax, Ås and Pudasjärvi) had 2, 6 and 19 identical MLGs, respectively (Table 2).

Population relationships. The eight AFLP primer combinations generated 64 polymorphic markers across 195 individuals. Only markers with a minor allele frequency >5% and samples without missing data were retained in the dataset. It should be noted that the single heteroecious sample in Pudasjärvi was omitted from the population genetic analyses because of missing data in the AFLP data set. The number of markers produced by the different primer combinations is listed in Table 1. There were no private bands (unique to a population) in any of the populations (Table 3). When comparing samples belonging to the autoecious and heteroecious forms, there were only two cases (out of 64 markers) where a band was absent in the autoecious form, while present in the heteroecious, with band frequencies of 0.168 and 0.232 (data not shown). The values of unbiased expected heterozygosity (uHe) ranged from 0.140 (Tjärby) to 0.248 (Övertorneå) (Table 3). The standardized index of association (RBarD) was significantly different from zero for all the four autoecious populations (Kallax, Pudasjärvi, Ås and Tjärby) and for four of the heteroecious populations (Ätnarova, Uppsala, Ar and Jokela) (Table 3) and thus, the hypothesis of random mating and no linkage between markers in these populations was rejected.

Analysis of molecular variance (AMOVA) revealed that 21% of

Table 2
Genotypic diversity and life cycle forms in *Cronartium pini* populations based on microsatellite marker analysis.

Population	# aecia	# unique MLGs[a]	# heterozygotic aecia[b] (heteroecious form)	# homozygotic aecia[c] (autoecious form)
Sweden				
Ätnarova	28	28	28	–
Övertorneå	12	12	12	–
Kallax	5	2	–	5
Ås	20	6	–	20
Uppsala	8	8	8	–
Sorunda	13	13	13	–
Ar	5	5	5	–
Ardre	6	6	6	–
Tjärby	5	5	–	5
Finland				
Kolari	21	21	21	–
Jokela	18	18	18	–
Juomukuru	6	6	6	–
Pudasjärvi	46	19	1	18
Naantali	3	3	3	–
Total	**196**	**152**	**122**	**48**

[a] MLGs = multilocus genotypes.
[b] Heterozygotic aecia, implying the heteroecious form of *C. pini*.
[c] Homozygotic aecia, implying the autoecious form of *C. pini*.

Table 3
Genetic diversity statistics of *Cronartium pini* populations based on AFLP data.

Population	N	No. bands	No. Private bands	uHe	rBarD	P (rBarD)
Sweden						
Ätnarova	35	59	0	0.235	0.010	0.001
Övertorneå	11	61	0	0.248	0.005	0.186
Kallax	9	47	0	0.172	0.273	<0.001
Ås	21	51	0	0.207	0.093	<0.001
Uppsala	10	43	0	0.186	0.045	<0.001
Sorunda	9	46	0	0.182	0.018	0.063
Ar	6	53	0	0.183	0.617	<0.001
Ardre	11	58	0	0.238	0.009	0.058
Tjärby	6	42	0	0.140	0.230	<0.001
Finland						
Kolari	19	57	0	0.221	0.004	0.149
Jokela	15	51	0	0.224	0.048	<0.001
Juomukuru	6	49	0	0.157	−0.003	0.561
Pudasjärvi	34	57	0	0.242	0.068	<0.001
Naantali	3	42	0	0.151	0.060	0.138
All populations	182	64	–	0.293	0.017	<0.001

rBarD = Standardized index of association.
uHe = Unbiased expected heterozygosity.

Table 4
Summary of Analysis of Molecular Variance (AMOVA) for the 14 *Cronartium pini* populations using amplified length polymorphism (AFLP) markers and clone corrected samples.

Source of variation		d.f.	Sum of squares	Variance components	Percentage of variation	p[a]
Two levels	Among populations	13	391.3	1.84	21	<0.001
	Within populations	168	1179	7.02	79	
Three levels	Between fungal forms[b]	1	129.1	1.27	13	<0.001
	Among populations within forms	12	262.2	1.23	13	<0.001
	Within populations	168	1180	7.02	74	<0.001
Only heteroecious form	Among populations	9	156.2	17.35	12	<0.001
	Within populations	115	773.0	6.72	88	
Only autoecious form	Among populations	3	106.0	35.33	22	<0.001
	Within populations	53	407.0	7.68	78	

[a] Levels of significance based on 999 permutations.
[b] The heteroecious and autoecious forms of *Cronartium pini*.

the variation was attributed to differences between populations and 79% to differences within populations in a two-level analysis (Table 4). When also variation between life cycle forms were included in a three-level AMOVA, the analysis showed that 13% of the variation was attributed to variation between the two forms and 13% of the variation among populations within the forms (Table 4). When AMOVA was done on the two lifecycle forms separately, differences among populations amounted to 12% within

the heteroecious form and 22% within the autoecious form (Table 4). Pairwise comparisons of all *C. pini* populations showed only little differentiation between northern heteroecious populations (PhiPT = 0.000–0.084) while the southern heteroecious populations were in some cases more differentiated (PhiPT = 0.071–0.238) and especially the Ar population showed higher levels of differentiation (Table 5). Pairwise comparison of northern and southern heteroecious populations also showed varying levels of differentiation (PhiPT = 0.091–0.283). The highest levels of pairwise differentiation were found between populations of the different life cycle forms (PhiPT = 0.109–0.448). Among the autoecious populations, Pudasjärvi was least differentiated to heteroecious populations (PhiPT = 0.109–0.213). Varying levels of pairwise differentiation were found between autoecious populations (PhiPT = 0.130–0.282).

In the Principal component analysis (PCoA) of individual samples, all heteroecious samples (from both northern and southern populations) were grouped together (Fig. 2). Samples from the autoecious populations (Kallax, Ås, Pudasjärvi and Tjärby) were more dispersed although a loose clustering of these populations could be distinguished. When PCoA was made on populations, the five northern and the five southern heteroecious populations formed two separate clusters, while the four autoecious populations were dispersed (Fig. 3).

Bayesian analysis as implemented in the software Structure was used to further investigate genetic groupings of the fungal individuals. The number of clusters were inferred from the slope of the LnP(D) curve and ΔK, and six clusters (K = 6) was concluded most adequate. Results on the proportion of the six clusters in individual samples are shown in the bar plot in Fig. 4A, and pie charts showing proportions of the clusters in each population are presented in Fig. 4B. The five northern heteroecious populations in Finland and Sweden (Ätnarova, Övertorneå, Kolari, Jokela and Juomukuru) showed a high similarity (with high proportions of the green, yellow and blue cluster; Fig. 4B). The three heteroecious populations in southern Finland and central Sweden (Naantali, Uppsala and Sorunda) were also similar (dominated by the purple cluster). The two heteroecious populations on the island Gotland (Ar and Ardre) appeared intermediate between the middle and northern heteroecious groups. The four autoecious populations differed from the heteroecious populations (by having a high proportion of the red and orange clusters) but also showed some dissimilarities among themselves.

4. Discussion

This study showed that the heteroecious and autoecious life cycle forms of *C. pini* usually exist at different locations, in separate populations. However, although the two forms occurred separated on a local scale, there is no obvious geographic separation of the two life cycle forms on a larger scale in Fennoscandia. Genetic analyses indicated a moderate differentiation (13%) between the heteroecious and autoecious forms, which was in the same range as the overall variation among *C. pini* populations. Most AFLP bands (62 out of 64) were present in both of the two forms and the overall result agrees with the classification of the two life-cycle forms as belonging to the same species, *C. pini*.

The epidemic area in northern Sweden and Finland was clearly dominated by the host-alternating, heteroecious form. These populations were very similar and genetically close to each other as shown by both PCoA and Structure analysis. The southern heteroecious populations were also relatively similar to one another but distinct from the northern ones. The geographical distance between southern and northern heteroecious populations is probably one reason for the differentiation. The Scots pine blister rust infections are sparse in large parts of the middle and southern parts of the countries which will result in discontinuous populations and restricted gene flow. Hamelin et al. (2000) showed a strong differentiation between eastern and western populations of *Cronartium ribicola* in North America, which was suggested to be caused by the Great Plains being a barrier to gene flow because of the lack of pine hosts.

The differentiation between southern and northern heteroecious populations is probably also due to environmental differences and local adaptation and dissemination pattern of the heteroecious populations. The northern populations have been promoted by silvicultural practices, where Scots pine have been frequently cultivated on nutrient-rich soils that are normally forested with Norway spruce. Nutrient-rich soils bear high frequencies of *Melampyrum sylvaticum* which is highly susceptible to the heteroecious form of *C. pini* and considered as the main alternate host in the North (Kaitera and Hantula 1998). Therefore, the northern heteroecious populations have acclimatized to spread efficiently via *M. sylvaticum* during the past decades.

Similarly, the southern heteroecious populations have spread commonly via *Vincetoxicum hirundinaria* Medicus at least for a century (Liro 1908; Kaitera et al., 2005), being acclimatized locally to reproduce on this alternate host. In Sweden and Finland, *V. hirundinaria* is restricted mainly to coastal regions of southern Finland and eastern and southern parts of Sweden, including the island Gotland. Geographic isolation and local adaptation probably also explains why the two populations on Gotland differed slightly from the rest of the heteroecious populations. It should be noted, however, that both northern heteroecious populations (eg.

Table 5
Pairwise PhiPT values between the studied *Cronartium pini* populations.

	Ätnarova	Kolari	Jokela	Juomukuru	Övertorneå	Naantali	Uppsala	Sorunda	Ar	Ardre	Kallax	Pudasjärvi	Ås	Tjärby
Ätnarova	–	0.009	0.000	0.184	0.025	0.001	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
Kolari	0.027	–	0.019	0.443	0.003	0.008	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
Jokela	0.059	0.044	–	0.321	0.005	0.010	0.000	0.001	0.000	0.000	0.000	0.000	0.000	0.000
Juomukuru	0.021	0.000	0.015	–	0.277	0.011	0.004	0.026	0.002	0.014	0.002	0.024	0.000	0.002
Övertorneå	0.032	0.064	0.084	0.019	–	0.003	0.000	0.000	0.000	0.001	0.000	0.000	0.000	0.000
Naantali	0.225	0.167	0.201	0.171	0.228	–	0.021	0.025	0.023	0.009	0.000	0.023	0.000	0.010
Uppsala	0.165	0.198	0.202	0.133	0.174	0.193	–	0.029	0.000	0.002	0.000	0.000	0.000	0.000
Sorunda	0.107	0.126	0.118	0.093	0.158	0.163	0.071	–	0.000	0.007	0.000	0.000	0.000	0.000
Ar	0.249	0.242	0.283	0.243	0.168	0.232	0.274	0.238	–	0.048	0.001	0.001	0.000	0.003
Ardre	0.164	0.138	0.165	0.091	0.092	0.131	0.100	0.087	0.071	–	0.000	0.000	0.000	0.000
Kallax	0.332	0.327	0.324	0.300	0.288	0.367	0.320	0.346	0.370	0.284	–	0.002	0.011	0.000
Pudasjärvi	0.182	0.143	0.169	0.109	0.167	0.165	0.195	0.161	0.213	0.171	0.189	–	0.000	0.014
Ås	0.375	0.362	0.332	0.286	0.313	0.323	0.306	0.334	0.312	0.275	0.168	0.244	–	0.000
Tjärby	0.319	0.321	0.356	0.322	0.293	0.448	0.398	0.392	0.403	0.319	0.282	0.130	0.277	–

Below diagonal are pairwise PhiPT values. Above diagonal are significance values based on 9999 permutations.

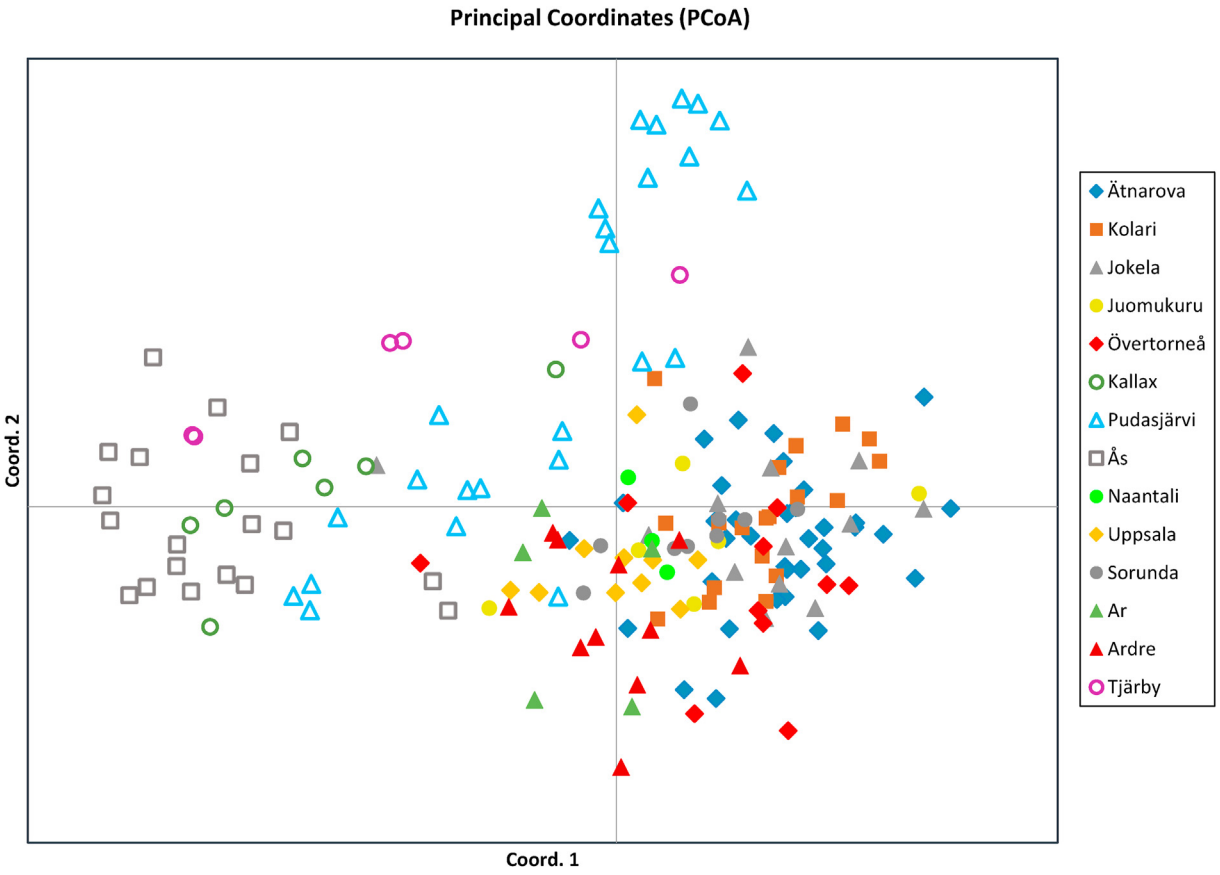

Fig. 2. Principal component analysis to visualize the pattern of genetic clustering among all individuals of *Cronartium pini* sampled at 14 locations in Finland and Sweden. Heteroecious individuals are designated by filled symbols and autoecious individuals by empty symbols.

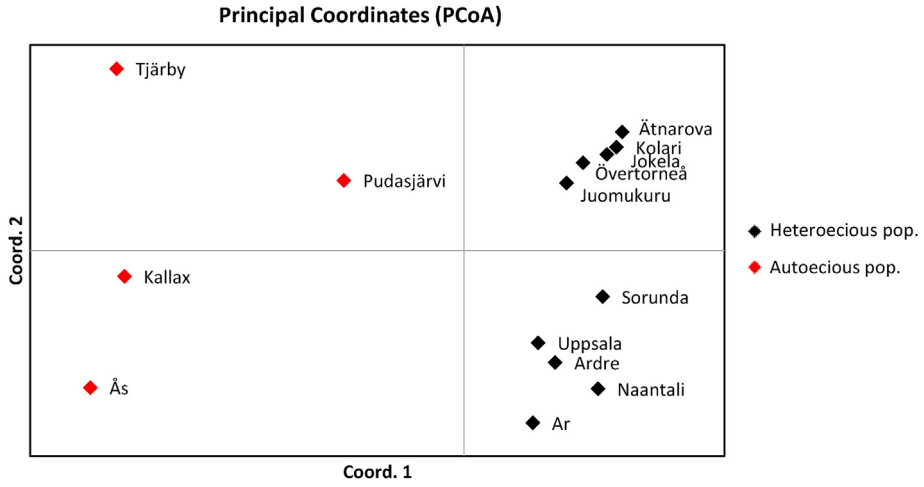

Fig. 3. Principal component analysis to visualize the pattern of genetic clustering among 14 populations of *Cronartium pini* in Finland and Sweden.

Övertorneå, Kolari and Juomukuru) and southern (eg. Naantali, Gotland, Uppsala, Sorunda and Tjärby) have wide, and to a large extent overlapping alternate host-ranges, as shown by artificial inoculations (Kaitera et al. 2012, 2015, 2018) as well as examination of natural infections in the field (Kaitera and Hantula 1998; Kaitera et al. 2005, 2017a, 2017b, 2018). Therefore, host-specificity by itself does not explain differences between the 5 northern and the 5 southern heteroecious *C. pini* populations in this study. Probably, it is rather the presence and distribution of habitats where pine and alternate host plants grow together in different regions that will lead to separated populations.

The autoecious *C. pini* populations in this study were highly differentiated among themselves, with AMOVA showing 22% variation among populations, as compared to 12% variation among the heteroecious populations. This is probably due to the dissemination pattern of rust aeciospores when infected trees are highly concentrated close to the primary spore source and most aeciospores are spread only short distance (e.g. van der Kamp 1968;

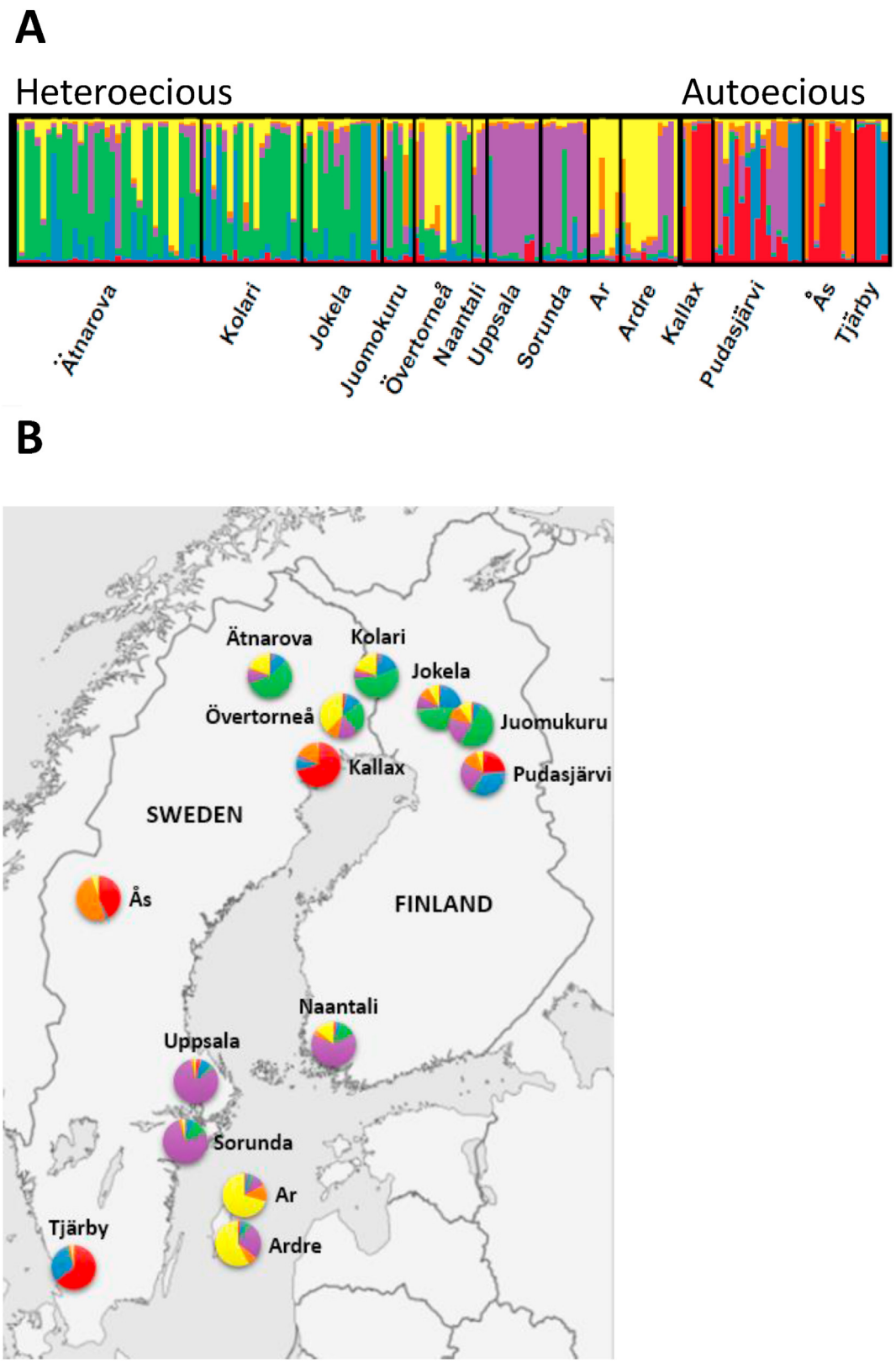

Fig. 4. Distribution of the six AFLP-based genetic clusters (K = 6) inferred by Structure 2.3.4 among 14 locations of *Cronartium pini* sampled in Finland and Sweden. (A) Bar plot showing the assignment of heteroecious and autoecious individuals to the six clusters. (B) Pie charts showing the frequencies of the six clusters in the *C. pini* populations.

Ragazzi et al., 1998). Lacking the ability to exploit an alternate host for spore multiplication and dissemination will certainly limit the dispersal capacity of the autoecious form. Moreover, the exclusively asexual reproduction mode of the autoecious form of *C. pini* will rule out sexual recombination as a way of genetic exchange and contribute to genetic isolation and differentation of autoecious populations. The benefits for autoecious *C. pini* in evolutionary terms will be that adapted genotypes can reproduce clonally and persist for unlimited time and, further, the fungus can survive and reproduce also in environments where alternate hosts are not available.

The heteroecious populations of *C. pini* showed high genotypic diversity, with all multilocus genotypes being unique, which is expected in a sexually reproducing rust fungus where aecia are formed after genetic recombination in meiosis and mating. In the autoecious populations, several lesions had identical multilocus genotypes, which agrees with clonal reproduction. Nevertheless, a relatively high genotypic diversity was unexpectedly found also in the autoecious populations, with many different multilocus genotypes present at the same site. The high genotypic diversity of the autoecious *C. pini* populations can be explained by plentiful infections in certain years, so called "wave years", when infection conditions are favourable in an area. Recently in 2017, the autoecious form was found to sporulate heavily on young Scots pines on dry soils in the Pudasjärvi area (Nevalainen et al., 2018, Kaitera pers. comm.), but in the following year, sporulation was very low (Kaitera pers. comm.). Due to several years' long life-cycle on the pine host, aecial sporulation of *C. pini* can be frequent, starting 2 y or more after infection for the autoecious form and lasting up to 8 y in single lesions (Kaitera 2003; Kaitera and Nuorteva 2008). The high diversity of the autoecious Pudasjärvi population may be explained by the long epidemical history of the rust in the area: the epidemics have continued on dry sites (where alternate host plants are rare or absent) for decades with continuous epidemical waves in the damage pattern in certain years. As also heteroecious populations occur in the same area on nutrient-rich sites where alternate hosts are frequent (Kaitera pers. comm.), mixed populations with both rust forms may occur especially at the edges of dry soils. This is probably the reason, why one heterozygotic spore sample was found in the Pudasjärvi population.

Non-random mating was indicated in the autoecious *C. pini* populations, which is normal in asexually reproducing populations. Unexpectedly, non-random mating was also indicated in some of the heteroecious populations. This may be explained by concentration of basidiospore dissemination from *V. hirundinaria*, *M. sylvaticum* or some other alternate host in specific wave years after good aeciospore dispersal from pines. In some years the frequency of *Melampyrum* is very high due to optimal growing and dormancy periods, which may add disease spread to pines in these years. The latest year of high *Melampyrum* frequency in the north was 2018 (Kaitera and Hiltunen pers. comm.) during which the temperature sum exceeded highest measured level over the past 60 y in Finland. Silvicultural practices also affect *Melampyrum* frequencies, whereas *M. sylvaticum* enriches in young Scots pine forests in nutrient-rich soils shortly after final-cutting (Tonteri et al., 2005). Hot periods during the growing period in some years may reduce aeciospore germination and uredinia and telia development on *V. hirundinaria,* resulting in inefficient disease spread to pines.

Another explanation for non-random mating might be that the autoecious form develops or mutates continuously from the heteroecious form: these non-random matings of the heteroecious form might be on the way to develop from the heteroecious to the autoecious rust form. The first steps in this life-cycle mutation would be non-random mating, or selfing, and losing of pathogenicity on alternate hosts. This could be an explanation of how autoecious populations originally develop from heteroecious populations. This hypothesis would agree with the observed homozygosity of the autoecious aeciospores, since repeated selfing will lead to homozygosity. An alternate hypothesis to explain the link between the two life cycle forms is that the autoecious form has its origin as a haploid mutant of the heteroecious form, as proposed by Vogler et al. (1997) for *C. querquum/P. harknessi* and Kasanen et al. (2000) for *C. pini*. Such a haploidization event may occur repeatedly.

We conclude that the host-alternating form of *C. pini* was dominating on Scots pine in the epidemic areas in Northern Sweden and Finland as well as in the heavily infected stands on the island Gotland in Sweden. The presence of alternate hosts in the vicinity of pine stands is thus an important factor in the Scots pine blister rust epidemics and habitats where an alternate host is common involving a high risk for infections. When regenerating Scots pine in the North it should be recommended to avoid nutrient rich sites where *M. sylvaticum*, the main alternate host, is common. *Cronartium pini* is a quarantine species in North America. Therefore, special attention should be paid by the international plant trade to avoid transporting of the rust from Europe to North America. In case *C. pini* were introduced to North America, the rust would potentially spread via hemiparasitic susceptible species like *Castilleja*, *Euphrasia*, *Rhinanthus* and *Pedicularis*. The connection between the two life cycle forms of *C. pini* is still not clear. Further research is needed to elucidate the processes and conditions for the transformation between the heteroecious and the autoecious form.

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