

Relationship between *Cronartium flaccidum*
and *Peridermium pini*

Risto Kasanen

Academic dissertation

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To Mia and Juho

Preface

This work started as a M.Sc. thesis at the University of Joensuu. I am still very thankful for Ari Pappinen for supervising my M.Sc. thesis with clear ideas of publishing my work in international peer-reviewed journals. In the summer of 1995, I was lucky to be chosen to the Graduate School of Biology and Biotechnology of Forest Trees. I also wish to thank my supervisor, prof. Kim von Weissenberg for encouraging me to keep the same topic and proceed with my work, now at the University of Helsinki. I also got a second supervisor from the Graduate School, Outi Savolainen from the University of Oulu. Thanks to Outi, I learned essential views of genetics, both during discussions and courses at the University of Oulu.

As I started with my thesis in Helsinki, I knew that Jarkko Hantula, together with Juha Kaitera and colleagues at the Finnish Forest Research Institute (FFRI), had studied almost the same topics. I met Jarkko, who was after short discussions convinced that we should start co-operation. He also kindly allowed me to use his laboratory facilities at Vantaa Research Center. I learned a lot. There is no doubt that Jarkko had more practical impact than any other person on my ideas about this thesis. Juha Kaitera's pioneering work had been a basement for many conclusions presented in this thesis.

In spring 1999 my Ph.D. work suddenly became a hobby. Professor Timo Kurkela and Jarkko Hantula had received funding from Metsäliitto Group, for studying pathogens of aspen. I was lucky to get the job, and a open-minded boss, Timo, who overlooked the fact that I will be writing my thesis also during the working hours.

I am sure this thesis has been so easy to put together because I have had priviledge to work with competent and nice people. I have made many friends during these years – both in the “old farm” of Viikki and Vantaa Research Center.

I am grateful to grants by Emil Aaltonen Foundation and The Finnish Society of Forest Science, since graduate school had no funding for laboratory work and traveling costs. A grant by Metsämiesten säätiö enabled the printing of this thesis. The reviewers of my thesis made several valuable comments on the manuscript.

I want to acknowledge my close relatives and friends who have supported me in so many ways. Escpecially I want to thank my wife Mia, who has always been sure I will make it.

Abstract

The resin top disease of Scots pine is caused by rust fungi that have been classified to two species, *C. flaccidum*, which is host-alternating and *P. pini*, which is capable of infecting only pine. Although several studies have been made, no clear differences (in addition to host specificity) between these fungi has been reported. The aim of this study was to elucidate the relationship between the two fungi. Variation of morphological and molecular data at several hierarchical levels of populations was studied in order to characterize the unknown sexual behaviour, and other differences within the *C. flaccidum*-*P. pini* complex.

The surface structure of aeciospores was analysed with scanning electron microscopy (SEM). Considering the variation of the surface warts on the individual spores, it was obvious that the morphology of the spores can not be used in identification of any of species studied (I). The haplotypes of *P. pini* were nonrandomly distributed in pine stands and the frequencies of haplotypes within and between stands were statistically different, which suggested asexual reproduction and some specific resistance interaction between pine and the fungus (II). The aecia within single cankers of *P. pini* were homogenous and homozygous. This indicated that single canker is caused by one haploid fungal individual. The cankers of *C. flaccidum* contained several genotypes and heterozygous aecia, which indicated mating of several haploid fungal strains within cankers. Thus, population fine structure of *C. flaccidum* was in accordance with a sexual life cycle (III). Mostly the same alleles of specific marker loci were observed in the two species. Although a low level of genetic differentiation occurred among the populations of both rust fungi, no overall differentiation between the two rusts occurred, and in this respect they resembled a single taxon. No genetic differentiation between *C. flaccidum* and *P. pini* was observed. The two fungi differed only by the existence of linkage disequilibrium within *P. pini* (IV).

The hypothesis was put forward that clonal *P. pini* strains have their origin as haploid life cycle mutants of *C. flaccidum*, that has a sexual life cycle. This hypothesis was in accordance with all the data available including the lack of morphological differentiation of the two fungi (I) haplotype distribution within stands (II), genetic fine structure of cankers (III), amount of genetic variation within populations (IV) and finally, the observation of linkage disequilibrium within *P. pini* populations (IV).

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Contents

LIST OF PUBLICATIONS.....	7
AUTHOR'S CONTRIBUTION.....	8
1. INTRODUCTION.....	9
1.1. <i>CRONARTIUM FLACCIDUM</i> AND <i>PERIDERMIIUM PINI</i>	9
1.1.1 <i>Resin top disease</i>	9
1.1.2 <i>Life cycle of Cronartium flaccidum</i>	9
1.1.3 <i>Life cycle of Peridermium pini</i>	10
1.2 SPECIES COMPLEX <i>C. FLACCIDUM-P. PINI</i> IS INADEQUATELY DESCRIBED.....	10
1.2.1 <i>Endocronartium or Peridermium ?</i>	10
1.2.2 <i>Relationship between C. flaccidum and P. pini is unknown</i>	12
1.3. POTENTIAL DIFFERENCES BETWEEN <i>C. FLACCIDUM</i> AND <i>P. PINI</i>	13
1.3.1 <i>Morphological characters</i>	13
1.3.2 <i>DNA markers and population structure</i>	13
1.3.2.1 <i>Analysis of pathogenic fungal populations</i>	13
1.3.2.2 <i>Structure of the known Cronartium populations</i>	14
1.3.2.3 <i>DNA markers in studies of C. flaccidum and P. pini</i>	15
2. AIMS OF THE STUDY.....	17
3.MATERIAL AND METHODS.....	18
3.1 FUNGAL SAMPLES.....	18
3.2 ANALYSIS OF FUNGAL SAMPLES.....	18
3.2.1 <i>DNA markers</i>	18
3.2.3 <i>Scanning electron microscopy of the spore surface</i>	19
4. RESULTS.....	21
4.1 POPULATION STRUCTURE OF <i>C. FLACCIDUM</i>	21
4.2 POPULATION STRUCTURE OF <i>P. PINI</i>	21
4.3 COMPARISON OF <i>C. FLACCIDUM</i> AND <i>P. PINI</i>	22
4.3.1 <i>Morphological characters</i>	22
4.3.2 <i>Genetic differentiation</i>	23
5. DISCUSSION.....	24
5.1 POPULATION STRUCTURE OF <i>C. FLACCIDUM</i> IS IN ACCORDANCE WITH SEXUAL LIFE CYCLE.....	24
5.2 POPULATION STRUCTURE OF <i>P. PINI</i>	25
5.2.1 <i>Population structure of P. pini suggests an asexual life cycle</i>	25
5.2.2 <i>Does meiosis occur during the life cycle of P. pini?</i>	26
5.3 MORPHOLOGICAL OR MOLECULAR EVIDENCE DOES NOT SUPPORT THE DIVISION OF <i>P. PINI</i> AND <i>C. FLACCIDUM</i> INTO TWO SPECIES.....	27
5.3.1 <i>Morphology</i>	27
5.3.2 <i>Molecular evidence</i>	28
5.4. HOW CAN THE CLOSE RELATIONSHIP OF <i>C. FLACCIDUM</i> AND <i>P. PINI</i> BE EXPLAINED ?.....	28
6. CONCLUSIONS.....	30
7. REFERENCES.....	31

List of Publications

This thesis is based on the following articles, which are referred to by their Roman numerals. The published papers are reprinted with the permission of the publishers.

- I** Kasanen, R. 1997. Aeciospores of *Cronartium flaccidum*, *Endocronartium pini* and *C. ribicola* show no differences in morphology. Eur. J. For. Path. 27:251-260.
- II** Pappinen, A., Kasanen, R., & Weissenberg, K. von. 1996. The identification of multiple infection of *Endocronartium pini* on Scots pine with PCR. Eur. J. For. Path. 26:183-191.
- III** Kasanen, R., Kaitera, J. & Hantula, J. 2000. The genetic composition of *Peridermium pini* and *Cronartium flaccidum* cankers on Scots pine as revealed by two multiallelic loci. Forest Pathology 30:221-231.
- IV** Hantula, J, Kasanen R, Kaitera J & Moricca S. Population genetics of *Cronartium flaccidum* and *Peridermium pini*. Submitted manuscript.

Author's contribution

The contribution of Risto Kasanen is presented here. The following text has been compiled from the documents where each of the authors' contribution to the above-mentioned publications were stated and signed.

The work presented in paper I was solely carried out by Risto Kasanen.

In paper II, Risto Kasanen participated planning of the experiment as a part of his M.Sc. thesis, collected the samples together with Ari Pappinen, analysed the samples in the laboratory, was responsible of statistical analysis of data and wrote the paper together with AP. Kim von Weissenberg and AP supervised the work.

In paper III, Risto Kasanen and Jarkko Hantula planned the experiment. The field sampling, laboratory work and data analyses were mainly carried out by Risto Kasanen. Risto Kasanen is responsible of writing the manuscript; JH and Juha Kaitera participated in the writing process and discussions of the results.

In paper IV Risto Kasanen provided DNA markers and Participated in guidance of technicians in analysis with the markers. Jarkko Hantula, Salvatore Moricca and Juha Kaitera were responsible of field collection of the samples. JH was responsible of statistical analysis as well as main lines of the manuscript. Risto Kasanen, SM and JK shared ideas of the manuscript and participated in the writing process. Risto Kasanen also participated in the analysis of the data, result interpretation and discussions.

1. Introduction

1.1. *Cronartium flaccidum* and *Peridermium pini*

1.1.1 Resin top disease

Dead tops or parts of otherwise green crowns of Scots pine caused by resin top disease are common in the Finnish landscape. Symptoms of the disease include lesion (canker) formation and resin flow on branches or stem. Canker formation usually kills branches or a top of a pine but if a canker is formed at the lower part of a stem, the whole tree can die. Two fungi cause the disease: the host-alternating fungus *Cronartium flaccidum* Alb. & Schw. and the pine-to-pine form, *Peridermium pini* (Pers.) Lev, syn. *Endocronartium pini* (Pers.) Y. Hiratsuka. In addition to host specificity, no clear differences between these fungi has been reported (see 1.2.2).

It has been estimated that 60% -20% of trees in single stands in Scandinavia may have resin-top damage, and the resin top disease has been shown to cause up to 7% loss of timber value (Kaitera et al. 1994). The pine-to-pine type *P. pini* was considered as the dominant type of resin top fungus in Northern Europe for decades, since the known alternate hosts of *C. flaccidum* were rare or absent. Although later studies have shown that *C. flaccidum* can also use common and widely spread *Melanpyrum* species as alternate hosts, most tested aeciospore sources have been autoecious *P. pini* (Kaitera and Hantula 1998, Kaitera et al.1999a).

In the past two-three decades *C. flaccidum* has caused epidemics on two-needle pines in the Mediterranean countries. The presence of pine hosts and the main alternate host *Vincetoxicum hirundinaria* Med. created conditions optimal for fungus spread (Raddi and Ragazzi 1982). Damages on plantations were so high that, in an attempt to stop the rapidly advancing epidemic of the pathogen, pine was replaced with other tree species on a number of forest sites (Raddi and Ragazzi 1982). Autoecious *P. pini* caused serious disease in the same period in Britain (Greig 1987).

1.1.2 Life cycle of *Cronartium flaccidum*

The hyphae of the resin top fungi overwinter in the pine bark. In early summer the aeciospores are released from aecia emerging in cankers on pine stems or branches. *C. flaccidum* is a macrocyclic rust fungus, which has a broad range of alternate hosts. Klebahn (1938) summarized infections reported on plant species in genera *Pedicularis*, *Paeonia*, *Vincetoxicum*, *Grammatocarpus*, *Impatiens*, *Loasa*, *Nemesia*, *Schicanthus*, *Tropaeolum* and *Verbena*. In addition to these, recent report by Kaitera and Hantula (1998) showed that *Melanpyrum sylvaticum* is probably one of the main telial hosts in Scandinavia.

The aeciospores of *C. flaccidum* infect the leaves of the alternate host. The uredia form within two weeks releasing urediospores. Several generations of urediospores can be formed, while urediospores infect leaves and new uredia emerge. Telial columns, containing the teliospores, develop 2-3 weeks after the aeciospore infection (Kaitera 1999). The meiosis is suggested to take place within these spores, which germinate immediately, each forming a basidium and four haploid basidiospores (Hiratsuka 1981). Basidiospores spread with wind and turbulences to pines where they germinate and infect. The mycelium kills cambium and a canker is formed. Spermagonia emerge in a canker during late summer. The margins of the spermagonia are suggested to function as receptive hyphae, which the spermatia fertilize. Accordingly, the fertilized hyphae in the canker form aecia, which contain dikaryotic aeciospores (Klebahn 1938).

It should be noted that the mechanism of spermatization has not been documented on *C. flaccidum*. Observations of spermatial crossings with receptive hyphae within the genus *Cronartium* has been made with only a few samples of the white pine blister rust, *Cronartium ribicola* Fisch. (Pierson 1933), although they have widely been accepted as a mechanism of mating, also in *C. flaccidum* (i.e. Hiratsuka 1981).

1.1.3 Life cycle of *Peridermium pini*

The only known spore stages of *P. pini* are aeciospores, which infect pine and spermatia, which in principle could be used for mating. Before Haack (1914) succeeded in infecting diseased *P. sylvestris* with aeciospores of *P. pini*, there had been attempts to find the telial host of this very common fungus (Liro 1907). Although the absence of alternate host and also uredinial and telial stages was assured, the details of life cycle have remained unknown. The aeciospores infect pines via wounds in the bark or needles (Klebahn 1918) or through direct stomatal penetration (Olembo 1971). The hyphae grow towards the stem in pine cortex (Olembo 1971). The result is a canker formed on a branch or stem. One to five years after the infection, the uninucleate, presumably haploid hyphae form spermagonia ((Klebahn 1918, 1924, Olembo 1971). Although *P. pini* has been shown to produce spermatia, studies of plasmogamia between two strains have not been conducted.

1.2 Species complex *C. flaccidum*-*P. pini* is inadequately described

1.2.1 *Endocronartium* or *Peridermium* ?

The genus *Endocronartium* was described as a new name for *Peridermium* species according to postulated endo-type life cycle. Hiratsuka (1966) studied cytology of *Peridermium harknessii* J.P. Moore, by staining nuclei of subsets of spores that were incubated for given time intervals. He observed that during aeciospore maturation and germination young, immature aeciospores had two nuclei, mature aeciospores were

uninucleate, and in the germ tubes there were two, three or four nuclei. This was considered as evidence of meiosis, although no actual phases of meiotic division were reported. Later Hiratsuka (1968) concluded, by the morphology of the germ tubes, that nuclei in the germ tubes of *Peridermium pini* (Pers.) Lev. followed the same mechanism, despite the same uncertainty of the occurrence of nuclear fusion and meiosis. It is noteworthy that Klebahn (1938) in his earlier, detailed study of the cytology of *P. pini* mycelia, spore-building structures and aeciospores, did not observe any decline in nuclear number, nor any nuclear phases that he could have connected to meiosis. Based on nuclear cycle, Hiratsuka (1969) placed *P. harknessii* and *P. pini* into a new genus, *Endocronartium*, which was characterized by still questionable meiosis within a germ tube; fusion of two haploid nuclei, followed by nuclear division into four daughter cells. Uninucleate branches of germ tubes would be functionally basidiospores, in which basidiomycetes generally establish the haploid life form. The theory was strongly criticized by Laundon (1976), who saw no evidence for meiosis in Hiratsuka's paper (1968). Meiosis within a germ tube was also suggested for *Peridermium yamabense* (Imazu et al. 1991), but again, the result was based on observed nuclear number and the details of meiosis remained unshown.

Especially in the case of gall rust, *P. harknessii*, several studies have suggested that the life cycle is asexual. Epstein and Buurlage (1988) observed only nuclear divisions in the germ tubes of mainly binucleate *P. harknessii* aeciospores and later population studies by Vogler et al. (1991, 1997) showed that *P. harknessii* comprised two distinct zymodemes (multilocus electrophoretic phenotypes), which also differed by the nuclear number of the spore. Predominantly binucleate type I was characterized by single marker at each isozyme loci, while uninucleate type II had additional markers. Based on photometric measurements, uninucleate zymodeme had the same amount of DNA in one nucleus as the binucleate had in two nuclei. Also number of nuclei in aeciospore germlings increased arithmetically over time. These data suggested that aeciospore nuclei, in both types I and II, divide mitotically, as is consistent with an asexual life cycle. It was interpreted that the uninucleate type was the result of karyogamy of two binucleate types or that the binucleate type was the result of haploidization of the uninucleate type (Vogler et al. 1997). Within these studies, no phases of cell divisions were documented. Neither was any mechanism for the haploidization of the fungus suggested, although it is known that *Peridermium* hyphae are uninucleate within pine tissue (Hiratsuka 1969). It can be considered that neither meiotic or mitotic events within germ tubes of *P. harknessii* are yet adequately reported. Even if cytological observations based on nuclear number were correlated, the actual phases of meiosis should still be documented microscopically, or evidence of meiosis should be obtained with analysis of genetic markers in controlled matings.

Even if the nuclear cycle of *P. harknessii* was well known, it would not be justified to suggest the same unique cell cycle with basidia-like germ tubes for *P. pini*. On the contrary, the interpretations of nuclear status of *P. harknessii* aeciospores or aecial germ tubes by Epstein and Buurlage (1988), Vogler et al. (1997), and Hiratsuka (1969) are clearly controversial.

1.2.2 Relationship between *C. flaccidum* and *P. pini* is unknown

In addition to clear difference of host specificity between *C. flaccidum* and *P. pini*, only few, strongly criticized and debated differences have been found. According to Hiratsuka (1968) aeciospores of *C. flaccidum* and *P. pini* are functionally different by the meiosis in the aeciospore germ tube of *P. pini* and thus they could be identified by the morphology of germ tubes. Tube of heteroecious *C. flaccidum* were dichotomously branched, no septa were observed and it contained two nuclei migrating along the germ tube. The cells in mature germ tubes of *P. pini* were uninucleate and shorter than those of *C. flaccidum* (Hiratsuka 1968). However, the morphology of aeciospore germ tubes of different origins, such as Great Britain (Gibbs et al. 1988) and Finland (Kaitera et al. 1999b) were different from Hiratsuka's (1968) original description of their distinguishable characters. Gibbs et al. (1988) found that *P. pini* samples that were identified according to characters described in Hiratsuka (1968), were not autoecious but capable to form sexual stages of *C. flaccidum* on *Peonia*. No evidence of nuclear fusion in germ tubes was found. Kaitera et al. (1999b) measured more morphological variation of aecial germ tubes within populations than between autoecious and heteroecious species. Germ tube morphology reported in Klebahn's work (1938) is strikingly similar to observations made by Gibbs et al. (1988) and Kaitera et al. (1999).

The controversies of the results of nuclear number of aeciospores and their germ tubes point out that evidence based on cytology is inconclusive. Even if cytological observations based only on nuclear number were correlated, the actual phases of meiosis would still need to be documented. The number of nuclei in germinating spores can not be used as evidence of genetic phase of a fungus. For example, haploid binucleate basidiospores of *Heterobasidion annosum* give rise to haploid homokaryotic multinucleate mycelia that following mating form multinucleate heterokaryotic mycelia (Korhonen and Stenlid 1998).

Genetic marker studies of the relationship between *P. pini* and *C. flaccidum* have also produced controversial results. Moricca and Ragazzi (1998) suggested that *P. pini* and *C. flaccidum* can be distinguished by the internal transcribed spacer genes of their ribosomal DNA, while Hantula et al. (1998) successfully amplified the same RAMS (Hantula et al 1996, see also 1.3.2.3) markers from both fungi. Interestingly, some of the RAMS markers were shown to be codominant, and these markers were heterozygous within all *C. flaccidum* isolates, whereas only homozygous *P. pini* samples were observed.

Comparison of previous studies of the differences between *C. flaccidum* and *P. pini* reveals that there is no single explanation covering the actual differences. Clearly, the relationship between these fungi needs further investigations. In spite of this, the two types of fungi have consistently been classified into two species, although in many cases morphologically similar fungi with different host specificity have been recognised as races, so called pathovars or forma specialis (f.sp.).

The most reliable way to answer the question of species division would be studies with controlled mating tests. Accompanied by analysis of progenies with genetic markers, mating tests could be used to study sexual behaviour of the fungi. While these tests have not been technically possible for *Cronartium/ Peridermium*, one has to conclude the relationship between *C. flaccidum* and *P. pini* by other characters.

1.3. Potential differences between *C. flaccidum* and *P. pini*

1.3.1 Morphological characters

Fungal taxonomy has traditionally been based on morphological characters. When studying the relationship between *C. flaccidum* and *P. pini*, the morphological studies can not be overlooked. A quantitative comparison of characters of the aeciospores between *C. flaccidum* and *P. pini* would reveal if the two fungi could be separated morphologically. By studying ecologically more distant species, it is possible also to contribute to the question whether the spore morphology is a relevant species marker within *Cronartium*.

A promising feature to study would be surface warts, shape and size of the aeciospores. Within some *Cronartium* species these have been used as taxonomical criteria (Peterson 1967, Hiratsuka 1971). Several interesting studies have been made by simple and effective methods of scanning electron microscopy (SEM). According to Hiratsuka (1971), some of the stem rusts in Canada (*E. harknessii*, *C. ribicola*, *C. comandrae* Peck, *C. comptoniae* Arthur and *C. coleosporoides* Arthur) could be distinguished by surface warts on the aeciospore. Grand and Moore (1972) came to partially different conclusion about the usefulness of these criteria, as they determined the aeciospore morphology of the *C. quercuum* complex. The comparison of these species revealed the similarity of surface morphology in most of the studied species, except *C. appalachianum*, which had no smooth areas on the spore surface. Imazu et al. (1989) found differences even at subspecies level, expressed in the surface warts and dimensions of the aeciospores of *C. ribicola*, *E. yamabense*, *E. sahoanum* var. *sahoanum* and *E. sahoanum* var. *hokkaidoense* in Japan.

Ultrastructure of hyphal walls and septa has also been used as a morphological key, and the ultrastructure of *P. pini* hyphae has been described by Walles (1974). It is noteworthy that Longo (1982) was not able to find any differences when he compared these results with the ultrastructure of *C. flaccidum*.

1.3.2 DNA markers and population structure

1.3.2.1 Analysis of pathogenic fungal populations

Because of various mating systems and possibility of asexual reproduction pathogens adapt to various situations with great flexibility. Due to this, structure of a pathogen population can vary from worldwide panmictic population to worldwide clonal

population. *Mycosphaerella graminicola*, wheat leaf blotch fungus is an example of a former (McDonald and Martinez 1990), while *Phytophthora infestans*, the fungus causing potato late blight is an example of a spread of worldwide clones (McDermott and McDonald 1993). Examples of intermediate types of population structure are chesnut blight fungus (*Chryptonectria parasitica*) (Liu et al. 1996) that has extremely local clonal populations and regional sexual populations. A few gigantic individuals of *Armillaria bulbosa* can locally be produced by vegetative propagation instead of spores (Smith et al. 1992).

Rust fungi do not grow well *in vitro*. The traditional ways of studying structure of fungal populations; vegetative compatibility or mating tests are thus not applicable for many species of rust fungi. Especially *in vitro* cultures of the aecial state of *Cronartium* and *Peridermium* have seldom been successful, and single-spore cultures have failed (Pei and Gibbs 1991, Moricca and Ragazzi 1994). Moreover, genetic marker techniques such as allozyme electrophoresis, or restriction fragment length polymorphism (RFLP) of whole genomic DNA that needed relatively large amounts of tissue, have not been possible to use in studying single aecia or aeciospores.

Studies done on mass collection of spores of a single gall or a canker (i.e. Tuskan et al. 1990, White et al. 1996, Vogler et al. 1997) can be criticized due to possibility of mixing the genotypes if the fine population structure of a given fungus is not known. Apparently, difficulties of interpretation of results have risen from bulking the genotypes; Moricca et al. (1996) reported variation of ITS genes within single DNA isolates of bulked *C. flaccidum* aeciospores, but they could not separate whether the different types were from genome of single fungal individual or several individuals. Also White et al. (1996) observed variety of length variants of IGS genes occurring within DNA samples of bulked aeciospores from *C. ribicola* cankers.

1.3.2.2 Structure of the known Cronartium populations

The best known fungi in the genus *Cronartium* are apparently *C. ribicola* and *C. quercuum* f.sp. *fusiforme*. Genetic fine structure of *Cronartium* fungi within cankers changes from haploid hyphae to multiple matings, due to the mechanism of mating. Between two fungal strains, germinating non-self spermatia (gametes) permit plasmogamy with receptive hyphae formed in spermagonia of the resident fungus in a host tissue (Pierson 1933). This dikaryotic mycelium produces aeciospores. From this mechanism it follows that genotypes of aecia of a canker are a combination of at least two fungal haplotypes; one that established hyphae on pine stem and another (or several) from spermagonia.

According to several studies, it appears that this mechanism works, and it probably needs insects as a pollinator (Hunt 1985, Hamelin 1996). It was observed that insects cross-fertilize *C. ribicola* by spreading the spermatia within and between cankers (Hunt 1985). Indeed, within an actively breeding population of a *C. ribicola*, perennial cankers which produce spores successfully, usually contain several genotypes as a result of multiple matings. The cankers of *C. ribicola* contained several RAPD

profiles and most of the genetic variation could be found within the cankers and the sites (Hamelin et al.1998). As spermatia and later formed aecia of individual cankers were studied, it was found out that on cankers with monomorphic spermatia, 75% of the aecidia were combinations of spermatial haplotypes. This indicated that *C. ribicola* had multiple matings between cankers via spermatization. Also White et al. (1996) came to the same conclusion by using slightly different approach; they found genetic variation of a ribosomal IGS region within bulked samples of aecia of single cankers. The haplotypes of *C. ribicola* were isolated from pine needles that are known to be infected by haploid basidiospores. Because the genotypes of cankers were apparently mixtures of haplotypes, it was stated that multiple spermatial fertilizations would have occurred within cankers. Kuhlman and Matthews (1993) obtained clear indication of the same type of population structure in *C. quercuum* since aeciospores of *C. quercuum* f.sp. *fusiforme* obtained from single cankers showed variation in pathogenicity. The observation also emphasizes that any variation that is studied between rust origins must also be determined within cankers. Concerning *C. flaccidum*, Moricca et al. (1996) suggested that several genotypes occur within bulked canker samples but they did not analyze aecia separately.

Basidiospore populations collected from single telia of *Cronartium ribicola* were shown to be in Hardy-Weinberg equilibria at several loci. High variability observed in ribosomal haplotype markers supported the hypothesis that the fungus is heterothallic (Gitzendanner et al. 1996).The authors conclude, that selfing practically does not occur in *C. ribicola*, and its mating system is overwhelmingly outcrossing.

1.3.2.3 DNA markers in studies of *C. flaccidum* and *P. pini*

Template DNA for PCR-based techniques can be isolated on small amounts of fungal tissue, even single spores (Lee and Taylor 1990). Arbitrary or random primers (Welsh and McClelland 1990, Williams et al. 1990) have been popular in population studies of plant pathogenic fungi. Recently, random amplified microsatellite (RAMS) technique originally described by Zietkiewics et al.(1994) has shown to be applicable within studies of fungal populations (Hantula et al.1996). In this technique, the distal ends of di- or trinucleotide repeats of nuclear DNA are primed with anchored primers (Hantula et al.1996). Part of the PCR amplified regions show codominance in RAMS patterns. Usually the above mentioned PCR based markers are dominant; it is not possible to differentiate homo- and heterozygous loci by the presence or absence of DNA markers. Dominant markers are applicable for studies of haploid organisms such as cultures derived from haploid spores of basidiomycetes or ascomycetes, like *Gremmeniella abietina* (Wang 1997). RAPDs or RAMS have advantage of technical simplicity. The most important theoretical disadvantage of the use of RAPD markers is that they are dominant and therefore presence of a band in dikaryotic or diploid organism may represent both a heterozygote or a homozygote. Therefore, determinations of gene frequencies are uncertain. Amplification and scoring of polymorphic markers such as RAPDs and RAMSs has an inherent problem of scoring the different alleles of approximately equal size and sequence as identical ones. The advantage of specific marker loci, such as microsatellites or SCARs (i.e. McDermott

and McDonald 1993, Schlötterer and Tautz 1992, Tenzer et al. 1999) is that the homology of amplified DNA is assured in advance by designing highly specific primers that amplify only the target region. The possibility to amplify the same marker loci within the two fungi facilitates also the studies of genetic differentiation between *P. pini* and *C. flaccidum*. The pilot studies of *P. pini* populations were made with RAPD markers in order to detect overall population structure, haplotype frequencies and spatial distribution in pine stands (I).

Relatively high amount of polymorphism could be observed by screening several RAPD or RFLP loci or alternatively, by effective screening for mutations of few marker loci. Codominant markers that facilitate scoring of heterozygous alleles have advantage in studying population structure of resin top fungi; the possibility to observe heterozygosity is especially valuable if conclusions of sexual behavior are to be drawn. Also, the results can be compared to earlier studies of populations of rust fungi, which has been based on codominant allozyme loci (Tuskan et al. 1990, Vogler et al. 1991, Burdon and Roelfs 1985).

The genetic composition of cankers is clearly one of the key factors in characterization of the sexuality of the *C. flaccidum*-*Peridermium pini* Pers. Lev. complex. If there is genetic differences between the aecia within a canker, it is most likely that the fungus had multiple matings within or between the cankers. Therefore, genetic variation, and the possible combinations between the aecial genotypes of cankers could be considered as evidence of the multiple matings. Another reason for genetic polymorphism within a canker could be infection by several aeciospores. By using codominant DNA markers, one should be able to detect whether the different genotypes are results of combination of nuclei between haploid branches of germ tubes from different aeciospores in a canker or of several infections without mating.

The only difference between *P. pini* and *C. flaccidum* scored by Hantula et al. (1998) was the heterozygosity of marker loci within the Italian *C. flaccidum* samples against the homozygosity within all populations of *P. pini*. Only marker of *P. pini* that could have been considered heterozygous was ITS2 locus (internal transcribed spacer) of ribosomal gene cluster (Moricca and Ragazzi 1988), but rDNA repeats can show variability within haploid DNA samples (White et al. 1996). The observation by Hantula et al. (1998) was a promising clue to differences in the life cycles of these fungi, because Kaitera (1999) showed later that fungal isolates from the two locations where heterozygotic individuals were observed, were also capable to infect *Melampyrum* spp. Thus the results suggested that *P. pini* aeciospores are exclusively homozygous, which can be an evidence of an asexual population with clonal lineages (i.e. Burdon and Roelfs 1985). The existence of clonal lineages within *P. pini* should be seen as linkage disequilibrium between different loci.

Intraspecific variation in genetic fingerprinting markers has been observed both in *P. pini* and *C. flaccidum* (Hantula et al.1998). However, according to the RAMS markers, this variation is evenly distributed and the level of genetic differentiation

among the Finnish populations of these fungi is low (Hantula et al.1998). It is not known whether genetic differentiation would exist on a more wide geographical scale.

Moreover, if *P. pini* and *C. flaccidum* are two different species, there should be DNA markers which would differentiate between them. Such a study was made by Moricca et al. (1998): they concluded that *P. pini* and *C. flaccidum* could be identified by the internal transcribed spacer (ITS) of ribosomal gene cluster. However, it must be noted that the study (Moricca et al. 1998) was based on eight samples only and the ITS region is known to be variable also between individuals of single species (Kasanen et al. 2000). Interestingly, an opposite conclusion was made by Hantula et al. (1998), who amplified the same type of RAMS markers from *P. pini* and *C. flaccidum*. If the two fungi are two different species, genetic differentiation would be obvious and easy to observe.

2. Aims of the study

The aim of this study was to elucidate the relationship between *C. flaccidum* and *P. pini*. Variation of morphological and molecular data at several hierarchical levels of populations is clearly one of the key factors in characterization of unknown sexual behaviour, and other differences within the *C. flaccidum*- *P. pini* complex. This was studied more specifically by testing the following hypotheses;

- *C. flaccidum* and *P. pini* can be separated by some specific characters of the aeciospores (I)
- The morphology of aeciospores is a valid taxonomic feature (I)
- Aecia of pine stem rust in a scots pine stand represent a polymorphic fungal population, and the populations are different between sites (II)
- The rust genotypes are statistically evenly distributed within a stand (II)
- The aecia formed within an individual canker are genetically different because of outcrossing (III)
- There are differences in population fine structure of *C. flaccidum* and *P. pini* (III)
- Population structure of *C. flaccidum* and *P. pini* supports the theory of multiple matings between cankers (III, IV)
- The population parameters between *C. flaccidum* and *P. pini* reflect differences, which can be connected to absence of meiosis and thus presence of clonality in the life cycle of *P. pini* (IV)
- The degree of genetic differentiation between *C. flaccidum* and *P. pini* suggests that they can be considered as distinct genetic entity (IV)
- The level of genetic differentiation among populations of *P. pini* in Finland as low as indicated by a previous RAMS analysis (IV)
- The Italian population of *C. flaccidum* is highly differentiated from the Finnish populations (IV)

3. Material and Methods

3.1 Fungal samples

For morphological studies (I), five aeciospore samples of *C. flaccidum* were obtained from Italy and one from Sweden, one *C. ribicola* sample from Finland and two samples of *P. pini* from Finland. In study II, samples of *P. pini* were collected in order to describe the fungal population of two stands located in Eastern Finland (37 and 40 samples). The stands were visited again to obtain 25-50 aecia from two perennial cankers (III), that were present and had produced aecia previously (II). No known telial hosts of *C. flaccidum* were found within these stands since they are located on poor sandy soils. Hierarchical sampling of populations was used in IV; 15 populations were collected, and 3-16 samples per population was used for population analysis. Unfortunately, some population samples were too small for statistical analysis. Only unopened aecia of *P. pini* and *C. flaccidum* were collected in studies I-IV in sterile microcentrifuge tubes or ampoules to avoid contaminants.

3.2 Analysis of fungal samples

3.2.1 DNA markers

Random amplified polymorphic DNA (RAPD) markers (Williams et al. 1990, Welsh and McClelland 1990) were used in study II. RAPD primers amplify target regions, where no *a priori* information is known. Technically RAPDs are easy to perform, but as discussed previously (1.3.2.3) they have methodological limitations (dominance) that hampered their usage for testing the hypotheses of studies III and IV. Both RAPD and RAMS (Hantula et al. 1996) fingerprinting methods occasionally produce markers that seem to have slightly different molecular weight between the sample lanes in agarose gels. These can be different loci or length variant alleles of a given locus. In study III two polymorphic RAMS markers were chosen for further analysis. Briefly, PCR products were cloned, sequenced and specific primers were designed in order to amplify each marker locus. Using the protocol described in Fig. 1, DNA fingerprints can be used for detection of polymorphic marker loci. The alleles of the marker loci were analysed with denaturing gradient gel electrophoresis (III). The separation of alleles during DGGE is based on differences in melting behavior of DNA fragments (Fischer and Lerman 1983).

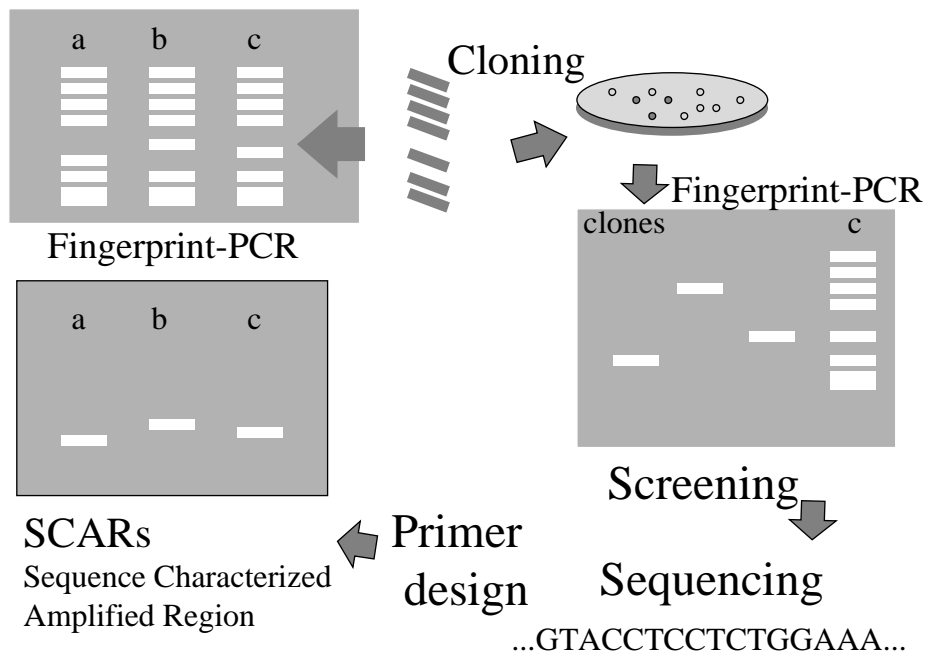
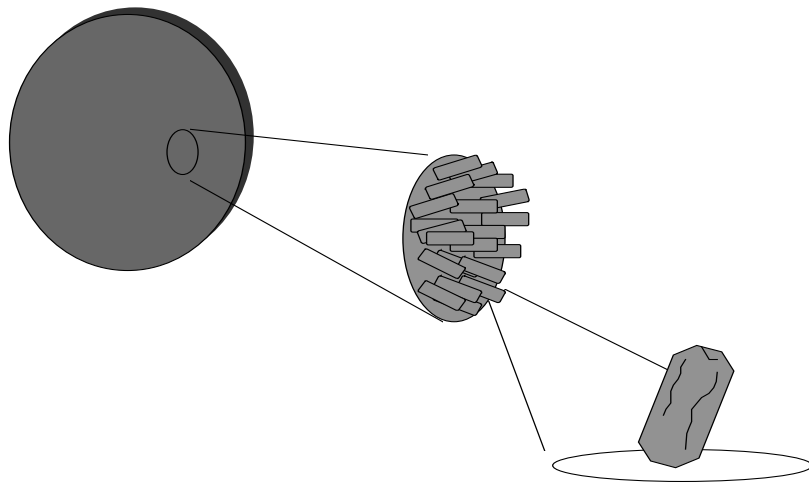


Fig.1. A schematic presentation of conversion of an individual band into a specific marker. The method includes cloning of an individual fingerprint, screening for correct clones, sequencing and designing primers.

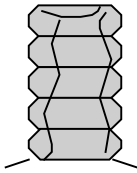
3.2.2 Scanning electron microscopy of the spore surface

Commonly, the surface of a rust spore is not smooth but roughened or verrucose. The wart-like structures of aeciospore surfaces were studied in order to identify *P. pini* and *C. flaccidum*, and to test these criteria with *C. ribicola*, that has been previously studied (Fig. 2).

Spores were chosen arbitrary and the warts were visually classified according to the three main types described by Imazu et al. (1989); clearly annulated, slightly annulated and smooth. The number of annulations in warts was also examined. The dimensions (width, length) of the spores were measured from the micrographs. In addition, the form of the spores was measured as the ratio length / width. Discriminant analysis was used in order to detect whether the classification by species or sample location was supported by the morphological data (I).

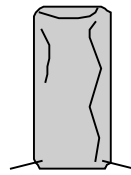


A



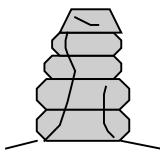
Cronartium ribicola

B



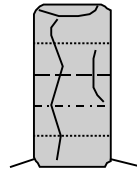
Endocronartium yamabense

C



Endocronartium harknessii

D



Endocronartium sahoanum
var. *hokkaidoense*

Fig. 2. The surface structures of *Cronartium* spores. A-D are drawn according to Hiratsuka (1971) and Imazu et al. (1989).

4. Results

4.1 Population structure of *C. flaccidum*

The 50 aecia collected from canker J of *C. flaccidum* all contained the same two alleles in the *Pp1* locus and also in the *Pp2* locus, and were therefore genetically uniform and heterozygous. The situation in the other canker P of *C. flaccidum* was different as it was not uniform and contained heterozygous aecia. The number of alleles observed in the *Pp1* locus was three (frequencies 0.5, 0.39 and 0.11) within the whole canker which included only heterozygotic aecia (Paper III, Fig. 1b). In the *Pp2* locus, the canker contained 14 homozygotic and 21 heterozygotic aecia. Thus allelic composition of *C. flaccidum* cankers suggested that aecia are composed of several haplotypes (III).

When the distribution of genetic variation within and among *C. flaccidum* populations (AMOVA; Excoffier et al. 1992) was analyzed using information from the *Pp1* locus, 8.62% of the variance was observed among and 91.38% within populations ($P=0.0378$). Such a result has to be attributed mostly to the differentiation between the Italian and Finnish populations, because the same analysis made by grouping the two Finnish populations together resulted in 15.64% of variation due to difference between Finnish and Italian populations ($P=0.0074$) (IV). As only two Finnish populations of *C. flaccidum* were analysed, -5.06 % of variation was observed between the populations, implying that two Finnish populations could be treated as one population in analysis.

In the *Pp2* locus of *C. flaccidum*, the allelic composition of all heterozygotes was not possible to be determined because in some cases two different allelic combinations were assumed to give the same banding pattern in an RFLP analysis. However, it was possible to score the heterozygotic state of the locus. This locus was not used to analyze the genetic composition of *C. flaccidum* populations, but the occurrence of heterozygotes among *C. flaccidum* aecia was recorded. In Kolari, Juomukuru and Naz most of the aecia were heterozygotic whereas in Åland only two heterozygous aecia were observed (III, IV).

4.2 Population structure of *P. pini*

Alltogether nine RAPD phenotypes (=haplotypes) were found within two populations of *P. pini* (II). The Kesälahti population comprised eight haplotypes, while in Kontiolahti only three haplotypes were found. One haplotype was most common in both stands (62% and 32 %), while the next most common haplotype in Kontiolahti was not found in Kesälahti. The results of visual examination of haplotype frequencies were verified by comparison of cumulative distribution functions with the Kolmogorov-Smirnov test. Frequencies of the haplotypes were significantly different ($p<0,03$) from the expected even distribution where each haplotype has an equal

possibility to infect pines, and thus reach equal frequency in a pine stand. The populations were also statistically different according to the same test ($p < 0,03$)(II).

Alltogether, fourteen alleles were observed within marker locus *Pp1*. According to the *Pp1* marker, the 38 aecial isolates of canker K1 were all identical, and contained only one allele (III). The same was observed also on cankers V, K2, L1, and L2, among which 24, 25, 30, and 30 isolates, respectively, contained only one allele in each canker. The analysis of variation within marker *Pp2* was carried out using two restriction endonucleases (*TaqI* and *HinfI*). The two digestions were in accordance with each other, but one of the alleles identified with *TaqI* was further separated into two alleles in *HinfI* digestion. Thus, based on this analysis altogether seven alleles of *Pp2* were observed in *P. pini*. Since genetic variation within cankers was not observed, the results of the present study suggest that the cankers of this rust usually include only one genotype (III).

The Nei's (Nei, 1973) measure for expected gene diversity (H_E) among *P. pini* was calculated for markers *Pp1* and *Pp2*. In order to obtain information about the possible existence of clonality within *P. pini* the linkage (i.e. the co-occurrence of different alleles) between *Pp1* and *Pp2* loci was analyzed among the Finnish isolates of this rust, but no statistically significant deviation from equilibrium was found. However, a simple visual examination seemed to indicate that some allele combinations would have been considerably more common than expected. Therefore the analysis was expanded to further markers by adding data from the two most variable, previously analyzed, RAMS-loci GAAA590 and ACA580 (Hantula et al. 1998). Data from all four loci (*Pp1*, *Pp2*, GAAA590 and ACA580) were available from 42 aecia, and visual analysis of the allelic distribution among them clearly indicated that several haplotypes occurred far more often than expected. This was confirmed by a statistical analysis which confirmed a nonrandom ($P=0.0433$, Fisher's exact test) distribution of markers and thus existence of linkage disequilibrium among the markers (IV).

4.3 Comparison of *C. flaccidum* and *P. pini*

4.3.1 Morphological characters

The size of aeciospores of *C. ribicola*, *C. flaccidum* and *P. pini* were 34,2-30,5 x 24,3-21,6 μm (values are averages of population samples). The discriminant analysis (Dillon and Goldstein 1984) of the grouping into nine sampling locations resulted in one statistically significant discriminant function, although some of the group centroids overlapped. However, at the species level, the discriminant analysis of the dimension variables did not reveal statistically significant axes in the data space, as seen in the plot of the centroids of the pre-defined groups (species) against the two discriminant functions. The surface warts of the spores of *C. ribicola*, *C. flaccidum* and *P. pini* from all the origins were classified as clearly annulated. The lowest layers of the annulations near the spore surface were not always visible because of the positions and the density of the warts. In addition, the number of the annulations/wart on the

spores varied, and thus, the wart type, or the number of the annulations/wart were not used as a variable in this study. The warts of *C. ribicola*, *E. pini* and *C. flaccidum* were approximately 1-1,5 μm high, uniform or slightly tapered ($<1\mu\text{m}$) and clearly annulated with 5-10 layers, having also longitudinal ditch-like formations. The surfaces of the spores had well defined smooth areas as well as annulate warts. Considering the variation of the surface warts on the individual spores, it was obvious that the morphology of the spores can not be used in identification of any of these species (I).

4.3.2 Genetic differentiation

Mostly the same alleles of *Pp1* were observed in the two rust species, as eight out of the ten alleles found among *C. flaccidum* aecia were observed also among aecia of *P. pini*, and eight out of the thirteen alleles found among *P. pini* aecia were observed also among aecia of *C. flaccidum*. All heterozygotic aecia originated from known locations of *C. flaccidum* (Juomukuru, Kolari and Åland archipelago in Finland as well as Naz in Italy (III, IV).

The Nei's (Nei, 1973) measure for expected gene diversity (H_E) among *P. pini* was calculated for markers *Pp1* and *Pp2* and among *C. flaccidum* it was calculated for marker *Pp1*. In the *Pp1* locus the expected gene diversity in *P. pini* and *C. flaccidum* was very similar ($H_E=0.785$ and $H_E=0.776$, respectively). Such diversity, calculated only for *P. pini*, was lower in locus *Pp2* ($H_E=0.559$), indicating somewhat lower degree of diversity for this locus (IV).

In addition to expected gene diversities also Nei's gene diversities (H_S) were calculated for all populations separately. Among populations of *C. flaccidum* and *P. pini* the gene diversity in the *Pp1* locus ranged from 0.642 to 0.748 and from 0.444 to 0.789, respectively. Therefore both the highest and the lowest values were observed among populations of *P. pini*, and the populations of the two species seemed not to differ in this respect. Among populations of *P. pini* the gene diversity (H_S) in the *Pp2* locus ranged from 0.198 to 0.660. Interestingly, the estimates for gene diversity in the two loci correlated statistically significantly with each other among the populations of *P. pini* ($P<0.01$, $df=7$), implying that the calculated indices would possibly reflect diversity differences among the populations of this species in general (i.e. not only among the loci studied) (IV).

The Nei's measure for gene differentiation (Nei, 1973) was calculated for populations of both *P. pini* and *C. flaccidum*. The two markers *Pp1* and *Pp2* produced higher values for *P. pini* ($G_{ST}=0.149$ and $G_{ST}=0.200$, respectively) than *Pp1* for *C. flaccidum* ($G_{ST}=0.090$) (IV).

To estimate the level of gene differentiation between the two species (i.e. considering all isolates of each species as a single population) the G_{ST} values using data from *Pp1*

were calculated. The value was very low ($G_{ST}=0.024$) indicating that there was practically no genetic differentiation between *C. flaccidum* and *P. pini* (IV).

5. Discussion

5.1 Population structure of *C. flaccidum* is in accordance with sexual life cycle

The aecia collected of *C. flaccidum* cankers all contained two alleles, ie. they were heterozygous. The existence of heterozygosity within cankers of *C. flaccidum* confirms the earlier observations (Hantula et al. 1998, Kaitera et al. 1999 a), but the difference in the genetic composition of the two cankers studied was surprising. The variable canker P can be taken as evidence for the occurrence of functional spermatogonia, and the occurrence of several aecial genotypes in this canker is in accordance with observations of the sexually outcrossing species *C. ribicola*, where cankers contained a single spermatial genotype, and aecial genotypes were combinations of spermatial genotypes (Hamelin et al. 1998, Hamelin 1996). The observed population fine structure supports well the hypothesis that the aecial genotypes of *C. flaccidum* probably are the result of haploid receptive hyphae fertilized by spermatia.

Observed homogeneity of one *C. flaccidum* canker can also be partly due to lack of suitable insect transfer, since the ability of spermatia to spread and cross-fertilize without insect transfer seems to be limited. It was also observed that cross-fertilization of *C. ribicola* increased aecial production, and isolated cankers failed to produce aecia (Hunt 1985). If this would also be the case of *P. pini* and *C. flaccidum*, the lack of cross-fertilization by insects could be an important factor regulating the epidemics. So far, the role of insect vectors has only been studied in the infection process (Pappinen & von Weissenberg 1994 a, b).

Also selection pressure provided by the pine host could prevent some strains of *C. flaccidum* to infect, and it is possible that only strains with the observed alleles would have been able to colonize the host tissue. Also, it cannot be ruled out that the marker system was simply not sensitive enough to reveal polymorphism.

So far analyzed aecia of *C. flaccidum* have been mainly heterozygous for several DNA markers, such as the sequence specific markers *Pp1* and *Pp2* (III, IV) or RAMS markers (Hantula et al. 1998). The earlier results of RAMS analysis (Hantula et al. 1998) were verified in this study, since only low level of geographical differentiation was observed within *C. flaccidum* (IV). The same type of population fine structure (Hamelin et al. 1998) and low level genetic differentiation (Gitzendanner et al. 1996, Et-touil et al. 1999) were detected for *C. ribicola*, and it was interpreted that the mating system of *C. ribicola* is highly outcrossing. The results indicate that population structure of *C. flaccidum* is in accordance with the sexual life cycle.

5.2 Population structure of *P. pini*

5.2.1 Population structure of *P. pini* suggests an asexual life cycle

Several fungal individuals of *P. pini* were found within a Scots pine stand. Some haplotypes were statistically far more common than expected and the haplotype composition within separate stands varied. One explanation would be that some haplotypes of *P. pini* have higher fitness (i.e. virulence or ability to produce more aeciospores) within these stands, but common haplotypes might be common for stochastic reasons. Such an observation was made by Vasiliauskas and Stenlid (1998), who observed that most common vc groups of *Stereum sanguinolentum* were growing shortest distance in wood i.e. had the lowest virulence. Interestingly, the dominant haplotypes of *P. pini* were not present in all trees, although the spatial distribution of haplotypes within stands and assumption of even spore load, where each haplotype has equal chances to infect (Van der Kamp 1993), would have suggested such a result (II). Considering this, it is also possible that some specific interaction of resistance and virulence exists. The haplotype distribution also suggests a scenario where few clones with different rates of fitness compete in a stand, since it would result in the observed haplotype composition, where only few clones per stand can be found and some clones are far more common than others. A sexual cycle would spread fitness within population and break down this clear pattern. It is also possible that the distribution of spores has varied over time, and the most common haplotypes had entered the stand earlier.

Basically, the polymorphism observed within populations in (II) can be due to existence of a sexual population or several clonal lineages within an asexual population (i.e. Burdon and Roelfs 1985). In addition to the observation that some haplotypes were far more common than expected (Fisher exact test, see 4.2), also the low number of haplotypes observed with seven polymorphic RAPD markers (nine out of 128 theoretical possibilities) suggested that the populations are composed of asexual clones. This was apparent especially in the stand where only three haplotypes were observed.

The same observation that some allelic combinations occurred considerably more common than expected, was made in IV. Thus linkage (i.e. the co-occurrence of different alleles) was analyzed between specific marker loci and RAMS loci from the data by Hantula et al. (1998). Statistical analysis confirmed a nonrandom distribution of markers and thus linkage disequilibrium between loci. It can be taken as the strongest evidence of clonality of *P. pini* populations (IV).

Another evidence of asexuality was the population fine structure of *P. pini*. The spatial distributions of haplotypes overlapped with obvious possibilities of mating, since even within single pine trees two haplotypes were found (II). As two perennial, actively spore producing cankers from each of two stands were studied in detail in (III), and no heterozygotic aecia nor polymorphism within cankers were observed, it can be concluded that pine stem rust fungi do not mate within these stands. The

homogeneity and homozygosity of the aecia within the cankers, in addition to the nonrandom haplotypic (II) and genotypic (IV) distribution of fungi in individual stands, agrees with the hypothesis that *P. pini* is asexual (clonal).

There are, however, observations that seem not to fit the hypothesis of the asexuality of *P. pini*: (i) The genetic variation observed with the same marker loci, is higher within *P. pini* populations than in populations of *C. flaccidum*, which evidently is a sexual fungus (see 4.3). A similar pair of populations was studied by Burdon and Roelfs (1985), who analyzed the variation within asexual and sexual populations of wheat stem rust. Asexual populations, although polymorphic, had lower amount of variation, measured by allelic richness (i.e. number of alleles per locus), gene diversity or frequency distribution of the number of heterozygous loci per individual. The remarkable diversity of asexual *P. pini* could be achieved if asexual *P. pini* lineages have their origin in polymorphic, sexual *C. flaccidum* population. (ii) Cankers of *P. pini* are known to produce spermatia (Klebahn 1918, 1924, Olembo 1971) that basically could be used for mating. However, to my knowledge it has not been studied, whether the spermatia are functional. Thus observations of genetic variation and spermatial production can not either be taken as evidence of sexual cycle.

To conclude, the asexuality of *P. pini* is supported by several observations. (i) No heterozygosity was observed within single aecia of *P. pini* (III, IV), (ii) single cankers of *P. pini* are homogeneous (III), (iii) the population structure of *P. pini* is most probably clonal (and therefore asexual) as linkage disequilibrium occurs between different alleles of four loci (IV), (iv) within both of the stands studied in detail in (II) some haplotypes were far more common than expected. Thus it can be considered highly probable that *P. pini* is asexual, but the mechanism behind observed high genetic variation and spermatial production and functionality needs to be studied. Such studies could reveal whether the basis for clonality in *P. pini* is self-mating, a misfunction in the fertilizing ability or a misfunction in the receiving fertilization.

5.2.2 Does meiosis occur during the life cycle of *P. pini*?

Klebahn (1938) suggested that germination of the aeciospores and growth of the germ tubes of *P. pini* would only have mitotic cell divisions. The same was described on *P. harknessii* by Epstein and Burlage (1988). The obvious result of such a growth, without fertilization by genetically different spermatia, would be a homogenic canker. However, the *P. pini* cankers studied here were not only homogenic, but also homozygotic. This could be explained by aecia carrying uninucleate and haploid aeciospores. However, the cankers L1, L2, K1, K2 (*P. pini*) and also canker P (*C. flaccidum*) were confirmed to contain dikaryotic aeciospores by fluorescence microscopy (Fig.2) as was detected by Hiratsuka (1968) and Gibbs et al. (1988). This is also in accordance with the observation of Vogler et al. (1997) that aeciospores of homozygous *Peridermium harknessii* (J.P. Moore) strains are predominantly binucleate. Therefore other explanation than the uninucleate aeciospores is needed to explain the lack of heterozygosity in the aecia of *P. pini*.

Hiratsuka (1981) proposed that in the genus *Endocronartium* (*Peridermium*) the spermatia would fertilize the haploid spermogonia, the resulting diploid hyphae would then form diploid aecia and haploidization would occur via meiosis in the germ tubes of aeciospores. The lack of variation in the cankers studied here clearly disagrees with the possibility of non-self spermatia fertilizing resident receptive hyphae, as such action should result in polymorphic aecia. However, if the spermatia would only be dispersed within a single canker, fusion between receptive hyphae and spermatia from the same haploid individual (e.g. selfing or homothallism) would result in homogenic and homozygotic aecia within single cankers (III). Thus the homozygosity of the samples could be explained by primary homothallism, which can not be ruled out only by the high genetic polymorphism of *P. pini* (IV). On the other hand, there is no known reason why spermatia of *P. pini* would not spread within stands and between trees as effectively as those of Finnish *C. flaccidum* (this study), North-American *C. ribicola* or Mediterranean *C. flaccidum* (Hamelin 1996, White et al. 1996, Moricca et al. 1996). Therefore, a canker located in a polymorphic *P. pini* population, can be expected to include several genotypes within single cankers. It can be concluded that neither of the hypotheses for the life cycle of *P. pini* does completely explain the observed gene composition of *P. pini* aecia.

5.3 Morphological or molecular evidence does not support the division of *P. pini* and *C. flaccidum* into two species

5.3.1 Morphology

The details on annulate warts of *C. ribicola* were similar to those described by Hiratsuka (1971) and Imazu et al. (1989). Although some *Cronartium* species reflect differences in spore morphology (Hiratsuka 1971, Imazu et al. 1989), Grand and Moore (1972) were not able to find specific characteristics between *Cronartium* species in North America. Also Imazu et al. (1989) observed similar, annulated warts on the aeciospores of *E. yamabense* and *C. ribicola*.

The average dimensions of aeciospores reveal some variation between the samples. According to Peterson (1967), the average dimensions of the spores of *P. pini* (*C. flaccidum* sensu lato) were 22-28 μm (length) and 17-21 μm (width); the ratio l / w was 1,2-1,4. These dimensions are consistently shorter than in this study. However, Peterson (1967) did not describe the origin of the spores. Although Imazu et al. (1989) observed some differences in the average dimensions of the spores from different locations, they did not test these differences statistically. The discriminant analysis of the grouping into nine sampling locations resulted in one statistically significant discriminant function, although some of the group centroids overlapped. However, at the species level, the discriminant analysis of the dimension variables did not reveal statistically significant axes in the data space, as seen in the plot of the centroids of the pre-defined groups (species) against the two discriminant functions. The differences in spore dimensions can also reflect some other difference than a genetic one. For example, the size of the spores most likely depends on the maturity of the spores,

which could not be standardized between the samples. The evident conclusion is that the analysis of the dimensions did not support the grouping of the spores into species. Despite of the clear difference in host specificity between *C. flaccidum* and *P. pini*, their close connection has already been demonstrated in a number of genetic and other studies. The ultrastructure of *P. pini* hyphae within pine cortex was studied by Walles (1974), and when Longo (1982) compared these results with the ultrastructure of *C. flaccidum* he was not able to find any differences in structure of septa or haustoria. The spore surface of these species was indistinguishable (II), and the germ tube morphology of the two fungi was highly variable but did not separate them (Gibbs et al. 1988, Kaitera et al. 1999).

5.3.2 Molecular evidence

Previously it has been observed that the IGS and ITS regions of the nuclear DNA of the two species are closely related (Moricca et al. 1996) and they contain similar RAMS markers (Hantula 1998). The results of III and IV show similar specific DNA markers in the two species. This observation indicates that they have identical marker alleles of marker loci and it further strengthens the close relationship between the two fungi. There were no observable population differentiation between *P. pini* and *C. flaccidum* (IV), which means that some gene flow exists or has recently occurred between these two types of fungi. In each respect, *P. pini* and *C. flaccidum* look like a single species. It is not known if spermatia of *P. pini* can be paired with receptive hyphae of *C. flaccidum* or vice versa. The possibility of these two types of fungi to pair would create gene flow enough to prevent observable genetic differentiation.

In the case of *P. harknessii* it has been suggested that its genetically different types (zymodemes) would have evolved as life cycle mutants of macrocyclic *C. quercuum* (Vogler et al. 1997). Although the case of *C. flaccidum*/*P. pini* complex differs in many details from that of *C. quercuum*/*P. harknessii* it can be considered highly probable that also *P. pini* would have its origin as a life cycle mutant of *C. flaccidum*. Combining this with the lack of any hypotheses capable to explain the origin of genetic variation in *P. pini* aecia (III) the following working hypothesis was proposed: *P. pini* would have originated as a haploid mutant of *C. flaccidum*, and the two nuclei observable in the aeciospore would be genetically identical. This hypothesis was in accordance with all the data available including the lack of morphological differentiation of the two fungi (I), haplotype distribution within stands (II), genetic fine structure of cankers (III), amount of genetic variation within populations (IV) and finally, the observation of linkage disequilibrium within *P. pini* populations (IV).

5.4. How can the close relationship of *C. flaccidum* and *P. pini* be explained ?

The hypothesis where *P. pini* has risen as a haploid mutant of *C. flaccidum* was suggested in III and tested by analysis of genetic differentiation in IV. The hypothesis

seemed to explain the observations but the actual mechanism behind the close relationship needs also to be explained.

The change of a pine stem rust strain from a heteroecious to an autoecious fungus could be explained by facultative heteroecism, a hypothetical mechanism where some strains of rust fungi are capable of infecting both pine and alternate host. Facultative heteroecism was described by Meinecke (1929) but has never been observed again. Wagener (1964) summarized several attempts to repeat the experiment, criticized strongly the Meinecke's experimental set-up, and finally concluded that the observation was false. It is noteworthy that facultative heteroecism could have been a true observation if it was caused by a rare mutation.

Another explanation for sudden and common host jumps could be virulence/ avirulence of aeciospores for different hosts. Resistance of pines or alternate hosts could thus explain local differences in distribution of fungi. Single genes coding for resistance in pine has been found in the pathosystem of white pine blister rust *C. ribicola* (Kinloch et al. 1999). It has also been observed that virulence in white pine blister rust could be coded by a single cytoplasmically inherited element (Kinloch and Dupper 1999). For example, let us make an assumption that a single gene coding avirulence for pine would exist in a *C. flaccidum* population, expressed only within aeciospores (if such a gene would be expressed in haploid basidiospores, it would disappear since basidiospore containing the gene could not infect pine or anything). Since avirulence is usually considered a dominant factor (Flor 1971), resistance of pine could be broken by a fungal strain that is recessive homozygote for pine virulence. Thus a portion (25%) of aeciospore population homozygous for virulence could infect pines, and thus turn to *P. pini*. Interestingly, observed *P. pini* genotypes were exclusively homozygotes for both marker loci. It must be noted that observation of avirulence or virulence factors by two neutral marker genes would be fortunate and practically not possible, since genes that could be involved within infection of pine or alternate host are most likely not linked with markers used in this study. On the other hand, genotypes that are homozygotes for virulence and neutral genetic markers could arise via homothallic mating (selfing) of a haploid virulent strain, but homothallism of *C. flaccidum* was not supported in this study. It is probable that the selection pressure caused by host resistance exists in this pathosystem, but it is difficult to conclude from the results of this study.

Although the hypothesis where *P. pini* has risen as a haploid mutant of *C. flaccidum* is otherwise well justified, the distribution of *P. pini* and *C. flaccidum* does not fit into this scenario. Thus a mutation that causes *C. flaccidum* to turn into *P. pini* should be very common in Finland and Great Britain, but absent in Mediterranean countries, where *C. flaccidum* is the dominant species. Also the ecological benefits that cause selection pressure against heteroecious life cycle are difficult to assess. Alternate hosts (especially *Melampyrum*) are common in pine stands, although locally there is plenty of stands where *Melampyrum* is absent. Some *Melampyrum* populations might be resistant to local *C. flaccidum*, which leads to selection pressure towards *P. pini*. A further selection benefit for an autoecious life cycle is that the infection of pines by

aeciospores released from cankers in pine crowns, probably is more efficient strategy of spreading than the basidiospore load from the forest floor.

It is obvious that the current knowledge and the results of this study do not explain totally the mechanism which makes it possible for *C. flaccidum* to turn into *P. pini*. It is highly probable that resistance of polymorphic host populations causes selection pressure on a fungal population, and, at least locally, autoecious life cycle has its ecological benefits that cause additional selection.

6. Conclusions

No overall morphological or genetic differentiation exists between *P. pini* and *C. flaccidum*. The two fungi differ only by the existence of linkage disequilibrium within *P. pini* and the population fine structure (i.e. genetic structure of aecia within cankers), which both suggested that *P. pini* is clonal. Thus it can be considered highly probable that *P. pini* strains are clonal and they have their origin as haploid life cycle mutants of *C. flaccidum*, that has a sexual life cycle. This hypothesis was in accordance with all the data available including the lack of morphological differentiation of the two fungi (I) haplotype distribution within stands (II), genetic fine structure of cankers (III), amount of genetic variation within populations (IV) and finally, the observation of linkage disequilibrium within *P. pini* populations (IV). The actual mechanism which causes *C. flaccidum* to turn into *P. pini* remains unknown.

7. References

- Boidin, J., Lanquetin, P. 1984. Indices of useful informations for intercompatibility tests in basidiomycetes III. Non poroid aphyllophorales. *Cryptogamie, Mycol.* 5: 193-245.
- Burdon, J. J., Roelfs A.P. 1985. The effect of sexual and asexual reproduction on the isozyme structure of populations of *Puccinia graminis*. *Phytopathology* 75: 1068-1073.
- Dillon, W.R., Goldstein, M. 1984. *Multivariate Analysis. Methods and Applications*. New York: John Wiley. pp. 395-429.
- Doudrick, R. L., Nance, W. L., Nelson, C. D., Snow, G. A., Hamelin, R. C. 1993. Detection of DNA polymorphisms in a single urediniospore-derived culture of *Cronartium quercuum* f. sp. *fusiforme*. *Phytopathology* 83: 388-392.
- Dusabenyagasani, M., Lecours, N., Hamelin, R. C. 1998. Sequence-tagged sites (STS) for studies of molecular epidemiology of scleroderma canker of conifers. *Theor. Appl. Genet.* 97: 789-796.
- Excoffier, L., Smouse, P.E., Quattro, J.M. 1992. Analysis of molecular variance inferred from metric distances among DNA haplotypes: application to human mitochondrial DNA restriction data. *Genetics* 131: 479-491.
- Et-touil, K., Bernier, L., Beaulieu, J., Berube, J.A., Hopkin, A., Hamelin, R.C. 1999. Genetic structure of *Cronartium ribicola* populations in Eastern Canada. *Phytopathology* 89: 915-919.
- Epstein, L., Buurlage, M.B. 1988. Nuclear division in germinating aeciospores and its taxonomic significance for the western gall rust *Peridermium harknessii*. *Mycologia* 80: 235-240.
- Fischer, S.G., Lerman, L.S. 1983. DNA fragments differing by single base-pair substitutions are separated in denaturing gels: Correspondence with melting theory. *Proc. Nat. Acad. Sci. USA* 80: 1579-1583.
- Flor, H.H. 1971. The current status of gene-for-gene concept. *Ann. Rev. Phytopath.* 9: 275-297.
- Gibbs, J.N., England, N., Wolstenholme, R. 1988: Variation in the pine stem rust fungus *Peridermium pini* in the United Kingdom. *Plant Pathology* 37: 45-53.
- Gitzendanner, M.A., White, E.E., Foord, B.E., Dupper, G.E., Hodgskiss, P.E., Kinloch, B.B. Jr. 1996. Genetics of *Cronartium ribicola*. III. Mating system. *Can. J. Bot.* 74: 1852-1859.
- Grand, L.F.; Moore, R.T. 1972. Scanning electron microscopy of *Cronartium* spores. *Can. J. Bot.* 50: 1741-1742.
- Greig, B.J.W. 1987. History of *Peridermium* stem rust of scots pine in Thetford forest, East Anglia. *Forestry* 60: 193-202
- Haack, H. 1914. Der Kieozopf (*Peridermium pini*) Willd. Kleb. und seine Übertragung von Kiefer zu Kiefer ohne Zwischenwirt. *Zeitschrift f. Forst- und jagdwesen* 46: 3.
- Hamelin, R.C. 1996: Genetic diversity between and within cankers of the white pine blister rust. *Phytopathology* 86: 875-879.
- Hamelin, R.C., Dusabenyagasani, M., Et-touil, K. 1998. Fine-level genetic structure of white pine blister rust populations. *Phytopathology* 88: 1187-1191.
- Hantula, J., Dusabenyagasani, M., Hamelin, R.C. 1996. Random amplified microsatellites (RAMS) - a novel method for characterizing genetic variation within fungi. *Eur. J. For. Path.* 26: 159-166.
- Hantula, J., Niemi, M.E., Kaitera, J., Jalkanen, R., Kurkela, T. 1998. Genetic variation of the resin top fungus in Finland as determined by random amplified microsatellites (RAMS). *Eur. J. For. Path.* 28: 361-372.
- Helfer, S. 1992. The rust diseases of willows in Britain. *Proc. of the Royal Soc. in Edinburgh.* 98 B: 119-134.
- Hiratsuka, Y., Morf, W., Powell, J.M. 1966. Cytology of aeciospores and aeciospore germ tubes of *Peridermium harknessii* and *P. stalactiforme* of the *Cronartium coleosporoides* complex. *Can. J. Bot.* 44: 1639-1643.
- Hiratsuka, Y. 1968. Morphology and cytology of aeciospores and aeciospore germ tubes of host-alternating and pine-to-pine races in *Cronartium flaccidum* in northern Europe. *Can. J. Bot.* 44: 1639-1643.
- Hiratsuka, Y. 1969. *Endocronartium*, a new genus for autoecious pine stem rusts. *Can. J. Bot.* 47: 1493-1495.
- Hiratsuka, Y. 1971. Spore surface structure of pine stem rusts of Canada as observed under a scanning electron microscope. *Can. J. Bot.* 49: 371-372.
- Hiratsuka, Y. 1981. Host relationship, life cycle, and species concept of *Cronartium* and *Endocronartium*. *Lev. Proc. XVII IUFRO World Congress.* p. 247-257.

- Hiratsuka, Y. 1995. Pine stem rusts of the world - frame work for a monograph. In: Proc. of the Fourth Conference of the IUFRO Rusts of Pines Working Party. Ed. by Kaneko, S.; Katsuya, K.; Kakishima, M.; Ono, Y. Tsukuba: IUFRO Rusts of Pines Working Party. p.1-9.
- Hunt, R. S. 1985. Experimental evidence of heterothallism in *Cronartium ribicola*. Can. J. Bot. 63: 1086-1088.
- Imazu, M., Kakishima, M., Kaneko, S. 1989. *Endocronartium sahoanum*, a new stem rust fungus on *Pinus pumila* in Japan. Trans. Mycol. Soc. Japan 30:301-310.
- Imazu, M. Kakishima, M., Katsuya, M. 1991. Morphology and nuclear cycle of *Endocronartium yamabense*. Trans. mycol. soc. Japan 32: 371-379.
- Kaitera, J., Aalto, T., Jalkanen, R. 1994. Effect of resin-top disease caused by *Peridermium pini* on the volume and value of *Pinus sylvestris* saw timber and pulpwood. Scand. J. For. Res. 9: 376-381.
- Kaitera, J., Hantula, J. 1998. *Melampyrum sylvaticum*, a new alternate host for pine stem rust *Cronartium flaccidum*. Mycologia 90: 1028-1030.
- Kaitera, J. 1999. *Cronartium flaccidum* fruitbody production on *Melampyrum* spp. and some important alternate hosts to pine. Eur. J. For. Path. 29: 391-398.
- Kaitera, J., Seitämäki, L., Hantula, J., Jalkanen, R., Kurkela, T. 1999a. Inoculation of known and potential alternate hosts with *Peridermium pini* and *Cronartium flaccidum* aeciospores. Mycol. Res. 103: 235-241.
- Kaitera, J., Seitämäki, L., Hantula, J., Jalkanen, R., Kurkela, T. 1999b. Morphological variation of *Peridermium pini* and *Cronartium flaccidum* aeciospores. Mycol. Res. 103: 677-683
- Kamp, B.J. van der. 1993. Limits to selection for disease resistance from natural tree populations. Can. J. For. Res. 23: 1944-1947.
- Kasanen, R., Hantula, J., Kurkela, T. 2000. The occurrence of a previously undescribed *Venturia* species in the blighted shoots of *Populus tremula*. Mycol. Res. 105: 338-343.
- Korhonen, K., Stenlid, J. 1998. Biology of *Heterobasidion annosum*. In: Woodward, S., Stenlid, J., Karjalainen, R., Hüttermann, A. *Heterobasidion annosum*- Biology, Ecology, Impact and Control. CAB International. University press. Cambridge.
- Kinloch, B.B. jr, Dupper, G.E. 1999. Evidence of cytoplasmic inheritance of virulence in *Cronartium ribicola* to major gene resistance in sugar pine. Phytopathology 89: 192-196.
- Kinloch, B.B. jr., Sniezko, R.A., Barnes, G.A., Greathouse T.E. 1999. A major gene for resistance to white pine blister rust in western white pine from the Western Cascade Range. Phytopathology 89: 861-867
- Klebahn, H. 1918. *Peridermium pini* (Willd.) Kleb. und seine Übertragung von Kiefer zu Kiefer. Flora 11: 194-207.
- Klebahn, H. 1924. Kulturversuche mit Rostpilzen XVII. Zeitschrift für Pflanzenkrankheiten und Gallenkunde. XXXIV:289-303.
- Klebahn, H. 1938. Offene fragen und neue beobachtungen über die rindebewohnenden Blasenroste der Kiefern nebst Bemerkungen über einige andere Rostpilze IX. Zeitschrift für Pflanzenkrankheiten und Pflanzenschutz 48: 369-410.
- Kuhlman, E.G., Matthews, F.R. 1993. Variation in virulence among single-aeciospore isolates from single-gall isolates of *Cronartium quercuum* f.sp. *fusiforme*. Can. J. For. Res. 23: 67-71.
- Laundon, M. 1976. *Peridermium* (fungi). Taxon 25: 186-187.
- Lee, S.B., Taylor, J.W. 1990. Isolation of DNA from fungal mycelia and single spores. In: Innis, M.A., Gelfand, D.H., Sninsky, J.J. ja White, T.J. (ed.). PCR Protocols. A Guide to Methods and Applications. San Diego. Academic Press. 282-287.
- Liro, J.I. 1907. Kulturversuche mit finnischen Rostpilzen. Acta Societatis pro Fauna et Flora Fennica 29-52.
- Longo, N. 1982. Ultrastructural observations on the septal pore in *Cronartium flaccidum* (Alb. et Schw.) Wint. also in relation to the taxonomy of the uredinales. Caryologia 35: 425-441.
- McDermott, J.M., McDonald, B.A. 1993. Gene flow in plant pathosystems. Ann. Rev. Phytopathol. 31: 353-373.
- McDonald, B.A., Martinez, J.P. 1990. DNA restriction fragment length polymorphisms among *Mycosphaerella graminicola* (anamorph *Septoria tritici*) isolates collected from a single wheat field. Phytopathology 80: 1368-1373.
- Meinecke, E.P. 1929. Experiments with repeating pine rusts. Phytopathology 19: 327-342.

- Millar, C., Kinloch, B.B. 1991. Taxonomy, phylogeny and coevolution of pines and their stem rusts. In: Rusts of Pine. Proc. of the IUFRO Rusts of Pine Working Party Conference. Ed. by Hiratsuka, Y., Samoil, J.K., Blenis, P.V., Crane, P.E., Laishley, B.L. For. Can. Northwest Reg., North. For. Cent. Inf. Rep. NOR-X-317. p.1-39.
- Moricca, S., Kasuga, T., Mitchelson, K., Ragazzi, A., Diamandis, S. 1996. Heterogeneity in intergenic regions of the ribosomal repeat of the pine-blister rusts *Cronartium flaccidum* and *Peridermium pini*. Curr. Genet. 29: 388-394.
- Moricca, S., Ragazzi, A. 1994. Axenic culture of the aecial state of *Cronartium flaccidum* from Italy. Mycol. Res. 98: 1258-1262.
- Moricca, S., Ragazzi, A. 1998. Use of RFLP and SSCP analysis to differentiate the pine rusts *Cronartium flaccidum* and *Peridermium pini*. Mycol. Res. 102: 666 -670.
- Nei, M. 1973. Analysis of gene diversity in subdivided populations. Proc. Natl. Acad. Sci. USA 70: 3321-3323.
- Pappinen, A., Weissenberg, K. von. 1994 a. Association of the pine top weevil with *Endocronartium pini* on Scots pine. Eur. J. For. Path. 24: 249-257.
- Pappinen, A., Weissenberg, K. von. 1994 b. The ability of the pine top weevil to carry spores and infect scots pine with *Endocronartium pini*. Eur. J. For. Path. 24: 258-263.
- Pei, M., Gibbs, J.N. 1991. Cultural variation in *Peridermium pini* from two parts of the United Kingdom. Eur. J. For. Path. 21: 164-167.
- Peterson, R.S. 1967. The *Peridermium* species on pine stems. Bull. Torr. Bot. Club. 94: 511-542.
- Pierson, R. K. 1933. Fusion of pycniospores with filamentous hyphae in the pycnium of the white pine blister rust. Nature 131: 728-729.
- Olembo, T.W. 1971. A study on the mode of infection of *Pinus sylvestris* L. by *Peridermium pini* Pers. Lev. Forestry 44: 67-79.
- Raddi, P., Ragazzi, A. 1982. Italian studies on resistance to pine blister rust (*Cronartium flaccidum*). In: Resistance to Diseases and Pests in Forest Trees. Ed. by Heybroek, H.M., Stephan, B.R. and Weissenberg, K. von. Wageningen: Pudoc. p. 236-242.
- Rennerfelt, E. 1943. Om vår nuvarande kunskap om törskatesvampen och sättet för dess spridning och tillväxt. Svenska Skogsvårdsföreningens Tidskrift 41, 305-324.
- Schlötterer, C., Tautz, D. 1992. Slippage synthesis of simple sequence DNA. Nucleic Acids Res. 20: 211-215.
- Smith, M.L., Bruhn, J.N., Anderson, J.B. 1992. The fungus *Armillaria bulbosa* is among the largest and oldest living organisms. Nature 356: 428-431.
- Swertz, C.A. 1994. Morphology of germlings of urediniospores and its value for the identification and classification of grass rust fungi. Institute of the Royal Netherlands Academy of Sciences and Lettres. Studies in Mycology 36.
- Tenzer, I., Ivanissevich, S.D., Morgante, M., Gessler, C. 1999. Identification of microsatellite markers and their application to population genetics of *Venturia inaequalis*. Phytopathology 89: 748-753.
- Tubeauf, C. von. 1917. Über das Verhältnis der Kiefern-Peridermien zu *Cronartium*. II. Studien über die infektion der Weymouthskiefern. Naturwissenschaftliche Zeitschrift für Forst- und Landwirtschaft 15: 274-307.
- Tuskan, G.A., Walla, J.A., Lundquist, J.E. 1990. Genetic-geographic variation in *Peridermium harknessii* in the north-central United States. Phytopathology 80: 857-861.
- Wagener, W.W. 1964. "Facultative heteroecism" Was it demonstrated in *Peridermium harknessii* in 1919-1920? Mycologia 56: 782-785.
- Vainio, E. J., Korhonen, K., Hantula, J. 1998. Genetic variation in *Phlebiopsis gigantea* as determined with random amplified microsatellite (RAMS) markers. Mycol. Res. 102: 187-192.
- Wallis, B. 1974. Ultrastructure of the rust fungus *Peridermium pini* (Pers.) Lev. Stud. For. Suecica 122: 1-30.
- Wang, X-R. 1997. Genetic variability in the canker pathogen fungus *Gremmeniella abietina*. Contribution of sexual compared with asexual reproduction. Mycol. Res 101: 1195-1201
- Vasiliaskas, R. Stenlid, J. 1998. Spread of *Stereum sanguinolentum* vegetative compatibility groups within a stand and within stems of *Picea abies*. Silva Fennica 32: 1-9.
- Welsh, J., McClelland, M. 1990. Fingerprinting genomes using PCR with arbitrary primers. Nucleic Acids Res. 19: 303-306
- White, E. E., Foord, B. M., Kinloch, B.B. Jr. 1996. Genetics of *Cronartium ribicola*. II. Variation in the ribosomal gene cluster. Can. J. Bot. 74: 461-468.

- Williams, J.G.K., Kubelik, A.R., Livak, K.J., Rafalski, J.A., Tingey, S.V. 1990. DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. *Nucleic Acids Res.* 18: 6531-6535.
- Vogler, D.R., Kinloch, B.B.Jr., Cobb, F.W., Popenuck T.L. 1991. Isozyme structure of *Peridermium harknessii* in the western United States. *Can. J. Bot.* 69:2434-2441.
- Vogler, D. R., Epstein, L., Cobb, F.W. Jr. 1997. Nuclear behaviour and evolution of two populations of the western gall rust fungus. *Mycol. Res.* 101: 791-797.
- Zietkiewics, E., Rafalski, A., Labuda, D. 1994. Genome fingerprinting by simple sequence repeat (SSR)-anchored polymerase chain reaction amplification *genomics* 20: 176-183