Population Genetics of the Red-listed Wood-decay Fungus *Phlebia centrifuga*

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Abstract

The aim of this licentiate thesis was to study the population genetic structure of the wood-decay fungus *Phlebia centrifuga* P. Karst. in northern Europe. The fungus, which is red-listed in Sweden, is dependent on dead wood of Norway spruce (*Picea abies*) and therefore rare in managed forest stands.

The isolates used in the studies included in this licentiate thesis were sampled from both continuous and fragmented populations of *P. centrifuga* in eight European countries and six North American states.

The first study included development of seven polymorphic microsatellite markers specific to *P. centrifuga*, using two different techniques. Of the seven primers, two varied only on a worldwide scale, whereas the other five varied both on a worldwide and a European scale.

In the second study, microsatellite markers and arbitrary primed PCR using the core sequence of the M13 minisatellite DNA as marker were used to study the genetic structure of eight populations of *P. centrifuga* in northern Europe. Here, the question studied was whether the dispersal of out-crossing basidiospores of the fungus manage to overcome the genetic isolation of populations that occur in fragmented habitats. No significant inbreeding was detected in any of the populations. The pair-wise comparisons of the fixation index (F_{ST}) generally revealed little to moderately low genetic differentiation. However, all comparisons with the southern-most population, on the edge of the distribution of the species, showed moderately high or even great genetic differentiation.

The results suggest that the basidiospores of the fungus do not completely overcome genetic isolation between fragmented populations, which might eventually lead to inbreeding. Different ways of assisting the persistence of these populations were discussed.

Keywords: wood-decaying fungi, *Phlebia centrifuga*, population genetics, old-growth forests, habitat fragmentation, conservation, forest management

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Dedication

To me, for making it ashore after getting myself into deep waters...

Contents

List of Publications

This licentiate thesis is based on the work contained in the following papers, referred to by Roman numerals in the text:

I Franzén, I., Slippers, B., Vasiliauskas, R. & Stenlid, J. 2006. Development of microsatellite markers for the red-listed wood-decay fungus *Phlebia centrifuga*. *Molecular Ecology Notes*, 6, 870-872.

II Franzén, I., Vasaitis, R., Penttilä, R. & Stenlid, J. 2007. Population genetics of the wood-decay fungus *Phlebia centrifuga* P. Karst. in fragmented and continuous habitats. *Molecular Ecology*, 16, 3326-3333.

For both papers, I have planned the research together with my co-authors. In paper I, I carried out the laboratory work and compiled the manuscript. In paper II, I performed a large part of the laboratory work, and took part in the data analysis as well as the compilation of the manuscript.

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1 Introduction

Fungi are very important as principal decomposers in terrestrial ecosystems and play the most important role in the decomposition of wood, particularly in boreal forests (Burnett, 2003; Richards, 1987). According to Gärdenfors (2000) there are about 4800 species of macrofungi, i.e. fungi with fruit bodies larger than ~1 mm, in Sweden. The majority of these are found in woodland and forest ecosystems and almost 63 % of them are associated with dead wood.

In natural forests, dead wood make up a considerable part of the wood volume, whereas the amount of dead wood is low in intensively managed forests. This affects the species richness in these habitats. For example, in European boreal forests, the diversity of wood-decay fungi is low in managed forests compared to unmanaged old-growth stands that harbor many rare and threatened species (Siitonen, 2001; Penttilä, 2004; Penttilä *et al.*, 2004).

This lack of substrate for wood-inhabiting fungi in managed forests restrict certain species to unmanaged virgin forests, where there is enough dead wood to sustain viable populations. Unfortunately, due to the long history of forestry in Sweden old-growth forests have become fragmented and larger areas with continuous natural forests can only be found in the north of the country (Angelstam, 1997).

Habitat fragmentation is one of the major threats to biodiversity in forests (Angelstam, 1997, and references therein). By reducing the habitat distribution and splitting the habitat into smaller and more or less isolated fragments, it causes a division of the population into several subpopulations. Small isolated subpopulations might suffer severe genetic consequences

(Hartl & Clark, 2007). The gene flow between different subpopulations may be constrained, reducing the genetic variation and causing genetic differentiation between the subpopulations. Inbreeding may also occur, impairing fitness and further reducing the genetic diversity. Ultimately, these genetic threats impose a danger of extinction on the species.

Studies on different species of Aphyllophorales have shown that the genetic differentiation usually is limited on a regional scale in Northern Europe. However, the results for *Fomitopsis rosea*, a rare polypore species, are ambiguous. Högberg and Stenlid (1999) observed that there was a significant heterozygote deficit among North European populations of *F. rosea*, whereas Kauserud and Shumacher (2003) found low levels of genetic differentiation, indicating that Fennoscandian populations of *F. rosea* all belong to a larger population. The apparent contradiction may be caused by differences in size of the sampled populations. In the study by Högberg and Stenlid (1999) considerably larger populations were sampled than by Kauserud and Schumacher (2003), and the population genetic consequences were thereby more easily expressed.

Several studies have shown that basidiospores of wood-decay fungi are widely and efficiently dispersed, for example by traveling by air over hundreds of kilometers (Risbeth, 1959; Hallenberg & Kuffer, 2001; Kallio, 1970; Stenlid & Gustafsson, 2001). However, a more recently performed study, using a species-specific spore trapping technique, showed that the ecologically effective spore spreading distances might only be a few kilometers (Edman *et al.*, 2004a; Edman *et al.*, 2004b). One consequence of restricted spore dispersal could be that population genetic bottlenecks might be the results of dispersal among geographically distinct habitats on a regional scale. To evaluate the risks, it is important to know if dispersal of out-crossing basidiospores overcomes the genetic isolation of populations that occur in fragmented habitats, and if so, on which spatial scales.

The focus of this licentiate thesis is on the wood-decay fungi *Phlebia centrifuga*, for which the effects of habitat fragmentation on the genetic population structures are studied.

2 Aims and questions

The aims of this licentiate thesis were to

> i. develop microsatellite markers specific to *Phlebia centrifuga* and study how their polymorphism varies on a worldwide and an European scale (paper I),
>
> ii. use the microsatellite markers developed to study the population genetic structures of *P. centrifuga* in fragmented and continuous habitats, and compare the results with those obtained with arbitrary primed PCR using the core sequence of M13 minisatellite DNA (paper II).

More importantly, the thesis also aims to answer the question whether the dispersal of out-crossing basidiospores of *P. centrifuga* manage to overcome the genetic isolation of populations that occur in fragmented habitats (paper II). If they do, there would be no differentiation in genetic variation between the populations (null hypothesis).

3 The species studied

Phlebia centrifuga P. Karst. is a corticioid fungus belonging to the genus Phlebia (order Aphyllophorales, family Corticiaceae). It has a circumboreal distribution and is known from northeast and central Europe, Siberia, and North America. In northern Europe, the fungus is found in northern Finland, south-east Norway, and Sweden, but it is absent in Denmark due to a lack of natural forests of its host species, Norway spruce (*Picea abies*) (Larsson, 1997).

In Sweden, *P. centrifuga* can be found through-out the whole distribution of Norway spruce except for the southernmost parts. However, it is a typical inhabitant of fallen decomposing trunks and as such dependent on coarse spruce logs, on which it causes white rot and produces annual fruit bodies where the bark is still attached. Therefore, it is only encountered in forest stands that have been unmanaged for a long time, which is the reason why it is used as a good indicator species for forests with high conservation values (Nitare, 2002). The distribution of the fungus is shown in figure 1.

Due to the fragmentation and decline of old-growth forests caused by the forestry, the fungus has been put on the Swedish red-list of threatened species as near-threatened (Gärdenfors, 2000). There are eight other species from the genus Phlebia on the Swedish red-list as well. *P. centrifuga* is also red-listed in Norway and Germany.

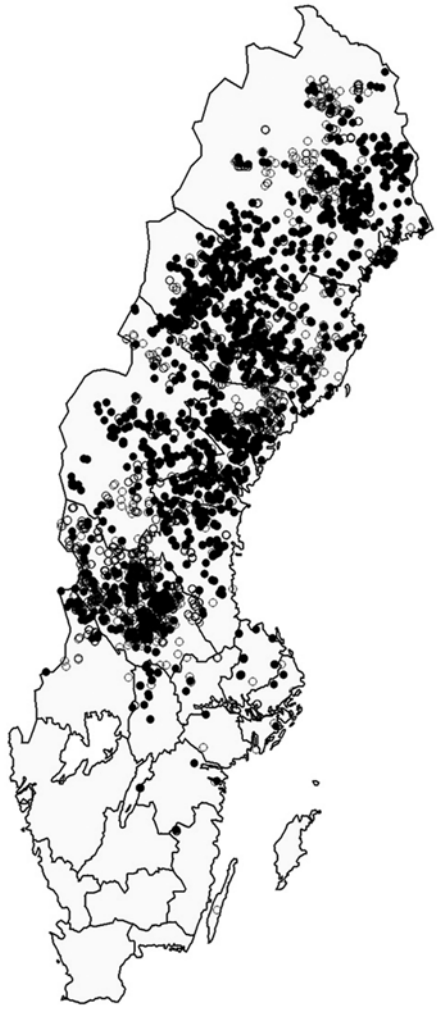

Figure 1. Map of localities where *Phlebia centrifuga* have been observed in Sweden. Filled circles are observations made after 1995 and hollow circles represent older observations made before 1995. The map was provided by Artdatabanken, 2008-01-10.

3.1 Populations

Paper II included single spore isolates of *Phlebia centrifuga* collected using spore prints at eight different sites in three different countries, shown in figure 2. The spore prints were gathered by attaching a piece of paper on the surface of the sporocarps. The spores were washed from the paper with

sterilized water, collected with a pipette and spread on Hagem agar (HA) medium (Stenlid, 1985) in Petri dishes. The dishes were examined with a microscope every day, and germinating spores were transferred individually to new dishes with HA medium, producing single spore cultures.

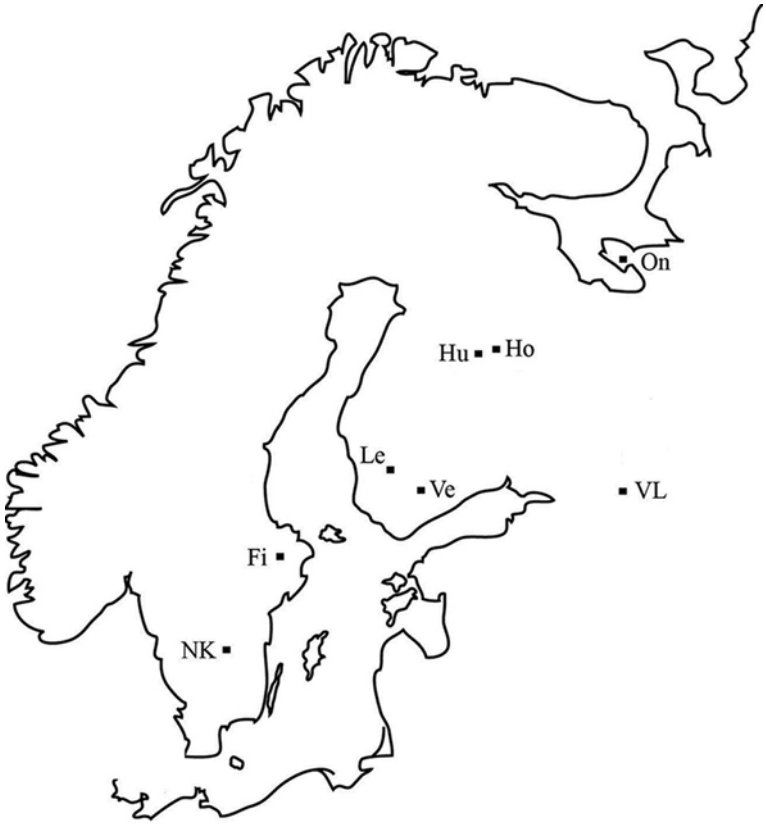

Figure 2. Map of northern Europe, showing the location of the eight European populations of *Phlebia centrifuga* included in this licentiate thesis: Norra Kvill (NK) and Fiby (Fi) in Sweden; Lehtivehmas (Le), Vesijako (Ve), Hukkapuro (Hu) and Honkavaara (Ho) in Finland; and Onega (On) and Vepskij Les (VL) in Russia.

In paper I a minor screening for microsatellite polymorphism was performed, including strains from the USA (Arizona, New York, Montana, and Michigan) and Canada (Ontario and Saskatchewan), provided by the Center for Forest Mycology Research of the United States Department of Agriculture, as well.

4 Summary of the papers

4.1 Paper I

Microsatellites are short reiterated sequences of DNA, usually 2 to 4 base pairs in length. They can be repeated up to 100 times and the number of repeats is variable between alleles. The population variation of microsatellites loci is usually high, because microsatellites exhibit rates of mutation that are as high as about 10^{-3} or 10^{-4} per locus per gamete per generation (Avise, 2004). Typically, microsatellites are neutral, co-dominant and highly abundant in the genome.

The characteristics of the microsatellite markers have made them one of the most popular and powerful of the molecular tools available today. They are useful in many fields of genetics, for example studies of kinship and population biology. Once primers for amplification are developed, large numbers of individuals can easily be screened for genotypes. Previously, polymorphic microsatellite markers specific to *Phlebia centrifuga* have not been available. Therefore, the aim of paper I was to develop such markers.

Two different techniques were used for the development of the microsatellite markers. Briefly, the first method was based on inter-simple sequence repeats (ISSR) (van der Nest, Bargelloni & Patarnello, 2000; Lian, Zhihua & Hogetsu, 2001) and the second method was based on amplified length polymorphism (AFLP) (Zane *et al.*, 2002). The ISSR-based technique was more time-consuming and costly than the AFLP-based technique, but gave 100 % specific primer sets, compared to the AFLP method that gave 70 % specific primer sets. About 40 % of the originally designed primers of both methods were usable as polymorphic markers.

In total, 17 primer sets were designed that could be used for amplification on *P. centrifuga* isolated from different European countries and North American states to test for size polymorphisms. Ten of the primer sets were shown to be polymorphic. They were used to screen a larger number of isolates for population variation using fluorescently labeled primers and AFLP analysis. Three of the primer sets were unspecific and had to be omitted, leaving seven polymorphic primer sets ready for use on *P. centrifuga* (amplifying loci Pcen1 through Pcen7). The primers for loci Pcen5 and Pcen6 were variable on a worldwide scale, but not on a European scale, whereas the remaining five primer sets were variable on both scales.

4.2 Paper II

In this paper, the question whether the dispersal of out-crossing basidiospores of *Phlebia centrifuga* manage to overcome the genetic isolation of populations that occur in fragmented habitats was addressed. In the study, two types of molecular markers were used in parallel to examine the population genetic structure of the eight northern European populations of *P. centrifuga* described above (see chapter 3.1 Populations). The first markers used were the microsatellites developed in Paper I. The second method was arbitrary primed PCR (AP-PCR) using the core sequence of the M13 minisatellite DNA as marker.

Generally, both markers gave the same pattern for the genetic population structure. No significant inbreeding was detected in any of the eight populations. The mean observed heterozygosity (H_O) was lowest in fragmented populations (for both markers in Norra Kvill and for microsatellites also for Lehtivehmas) and highest in the continuous population Onega, but did not differ from the expected heterozygosity (H_E). The highest number of non-variable loci (fixed alleles) was detected in the fragmented population Fiby for the microsatellites, and in both the Swedish populations (fragmented) for the AP-PCR. The pair-wise comparisons of the fixation index (F_{ST}) generally revealed little to moderately low genetic differentiation (both markers). However, all comparisons with the southern-most fragmented population Norra Kvill showed moderately high or even great genetic differentiation.

The results implicate that the genetic population structures observed in this study might be related to different forest landscape dynamics in southern and central Sweden compared to southern Finland and northwest Russia. The fragmentation of old-growth forests is indicated to be more recent in Finland and Russia than in Sweden. In addition, the Swedish populations are likely to have undergone a genetic bottleneck during the historical fragmentation. Consequently, the current genetic structure of the fungus could be seen as the result of a mix of population bottlenecks caused by colonization history and recent fragmentation due to anthropogenic causes, e.g. forestry.

The fungus currently shows a considerable amount of genetic variation even in the fragmented populations. However, the abundance of airborne spores, providing new migrants to the populations, has been found to be low or absent towards the south of Sweden (Edman *et al.*, 2004a, b), which implies decreased gene flow and an increasing likelihood of mating between genetically related fungal genets. Even though no population in this study showed signs of non-random mating, this might potentially change in the course of time.

5 Discussion

The study presented in paper II shows that, even though the genetic structure among most of the studied populations was moderately continuous, there is in fact some genetic differentiation between the northern European populations of *Phlebia centrifuga*, especially in the southern-most Swedish population Norra Kvill. Therefore, it can be hypothesized that the dispersal of out-crossing basidiospores of the fungus does not completely manage to overcome the genetic isolation that occurs in fragmented habitats. Further, despite the fact that no population in this study showed signs of non-random mating, there is potentially a risk of inbreeding occurring in the fragmented populations because of the low chances of new migrants arriving in the form of airborne spores. Provided that inbreeding depression is present, this indicates that the smaller, fragmented populations of *P. centrifuga*, especially in the south of Sweden, may eventually have difficulties to persist.

The persistence of these populations may be assisted by nature conservation and forest management in at least two ways. First, the requirements of substrate for the fungus to grow on need to be satisfied. This could be achieved either by leaving more coarse woody debris (CWD) in the managed forest stands or allowing more forest stands to become old-growth forests. Second, areas of suitable habitat, i.e. old-growth forests with larger amounts of CWD, need to be located close enough for the fungus to be able disperse between them. The difficulties lay in combining the goals of production and biodiversity preservation in forestry. Högberg (1998) discussed three different strategies for this, of which two physically separates production and nature conservation. The third strategy combines the two goals by creating structures to maintain biodiversity within the managed forest stands. However, many questions needed to determine which strategy

would be the most efficient are still unanswered. For example, more information is needed about the capacity of the fungus to disperse and how it is distributed in the landscape. More studies of the qualities of the structures needed to maintain viable populations are also necessary.

6 Prospects for the future

As stated earlier, there is potentially a risk of inbreeding occurring in fragmented populations of *Phlebia centrifuga* in northern Europe. To date, studies of the effects of inbreeding on wood-decaying fungi are scarce, but nevertheless indicate reduced viability of the basidiospores (Högberg, 1998; Edman *et al.*, 2004a). Possible effects of inbreeding on other characters of fitness of the fungi are basically unknown. It would therefore be of great interest to further study how inbreeding affects for example the capacity of the basidiospores to germinate, the ability of the fungus to mate and produce sporocarps, as well as the capacity of the fungus to grow, compete, and decay wood.

Another interesting aspect of fitness would be to investigate how it relates to the genetic differentiation. This type of study would, in other words, give information about the consequences of the genetic processes on the phenotype and performance of the fungus. It can be achieved by measuring different fitness characters, such as growth rate and capacity to decay wood, and calculating Q_{ST} values for these characters (McKay & Latta, 2002). It would then be possible to relate F_{ST} with Q_{ST}, and evaluate if the pattern of the phenotypic characters correlates to the genetic patterns.

To assist in finding the most efficient strategy for conservation of rare wood-decay fungi it would be of great use to develop metapopulation models based on dispersal data, as suggested by Högberg (1998). One such demographic model was presented by Gustafsson (2002), but the data put into the model was limited. The model could be improved and made more powerful by adding more data, such as information on parameters affecting the germination and fruit body development, demographic patterns, and

spore production. Perhaps it would be possible to attain some of this information from the fitness studies mentioned above.

In paper II, we hypothesized that the current genetic structure of *P. centrifuga* in northern Europe can, in part, be a result of population bottlenecks caused by the colonization history of Norway spruce, the host species of the fungus. To further understand the genetic structure of the fungus it would be interesting to study how it has followed the spread of the spruce over historic time, possibly by conducting phylogeographic analyses.

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