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Population genetics and reproductive biology of *Thekopsora areolata* in *Picea abies seed orchards*

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Department of Forest Mycology and Plant Pathology
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Abstract

Norway spruce seed production is strongly affected by fungal rust pathogens in northern Europe. To set up a background to better understand the Cherry – Spruce Rust disease, the genotypic diversity and mode of reproduction of *Thekopsora areolata* was investigated using microsatellite markers, a hierarchical sampling design and data analyses tools. Cones were collected at 8 different locations from Sweden, Norway and Finland and single aecium DNA extraction were performed in aecia sampled across locations, plots, cones and scales. Nine microsatellite primer pairs were designed and extracted DNA was amplified in 3 multiplex reactions. The results obtained in this study showed a high genotypic diversity, no apparent genetic structure among locations and random mating, which indicates a constant gene flow and frequent sexual events. Furthermore, it was observed that non-random mating occurs in cones under different hypothetic scenarios, namely: systematic colonization of the cone by more than one basidiospore, multiple cross- and self- fertilization events in pycnia and possible infection by previous years' aeciospores. In general, these results are in line with previous hypothesis and propose scenarios that should be tested in future studies. Additionally, it highlights the importance of understanding the reproductive biology of rust fungi to propose effective disease management solutions.

Keywords: cherry-spruce rust, population genetics, microsatellites, seed orchard, life cycle

Sammanfattning

Produktion av granfrö påverkas starkt negativt av rostsvampen *Thekopsora areolata* som orsakar grankotterost i norra Europa. För att bättre förstå grankotterostsvampen, dess genotypiska mångfald och reproduktionsform användes en hierarkisk provtagningsdesign, nya mikrosatellit markörer och flera data-analysverktyg. Kottar samlades på 8 olika ställen i Sverige, Norge och Finland, varpå DNA-extraktion av enskilda aecidier utfördes. Nio mikrosatellit-primerpar konstruerades och extraherat DNA amplifierades i 3 multiplexa PCR-reaktioner. Resultaten visar på en hög genotypisk mångfald och ingen uppenbar genetisk struktur mellan platser kunde påvisas, vilket indikerar frekvent sexuell reproduktion och ett stort genflöde. Vidare observerades att icke-slumpmässig parning sker i kottar. Tre olika hypoteser presenteras för att förklara detta, nämligen: systematisk kolonisering av kotten med fler än en basidiospor, flera kors- och självbefruktningshändelser i pyknia och eventuellt infektioner av föregående års aeciosporer. Generellt är dessa resultat i linje med tidigare hypoteser och ger uppslag till några scenarier som bör testas i framtida studier. Dessutom framhävs vikten av att förstå reproduktionsbiologin hos rostsvampar för att föreslå effektiva lösningar för att begränsa deras skadeverkningar..

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Abbreviations

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AMOVA	Analysis of Molecular Variance
DAPC	Discriminant Analysis of Principal Components
HWE	Hardy-Weinberg equilibrium
Ia	Index of Association
MLG	Multi locus genotype
P.Ia	P values for Index of Association

1 Introduction

1.1 Research problem

In Sweden, Norway spruce (*Picea abies*) is the most economically important conifer for the forest industry with more than 200 million trees planted in managed forests from which 70% originated from improved seeds (Haappanen et al., 2015). Genetic improvement of spruce started in Sweden around the 1940s with selection of plus trees and establishment of seed orchards for mass production of seeds. Seeds coming from seed orchards have an estimated genetic gain of up to 20%, a more even germination and are expected to be more resilient to environmental changes, since the mother trees consist of genotypes that have been selected for the specific areas in Sweden and crosses between good genotypes. These mother trees have been randomly distributed in rows within a size-variable area within each seed orchard. However, mass production of improved seeds for *P. abies* is usually lower than the demand (Haapanen et al, 2015). This is mainly due to a high interest in improved seeds (Haapanen et al, 2015), irregular production of cones and occurrence of pests and diseases (Kaitera et al 2014). In Finland expected good seed years have been repeatedly affected by *Thekopsora areolata* (Fig. 1), a rust fungus which grows in *P. abies* cones and reduces seed viability and production up to 10-fold (Kaitera & Tillman-Sutela 2009). One of the main proposed strategies to improve the production of improved seeds is by intensive seed orchard management (Haappanen et al., 2015).

However, pathogens such as *T. areolata* impose a challenge in disease management due to the complexity of their life history and reproductive biology. Population genetics can be one approach to better understand fungal plant pathogens and obtain results that could be translated into better disease management strategies.

The use of microsatellite molecular markers with a correct sampling strategy can help to answer ecological questions in fungal population biology (Dutech et al., 2007; Lim et al., 2004) such as population differentiation(Ali et al., 2014), genotypic and genetic diversity (Barres et al., 2012), heterozygosity and life-history characteristics like mode of reproduction (Danies et al., 2014) and host selection (Berlin et al., 2012). Microsatellites markers are highly variable short nucleotide motifs that are tandemly repeated at least 5 times and found abundantly in most eukaryotic genomes. These markers have been successfully isolated from fungal genomic DNA (Dutech et al., 2007).

Therefore, this thesis aimed to investigate the population genetics and reproductive biology of the rust fungi *T. areolata* using a hierarchical sampling strategy and microsatellite molecular markers to shed more light into the reproduction mode of the pathogen and therefore generate a better background to propose better disease management strategies. The specific objectives of the thesis were:

- Asses the genotypic variation of *Thekopsora areolata* across different hierarchical levels in *Picea abies* seed orchards in Sweden.
- Investigate the mode of reproduction of *Thekopsora areolata* in *Picea abies* seed orchards in Sweden.

Figure 1. Thekopsora areolata in *P. abies* (a) cones and (b) scales. The fungus grows throughout the cone forming aecia (arrow), blister-like structures that crow most of the scale surface and affects seed viability

1.2 Rust fungi

Rust fungi are responsible for some of the most economically compromising diseases in tree crops in the northern hemisphere. These fungi can produce up to five spore stages in the same host (macrocyclic and autoecious), three spore stages in one or two hosts (demicyclic and auto- or heteroecious, respectively) and produce all five spore stages in two alternating hosts (macrocyclic and heteroecious). The usual spore cycle and ploidy level sequence (in parenthesis) is: basidiospores (N), pycniospores (N), aeciospores (N+N), urediospores (N+N) and teliospores (N+N, karyogamy-> 2N, meiosis->N) (Littlefield & Heath, 1979). The loss of a part of the life cycle is a common event in rusts and it is thought to have occurred repeatedly and independently (Ono, 2002). Actually, it has been hypothesized that short-cycling in rust fungi might be a strategy in northern latitudes to survive in the absence of alternate hosts and harsh conditions (Savile, 1953).

The intricate life history and complexity of its reproductive biology has motivated studies in different rusts affecting tree crops around the world. In Europe, rusts from the genus *Melamspora* sp that infect broad leaved plants have been

studied. *Melamspora epitea* infects *Salix* spp. during its uredinial stage and larch (*Larix* spp.) during its aecial stage, with highly sexual and diverse populations and poor geographic differentiation in Sweden because of the well documented long distance migration of urediniospores (B. Samils et al., 2001). Likewise, *Melampsora larici-populina* grows on larch during its aecial stage, but in poplar (*Populus* spp) during the uredinial stage. Recently, a study across hierarchical levels, time points and wild/managed stands in *M. larici-populina* in the uredinial host showed that the fungus tends to be more clonal and less diverse at the leaf level, at the start of the epidemic and in the managed stand than in the site level, at the end of the epidemic and in the wild stand, where diversity is at its highest (Barres et al., 2012).

Similarly, rusts genera causing diseases on coniferous trees such *Cronartium* sp, *Peridermium pini* and *Thekopsora* sp have been studied. *Cronartium ribicola, Cronartium flaccidum* and *P. pini* develop their aecial stage in *Pinus* spp and cause important diseases across the northern hemisphere (Samils et al., 2011). The diseases caused by these fungi have usually been managed with eradication of alternate hosts and resistance breeding (Hansen et al., 1997). However, it has been shown recently that *Cronartium* species have a wide spectrum of alternate hosts, with *C. flaccidum* being able to infect 25 different plant species distributed in 11 different families, which creates more challenges for the disease management (Kaitera et al., 2012). Interestingly, *Peridermium pini* has shown a different mode of reproduction in which apparent clonal reproduction that has been proved to occur between trees (van der Kamp, 1968).

1.3 Thekopsora areolata

Likewise, species of the genus *Thekopsora* appear to be macrocyclic and heteroecious, causing diseases in coniferous trees (Leppik, 1973), with a part of their life cycle in the family *Pinacea* and the other part on plants of the families *Ericaceae, Rubiaceae, Asteraceae, Boraginaceae* and *Rosaceae* (Hansen et al. 1997). This genus belongs to the division Basidiomycota, order Pucciniales and family Pucciniastraceae and was firstly described by Magnus in 1875 on *Prunus padus*, with *Thekopsora areolata* (Fr.) Magnus as the type species (Yang et al 2014).

Specifically, *T. areolata* has a two years long life/disease cycle, with all five different spore stages and host alternation between *Picea* spp. *and Prunus* spp. (Figure 2). The cycle has been described previously by Gäumann (1967) and

Kuporevich and Transhel (1957), but no recent studies have described its biology as detailed as many other rusts. So far it has been shown that in spring monokaryotic basidiospores infect *P. abies* female flowering cones, where mycelium grows through the axis of the developing cone and forms pycnia composed of several flexuous hyphae and pycniospores on the outer surface of scales (Kuporevich & Transhel, 1957). Generally, rust pycniospores and flexuous hyphae fuse and develop into heterokaryotic mycelium undergoing dicaryotization(Littlefield & Heath, 1979). The resulting heterokaryon will in turn form aecia, globoid structures composed of a thick walled peridium that bear inside chains of heterokaryotic aeciospores (Kuporevich & Transhel, 1957). Aecia usually crowd the adaxial and often the abaxial surface of the majority of scales in a cone within months, but only break and release spores in spring next year (Juha Kaitera & Tillman-Sutela, 2014). These aeciospores infect *Prunus* leaves, where uredia bearing heterokaryotic urediniospores are formed. Urediniospores can spread and re-infect *Prunus* leaves during the same season until autumn. Thereafter, the fungus overwinters in fallen *Prunus* leaves and develops telia and teliospores. Basidia and basidiospores will germinate from the overwintering teliospores as the result of karyogamy and meiosis, and disseminate in spring to infect *Picea* again (Gäumann 1959; Littlefield & Heath 1979;Kaitera & Tillman-Sutela, 2014; Kuporevich & Transhel, 1957).

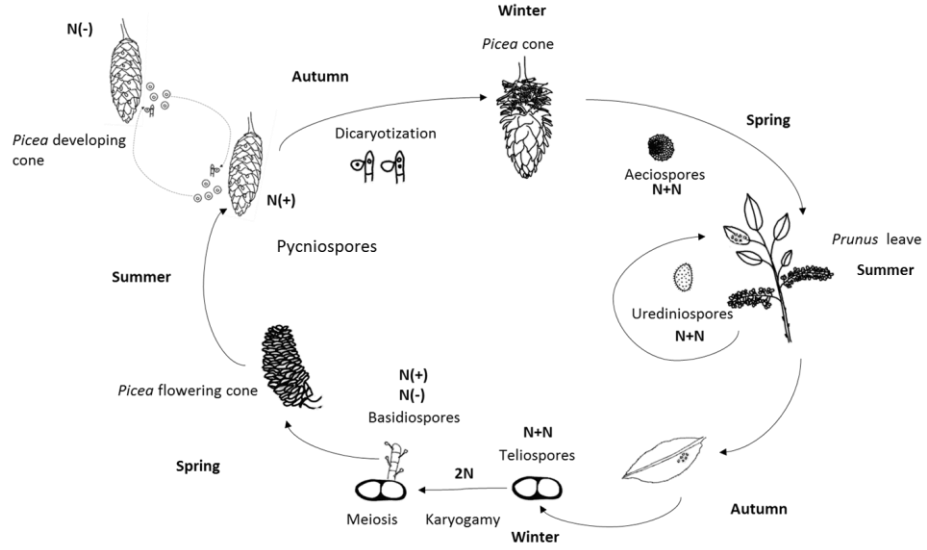

Figure 2. Life cycle of *T. areolata.* Basidiospores of different mating types (N+; N-) infect young *P. abies* cones. Pycniospores (N+, N-) and flexuous hyphae of different mating types mate and go through dicaryotization. Aeciospores (N+N) develop on spruce cones and infect *Prunus* leaves. Urediniospores (N+N) are produced on *Prunus*, where re-infections can take place. Teliospores develop on fallen leaves and go through karyogamy and meiosis (N+N -> 2N -> N) to give place to basidiospores (N+; N-) that will infect spruce again.

Lately, *T. areolata* has gained special attention because its growth on *Picea* cones harms seeds significantly and reduces their viability (Juha Kaitera & Tillman-Sutela, 2014). Actually seed production in Finland -especially in 2006 and 2009, has been considerably damaged (Juha Kaitera, 2013). This has motivated a diverse set of studies in recent years that have brought suggestions for management of the disease in orchards and formulated new hypothesis on the fungus' mode of reproduction. Cone bagging has prevented *T. areolata* infections in the majority of cases, but also affected development of the seeds, since bagging hinders pollen to enter the cone (Kaitera et al., 2009). Noteworthy, it has been proposed that infections in cones can appear without the need of basidiospores, since in recent studies in Finland no pycnia were found in cones nor basidiospores on fallen *Prunus* leaves (Kaitera et al., 2009). Based upon this, Kaitera and collaborators have hypothesized that aeciospores from old infected cones that remain in the trees can re-infect flowers or developing young cones (J. Kaitera et al., 2014; Juha Kaitera, 2013; Juha Kaitera & Tillman-Sutela, 2014) Consequently, it has been suggested to remove all infected cones from orchards to improve the health and quality of seeds and reduce the risk of outbreaks. This has been supported by aeciospore germination experiments that have shown how aeciospores remain viable even after 3 years in stands (Kaitera & Tillman-Sutela, 2014).

Also, *T. areolata* has been proved to infect different species of the genera *Picea* spp. and different varieties and cultivars of Russian and Finish *Prunus* trees, but not exotic ones (Juha Kaitera & Tillman-Sutela, 2014). Interestingly it has been shown that young leaf-bearing shoots of spruce can also be infected and in some cases show pycnia and aecia on them, however this occurs more seldom than in cones (Hietala et al., 2008; Kuporevich & Transhel, 1957). Apparently, aeciospores can also germinate and form mycelium on spruce shoots but no formation of other structures has been reported recently (Kuporevich & Transhel, 1957).

2 Materials and Methods

2.1 Cone collection and sampling

To investigate the population genetics and reproductive biology of *T. areolata*, a hierarchical sampling of single aecia was performed across different levels and at eight different locations in Sweden, as well as in one location in Norway and two in Finland (Table 1 and Figure 3). At each location at least 30 Norway spruce cones were collected, one scale per cone was randomly selected and one aecium per scale was randomly sampled. At the plot level, 25 cones were sampled from 4 different plots in Ålbrunna, Sweden (I ca. 100 m from II, II ca. 250m from III, III ca. 250m from IV, IV ca. 400m from I; fig. 3b) where one scale per cone was randomly selected and one aecium per scale was randomly sampled. At the cone level, 5 cones from Ålbrunna (Sweden), 4 cones from Bredinge (Sweden) and 1 cone from Rörby (Sweden) were randomly selected and split longitudinally to select 10 scales across each cone, from which one aecium per scale was randomly sampled. At the scale level, one scale per each of the aforementioned cones was selected and 5 aecia per scale were randomly sampled. All cones, scales and aecia were kept at -20°C.

Table 1. *Cone collection and sampling strategy across hierarchical levels: location, plot, cone and scale*

Hierarchical level											
Country	Location (1 aecium /cone)				Plot (1 aecium/cone)			Cone (1 aecia/10 scale/cone)		Scale (5 aecia/1 scale/cone)	
		Year	Cones	Aecia	Name	Cones	Aecia	Cones	Aecia	Cones	Aecia
Sweden	Ålbrunna	2015	30	30				5	50	5	25
					Ålbrunna I	25	25				
					Ålbrunna II	25	25				
					Ålbrunna III	25	25				
					Ålbrunna IV	25	25				
Sweden	Bredinge	2016	30	30				4	40	4	20
Sweden	Rörby	2016	30	30				1	10	1	5
Sweden	Söregärde	2016	30	30							
Sweden	Domsjöänget	2016	30	30							
Norway	Ås	2016	30	30							
Finland	Muhos	2009	30	30							
Finland	Ahvenlampi	2006	30	30							
	Total		240	240		100	100	10	100	10	50

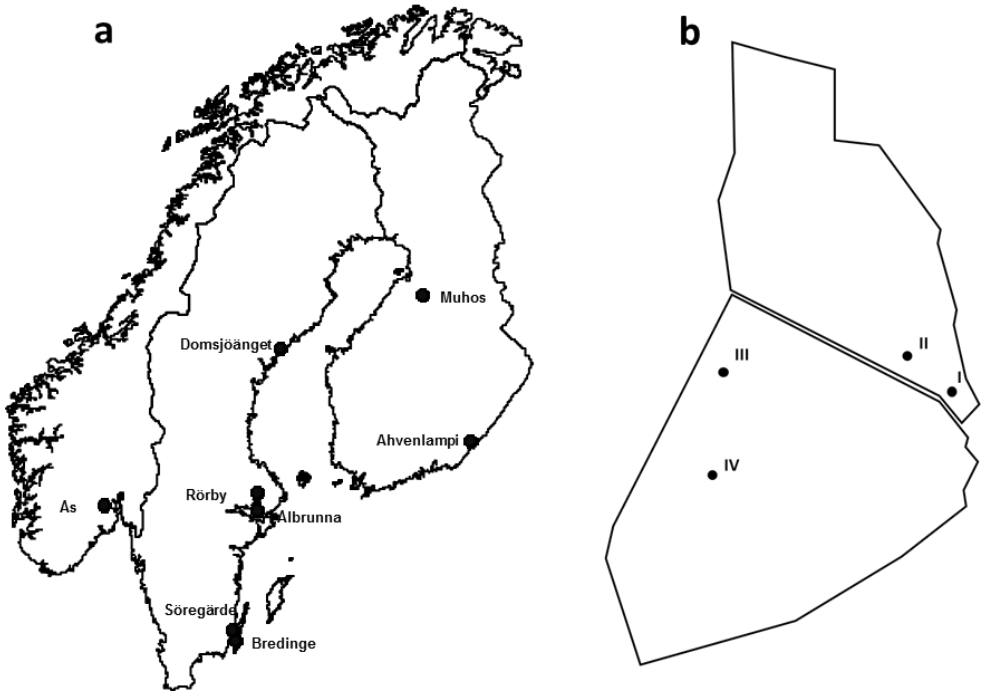

Figure 3. (a) Cone collection sites. Cones were collected or gently provided from 8 sites: Ålbrunna (Uppland, Sweden), Bredinge (Öland, Sweden), Söregärde (Småland, Sweden), Domsjöänget (Västerbotten, Sweden), Rörby (Uppland, Sweden), Ås (Akerhus, Norway), Muhos (Oulu, Finland) and Ahvelampi (South Karelia, Finland). (b) Location of plots within Ålbrunna.

2.2 Microsatellite primer design and primer testing

Bacterial colonies containing DNA fragments with putative microsatellites isolated with FIASCO (Zane et al. 2002; see supplementary material: report) were Sanger sequenced (Macrogen). Sequences were aligned with SeqMan Pro (DNASTAR), and primers were designed from the flanking region of the sequences repeats using PRIMER3 (Untergasser et al. 2012). These primers were tested in DNA extracted from single aecia (see above) using Inhibitor Resistant Genotyping PCR Readymix (PIR00, SIGMA) with the following PCR conditions: 95°C for 5 min, 35 cycles of 94°C for 30 sec, 60°C for 30 sec, 72°C for 30 sec and a final extension at 72°C for 7 min. Primer pairs with an efficient amplification were tagged with FAM or HEX on one primer of each pair (TAG Copenhagen) and 3 multiplex combinations were arranged based on fragment size and fluorescent tag (Table 2).

2.3 Genotyping

450 DNA extractions were performed, and each extract was amplified with 3 multiplex primer combinations (Table 2) with the same conditions aforementioned. PCR products were diluted 1:20 and analysed at SciLifeLab (Uppsala, Sweden) by capillary electrophoresis on ABI3730XL DNA Analyzer (Applied Biosystems), and peaks were scored using GeneMarker V2.7.0 (SoftGenetics).

Table 2. *Description of 9 microsatellites designed from FIASCO libraries*

Name	Sequence (5'-3')		Motif	Dye	Multiplex combination
Tha9	F	AAGGCAGATGACAGTCGTGA	AC	FAM	A
	R	TCCTCTGTCCAAAGCGTCTT			
Tha61	F	TGGGTAATTTGGGGTGTTTGT	AC	FAM	C
	R	ACAGAAGTTACTCCGCCCTT			
Tha91	F	GTCTGTGTCTCTGGTGTCGA	AG	FAM	C
	R	ACCAAAGTTCCCTGATATCCC			
Tha92	F	TTCTCGGGAATGGTGTGGAA	AACAAT	FAM	B
	R	CCCCACAAATCTTACGAGCTG			
Tha96	F	ATCACAACGCCTGATGG	AAG	FAM	A
	R	GCTCACAACATTCGCAATCC			
Tha105	F	GCCGATTCTCAAACCTACACC	AG	FAM	B
	R	TGCTGCCAACTTTTCACGTT			
Tha109	F	TGAAGTTCTACATGCACCGG	AC	HEX	A
	R	TTTGTCGTGGGATGCAGAAC			
Tha136	F	CAAGCACAACCTTCACCACA	AG	HEX	C
	R	TGGGTCATCAGCTTTACGGA			
Tha137	F	AAAGGGTTTTCAGGAGGGGC	AG	HEX	B
	F	CCAGTGCAAATCAAACGTCC			

2.4 Data analysis

A dataset for all samples containing the allelic size for each locus was created in Microsoft Excel following the GenAlEx data format (Peakall & Smouse, 2006). For each analysis, data was filtered and corrected using the population genetic analysis software POPPR 2.3.0 (Kamvar et al., 2014). Populations were set at each hierarchical level, and non-relevant populations were excluded together with uninformative loci (Figure 4). Thereafter a threshold for allowed missing data was defined for each analysis (25% - 0%), and samples above that threshold were excluded.

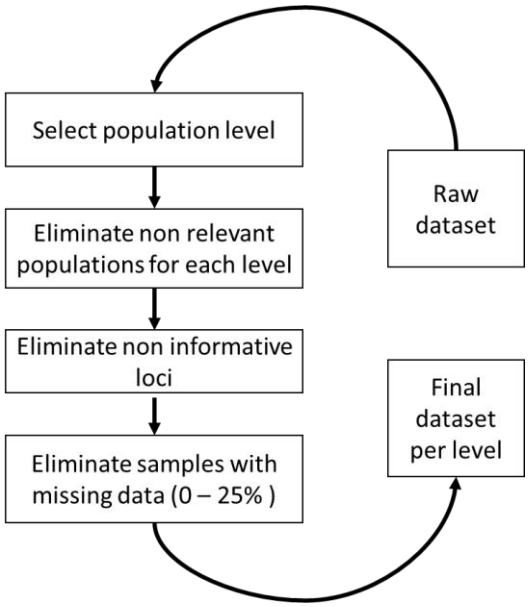

Figure 4. Data analysis. Dataset was filtered and organized according to each hierarchical level

To estimate the power of the microsatellites to distinguish multi locus genotypes (MLGs) in hierarchical levels, genotype accumulation curves were plotted using POPPR. In this analysis random loci were sampled from 1 to n-1 (n=number of informative loci) in the horizontal axis 999 times, and the number of MLGs observed on each permutation are plotted per column (Kamvar et al., 2015). Additionally, each locus was tested for the probability for being under Hardy-Weinberg equilibrium (HWE) with a X^2 test with POPPR.

Genetic distance among and within locations, plots, cones, scales and samples was tested with Analysis of Molecular Variance (AMOVA) using POPPR. Differentiation is calculated based on a Euclidean distance matrix at each level and variation within samples can be interpreted as heterozygosity, because it measures differentiation between the alleles between each loci across each sample, P values were computed based on 999 permutations (Excoffier et al., 1992; Kamvar et al., 2014, 2015).

Additionally, Discriminant Analysis of Principal Components (DAPC) was tested using POPPR between countries and locations. In DAPC the ability of identify genetic structure form a Principal Component Analysis (PCA) is combined with a Discriminant Analysis (DA) that accounts variability between previously defined populations more than variability within them.

Genotypic diversity was estimated as the number of multi locus genotypes (MLGs) per population in all hierarchical levels using POPPR. To test the reliability of identical MLG, the probability of finding the same MLG more than once by chance (Psex) at each location was calculated across different hierarchical levels using POPPR. Psex was calculated based on Pgen, which is the probability of a given genotype occurring assuming Hardy-Weinberg equilibrium.

Also, the Index of Association (Ia) was calculated at all hierarchical levels with POPPR and P values for Ia (P.Ia) were computed based on 999 permutations.

3 Results

3.1 Single T. areolata aecium were genotyped with sufficient microsatellite markers

To develop polymorphic and codominant microsatellites, colonies cloned with fragments containing repeats (FIASCO method (Zane et al., 2002)) were sequenced. Out of 288 colonies sequenced, 20 primer pairs were designed and 9 microsatellites were selected based on amplification of bands from DNA samples. The allele numbers varied per loci, location and hierarchical level (Table 3). Locus Tha109 was monomorphic for all locations and levels and therefore considered uninformative. In genotype accumulation curves across the levels the number loci analysed were sufficient to observe the total number of MLGs (Figure 5). Most of the polymorphic microsatellite loci (5 out of 8) were on HWE, while number of loci deviating from it increased from the scale level to the location level (Figure 6).

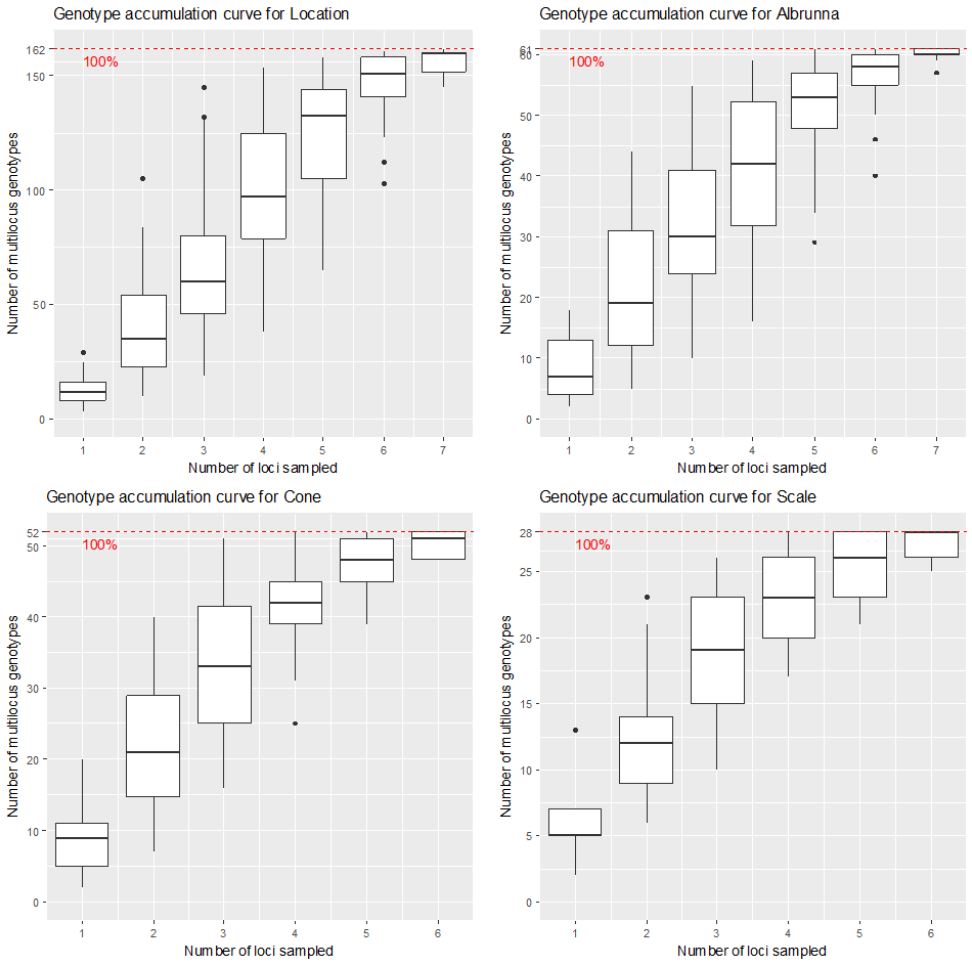

Figure 5. Genotype accumulation curve across all hierarchical levels.

After the dataset was filtered and corrected, 22 - 36% of the individuals were removed due to the number of missing data above the threshold. The missing data was spread across levels and locations and was mainly due to difficulties with the non-uniformity of the PCR plates and the amount and quality of DNA, even though a polymerase resistant to inhibitors was employed.

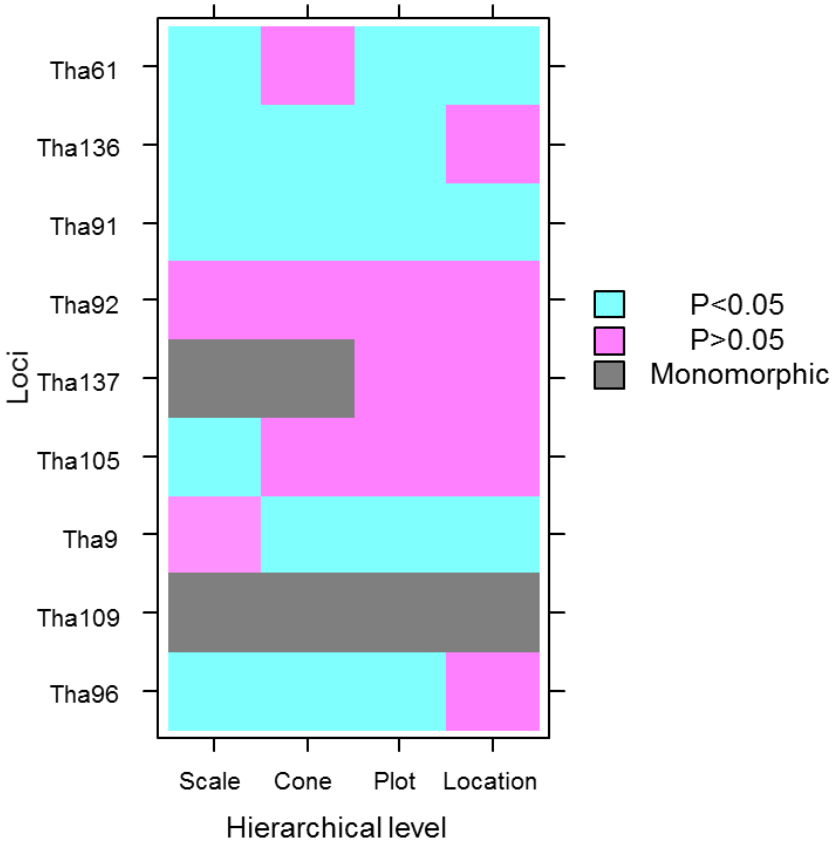

Figure 6. Hardy-Weinberg equilibrium per loci across different hierarchical levels. Loci coloured in pink are suspected to not be under Hardy-Weinberg equilibrium (P>0.05), while loci in cyan are suspected to be (P<0.05). Loci in grey are fixed (monomorphic) at that level.

Table 3. *Allelic variation through different locations. Tha109 was monomorphic at all levels and locations.*

Locus	**Location**							
	Ålbrunna	**Bredinge**	**Rörby**	**Söregärde**	**Domsjöänget**	**Ås**	**Muhos**	**Ahvenlampi**
Tha96	8	6	4	10	6	6	6	4
Tha9	2	2	3	2	3	2	3	2
Tha109	1	1	1	1	1	1	1	1
Tha105	8	6	6	6	7	7	7	5
Tha137	3	2	4	3	1	2	2	1
Tha92	2	2	1	2	1	2	1	2
Tha91	5	4	4	6	5	5	4	5
Tha136	4	5	5	4	6	6	5	2
Tha61	2	2	2	3	1	2	4	1

3.2 There is no genetic population structure among locations

To investigate the structure of *T. areolata* populations at country and location level, AMOVA and DAPC tests were performed. The results of AMOVA (Table 4) indicate that there is not a significant difference between countries or locations (P>0.05), whereas most of the variation is explained by variation among samples within locations. This is supported by the results of DAPC (Figure 8), where samples from different locations and countries overlap each other with a small separation

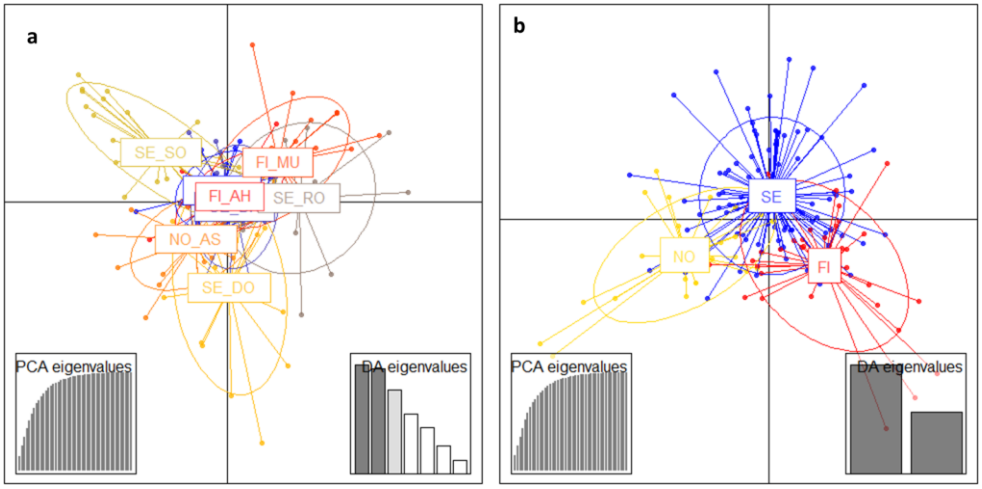

Figure 7. Discriminant Analysis of Principal Components (DAPC) between locations (a) and countries (b). Locations and countries are coloured differently and abbreviated. PCA and DA eigenvalues selected for analysis are shown in the lower corners in grey. SE= Sweden, NO= Norway, FI= Finland, SE_AL=Ålbrunna, SE_BR =Bredinge, SE_RO = Rörby, SE_SO = Söregärde, SE_DO = Domsöänjet, NO_AS = Ås, FI_MU = Muhos, FI_AH = Ahvelampi.

Similarly, no significant differences were found between plots within Ålbrunna with AMOVA, and most of the variability was explained by differences between samples. The case was the same for comparisons between cones and scales, where most of the variability was explained within and not between them. Across all the hierarchical levels the greatest fraction of variation was explained by differences within samples, which indicates difference between alleles (ergo, heterozygosity) within each sample.

Table 4. *AMOVA test for each hierarchical level. Country and location levels were run together, and the percentage of variance explained at each level within and between populations is shown together with the P value based on 999 permutations.*

Hierarchical level	**Variance (%)**	**P value**
Between countries	0.159	0.641
Between locations, within country	0.310	0.237
Between samples, within location	19.000	0.001**
Within samples	80.516	0.001**
Between plots	-0.103	0.535
Between samples, within plot	21.605	0.001**
Within samples	78.490	0.001**
Between cones, within location	-1.470	0.805
Between samples, within cone	19.911	0.001**
Within samples	79.993	0.001**
Between scales, within location	-5.471	0.983
Between samples, within scale	14.095	0.018
Within samples	88.221	0.036

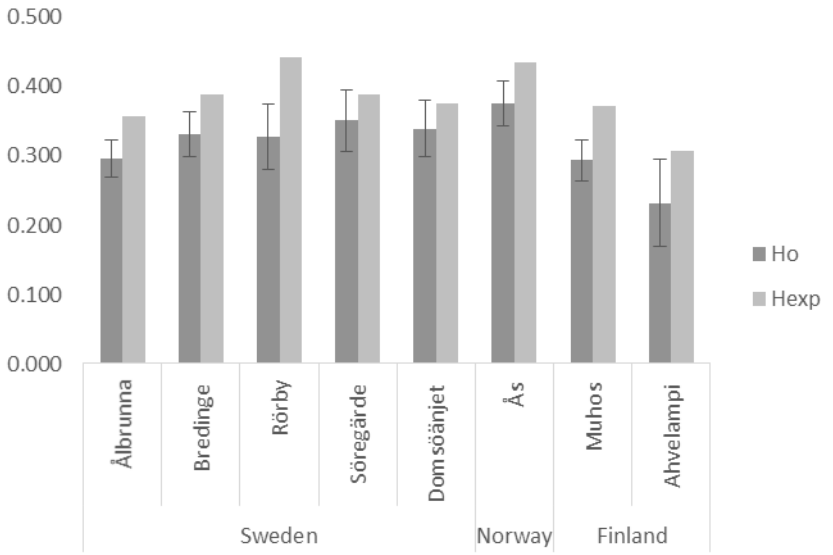

Figure 8. Expected heterozygocity (Hexp) and observed heterozygocity (Ho) across different locations. Ho is the mean heterozygocity across loci per each sample and error bars indicate standard error.

3.3 Thekopsora areolata seems to reproduce sexually across most hierarchical levels with a mixed mode of reproduction in cones

To estimate the mode of reproduction of *T. areolata* and the probability of random mating between each hierarchical level, Ia was calculated and the probability of observing its value was estimated with 999 permutations (Table 7). At country, location and plot level, the hypothesis of random mating and no linkage between markers was accepted, indicating common sexual events. Contrastingly, the null hypothesis was rejected ($P<0.01$) in the cone, which indicates linkage disequilibrium and non-random mating in this level. This was also the case for two individual cones (AL_37 and BR_231) and in one scale (AL_69_1).

Genotypic diversity was high across all hierarchical levels, since most of the samples had unique genotypes (Table 5). Interestingly, identical MLGs were found within and across locations, plots, cones and scales. Psex was used to test the probability of those MLGs being true clonemates (i.e. identical genotypes; $P<0.05$), rather than just identical MLGs by chance (through sexual recombination; $P>0.05$) at the investigated microsatellite loci (Figure 8). At the location level, MLGs 278 and 221 were found across and within locations, but they do not seem to be clonemates ($Psex>0.01$). At the plot level, MLG 221 was found between plots, and a high probability of being found by chance. At the cone level 5 MLGs were found more than once across different cones, of which only 2 MLGs seem to be true clonemates ($Psex<0.01$). MLG 67 was found 3 times in Bredinge (twice in cone 231 and once in cone 285) with a considerably low Psex ($P<0,0001$), while MLG 112 was found four times in different cones in Ålbrunna and two times in cone 202 in Bredinge. At the scale level, 4 MLGs were found across scales, but not within the same scale. MLG 262 and 129 where both found in two different cones in Bredinge. Additionally, MLGs were studied in detail and samples homozygous for all loci were marked with triangles. Interestingly, some of those homozygous samples have low probability of being found by chance.

Table 5. *Genotypic diversity (No. samples – No. MLG), expected heterozygosity (Hexp) and index of association across five different hierarchical levels. Genotypic diversity is explained as the number of samples in relation to the MLG.*

Hierarchical level	No. Samples	No. MLG	Hexp	Ia	p.Ia

Country					
Sweden	102	101	0.389	-0.094	0.957
Norway	25	25	0.434	0.210	0.074
Finland	38	37	0.357	0.047	0.415
Total	165	162	0.390	-0.021	0.807
Location					
Ålbrunna (S)	25	24	0.356	0.146	0.222
Bredinge (S)	23	23	0.389	-0.065	0.734
Rörby(S)	15	15	0.443	-0.099	0.576
Söregärde (S)	22	22	0.388	-0.146	0.868
Domsöänjet (S)	17	17	0.375	-0.192	0.85
Ås (N)	25	25	0.434	0.210	0.059
Muhos (F)	24	24	0.371	0.069	0.343
Ahvelampi (F)	14	13	0.308	0.013	0.485
Total	165	162	0.390	-0.021	0.777
Plot					
AL_1	24	24	0.386	0.150	0.24
AL_2	7	7	0.361	0.612	0.08
AL_3	20	20	0.376	-0.074	0.642
AL_4	11	11	0.349	-0.037	0.519
Total	62	61	0.376	0.016	0.523
Cone					
AL_6	7	7	0.391	0.183	0.246
AL_37	6	6	0.492	1.158	0.006**
AL_46	7	6	0.392	0.155	0.296
AL_69	7	7	0.337	-0.078	0.598
AL_89	5	5	0.436	-0.395	0.791
BR_202	2	1	0.190	NA	7
BR_213	8	8	0.394	0.042	0.37
BR_231	7	6	0.419	0.560	0.042**
BR_285	9	9	0.442	0.144	0.245
RO_417	6	6	0.374	-0.393	0.843
Total	64	52	0.409	0.259	0.004**
Scale					
AL_6_1	3	3	0.533	0.167	0.477
AL_37_1	3	3	0.505	0.583	0.266
AL_46_1	3	3	0.429	0.000	0.454
AL_69_1	3	3	0.405	2.000	0.035**
AL_89_1	5	5	0.432	-0.778	0.993

BR_202_1	4	4	0.474	0.023	0.419
BR_213_1	2	2	0.286	NA	NA
BR_231_1	3	3	0.438	-0.200	0.361
BR_285_1	4	4	0.413	0.657	0.129
RO_417_1	2	2	0.238	NA	NA
Total	32	28	0.430	0.084	0.177

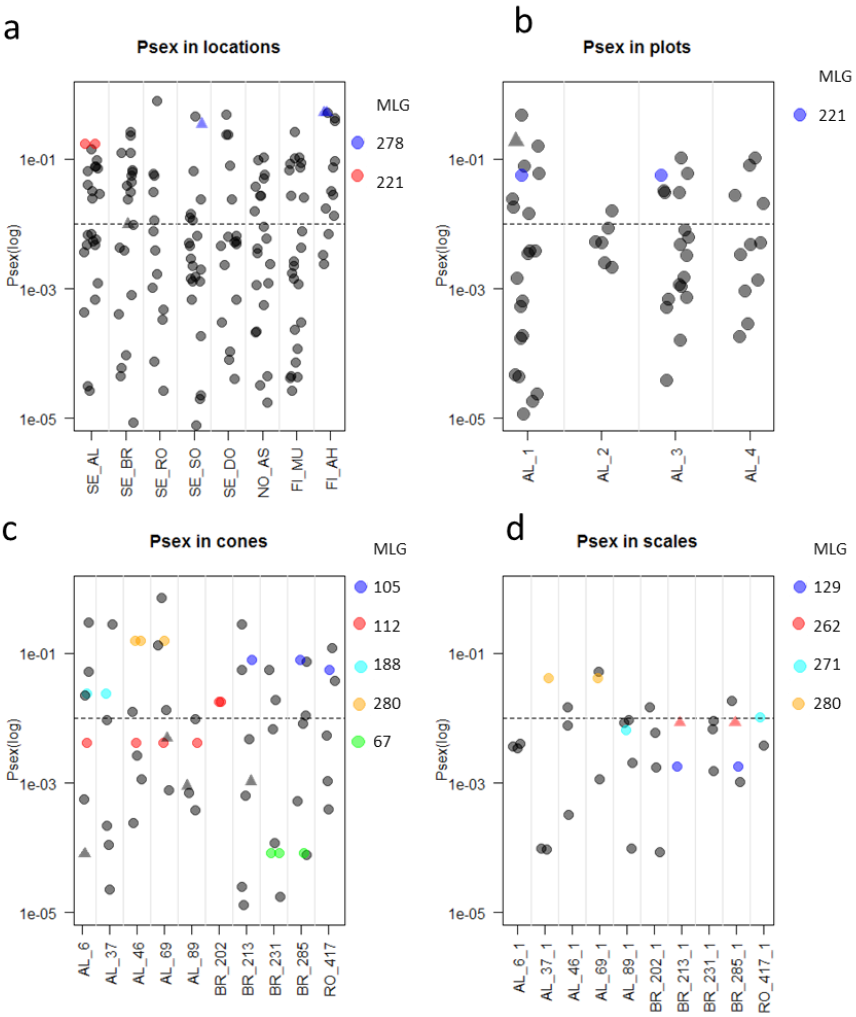

Figure 9. Psex on samples across hierarchical levels and between (a) locations, (b) plots, (c) cones and (d) scales. Triangles (▲) stand for samples homozygous for all loci, while circles (●) stand for heterozygous samples for at least one loci. Samples above the dashed line (Psex>0.01) are more likely to be found more than once by chance within its location. Samples are aligned in the X axis according to each location, plot, cone and scale. Colours correspond to MLGs that are found more than once within or between each hierarchical level.

4 Discussion

This thesis project described the genotypic diversity of *T.areolata* in *P. abies* seed orchards and highlighted its complex mode of reproduction. Moreover, it confirmed the usefulness of microsatellite markers and hierarchical sampling to study the population genetics of non-model pathogenic fungi and set the stage for new hypothesis that can lead novel management strategies in seed orchards.

4.1 There is high genotypic diversity and no genetic differentiation of *T. areolata* samples across levels and among different seed orchards in Sweden

The most obvious finding was that across all hierarchical levels and among locations genotypic diversity was high and no genetic differentiation was observed. This result is consistent with the high genotypic diversity and apparent random mating found in this study, which altogether suggest a constant gene flow and common sexual reproduction of *T. areolata* in Sweden. A constant gene flow over large distances is not surprising, given that rust uredinospores are robust and disperse over long distances- even across continents (Brown, 2002). One example of this phenomena is *Melampsora epitea* which is highly diverse with small geographic differentiation in Sweden due to the constant flow of urediniospores (Samils et al., 2001). Likewise, samples of *M. larici-populina* from European poplars have low genetic differentiation between them and common sexual reproduction events inferred by no linkage between markers (Barres et al., 2008).The results of this study are further supported by the widespread presence of *Prunus* in northern Europe that not only increases the chances of long dispersed spores to survive by landing in a susceptible host, but also augments genotypic

diversity, since sexual reproduction and recombination takes place on *Prunus* leaves.

The role of sexual reproduction in *T. areolata* appears to be important given the results obtained in this study. Firstly, genotypic diversity was high across all levels and identical MLGs with a high probability of being true clonmates were rare within locations and plot. Furthermore, the hypothesis of random mating and no linkage between markers was accepted significantly in countries, locations, and plots. In rusts, similar evidence has been found on populations known to be reproducing sexually (Barres et al., 2012), where sexually derived basidiospores with unique genotypes are the main drivers of genotypic diversity, but also responsible for the observed linkage equilibrium(Rodriguez-Algaba et al., 2014). The high linkage equilibrium across levels strengthens the hypothesis of sexual reproduction playing a crucial role in reproduction of *T. areolata* and is in concordance with the observed high genotypic diversity. Contrastingly, observed heterozygocity was lower than expected heterozygocity at each location which is not common in sexually reproducing populations. This inconsistency may be explained by random drift, since a decrease in heterozygosity relative to the expected heterozygocity assuming Hardy-Weinberg equilibrium is the main effect of subpopulation formation. This would indicate that the locations analysed could belong together to one subpopulation that is randomly mating, but possibly isolated from other subpopulations (Hartl & Clark, 1997). Another likely explanation for this could be related to the nature of the technique, since not optimal primers with variation at the binding site can lead to low heterozygosity (Guichoux et al., 2011).

Even though it is somehow surprising that genotypic diversity was high in scales - where the distance between samples is shortest, the case is similar in other rusts growing on different trees. In *Cronartium*, most aecia sampled within lesions has different MLGs (Berit Samils et al., 2011) and in *P. graminis*, *P. coronata* and *P. striitiformis* diversity was also high within lesions (or aecial clusters) in *Berberis* (Berlin et al., 2017; Rodriguez-Algaba et al., 2017). It has been hypothesised that the high variability is due to single different cross-fertilization events (fusion of pycniospores and flexuous hyphae, or dicaryotization) from the same pycnia and/or adjoining ones. Berlin and collaborators (2017) showed that MLGs at each pycnidial cluster share at least one haplotype. This strengthens the hypothesis of aecium formation due to single cross-fertilizations between different flexuous hyphae from the same pycnia (identical haploid genotype) and different pycniospores (different haploid genotypes), resulting in different MLG within the same aecial cluster (Berlin et al., 2017). However, this study showed that even within scales and cones,

aecia share more than one common haplotype, what implies directly the presence of more than one different pycnia at each scale and cone.

4.2 *Thekopsora areolata* seems to have a mixed mode of reproduction in *P. abies* cones.

At the cone level genotypic diversity was lower with identical MLGs found with a high probability of being true clonemates and the random mating hypothesis was rejected (P.Ia<0.05). This suggests clonal reproduction at the cone level, which could be explained by a few scenarios summarized below.

4.2.1 Markers are not sufficient to differentiate between very similar, but non-identical samples.

When estimating clonality, a common problem can arise when low number of markers or limited polymorphisms prevent the discrimination of MLGs that are different but appear to be identical (Guichoux et al., 2011). Though this scenario could be feasible, the genotype accumulation curves show that the set of microsatellite markers is sufficient to determine the number of genotypes in the sample.

4.2.2 Self-fertilization of identical pycniospores and flexuous hyphae occurred more than once

In general it has been assumed that cross-fertilization is needed for dicaryotization and that insects play a key role on the transport of pycniospores to cross-fertilize flexuous hyphae (Naef et al., 2002). This has been supported by experiments in rusts, where insects were attracted by the honeydew matrix (in which the pycniospores are embedded) and were required for the formation of the dikaryon (Pfunder & Roy, 2000). Additionally, it has been shown that either presence of insects or manual cross-fertilization among different pycnia resulted in successful aecia formation, while caged and self-fertilized pycnia formed a significantly lower number of aecia (Pfunder & Roy, 2000). From a genetic standpoint, the easiest way to look for self-fertilization events would be to look at duplication of the same

haplotype in the dikaryon, what would result in homozygote MLGs. Interestingly, this study showed the presence of MLG homozygous for all loci across all hierarchal levels, where identical homozygous MLG were found among different locations with a high Psex value (high probability of being identical by chance) and at the scale level in different scales with a Psex value just below the threshold. These results further support the hypothesis of rust fungi being able to self-fertilize themselves, as shown in *Peridermium pini*, where lesions are composed of identical homozygote aecia, with reported appearance of pycnia (Samils et al., 2011).

4.2.3 More than one systemic infection take place in the same cone

In the book *"Cryptogamic Plants of the USSR"* Kuporevich and Transhel (1957) give evidence of a systemic infection in cones, where mycelium grows through the axis (Kuporevich & Transhel, 1957). This find is in line with the results obtained in this study, and is the most likely explanation for rejection of random mating at the cone level since the same haplotype would be spread through the cone forming identical pycnia hypothetically in different scales. However, it remains strange to find the same heterozygote MLG within and among cones in the same location. This specific observation would confirm what has been hypothesized repeatedly by Kaitera and collaborators in Finland (J. Kaitera et al., 2014; Juha Kaitera, 2013; Juha Kaitera & Tillman-Sutela, 2014), since the only feasible explanation (besides low resolution of microsatellites, see above) would be that identical heterokaryotic aeciospores infect different cones systematically and result in the formation of identical aecia in different scales within and among cones. Actually, it has been reported that aeciospores can germinate in young shoots without forming other structures (Kuporevich & Transhel, 1957), but this seems to be a rare finding since experimental infections have been tested in Sweden without success (personal communication) and the biological mechanisms by which aeciospores could colonize cones remain unknown. However there is evidence of colonization of other tissues by *T. areolata* as reported by Hietala and collaborators (2008) who found the fungus in 100 *P. abies* symptomatic young seedlings by quantitative PCR and aecia growing in one of the leading shoots of the reference material (Hietala et al., 2008). Much biological and genetic evidence is still needed to fully understand the mode of reproduction of this fungus in *P. abies*.

4.3 What disease management strategies can be used considering these results?

A first general consequence of random mating of *T. areolata* over hundreds of kilometres for management of the disease is that the presence of *Prunus* trees is important in the disease cycle. However, studies aimed to confirm the influence of *Prunus* trees near to seed orchard together with the ability of basidiospores to travel long distances would give a better evidence to decide whether *Prunus* eradication near orchards is a feasible option.

Control of insect pests has been suggested as a mandatory management strategy in seed orchards (Haappanen et al., 2015). The present study showed that at the cone level most of the MLG originated from cross fertilization events of pycnia, hypothetically carried out by insects. This finding would support the control of insects in seed orchards to also prevent cross fertilization of pycniospores and flexuous hyphae. However, the relationship between insects and *T. areolata* needs further support and should be tested in the future.

In the literature, it has been suggested that removal of all the cones bearing old infections could be a good management strategy (Kaitera & Tillman-Sutela, 2014). However, another consequence of the random mating over hundreds of kilometres is that removal of cones is worthless, since fungal spores from non-managed fields can still travel long distances.

5 Conclusions

The main objective of this study was to investigate the population genetics and reproductive biology of the rust fungus *T.areolata* in Swedish seed orchards. The analysis of microsatellite data, together with a hierarchical sampling showed apparent random mating and common sexual events. Moreover, it showed that the fungus in the locations sampled seem to belong to the same population with not obvious genetic structure. Additionally, it highlighted the complex mode of reproduction of *T. areolata* in *P. abies* cones that appeared to be mixed, with evidence of probable clonality, self-fertilization and sexual reproduction. Overall, these findings show the high diversity and gene flow of *T. areolata* in Fennoscandinavia at all hierarchical levels and set the ground for the formulation of new studies aiming to confirm the clonal spread of *T. areolata* in seed orchards, or to answer more specific questions such as the exact biological mechanism by which *T. areolata* is reproducing clonally in *P. abies*, the role of insects in cross- and self-fertilization ability of pycnia, and the ability of the fungus to colonize branches or even trees systemically.

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