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Differences in population structure and zygosity between heteroecious and autoecious forms of *Cronartium pini* suggest selfing in the autoecious form

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ABSTRACT

Cronartium pini causes Scots pine blister rust. This rust fungus has two different forms without differentiation in morphology and internal transcribed spacer: the heteroecious form has a macrocyclic life cycle and infects pine and an alternate host; the autoecious form only infects pine. Epidemics caused by these two forms impose severe risk on the pine forest in Sweden, therefore knowledge of their distribution and diversity is needed for strategic disease management. We designed microsatellite markers with improved resolution based on the *C. pini* genome, developed a multiplex amplification system, and analyzed the *C. pini* population diversity and structure in Sweden using 396 isolates. The heteroecious and autoecious populations showed clear differences in diversity, linkage disequilibrium, and structure. The heteroecious isolates had unique multilocus genotypes. Autoecious isolates shared the same genotypes more frequently, especially three autoecious multilocus genotypes that were commonly found over a in northern Sweden. The genetic distances among autoecious isolates are closer than those among the heteroecious isolates. The results confirmed that heteroecious *C. pini* populations were sexual and autoecious *C. pini* populations were clonal. We further discussed the hypothesis that autoecious *C. pini* originated from self-fertilization, and frequent self-fertilization and infrequent mutation generate homozygous but diverse genotypes.

1. Introduction

Cronartium pini is a rust fungus that infects two-needle pines such as Scots pine (*Pinus sylvestris*) (Kaitera and Nuorteva, 2008) and causes Scots pine blister rust (SPBR). The infection starts from young pine needles and continues to grow towards the trunk. Symptomatic trees have swollen and deformed branches and trunks with aecia-bearing lesions. Infected trees have reduced radial stem increment (Martinsson and Nilsson, 1987) and increased resin accumulation (Kaitera et al., 2021), so the timber value is reduced. Severe *C. pini* infection can girdle the stem and cause death at the top or the entire tree (Samils and Stenlid, 2022). Scots pine accounts for 39.8 % of the standing volume in Swedish forests (SLU National Forest Inventory, 2023), therefore recent SPBR epidemics impose severe economic and ecological risks to the production and natural forest in Sweden and other Nordic countries.

Cronartium pini has two different forms. The life cycle of the heteroecious form (synonym *Cronartium flaccidum*) is macrocyclic: haploid basidiospores infect Scots pine. After one to two years, the mating structures, spermogonia, with haploid spermatia are produced (Moriondo, 1980). The spermatia can mate with compatible receptive hyphae to produce aecia with dikaryotic aeciospores, which infect alternate hosts/telial hosts. The heteroecious *C. pini* has many alternate host species, among which *Melampyrum* spp. Have the major roles in disease epidemics in northern Fennoscandia (Kaitera and Nuorteva, 2010; Kaitera and Hantula, 1998). Infected alternate hosts produce uredinia with dikaryotic urediniospores that can re-infect the alternate host. After one to two weeks, telia with dikaryotic teliospores are produced from the same area as uredinia (Ragazzi, 1983). Haploid basidiospores are produced after meiosis in the teliospores and they infect pine needles. The life cycle of the autoecious form (synonym *Peridermium pini*) is much simpler: In infected pine, the fungus produces dikaryotic aeciospores, and these aeciospores can only re-infect pine. No alternate host is required in its life cycle. Spermatia have been reported from pine inoculated by autoecious aeciospores (Kaitera and Nuorteva, 2008), but their role, such as their function in sexual reproduction, is still unknown.

The two forms of *C. pini* were previously described as two different species, *C. flaccidum* first from peony (Winter, 1880) and *P. pini* first

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from Scots pine (Schmidt and Kunze, 1817). Later morphological studies showed that these two forms have no morphological difference in aeciospores and germ tubes (Van Der Kamp, 1968; Kasanen, 1997; Kaitera et al., 1999a). The high similarity of the internal transcribed spacers (ITS) between the two forms and no overall differentiation based on molecular markers (Moricca et al., 1996; Hantula et al., 2002) provided more evidence to confirm that these two forms are the same species.

Rust aeciospores are usually more resilient in harsh environments, such as dryness and UV light, than basidiospores, therefore they stay viable for a longer time and spread further (Zhao et al., 2016). SPBR epidemics caused by aeciospores of the autoecious form and epidemics caused by basidiospores of the heteroecious form may differ in disease severity and distribution pattern. Therefore, identifying the form is critical to studying the *C. pini* epidemics. However, methods based on morphology or ITS sequences cannot distinguish the two forms. Since the aeciospores of the autoecious form can only infect pine and the aeciospores of the heteroecious form can only infect the alternate host, inoculation tests can be used for identification. However, the growth of *C. pini* hyphae in pine had a long latent period; first spermogonium was produced one to two years after inoculation (Ragazzi, 1989), and the first aecia were produced two to six years after inoculation (Kaitera, 2007). Accordingly, identification based on inoculation results is laborious and time-consuming.

Simple sequence repeat (SSR), or microsatellite, are tandem repeats of short di-, tri-, tetra-or penta-nucleotide motifs. Kasanen et al. (2000) tested aecia from autoecious and heteroecious *C. pini* canker with two highly variable SSR markers, Pp1 and Pp2, and showed that the autoecious form has only one allele in each locus and the aecia were homozygous, while aecia of the heteroecious form were heterozygous (Kasanen et al., 2000). The homozygosity in autoecious *C. pini* and heterozygosity in heteroecious *C. pini* were further investigated by Samils et al. (2011) with five additional SSR markers originally developed for *Cronartium quercuum* f. sp. *fusiforme* (Burdine et al., 2007). The autoecious *C. pini* was homozygous for all seven loci, and all aecia from one lesion were the same multi-locus genotype (MLG). The heteroecious *C. pini* was heterozygous for at least one locus, and aecia in one lesion could have several multi-locus genotypes. Therefore, the seven molecular markers have been used to distinguish heteroecious and autoecious *C. pini* (Samils et al., 2011, 2021).

C. pini is widely distributed in Euroasia (CABI Compendium, 2022), but its zygosity and genetic diversity are rarely investigated in areas other than the Fennoscandia. In the most recent study, 14 *C. pini* populations from Sweden and Finland were genotyped with SSR and amplified fragment length polymorphism (AFLP) markers (Samils et al., 2021), and AFLP markers were used in the analysis of genetic diversity and population structure. The heteroecious populations had much higher diversity than the autoecious populations, but unexpectedly high genotypic diversity was still found in autoecious populations. Compared to AFLP, SSR markers are more robust, variable, informative, and reproducible (Powell et al., 1996; Jones et al., 1997). Recent advances in high-throughput sequencing make genome-wide genotyping and single-nucleotide polymorphism (SNP) more accessible, but SSR makers are still popular for their hyper-variability and multi-allelic nature (Mishra et al., 2022). Genotyping based on SSR is widely used to study the genetic diversity, population structure, evolution and worldwide migration of many rust fungi in wheat, coffee, and trees (Kolmer et al., 2020; Weng et al., 2020; Czajowski et al., 2021).

SPBR disease severity in young Scots pine forests (10–30 years old) in Northern Sweden was surveyed by the Forestry Research Institute of Sweden in 2021 and 2022 (Svennerstam 2023). Among the surveyed stands, 87 % in Norrbotten, 60 % in Västerbotten, 43 % in Jämtland, and 32 % in Västernorrland were infected by SPBR (Svennerstam 2023). Many *C. pini* isolates were collected during the surveys. These samples are valuable resources for studying the diversity of *C. pini* populations since they cover a larger geographical region than the previous studies. Among the previous seven SSR markers for identification, five markers were based on the *C. quercuum* genome. Pp1 is a marker with long mononucleotide repeats which can make it hard to determine the size differences (score the peaks) in genotype analysis software. In addition, individual PCR and fragment length analysis need to be done for each marker and each isolate, which makes the genotyping laborious and expensive. In this study, we aimed to: 1) develop new SSR markers based on the *C. pini* genome to improve the robustness and resolution, 2) develop a multiplex genotyping protocol to reduce the labor and economic costs, and 3) investigate the distribution and population structure of *C. pini* in Sweden with high-resolution markers and compare the heteroecious and autoecious populations.

2. Material and methods

2.1. Sample collection and DNA extraction

All samples were collected from Scots pine (*Pinus sylvestris*). According to the previous study, aecia of autoecious *C. pini* in the same lesion have the same genotype, while aecia of heteroecious *C. pini* in the same lesion may have different genotypes (Samils et al., 2011). Therefore, most *C. pini* isolates included in this study were aeciospores collected from single aecium. All samples were collected from June to July from unopened aecia when it was possible. Sterilized forceps were used to break the peridium and the aeciospores were collected into a 1.5 mL centrifuge tube.

Only samples with no more than one missing SSR locus in later analysis were kept. The collection used in this study included 179 isolates collected in 2011 and 2014 in Southern and Northern Sweden and Northern Finland (Samils et al., 2021), and 217 isolates collected in 2021 in Northern Sweden. The number of samples collected from each location was listed in Table 1.

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 a 1.5 mL Eppendorf tube together with three 3-mm glass beads, twenty 2-mm glass beads and 200 μL of lysis buffer (50 mM Tris–HCl pH 8.0, 150 mM NaCl, and 100 mM EDTA, or Buffer AP1). DNA from isolates collected in 2011 and 2014 were extracted with CTAB procedure which is included in a previous study (Samils et al., 2021). DNA from isolates collected in 2021 were extracted with DNeasy Plant kit (Qiagen, Germany) according to the manufacturer's instructions. DNA products were stored at −20 °C until further processing.

2.2. SSR marker development

Novel *C. pini* SSR markers were developed with a *C. pini* draft genome assembly based on PacBio long-read sequencing (unpublished data). The genome was screened with Krait (Du et al., 2018) to identify SSR loci and develop corresponding primers. The application generated a long list of 880,472 perfect SSR loci (8121 dinucleotide, 9758 trinucleotide, 9446 tetranucleotide, 4572 pentanucleotide and 78,596 hexanucleotide) (Supplementary file 1). The default settings were used for primer design (amplicon size 100–300 bp, primer length 18–27 bp, Tm 58–65 °C). Primers were designed automatically for 1398 loci. One locus was picked from each contig to minimize linkage between loci. In addition, longer SSR loci with higher number of motifs were favored since they potentially have higher allele numbers; and tri-to hexa-nucleotide loci are favored since they are easier to score in the GeneMarker (SoftGenetics) software. This manual screening process selected 90 loci. After primer oligo analysis with Beacon designer® (http://www.premierbiosoft.com/qpcr/) for cross and self-dimers and hairpin formation, a shorter list of 40 SSR loci with primers was ready to be tested in the lab.

Twelve *C. pini* isolates from various locations in Finland and Sweden were selected as a panel to test the polymorphism and robustness by standard PCR with unlabeled primers. Each 15 μL reaction mixture included 1–10 ng DNA template, 0.5 μM forward primer, 0.5 μM reverse

Table 1
Number of *Cronartium pini* isolates collected from each location. All locations are in Sweden except Pudasjärvi, Finland. Letters after each county are the Swedish county letter codes (Länsbokstäver).

Year	County/Region	Municipality	Number of isolates
2011	Pudasjärvi, Finland	Pudasjärvi	45
	Gotland (I)	Gotland	18
	Halland (N)	Laholm	7
	Jämtland (Z)	Berg	2
		Krokom	1
	Norrbotten (BD)	Gällivare	58
		Jokkmokk	3
		Luleå	11
		Övertorneå	1
	Stockholm (B)	Nynäshamn	12
	Uppland (C)	Uppsala	8
2014	Jämtland (Z)	Krokom	13
2021	Jämtland (Z)	Berg	14
		Bräcke	1
		Krokom	10
		Östersund	2
		Ragunda	3
	Norrbotten (BD)	Älvsbyn	13
		Arjeplog	7
		Arvidsjaur	16
		Boden	1
		Gällivare	3
		Haparanda	1
		Jokkmokk	5
		Kalix	7
		Luleå	6
		Överkalix	7
		Övertorneå	12
		Pajala	14
		Piteå	16
	Västerbotten (AC)	Åsele	10
		Bjurholm	2
		Lycksele	19
		Malå	2
		Nordmaling	1
		Norsjö	1
		Örnsköldsvik	1
		Skellefteå	10
		Sorsele	10
		Umeå	3
	Västernorrland (Y)	Ånge	5
		Sollefteå	4
		Sundsvall	11

primer, 0.2 mM each dNTP, 1 × PCR buffer and 0.5 U DreamTaq DNA polymerase (Thermo Fisher Scientific). Cycling conditions were: 95 °C for 5 min; 40 cycles of 95 °C for 30 s, 55 °C for 30 s and 72 °C for 30 s; followed by a final extension of 10 min at 72 °C. Amplicons (5 μL) were loaded on a 2.5 % (m/v) agarose gel plate (Electran®, VWR Life Science) for electrophoresis (120–160 V, 1.5–2 h), where small amplicon length variations could be observed due to the low voltage, long electrophoresis time and dense agarose gel. Ten new SSR loci with high polymorphism and strong amplicon bands and two loci from a previous study, Pp2 and CqfSI_AAG13 (Samils et al., 2011) were selected (Table 2).

2.3. Marker amplification and genotyping

SSR markers were amplified three by three in multiplexed reactions (a total of 4 multiplexed PCR for 12 markers). Before multiplexing, primers from each three loci were checked with Multiple Primer Analyzer (Thermo Fisher Scientific) for cross dimers. Suitable combinations of loci were then grouped in each multiplex reaction, and forward primers were labelled with fluorescein amidite (FAM), hexachlorofluorescein (HEX), or ATTO 550 (Table 2). Three loci were amplified in a multiplex PCR in 25 μL reaction mixture (1–10 ng DNA template, 0.2 mM each dNTP, 1 × PCR buffer and 1 U DreamTaq DNA polymerase [Thermo Fisher Scientific]), primer concentrations and multiplex PCR combinations were listed in Table 2. The cycling conditions were the same as described above. The amplicons were processed with an ABI 3730xl DNA analyzer (Applied Biosystems) and GeneScan 400HD ROX as size standard for fragment length analysis (Macrogen Europe). Raw data were analyzed with GeneMarker v. 3.0.1 (SoftGenetics) to determine the SSR allele sizes.

2.4. Data analysis

The forms of the isolates (heteroecious or autoecious) were determined based on the heterozygosity of the SSR markers (Samils et al., 2011). Isolates were assigned to two populations based on forms (heteroecious and autoecious), nine populations based on forms and locations, and twelve populations based on form, location, and collection year. Samples from Uppland and Stockholm, and samples from Jämtland and Västernorrland were pooled due to close geographical distance and small sample size. SSR allele sizes of all isolates were summarized in GenAlEx (Peakall and Smouse, 2006). The SSR loci statistics and the diversity of the populations were analyzed with the R package *poppr* v. 2.8.0 (Kamvar et al., 2014). Data was analyzed both before and after clone correction, where clone correction was applied at the municipality level, i.e. isolates collected from the same municipality with the same MLG were treated as one clone.

Eight sub-populations were used to compare the standardized index of associations ($\bar{r}_{d)}$) (Agapow and Burt, 2001) in *poppr*: the heteroecious populations of 2011 Norrbotten, 2021 Norrbotten, 2021Västerbotten, and 2011 Uppland-Stockholm; the autoecious populations of 2021 Norrbotten, 2021 Jämtland-Västernorrland, 2021 Västerbotten, and 2011 Finland. The autoecious populations were analyzed before and after clone correction. The populations were re-sampled 999 times under the assumption of no linkage between the markers, and the $\bar{r}_{d}$ distribution was compared with the observed $\bar{r}_{d.}$

Data without clone correction was used in the following analysis: discriminant analysis of principal components (DAPC) as implemented in R package *poppr* was performed with a model with 41 principal components determined based on highest mean success and lowest mean squared error. Genetic distances between populations were calculated using Nei's distance and Edwards' angular distance and illustrated with the neighbor-joining method. SSR motif length and copy number were used to calculate the Bruvo's distance between isolates. The minimum spanning networks were calculated for all isolates and heteroecious and autoecious isolates separately.

Structure 2.3.4 was used to analyze the clustering of *C. pini* isolates with the admixture model. In preliminary analysis, 20 iterations with K from 1 to 12, burn-in period of 10,000 and a run length of 50,000 were used in Markov Chain Monte–Carlo (MCMC) simulation. The best number of clusters (K = 4 to 6) was obtained in StructureSelector (Li and Liu, 2018), and the selection was based on Puechmaille's method (Puechmaille, 2016) since the sample sizes are unbalanced. Then the analysis was repeated with 20 iterations of K from 3 to 8, burn-in period of 200,000 and a run length of 1,000,000 in MCMC simulation. The best K = 6 is supported by both Puechmaille's method and Evanno's method (Evanno et al., 2005). The population structure was visualized with *pophelper* (Francis 2017), where individuals are grouped by populations and then sorted by Q-value.

The relative migration network between populations was visualized by divMigrate (Sundqvist et al., 2016) based on Jost's D, Nei's Gst, and the effective number of migrants per generation (Nm) (Alcala et al., 2014). The bootstrap value was 1,000, the alpha level as confidence interval was 0.05, and relative migration values lower than 0.05 were filtered and not shown in the network.

Table 2
Characteristics of polymorphic simple sequence repeat (SSR) markers and primers, and their fluorescent dye labelling in multiplexed PCR.

Multiplex PCR	Locus	Primer sequence (5′–3′)	Motif	No. of repeats in *C. pini* reference genome	Amplicon size in *C. pini* reference genome (bp)	Fluorescent dye	Primer concentration in multiplex PCR (μM)
1	Pp2	CTGGGTCAAGTCAAATCTCC			260–300	FAM	0.25
		GGACCAAATTCGATCATAGG					0.25
	CqfSI_AAG13	AGCAGCACAAGCTGAGAATG	AAG		104–107	HEX	0.25
		CGTTCTCATCCGAATCCATC					0.25
	C3225	TTCTCAAGTACTGCGCCGAG	CCAG	10	110	ATTO 550	0.25
		TCGATCCATACACCACTGGC					0.25
2	C4566	CGTCTCTCATCATCCGCTCC	TCTCAC	6	185	FAM	0.25
		AGGAGATGTCAGTCAGCAGAC					0.25
	C5332	TAAGAGCGTGGAAGTGGTGG	TGGAGG	7	153	HEX	0.25
		AAGCGTGTGGTCCATCCTTG					0.25
	C10864	TTGCGAGATGCTATCCTGCC	TG	19	165	ATTO 550	0.25
		AGACAGTTGACGCTCGAAGG					0.25
3	C27695	CCGCCTGATCAGAGTGGATC	AAAG	10	116	FAM	0.5
		TGGTCTCGTTGAGGTAAGTGAAC					0.5
	C29658	AGGTTGGCGAGAGTTGGATG	GTGGAT	7	186	HEX	0.25
		AGCATTCTCCACTGACTGCC					0.25
	C31141	GGACAGCACACTCCATCCTC	TCCAC	7	154	ATTO 550	0.5
		TCGAGCTATGTACGAAGCGG					0.5
4	C33158	CAGCTAGACTGCGCATTGAC	CCGA	9	145	FAM	0.25
		ACCGACGAGAGATGCTCTTC					0.25
	C36856	GCATCAGCATCAGCATCAGC	CATC	7	142	HEX	0.25
		TTGGTTGGCACTATCGGACC					0.25
	C41628	TGCGATCGGAGTCTTGTACG	GAAA	8	124	ATTO 550	0.25
		CATCATCCATCCATCGTGCG					0.25

3. Results

3.1. Distribution of heteroecious and autoecious C. pini in Sweden

The forms of samples collected in 2011 and 2014 were identified by the old markers (Samils et al., 2011). Among these, the populations from Pudasjärvi Finland were known to be autoecious based on inoculation tests (Kaitera and Nuorteva. 2008). The homo-/heterozygosity of new SSR loci in these samples were used as references. Among the twelve SSR markers, eleven were always homozygous in autoecious samples and at least one marker was heterozygous in heteroecious samples. However, the last marker, C41628, could be heterozygous in some autoecious samples. Therefore, C41628 was ignored when determining the forms of *C. pini* isolates, but the locus was kept in later population genetics analysis.

Only the isolates with no more than 10 % missing data (1 out of 12 markers) were included in the analysis. Among these isolates, 197 were heteroecious and 199 were autoecious. Their distribution is shown in Fig. 1. In Northern Sweden, autoecious *C. pini* isolates dominated Jämtland-Västernorrland, while heteroecious *C. pini* isolates dominated Norrbotten. There was no clear geographical boundary for the distribution of the two forms. Occasionally, both forms could be found at the same sampling location, such as the overlapping data points in Västerbotten.

3.2. SSR marker diversity in the populations

The 12 polymorphic SSR markers revealed a total of 102 alleles in 396 isolates, ranging from 2 to 20 alleles for each marker (5–20 alleles for the new markers) (Table 3). The heteroecious population always had a higher, if not equal, number of observed alleles and private alleles in each marker than the autoecious population (Table 4). The autoecious population in Norrbotten showed the highest allele diversity by most observed alleles and private alleles, but this could be explained by the highest number of isolates in this population. The Simpson's diversity indices of the four heteroecious populations are similar (0.44–0.48), and the index was lower in the five autoecious populations (0.24–0.40) (Supplementary Table 1).

3.3. Genotypic diversity in C. pini populations

The twelve SSR markers identified 271 multilocus genotypes (MLGs) from the 396 isolates. The genotype accumulation curve (Supplementary Fig. 1) is close to a plateau. This suggests twelve makers can distinguish the MLGs with ideal resolution; in addition, nine to ten markers can distinguish 95 % of the MLGs.

Among the 197 heteroecious isolates, 196 MLGs were identified (Table 5, Supplementary Fig. 2). Particularly, almost every heteroecious isolate had a unique MLG, and only two Gotland isolates collected from two lesions from the same tree shared the same MLG. Among the 199 autoecious isolates, only 75 MLGs were identified (Table 5; Supplementary Fig. 3). There were three most common autoecious MLGs: MLG248 included 31 isolates collected from 14 municipalities and 5 counties in 2011 and 2021, MLG251 included 18 isolates collected from 8 municipalities and 3 counties in 2011, 2014, and 2021, and MLG263 included 16 isolates collected from 8 municipalities and 3 counties in 2011, 2014, and 2021 (Supplementary Table 2). The distribution of these isolates is shown in Fig. 2.

Shannon–Wiener Index of MLG diversity (H) and expected heterozygosity (H_{exp}) of heteroecious populations were generally higher than those of autoecious populations. Exceptionally, the heteroecious population in Gotland was less diverse than the autoecious populations in Pudasjärvi, Finland and Norrbotten, both before and after clone correction. This could be caused by the smaller sample size and limited gene flow between the island of Gotland and the main land.

3.4. Index of association and reproduction mode in C. pini populations

Since every isolate in the four heteroecious populations was a unique MLG, clone correction was not required. Three heteroecious populations, 2021 Västerbotten, 2011Uppland-Stockholm, and 2011 Norrbotten, had a low standardized index of association $\bar{r}_d$ ($p > 0.05$) (Fig. 3). Thus, the null hypothesis that the markers were unlinked could not be rejected, which indicated sexual populations under random mating. In the heteroecious 2021 Norrbotten population some degree of linked markers and thus non-random mating was indicated by a significant *p*-value (0.004) although $\bar{r}_d$ was relatively low.

All $\bar{r}_d$ were high ($p < 0.05$) in autoecious populations before and

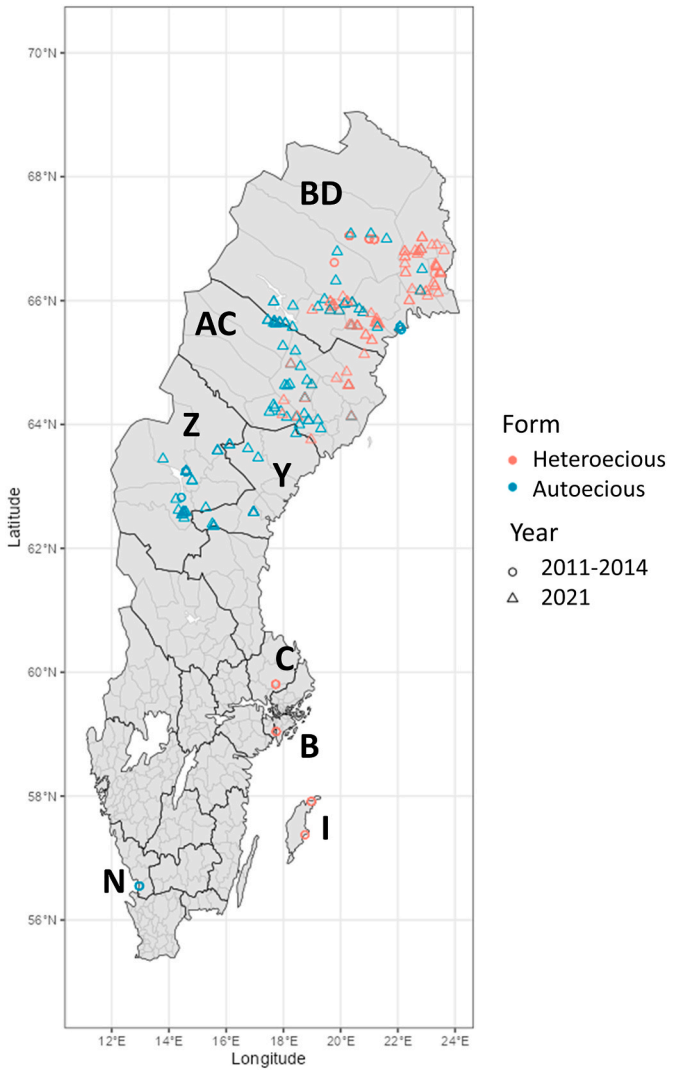

Fig. 1. Distribution of heteroecious and autoecious *Cronartium pini* isolates in Sweden. AC: Västerbotten, B: Stockholm, BD: Norrbotten, C: Uppland, I: Gotland, N Halland, Y: Västernorrland, Z: Jämtland.

after clone correction (Fig. 3), indicating linkages between markers. The populations in 2011 Finland and 2021 Norrbotten were rather diverse with lower $\bar{r}_d$ after clone correction, but the null hypothesis of random mating was still rejected. The results suggested that all autoecious populations lack random mating.

3.5. Structure and relationships of C. pini populations

In the discriminant analysis of principal components (DAPC) (Fig. 4 A), a separation of the two forms could be found. The four heteroecious populations were overlapped with each other. Their close genetic distances were also shown in the Nei's and Edwards' neighbor-joining trees (Fig. 5B and C). The heteroecious population from Västerbotten had an overlap with some autoecious populations (Fig. 4 A), and there was a discrepancy in its distances to other populations between the Nei's and Edwards' neighbor-joining trees (Fig. 4B and C). The autoecious populations, especially the three from northern Sweden (Norrbotten, Västerbotten, Jämtland-Västernorrland), were closely clustered in DAPC and genetic distance, while the two autoecious populations from Finland and Southern Sweden (Halland) were more diverged from the other autoecious populations (Fig. 4A and B and C).

Six ancestral clusters (K = 6) of *C. pini* were found in the Bayesian analysis in STRUCTURE (Fig. 5). The bar plots of individuals in all heteroecious populations and autoecious populations showed typical patterns of sexual populations and clonal populations (Grünwald et al., 2017). The heteroecious populations were mainly derived from clusters 5 and 6, while the autoecious populations were mainly derived from clusters 1 to 4. Being located in between the heteroecious-dominated Norrbotten populations and the autoecious-dominated Jämtland-Västernorrland populations, the *C. pini* populations in Västerbotten showed a more complicated genetic background (Fig. 5).

Only significant net directional gene flow, instead of both directions, was mapped in the relative migration network. The network based on Jost's D is in Fig. 6, and networks based on Nei's Gst and Nm are in Supplementary Fig. 4. The strongest directional gene flow was found from the autoecious population in Jämtland-Västernorrland to the heteroecious population in Västerbotten and Norrbotten, and autoecious populations in Norrbotten and Finland. Net gene flow was only found from autoecious populations to auto- or hetero-ecious populations in the network based on Jost's D.

3.6. Genetic distance and clusters of C. pini isolates

In minimum spanning networks, isolates in the heteroecious populations were rather dispersed; they had longer genetic distance from each other, while isolates in the autoecious populations were more closely linked (Fig. 7). The autoecious isolates could be divided into three clades marked as I, II and III. Most isolates from the Finnish population composited clade II, and most isolates from the Jämtland-Västernorrland population composited clade I. The three most common MLGs (Fig. 3) were all located in Clade I (Fig. 7). In the network with all isolates (Supplementary Fig. 4), heteroecious isolates still had a

Table 3
Simple sequence repeat (SSR) information in all isolates, heteroecious isolates, and autoecious isolates. Data before clone correction was used for the analysis. 1-D = Simpson index, Hexp = Nei's gene diversity.

Locus	All isolates				Heteroecious isolates				Autoecious isolates			
	Allele No.	1-D	Hexp	Evenness	Allele No.	1-D	Hexp	Evenness	Allele No.	1-D	Hexp	Evenness
Pp2	6	0.545	0.546	0.766	5	0.449	0.450	0.615	5	0.526	0.527	0.799
CqfSI_AAG13	2	0.481	0.482	0.964	2	0.496	0.497	0.993	2	0.391	0.392	0.817
C3225	12	0.441	0.441	0.497	12	0.493	0.494	0.486	4	0.376	0.377	0.588
C4566	7	0.088	0.088	0.348	7	0.145	0.145	0.367	2	0.030	0.030	0.376
C5332	9	0.681	0.681	0.685	9	0.753	0.755	0.786	6	0.537	0.538	0.629
C10864	11	0.650	0.651	0.715	10	0.709	0.711	0.772	6	0.526	0.533	0.611
C27695	20	0.777	0.778	0.571	19	0.866	0.869	0.740	10	0.584	0.585	0.493
C29658	8	0.533	0.534	0.660	8	0.589	0.591	0.758	4	0.390	0.391	0.593
C31141	5	0.076	0.076	0.376	5	0.084	0.084	0.366	3	0.068	0.068	0.403
C33158	11	0.569	0.569	0.721	11	0.599	0.600	0.676	5	0.535	0.536	0.855
C36856	5	0.379	0.380	0.652	5	0.461	0.462	0.687	2	0.281	0.282	0.680
C41628	5	0.529	0.530	0.764	4	0.263	0.263	0.587	4	0.584	0.585	0.779
Mean	8.5	0.479	0.480	0.643	8.083	0.492	0.493	0.653	4.417	0.402	0.403	0.636

Table 4
Observed alleles and private alleles (in parenthesis) in *Cronartium pini* populations. Data before clone correction was used for the analysis.

Population	Markers												mean
	Pp2	CqfSI_AAG13	C3225	C4566	C5332	C10864	C27695	C29658	C31141	C33158	C36856	C41628	
Heteroecious	5 (1)	2	12 (8)	7 (5)	9 (3)	10 (5)	19 (10)	8 (4)	5 (2)	11 (6)	5 (3)	4 (1)	8.083
Autoecious	5 (1)	2	4	2	6	6 (1)	10 (1)	4	3	5	2	4 (1)	4.417
Heteroecious-Stockholm-Uppland	5	2	3	3	4	8 (1)	12 (1)	3	2	6	2	1	4.25
Heteroecious-Gotland	5	2	5	3	5	6	11	3 (1)	3	4	3 (1)	3 (1)	4.42
Heteroecious-Norrbotten	5	2	12 (5)	7 (2)	8 (2)	7 (2)	15 (3)	8 (1)	4 (1)	11 (3)	4 (1)	3	7.167
Heteroecious-Vasterbotten	4	2	6	4	7	4	11 (1)	4	2	3	3	2	4.33
Autoecious-Pudasjärvi (Finland)	2	2	2	1	5	4	7	2	1	3	2	3	2.83
Autoecious-Halland	3 (1)	2	1	1	2	3	3	2	1	1	2	1	1.83
Autoecious-Norrbotten	4	2	4	2	4	3	6 (1)	3	2	2	2	3	3.083
Autoecious-Vasterbotten	3	2	4	2	4	4 (1)	5	3	3	3	2	3	3.167
Autoecious-Jämtland - Västernorrland	3	2	2	1	3	4	2	4	1	4	2	4	2.667

Table 5
The diversity of multilocus genotypes of *Cronartium pini* populations in Sweden and Finland. Data before and after clone correction were both used for the analysis. MLG = number of multilocus genotypes. H = Shannon–Wiener Index of MLG diversity. Hexp = Nei's gene diversity.

Population	Before clone correction					After clone correction				
	Isolate number	MLG	H	Evenness	Hexp	Isolate number	MLG	H	Evenness	Hexp
Heteroecious	197	196	5.28	0.997	0.493	196	196	5.28	1.000	0.493
Autoecious	199	75	3.70	0.493	0.403	124	75	3.96	0.595	0.428
Heteroecious-Stockholm-Uppland	20	20	3.00	1.000	0.463	20	20	3.00	1.000	0.463
Heteroecious-Gotland	18	17	2.81	0.970	0.481	17	117	2.83	1.000	0.478
Heteroecious-Norrbotten	139	139	4.93	1.000	0.487	139	139	4.93	1.000	0.487
Heteroecious-Vasterbotten	20	20	3.00	1.000	0.449	20	20	3.00	1.000	0.449
Autoecious-Pudasjärvi (Finland)	45	26	2.99	0.733	0.361	26	26	3.26	1.000	0.366
Autoecious-Halland	7	3	1.00	0.913	0.341	3	3	1.10	1.000	0.378
Autoecious-Norrbotten	42	24	2.89	0.717	0.379	31	24	3.06	0.843	0.419
Autoecious-Vasterbotten	39	20	2.62	0.605	0.407	30	20	2.78	0.741	0.444
Autoecious-Jämtland & Västernorrland	66	19	2.37	0.655	0.247	34	19	2.71	0.751	0.273
Total	396	271	5.18	0.411	0.480	320	271	5.44	0.654	0.493

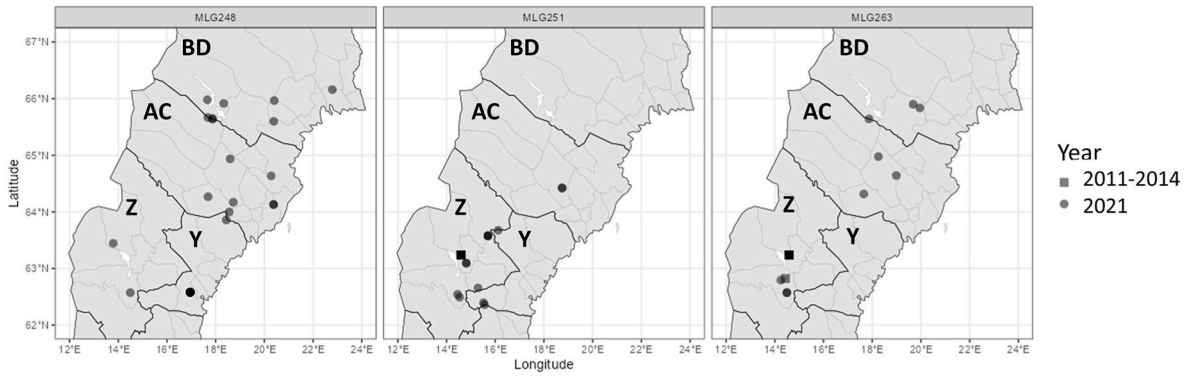

Fig. 2. Distribution and collection year of the most common *Cronartium pini* multilocus genotypes in northern Sweden. AC: Västerbotten, BD: Norrbotten, Y: Västernorrland, Z: Jämtland.

dispersed pattern, autoecious isolates were still aggregated on three clades with varied topology.

4. Discussion

In this study, we developed a new SSR maker panel, which includes ten new markers, for the form identification and genotyping of *C. pini*. Compared to the previous protocol (Samils et al., 2011), multiplex PCR can generate more informative data with less time and resources. Our population genetics data suggested sexual reproduction with random mating in heteroecious *C. pini* and clonal non-random reproduction in autoecious *C. pini*, which led to high genotypic diversity in heteroecious *C. pini* while there was a wide distribution of the same MLG in autoecious *C. pini*.

Scots pine can be infected by the basidiospores of heteroecious *C. pini* or the aeciospores of autoecious *C. pini*. These two types of spores are produced at different times of the year and have various levels of resilience against harsh environments (Zhao et al., 2016). Hence, identification and knowledge of the distribution of the heteroecious and autoecious forms of *C. pini* are critical for disease management. Identification criterion based on the heterozygosity/homozygosity of SSR markers reduced the time required for distinguishing these two forms from years to days (Samils et al., 2011). The underlying rationale is that the heteroecious form in the alternate host produces dikaryotic teliospores that undergo karyogamy as they mature (n + n to 2n). Then monokaryotic basidiospores (n) are produced after meiosis (Fig. 8). In

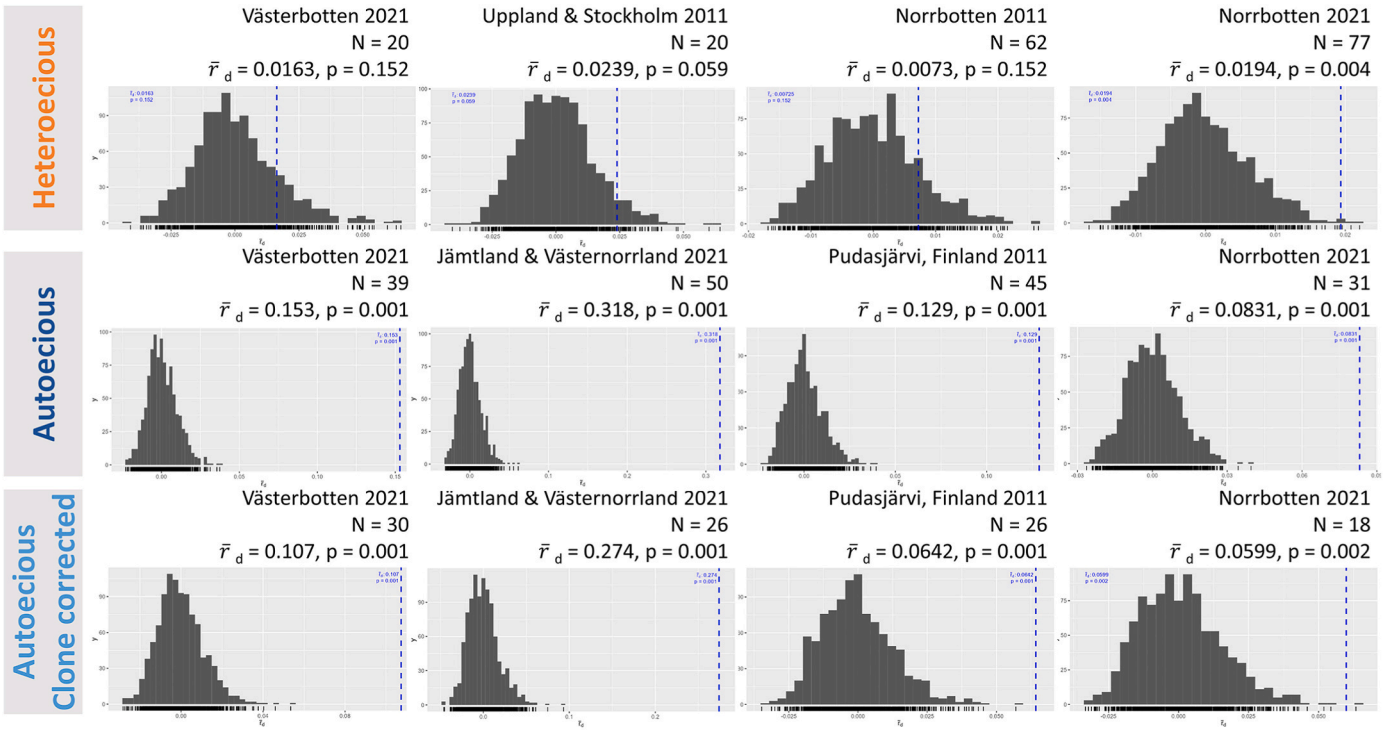

Fig. 3. Standardized index of association $\bar{r}_d$ of four heteroecious and four autoecious populations. The autoecious populations were analyzed before and after clone correction.

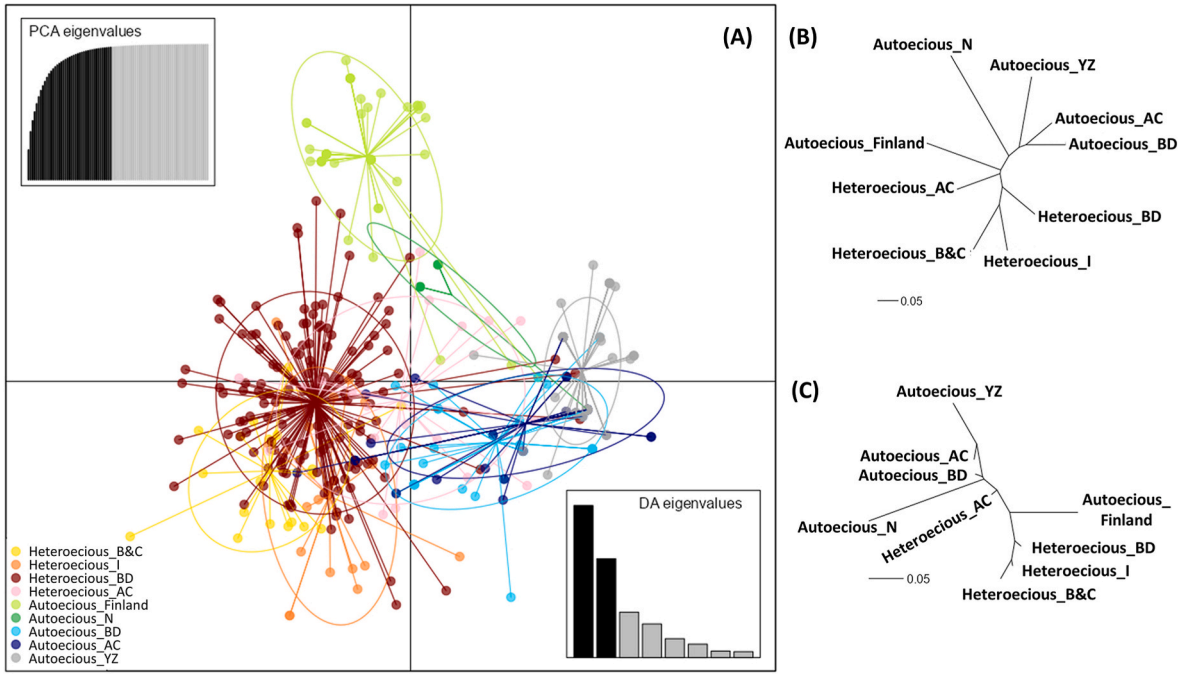

Fig. 4. Discriminant analysis of principal components (DAPC) (A), and the population genetic distance trees based on (B) Nei's method and (C) Edwards' method. AC: Västerbotten, B: Stockholm, BD: Norrbotten, C: Uppland, I: Gotland, N Halland, Y: Västernorrland, Z: Jämtland.

this process, sexual recombination will create high heterozygosity in the heteroecious *C. pini* genome. However, some limitations exist. i) Using insufficient markers may not fully address the heterozygosity in heteroecious *C. pini*. In this study, some isolates identified as homozygous/autoecious in the previous study were heterozygous in several new marker loci. ii) Using too many markers may over-interpret the heterozygous region in the autoecious form. Since the *C. pini* aeciospores are dikaryotic (n + n), mutations may happen in one of the two nuclei independently during evolution. In this study, we found that one marker C41628 could be either heterozygous or homozygous in the population from Pudasjärvi Finland. This area is known to be dominated by the autoecious form (Kaitera, 2003; Kaitera and Nuorteva, 2008). Therefore, we ignored this marker in the form identification. Ideally, identification of the *C. pini* forms should be based on comprehensive information on

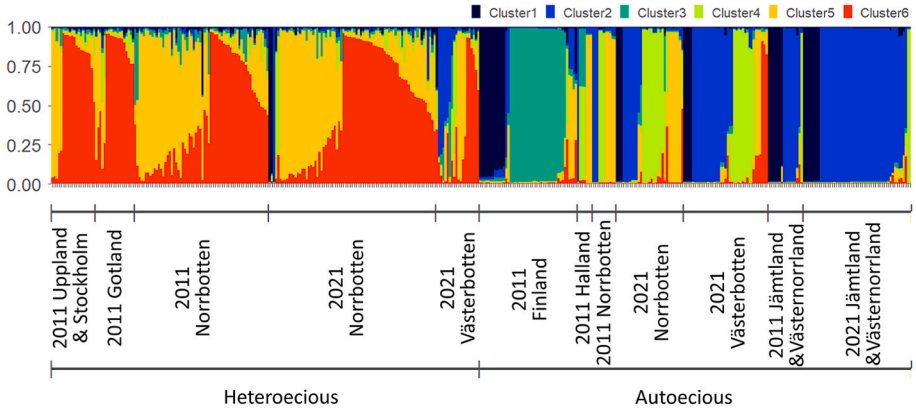

Fig. 5. STRUCTURE barplot of heteroecious (left) and autoecious (right) *Cronartium pini* populations.

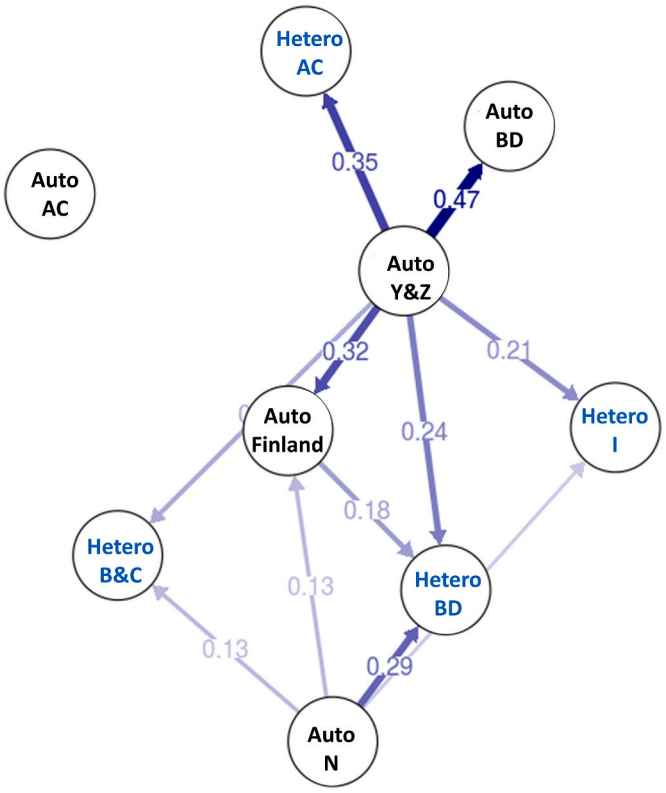

Fig. 6. Relative migration, i.e. net migration, of heteroecious (Hetero) and autoecious (Auto) *Cronartium pini* populations based on Jost's D. AC: Västerbotten, B: Stockholm, BD: Norrbotten, C: Uppland, I: Gotland, N Halland, Y: Västernorrland, Z: Jämtland.

the genotyping of a single aecium, genotyping of aecia from the same lesion or tree, and infectivity of the alternate/telial host. More knowledge on the overall homo- and hetero-zygosity in the autoecious and heteroecious *C. pini* genomes is needed.

We used the new SSR markers with better resolution to revisit the diversity and structure of the *C. pini* populations in Sweden and Finland. With samples from a wider geographical region and more SSR markers, the MLGs of almost all heteroecious isolates (195 out of 197) were unique (Table 5), except for two isolates from the same tree from Gotland that had the same MLG. New markers can further identify MLGs that were previously indistinguishable. For example, 19 MLGs were identified from 46 isolates from Pudasjärvi Finland in the former study (Samils et al., 2021), but the number increased to 26 MLGs from the 45 isolates with new markers (Supplementary Table 3). Nonetheless, the autoecious isolates still had much lower genetic diversity than the heteroecious isolates (Samils et al., 2021).

Only four autoecious populations were included in the previous study with samples collected from 2011 to 2014, and no MLG shared by different populations were found (Samils et al., 2021). In the present study, three MLGs were commonly found in northern Sweden in 2021. It is not known if these MLGs were already widely distributed in 2011, but they had been found at least one time in southern or northern Sweden. This evidence confirmed clonal reproduction over a relatively long period. The aeciospores of tree rusts, such as *Cronartium comandrae* and *Cronartium ribicola*, are capable of long-distance dissemination, where the distance can achieve several or even hundreds of kilometres, to reach between the alternate hosts and pine trees, depending on the landform and airflow pattern (Jacobi, 1993; Frank et al., 2008). The longest distance between isolates with the same MLGs in northern Sweden was around 500 km. Due to the complicated mountainous terrain, the wide distribution could be a result of recurring dissemination over many years. Two of the three common MLGs are genetically close, the other one is located in the same clade (Fig. 7). It is not known where these MLGs originated. Their wide geographical distribution could be a result of higher virulence, higher viability after long-distance distribution, and adaptation to several factors such as the climate condition and the managed Scots pine forests in northern Sweden. It is important to monitor the progression of these MLGs in the future for SPBR management, and the hypothesis shall be tested with inoculation experiments or surveys.

Most patterns in the standardized index of association $\bar{r}_d$ (Fig. 3), STRUCTURE bar plot (Fig. 5), and minimum spanning network (Fig. 7) showed that heteroecious *C. pini* populations were sexual, while the autoecious *C. pini* populations were clonal. The only exception is that the heteroecious population from 2021 Norrbotten had a higher $\bar{r}$ d than that from 2011 with low *p*-value, and therefore, we had to reject the null hypothesis of random mating. This may be explained by the different sampling setups and rather arbitrary prior population information. The majority of 2011 Norrbotten isolates (58 out of 73) were from 3 forest stands in Gällivare municipality and thus represented a population in which haplotypes could mate and reproduce relatively easily. However, the samples collected in 2021 were from a wider region, where some samples from southern Norrbotten could be closer to Västerbotten than northern Norrbotten (Fig. 1), and therefore, a higher level of geographical isolation existed in the 2021 Norrbotten population. A STRUCTURE bar plot showed a clear overall separation between the heteroecious and autoecious *C. pini* populations in Sweden. Similar cases can be found in the sexual and clonal populations of *Phytophthora infestans*: where the sexual population had high admixture, while the clonal population had little or no admixture (Goss et al., 2014). A few isolates collected in 2021 in Norrbotten and Västerbotten and identified

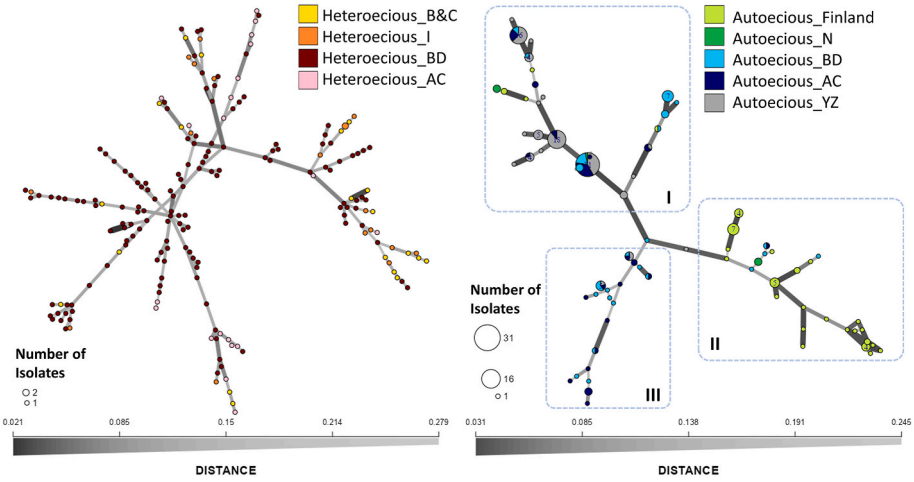

Fig. 7. Minimum spanning networks of heteroecious (left) and autoecious (right) *Cronartium pini* populations based on Bruvo's distance. AC: Västerbotten, B: Stockholm, BD: Norrbotten, C: Uppland, I: Gotland, N Halland, Y: Västernorrland, Z: Jämtland.

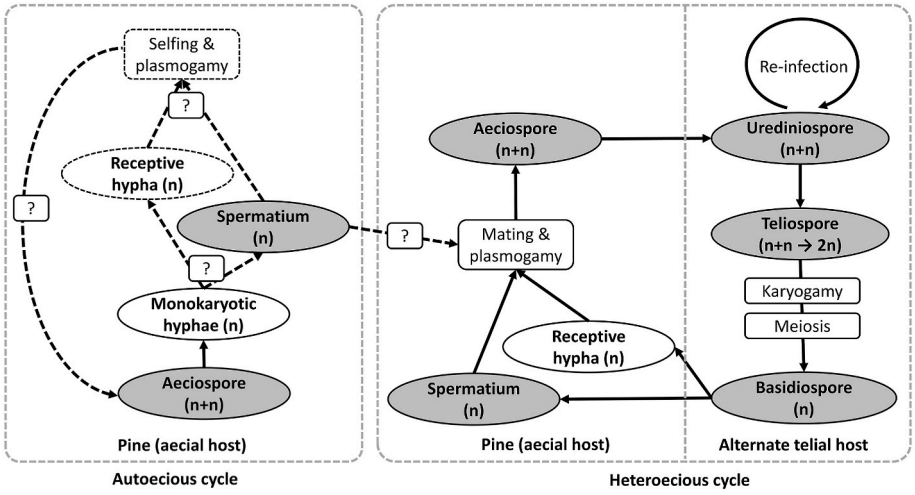

Fig. 8. Hypothesis life cycle of the autoecious *Cronartium pini* (left) and the macrocyclic heteroecious *C. pini* (right). Dash lines and question marks indicate biological events or structures which should be confirmed in future studies.

as autoecious, showed a higher admixture of clusters 5 and 6, and they were closer to the majority of heteroecious isolates (Fig. 5). This could indicate a few misidentifications of the two forms as we discussed above.

The macrocyclic rust life cycle with five different types of spores is complicated. For specific species or lineages within a species, the life cycles can be reduced with some types of spores skipped or missing, and there is different terminology to describe such specific life cycles (Petersen, 1974). For instance, the complete macrocyclic life cycle (heter-eu-form) of *C. quercuum* (Berk.) Miyabe ex Shirai (synonym *C. quercuum* f. Sp. *banksianae* Burds. & G.A. Snow) includes five spore types on pine and oak (Zhao et al., 2022). Within the species, lineages that cause pine–pine gall rust (synonym *Endocronartium harknessii* (J.P. Moore) Y. Hirats, *Peridermium harknessii* J.P. Moore) only infect pine and produce dikaryotic aecioid teliospores, and monokaryotic modified basidia can be produced from the aecioid teliospores (Epstein and Buurlage, 1988). This reduced life cycle is called the auto-endo cycle (Petersen, 1974). The complete macrocyclic and hypothesized reduced life cycle of *C. pini* is illustrated in Fig. 8. Previous studies showed that autoecious *C. pini* aeciospores are dikaryotic (Pei and Pawsey, 1991; Samils et al., 2011), but the vegetative hyphae in axenic culture from these aeciospores are monokaryotic (Pei and Pawsey, 1991). The mechanism by which dikaryotic aeciospores produce monokaryotic hyphal cells is still unknown. In addition, spermatia have been observed from autoecious *C. pini* inoculation (Kaitera and Nuorteva, 2008). We hypothesize that the autoecious isolates in *C. pini* and *C. quercuum* have the same mode of sexual reproduction of self-fertilization (Fig. 8): i) Dikaryotic aeciospores or aecioid teliospores germinate and produce monokaryotic hyphae or modified basidia to infect pine. ii) Hyphae continue to grow in the infection site and produce receptive hyphae and spermatia with the same haplotype. iii) Spermatia and receptive hyphae in the same site mate and finish plasmogamy, and produce dikaryotic aeciospores. Therefore, the diverse but homozygous genotypes of autoecious *C. pini* in this study and *C. quercuum* in previous studies (Tuskan, 1989; Vogler et al., 1991) can be explained by mutations in the aeciospores before infection or monokaryotic hyphae. Mutations that only occurred in the receptive hyphae or spermatia can explain the occurrence of minor heterozygosity in putatively autoecious *C. pini* in this study.

Nonetheless, there should be one obstacle in self-fertilization described above. Most Basidiomycota fungi including rusts are heterothallic, which means sexual reproduction only occurs between two individuals with different mating-types (Kües et al., 2011). The mating-types are determined by two mating-type loci, P/R and HD. The P/R locus encodes a pheromone precursor and a seven-transmembrane-domain pheromone receptor that can bind to the appropriate non-self-produced pheromone, and plasmogamy occurs

after compatible recognition (Raudaskoski and Kothe, 2010). The HD locus encodes two proteins, HD1 and HD2. HD1 and HD2 from two compatible individuals form a heterodimer that binds to promoters and induces other genes in sexual reproduction and pathogenic lifestyle (Kahmann and Bölker, 1996; Cuomo et al., 2017). If self-fertilization can occur in autoecious *C. pini*, this suggests malfunction of at least P/R recognition. We hypothesize that this P/R mutation in heteroecious *C. pini* happened once or several times, and is the fundamental event that generated autoecious *C. pini*. The molecular characterization of either loci has not been done in *C. pini* or any *Cronartium* spp. yet, further studies need to be carried out to validate this hypothesis in genetics and molecular biology.

In this study, we found relative migration between the autoecious populations and from the autoecious populations to heteroecious populations, and the heteroecious populations appeared to be sinks in the migration network (Fig. 6). The gene flow between autoecious populations may reflect the long-distance dissemination over time. The gene flow from autoecious populations to heteroecious populations brings up the possibility that the haploid spermatia of autoecious *C. pini* are functional to mate with receptive hyphae of heteroecious *C. pini*. However, this result can be rather limited since we only investigated the *C. pini* populations in Sweden, and the populations were arbitrarily divided based on counties.

The heteroecious *C. pini* (syn. *C. flaccidum*) has been reported in many countries in Europe and Asia (USDA Fungal Databases 2024). This heteroecious form can be easily confirmed if the record is from a telial host such as *Paeonia* spp., *Melampyrum* spp., or *Vincetoxicum* spp. (Ragazzi 1983; Kaitera et al., 2012; Zhao et al., 2022). The existence of the autoecious from (syn. *P. pini*) can be harder to confirm especially in early records due to the following reasons: i) the two forms lack morphological differences and variation in ITS (Hantula et al., 2002). ii) many alternate/telial hosts of *C. pini* have only recently been found (Kaitera et al., 1999b, 2015; Kaitera and Hiltunen 2011) and are unknown in previous publications. Therefore, a record from *Pinus* spp. without adequate tests, such as inoculation (Kaitera and Nuorteva 2008) or molecular markers (Kasanen et al., 2000; Samils et al., 2011), can be unreliable to confirm the form of *C. pini*. To our knowledge, *C. pini* population genetics studies have not been published from regions other than Fennoscandia. The *Cronartium* genus has many closely related species with various forms of life cycles that pose serious risks to many pine species. A global perspective on the distribution and diversity of autoecious and heteroecious *C. pini* will help us understand the origin and evolution of the two forms. Such studies on the autoecious and heteroecious *C. pini* will elucidate the mechanism of host–pathogen interaction in this genus, which is valuable information in forest disease management and resistance breeding in pine.

Data availability statement

The datasets used and/or analyzed during the current study are available from the corresponding author upon reasonable request.

CRediT authorship contribution statement

Ke Zhang: Writing – review & editing, Writing – original draft, Visualization, Software, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Berit Samils:** Writing – review & editing, Resources, Methodology, Investigation.

Declaration of competing interest

The authors, KZ and BS, declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.funbio.2024.09.007.

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