Ecology of *Daldinia* spp. with Special Emphasis on *Daldinia loculata*

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Abstract

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This thesis comprises studies on the xerophytic ascomycete genus *Daldinia.* By studying ecological, morphological and molecular characters of herbarium specimens, five distinct *Daldinia* species were identified from northern Europe: *D. concentrica, D. fissa, D. grandis, D. loculata* and *D. petriniae.* The last two mentioned have previously been referred to as *D. concentrica* in the literature. The distinct stromatal host-specificity of the three species: *D. concentrica, D. loculata* and *D. petriniae* was studied in an inoculation experiment. The results indicate that the observed substrate-specificity is not a result of enhanced wood-decaying capability in the substrate on which stromata are usually found.

Reproducible PCR-based molecular markers were developed for the postfire species *D. loculata* by using available sequence data of nuclear genes from species closely related to *D. loculata.* Each marker spans over one or several introns and showed relatively high intraspecific variability. The genetic variation of *D. loculata* was studied both within and among forest sites. In a local population structure study of *D. loculata* in burned birches, we found that it can establish endophytically in sound-looking wood of living trees, most probably by sexual ascospores. In birches killed by forest fire, each haploid genet occupied an extensive volume of wood. Multilocus genotyping of vegetative mycelia and offspring of the attached stromata indicated multiple matings of the relatively large vegetative mycelia in wood by several other, very small, genets. The genetic structure of six Fennoscandian and one Kamchatkan *D. loculata* subpopulation was studied in order to reveal any differentiation on a continental scale. Low levels of genetic differentiation among the Eurasian subpopulations of *D. loculata* were found, and the differentiation did not increase with distance; the Kamchatkan subpopulation, sampled more than 7000 km from the Fennoscandian subpopulations, was only moderately differentiated from the others. These results suggest that *D. loculata* consists of a large, coherent Eurasian population of latent mycelia in unburned forests, established by ascospores dispersed from scattered burned forest sites. A tentative life cycle of *D. loculata* is presented.

Key words: Xylariaceae, host-specificity, forest fire, postfire fungi, molecular markers

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Contents

Appendix

Papers I-V

The present thesis is based on the following papers, which will be referred to by their Roman numerals.

I. Johannesson, H., Læssøe, T. & Stenlid, J. 2000. Molecular and morphological investigation of *Daldinia* in northern Europe. *Mycological Research* 104: 275-280.

II. Johannesson, H., Ihrmark, K. & Stenlid, J. Differential decay extension capability of *Daldinia* spp. in wood of *Betula pendula*, *Alnus glutinosa* and *Fraxinus excelsior*. Manuscript.

III. Johannesson, H.S., Johannesson, K.H.P. & Stenlid, J. 2000. Development of primer sets to amplify fragments of conserved genes for use in population studies of the fungus *Daldinia loculata*. *Molecular Ecology*, 9, 375-377.

IV. Johannesson, H., Gustafsson, M. & Stenlid, J. Local population structure of the wood decay ascomycete *Daldinia loculata*. *Mycologia*, submitted manuscript.

V. Johannesson, H., Vasiliauskas, R., Dahlberg, A., Penttilä, R. & Stenlid, J. Genetic differentiation in Eurasian populations of the postfire ascomycete *Daldinia loculata*. *Molecular Ecology*, submitted manuscript.

Introduction

Fire is considered to be the most important natural disturbance in the boreal forest, with decisive impact on forest structure and dynamics (Esseen *et al.*, 1997). This is reflected by the large number of organisms, especially fungi and insects, which are associated with postfire conditions (Esseen *et al.*, 1992). Attention has recently been called to the decrease in abundance of organisms associated with postfire habitats, which most probably is due to the efficient fire control during the last century. The postfire fungus *Daldinia loculata*, a member of the xerophytic genus *Daldinia*, is a central species utilising postfire habitats since it is associated with a fauna of rare, pyrophilous, insect species. This thesis aimed primarily at revealing the underlying causes of substrate-specificity of *Daldinia* species, and to infer the life cycle of *D. loculata* by using a population genetics approach.

The genus *Daldinia*

The genus *Daldinia* Ces. & De Not. was named by the mycologists Cesati and De Notaris to honour their friend, the Swiss catholic monk Agosto Daldini (1817-1895)(Crivelli *et al.*, 1981). It is a member of the family Xylariaceae, and comprises approximately 20 taxa of wood-inhabiting pyrenomycetes with perithecia embedded in large stromata, that are internally concentrically zoned (Ju *et al.*, 1997). The type species of the genus is *D. concentrica* (Bolt.: Fr.) Ces. & De Not., and was originally described by Bolton (1789) as the common fungus growing on ash (*Fraxinus excelsior*) in England (Fig. 1). Stromata of *D. concentrica* have been called "cramp balls" in Great Britain because of its assumed positive relieving effect on leg cramps (Ainsworth, 1976). They have also been called "King Alfred's cake" because of their supposed resemblance to some pastries that this Saxon king reputedly burnt while falling asleep during a baking session (Ju *et al.*, 1997).

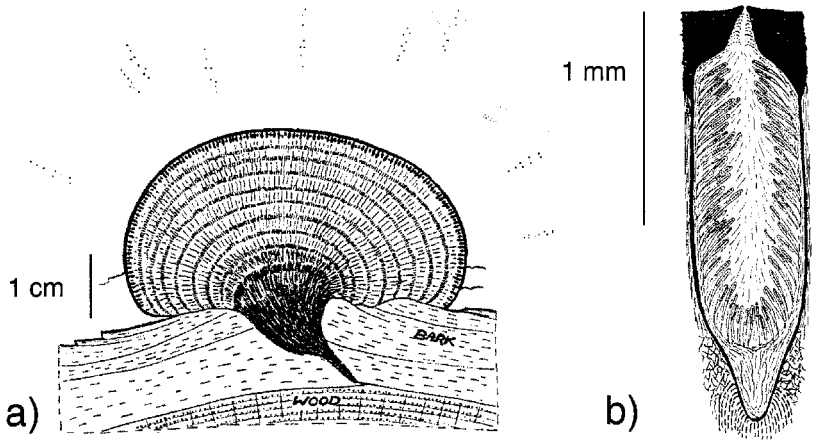

Fig. 1. a) Section through perithecial stroma of *Daldinia concentrica*. Discharged spores are seen in the air. b) A single perithecium. Modified after Ingold (1959).

Daldinia spp. as endophytes

Species of *Daldinia*, along with a large number of species belonging to the Xylariaceae, are suggested to be endophytes, i.e. organisms that live inside the plant tissue for at least part of their life cycles without causing any disease symptom in the host (Whalley, 1996 and references therein). Several endophytic fungi are suggested to be present as latent propagules, such as yeast spores or hyphal fragments, in the sapstream of the living host. The propagules remain passive when the sapwood is functional in water conduction, but develop into mycelia and start to decay dysfunctional sapwood when this begins to dry out. This phenomenon is a possible explanation of the rapid development of extensive columns of decay caused by a single fungal genet, in the sapwood and trunks of angiosperm trees with no sign of exterior wounds (Boddy, 1994 and references therein). The initial phase of endophytic interactions is largely unknown, and there are only few examples studied. In the xylariaceous endophytes *Hypoxylon mediterraneum, H. fuscum* and *H. fragiforme*, contact with tissue of the most common host has been shown to trigger germination or increase the germination rate of ascospores (Chapela *et al.*, 1991; Vannini *et al.*, 1996). In the latter species, ascospores germinate rapidly on the specific host, *Fagus sylvatica*, by a mechanism mediated by monolignol glucosides in the host acting as specific recognition messengers (Chapela *et al.*, 1990; 1993).

Wood decomposition

Daldinia spp. cause a white rot of host wood, i.e. both cellulose and lignin are degraded (Cartwright & Findlay, 1958; Rogers, 1979). *D. concentrica* is an effective wood-decayer; in two months it can cause a 62,9 % weight loss in sapwood blocks of birch (Nilsson *et al.*, 1989). Wood decayed by *D. concentrica* characteristically has a gross appearance of concentric dark speckled rings, resulting from dark coloured mycelium in the vessels of spring wood, and is sometimes termed Calico wood (Panisset, 1929; Cartwright & Findlay, 1958). In later stages of decomposition, the wood exhibits a patchy appearance as a result of some areas of wood being more decayed than others (Boddy *et al.*, 1985).

Xerophytic habit

Ingold (1965), was the first to recognise that *Daldinia*, as represented by *D. concentrica*, is ecologically and physiologically xerophytic (i.e. adapted to arid conditions). It has been suggested that the gel part of the concentric rings of *Daldinia* stromata is the key to its xerophytic habit, by acting as a water reserve for spore discharge of the stroma in dry conditions (Ju *et al.*, 1997). Ingold (1946) noted that stromata, detached from their host and brought indoors, discharge ascospores for 3 or 4 weeks without additional water, with a significant loss of water in the stroma as a consequence. The waxy to carbonaceous crust encasing the ringed stroma and bearing the perithecia probably retards evaporation from the interior (Ingold, 1971; Ju *et al.*, 1997). An attached stroma of *D. concentrica* remains active much longer than a detached one; it discharges ascospores from

the beginning of May to the end of September (Ingold, 1946). A nocturnal discharge of ascospores (Ingold, 1946) coupled with observations that ascospores can germinate within a few hours of discharge, is suggested as ecological adaptations to utilise night dew for hydration, i.e. even in very dry areas where rainfall is sporadic (Ju *et al.*, 1997). Other indications of their xerophytic life style are a high tolerance of *D. concentrica* to grow in low water potentials as compared to wood-rotting basidiomycetes (Abe, 1989; Boddy *et al.*, 1985), and the propensity of several of the *Daldinia* species, e.g. *D. loculata*, to colonise burned substrates (Rhoads, 1918; Whalley & Watling, 1980; Ju *et al.*, 1997, Paper I).

Host-preference

Teleomorphs (i.e. the sexual stage) of *Daldinia* species are believed to be exclusively angiosperm associates, and probably all produce conidia prior to, or on very young, stromata. However, the *Daldinia* anamorphs (i.e. the asexual stage), found in the genus *Nodulisporium* Preuss., are also free-living in nature. *Daldinia* spp. are found to occur in a diverse range of host plants in which they fail to produce a teleomorph (Petrini & Petrini, 1985; Petrini *et al.*, 1995), indicating a broader host-range than reflected by collections of stromata. However, several difficulties are encountered when performing studies of the host-specificity and distribution of *Daldinia* species. First, species of *Daldinia* are morphologically similar and the interpretations of taxa are somewhat confused. Thus, the name *D. concentrica* has been used for almost any entity within the genus, including taxa growing on both burned and non-burned wood of *Alnus*, *Betula, Corylus* and *Fraxinus* (Paper I). Moreover, apart from the difficulties of isolating endophytic fungi from wood (Chapela & Boddy, 1988; Chapela, 1989), anamorphs of *Daldinia* species can be difficult to identify to species level because the cultural characteristics are few and have been described for only a limited number of species (Petrini & Petrini, 1985; Petrini & Müller, 1986; Petrini *et al.*, 1995; Ju *et al.*, 1997). The problem with limitations of cultural characteristics has been solved for different genera of the Xylariaceae, by careful investigation and description of a combination of cultural and e.g. biochemical or molecular characteristics of single ascospore isolates, obtained from investigated teleomorphs (Gowan & Vilgalys, 1991; Brunner & Petrini, 1992; Rodrigues *et al.*, 1993, Whalley & Edwards, 1995, Paper I).

Teleomorph formation

The mechanism behind formation of *Daldinia* teleomorph is unknown, but have been suggested for xylariaceous fungi in general to be linked to a narrow range of host species, as well as the stage of decomposition and water-potential of the wood (Whalley 1985, 1996). Perithecial development of *Daldinia* has not been studied in detail, but Ingold (1954) described the morphology of the ascogenous system of *D. concentrica*. He found that the special feature of this extensive system of straight, unbranched, non-septate ascogenous hyphae is the elongation

of the growing tip between successive stages of ascus formation. In the base of each perithecium, a coiled archicarp can be observed, from which the ascogenous hyphae most probably arise. The young perithecium is criss-crossed with ascogenous hyphae from which asci grow into the mucilage-filled perithecial cavity (Ingold, 1954). The finding of a single archicarp in the base of each perithecium, suggests that asci of one perithecium result from only one mating event. However, result of Paper IV shows that different perithecia of stromata may be the result from different mating events.

Daldinia stromata as habitat for animals

It is well documented that stromata of *Daldinia* serve as a habitat for several insect species (Hingley, 1971; Lundberg, 1984; Wikars, 1992). In an analysis of about 1000 stromata of *Daldinia* spp., lumped under the name *D. concentrica*, Hingley (1971) found eggs, larvae, pupae and adults of 120 species of insects and arthropods inside the stromata. He found a gradually increasing number of animals in stromata during the growing season, a finding he explained with the coincident decrease in stromata density with the increased number of insects and arthropods that are in search for food, shelter or a site for egg-laying (Hingley, 1971). He concluded that "atypical forms" of *D. concentrica* found on burned birches, and interpreted by me as *D. loculata* (see Paper I), were colonised with an insect fauna different from that found in "typical forms" on non-burned *F. excelsior*. Difference in the range of secondary metabolites produced by *Daldinia* spp. (M. Stadler, personal communication) may possibly explain the observed difference in fauna.

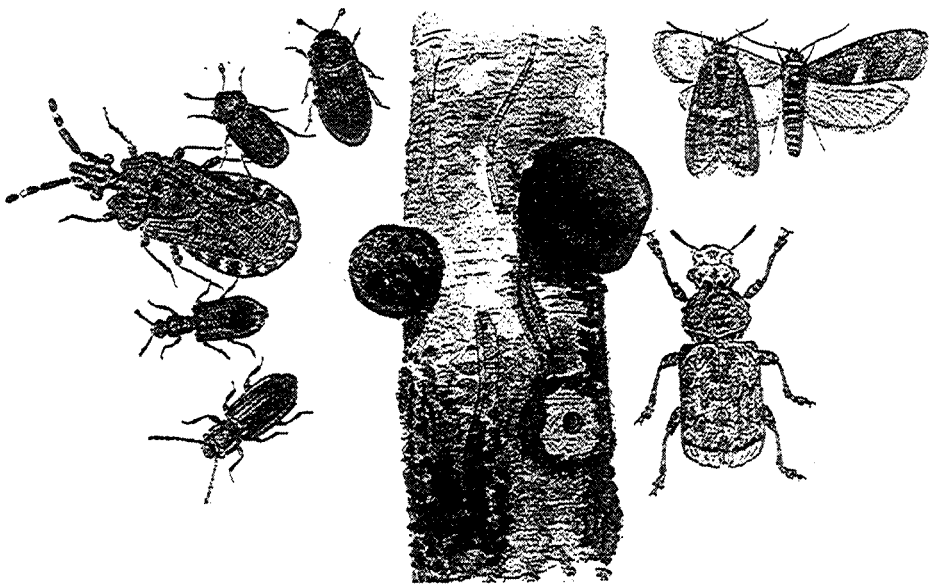

Fig. 2. A rich fauna of rare pyrophilous insects is associated with *Daldinia loculata*. *From*: Wikars & Ås, 1999. Reprinted with kind permission from the publisher.

Several insect species associated with *D. loculata* on burned substrates are both rare and pyrophilous, i.e. strongly attracted to burning or newly burned areas and have their main occurrence in burned forest 0 - 5 years after the fire (Lundberg, 1984; Wikars, 1992, Fig 2). The frequency of burned birches hosting stromata of *D. loculata* at a burned forest one the year after a fire is positively correlated with the number of pyrophilous insect species present at the site, even when the insect species directly associated with the fungus are excluded from the analysis (Wikars & Ås, 1999). This finding makes *D. loculata* suitable as an indicator species of a high species richness of pyrophilous insect (Wikars & Ås, 1999).

Fire ecology

Disturbance and fungal diversity

Fire is the most important natural disturbance in the boreal forest, with decisive impact on forest structure and dynamics (Moore, 1996). Until the beginning of the last century, fires reoccurred in Fennoscandian forests once to twice per century, annually affecting about 1% of the forest landscape (Esseen *et al.*, 1997). In contrast to northern Russia and Siberia, the active fire prevention in Fennoscandia, starting at the end of the 19th century, have almost eradicated fire as a disturbance factor in forests of this region (Vakurov, 1975; Parviainen, 1996; Niklasson & Granström, 2000).

The direct effect of fire can be destructive for the existing fungal community, particularly of wood and litter colonising fungi (Parmeter, 1977; Pugh & Boddy, 1988; Watling, 1988; Wicklow, 1988). At the same time, fire may act as an "enrichment disturbance", providing large inputs of new, mainly competition-free resources and substrates for fungi (Pugh & Boddy, 1988). The resources created by forest fires are quite homogenous, and the diversity of fungal communities on a local scale typically decreases (Zak, 1991). On the other hand, the diversity on a landscape level usually increases as the mosaic of burned and unburned areas in different successional stages is more diverse than totally unburned or burned areas (Levin & Paine, 1974; Picket & White 1985). Prescribed burnings of both forests and clear-cut areas have been introduced to forestry during the last years as an action to maintain biodiversity in the Fennoscandian boreal forest.

Fire-adapted fungal species

Importance and regularity of forest fires in boreal forests is reflected by a certain number of organisms, especially invertebrates and fungi, that are directly or indirectly dependent on, or favoured by, fire (Esseen *et al.*, 1992). As mentioned above, several *Daldinia* species are documented to exhibit a preference for burned woody substrates (Rhoads, 1918; Whalley & Watling, 1980; Ju *et al.*, 1997, Paper I). In Sweden, 75 out of 2500 species of boreal macrofungi are considered favoured by fire, of which 35 are fruiting exclusively at postfire conditions (Hallingbäck & Aronsson, 1998). The specific set of soil-dwelling discomycetes that characterise the fungal community after fire is well described (e.g. Moser,

1949; Petersen, 1970; Wicklow and Wittingham, 1978). However, only a few studies have been devoted to the effect of forest fires on wood-inhabiting fungi (Eriksson, 1958; Penttilä & Kotiranta, 1996). Studies of postfire fungi have until recently been focused mainly on monitoring of these species and the required conditions for their fruiting and to my knowledge, extensive life cycle studies have only been carried out on one postfire species, the discomycete *Geopyxis carbonaria*. *G. carbonaria* is suggested to be a common biotrophic associate of Norway spruce roots in unburned forests. It survives a forest fire at depths below the detrimental heat of a forest fire, after which an extensive production of ascocarps is triggered (Vrålstad *et al.*, 1998). A biotrophic association is subsequently re-established by the ascospores, either in the surrounding forest, or in the spruce seedlings revegetating the fire site (Vrålstad *et al.*, 1998).

Insect-fungal associations

Insects interact with a large number of fungi in several ways (Crowson, 1984; Gilbertson, 1984). In *D. loculata*, in addition to utilise the sporocarps as habitats (Lundberg, 1984; Wikars, 1992), insects may act as vectors for *dispersal* of fungal propagules to new substrates, or "*pollinate*" the fungus by bringing together two genets with different mating types (Paper IV, V).

Dispersal

Well studied examples of insects that function as vectors for fungal dispersal are the spread of wood-rotting basidiomycetes by wood wasps (Stillwell, 1966; Talbot, 1977) and of blue stain ascomycetes by bark beetles (Solheim, 1991). Wood wasps generally attack already weakened trees by simultaneously deposition of eggs and fungal oidia, mainly belonging to the genera *Amylostereum*, into the wood by a long ovipositor, after which the larvae feed on the mycelia, or the decayed wood. A wide geographical distribution of clones of *A. aerolatum* and *A. chailletii* has been attributed to the insect-fungus interaction (Vasiliauskas *et al.*, 1998; Vasiliauskas & Stenlid, 1999; Thomsen & Koch, 1999). The bark beetles act as vectors for bluestain fungi, mainly members of the family Ophiostomtataceae, by carrying either spore bearing mites or spores alone. The fungus is food for the mites and probably aids in beetle development by rapidly killing the tree (Blackwell *et al.*, 1986; Moser *et al.*, 1989; Solheim, 1991).

Pollination

The phenomenon of "pollination" of a fungus by an insect is known from diverse systems. A well-studied system is that of basidiomycete rust fungi infecting different plant species. The rust fungi modifies the host leaf morphology to produce fungal "pseudoflowers" that attract insects by visual floral mimicry, presence of nectar rewards and floral fragrances, and aid in the sexual reproduction of the fungus through the movements of the foraging insects (Roy, 1993, 1994, Pfunder & Roy, 2000). Another example constitutes the grass

endophyte *Epicloë typhina* that is cross-fertilised by female *Phorbia* flies. The flies ingest spores when they visit the fungal stromata for egg laying. Immediately following oviposition, the flies stereotypically drag their abdomen across the fungus in a spiral pattern while excreting faeces, resulting in cross fertilisation of unfertilised stromata (Bultman *et al.*, 1998). A final example given here is of the oak wilt fungus, *Cryphonectria fagacearum*. The sporulating mats of the fungus emit a fruity odour that attracts nitidulid beetles. The insects visit a number of fungal mats on different trees, and by transferring the fungus between the trees allow genets with different mating types to meet (Webber & Gibbs, 1989).

Compatibility and sexuality in ascomycetes

Two compatibility systems regulate vegetative integrity and mating of an ascomycete. The *vegetative compatibility* system maintains the genetic integrity of vegetative mycelia, while the *mating compatibility* system regulates the fusion between gametes or reproductive structures, and controls the potential for outbreeding within the species.

Vegetative compatibility

Filamentous ascomycetes are generally considered to grow vegetatively as homokaryotic mycelia, and to possess vegetative compatibility systems to prevent the coexistence of genetically different nuclei within a common cytoplasm (Rayner, 1991; Glass & Kuldau, 1992; Bégueret *et al.*, 1994; Leslie, 1996). Vegetative compatibility of ascomycetes is commonly based on biallelic interactions of many loci. For example, vegetative compatibility of *Neurospora crassa* is regulated by at least 10 biallelic loci (Perkins & Turner, 1988), which gives a potential of over a thousand (2^{10}) vegetatively compatible (vc) groups. In the filamentous fungi, compatible genotypes share alleles at all loci and fuse smoothly (Perkins & Turner, 1988), while incompatible genotypes can be identified by either a gap between the two genets, or by an interaction zone of vacuolation, hyphal deformation or death (Lane, 1981; Rayner *et al.*, 1984). The strength of the reaction generally increases with the number of allelic differences (Caten, 1972).

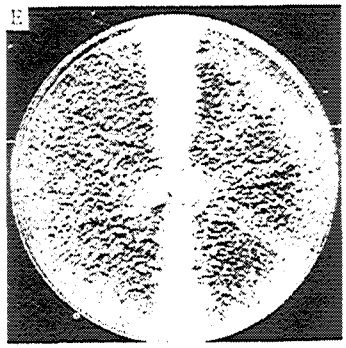

Fig. 3. Vegetative incompatibility observed between two isolates of *Daldinia concentrica*. *From*: Sharland & Rayner, 1986. Reprinted with kind permission from the publisher.

In general, vegetative compatibility provides a basis for distinguishing isolates within a species, and has been used extensively to characterise fungal populations (e.g. Brayford, 1990; Leslie, 1993; Vasiliauskas & Stenlid, 1997). However, vegetative compatibility groups do not always show a clear association with genetic individuals, as is exemplified by a study of populations of *Sclerotinia sclerotiorum* on *Ranunculus ficaria*, in which DNA fingerprint and vegetative compatibility group affiliation were found to be decoupled (Kohn, 1995).

Mating systems

The characteristic mating system of heterothallic ascomycetes is a one locus, two-allele system called bipolar. Mating in a heterothallic species is only possible between mycelia of the two opposite mating types. On the contrary, in homothallic ascomycetes a given strain is self-fertile and can give rise to sexual spores without mating with a different individual (Fincham *et al.*, 1979). Thus, in a heterothallic species, progeny from a fruitingbody consists of genetically distinct individuals, in contrast to progeny from a homothallic species. In a secondary homothallic ascomycete, a single culture gives the appearance of self-fertility because nuclei of opposite mating type are compartmentalised within a single ascospore (Fincham *et al.*, 1979). Observed vegetative incompatibility between progeny of a single perithecia of *D. concentrica* and *D. loculata* has revealed that they are heterothallic species (Sharland & Rayner, 1986; Paper IV).

Genetic structure of fungal populations

The genetic structure of populations can be affected by a number of factors: *mutations* are the source of new alleles in populations while *the reproductive mode* of the population affects the combination of the alleles. *Gene flow* refers to the spread of genes between populations, and tends to equalise gene frequencies in interacting populations. *Selection* can be a powerful agent for changing allele frequencies in the population for genes whose alleles differ in fitness when expressed in the phenotype, while *genetic drift* tend to change allele frequencies within the small population as a result of random gamete sampling. These dynamic processes may vary in time and space, making it difficult to separate and measure them accurately.

Mutations

Mutation is the ultimate source of genetic variation in organisms, and is caused by e.g. errors occurring during the process of chromosome replications or by certain chemicals or types of radiation. A mutation could be a single nucleotide substitution, i.e. a point mutation, or larger rearrangements such as deletions, insertions, substitutions or inversions. Most mutations are deleterious – while advantageous changes are considered to be much more infrequent (Kimura, 1983).

Reproductive mode

Fungal population structures range from clonal to panmictic, and the population structure of a fungus is largely dependent on its reproductive mode (Milgroom, 1996; Brown, 1999). It should be noted that, for some fungal species, genetic exchange and recombination are not necessarily limited to the sexual cycle. Pontecorvo (1956) described the parasexual cycle, in which genotypically different nuclei coexist within the same hyphal compartments following hyphal fusion. The nuclei occasionally fuse with one another, and new genotypes are then produced by two independent processes: mitotic crossing-over and haploidisation with segregation of whole chromosomes. However, these processes of recombination occur relatively infrequently, and their occurrence in natural populations has not been proven conclusively (Anderson & Kohn, 1998).

Two kinds of clonal reproduction can be distinguished: long-term and short-term clonality. In the long-term case, a population exists as distinct clones, either because there is no opportunity for sexual reproduction or because the fungus has lost the biological ability to reproduce sexually (Brown, 1999). An extreme example of long-term clonality is clonal species, or a clonal population within a species, as exemplified by the rice blast fungus, *Pyricularia grisea* (Correll & Gordon, 1999 and references therein). In short-term clonality, there is a dynamic relationship between sexual and asexual reproduction, within or between populations. Reproduction in the chestnut blight fungus, *Cryphonectria parasitica*, is characterised by self-fertilisation and outcrossing within the same population (Milgroom, 1993).

Most ascomycetes, including species of *Daldinia*, produce both sexual and asexual spores. The relative importance of the different spores for dispersal of a fungus can be estimated from the frequency of recombination in a population. First, information about association of genes at different loci can give indications of the frequency of sex in a population. In a long-term clonal population, the frequencies of alleles of different loci covaries, i.e. alleles are in gametic disequilibrium (Lewontin, 1988). However, sexual recombination breaks up associations between genetically unlinked loci, so that one complete cycle of reproduction halves the value of gametic disequilibrium. Random mating in a sexually reproducing population results in high levels of genotypic diversity, while repeatedly sampled multilocus genotypes, unlikely to arise by chance in sexual reproduction, is a result from asexual reproduction (Anderson & Kohn, 1995; Milgroom, 1996; Brown, 1999). Secondly, alleles at a locus in sexually propagating populations are expected to be in Hardy-Weinberg equilibrium, i.e. the frequency of each homozygote genotype is the square of the frequency of the relevant allele. Even a rather low frequency of sex may soon give a population the characteristics of mating; one cycle of fully sexual reproduction restores a clonal population to Hardy-Weinberg equilibrium (Hartl & Clark, 1997). Hardy-Weinberg equilibrium tests have been used to investigate random mating in for example the wood-decay basidiomycetes *Fomitopsis pinicola* and *F. rosea*

(Högberg *et al.*, 1999; Högberg & Stenlid, 1999), but provide limitations in that it is confined to diploid or dikaryotic phases of fungal life cycles. Phylogenetic approaches to detect recombination complement tests for linkage disequilibrium or association (Guttman & Dykhuizen, 1994). If populations are clonal, all genomic regions are linked and share the same evolutionary history, and gene genealogies from two or more physically unlinked loci should be congruent. A tree inferred from the combined dataset of two or more loci should not be significantly longer than the sum of the lengths of trees inferred for each locus alone. In contrast, the signature of recombination is incongruency in the genealogies of different genomic regions of DNA, and the consensus trees from the observed genotypes of a sexual population has essentially no resolution (Anderson & Kohn, 1998). This method has been used to discover recombined population structures in fungal species that present no morphological evidence of mating and meiosis (e.g. Burt *et al.* 1996; Koufopanou *et al.*, 1997; Geiser *et al.*, 1998). Finally, mating type ratios can give indications of the reproductive mode of a population. In heterothallic ascomycete species, populations that have only one mating type can obviously not be mating randomly. In sexual ascomycete populations with bipolar mating system, the mating type gene segregates in sexual crosses, so that equal numbers of progeny from the two mating types should be produced in a 1:1 ratio in a random mating population. Mating type ratios have been used to reveal a contrasting structure of European and North American populations of *Ophiostoma ulmi* (Mitchell & Brasier, 1994).

Gene flow

Gene flow is a collective term that includes all mechanisms resulting in the movement of genes from one population to another (Endler, 1977; Slatkin, 1985). Thus, gene flow is a uniting force that keeps populations or species from diverging, since the boundaries between them are crossed. Alternatively, when gene flow is blocked and populations are isolated from one another, increased differentiation will become established and may eventually result in speciation. Gene flow may be influenced by a number of factors, including physical barriers, e.g. mountains and oceans, and biological features of the fungal species, e.g. spore discharge mechanisms and dispersal capacity (Rogers & Rogers, 1999).

Population biologists use two classes of methods to estimate the amount of gene flow in natural populations. "Direct methods" use estimates of dispersal distances, and reproductive success of dispersers, to infer how much gene flow that occurs at the time of observation. "Indirect methods" often use allele frequencies to estimate the levels of gene flow that must have occurred in order to produce the observed allelic distributions (Stenlid, 1994).

Direct measures of gene flow in fungal populations includes studies of spore dispersal mechanisms such as spore production, transport, survival and settlement (Ingold, 1971; Lacey, 1996). According to Ingold (1971), 10^8 ascospores can be actively discharged from a single perithecial stroma of *D. concentrica* every day

throughout the summer. Fungal spores can be elevated above 1 km in the atmosphere and travel more than 700 km over seas (Lacey, 1996), although the vast majority of spores seems to fall within a few meters from the fruiting body (Malloch & Blackwell, 1992).

A number of indirect ways have been used to estimate levels of gene flow of alleles at independently inherited loci. Wright (1921) was the first to develop methods to describe population structure. His measurements, often called the F-statistics, are based on the idea of inbreeding in a diploid mating population. Wright's fixation index, F_{ST}, is a measure of the genetic differentiation of subpopulations relative to the total population due to non-random mating. Nei (1973) generalised Wright's population subdivision concept to haploid and asexual populations. Nei's G_{ST}, like Wright's F_{ST}, describes the average amount of genetic variation attributed to a particular subdividing factor relative to the total genetic variation. Values of F_{ST} and G_{ST} theoretically range between 0 and 1, where 0 indicate no genetic divergence and 1 indicate fixation of alternative alleles in different subpopulations. Other indirect measurements of gene flow is based on DNA sequence data of nonrecombining segments of DNA, and is based on the analysis of the phylogenies of the alleles (Slatkin & Maddison, 1989, 1990). Indirect measurements have been used to assess the genetic structure of several wood-inhabiting fungi. A high variation and low genetic differentiation both within large and small geographical scales was revealed for *F. pinicola* (Högberg *et al.*, 1999) by using Wrights F-statistics.

Selection

Selection for genes whose alleles differ in fitness when expressed in the phenotype, can be a powerful force for changing allele frequencies in the population. In recombining populations, an advantageous allele can increase in frequency because of selection, and still remain in random association with alleles at other loci. This is in contrast to clonal populations, in which the entire genotype including the advantageous allele would also increase in frequency. The same phenomenon, known as "hitch-hiking", also apply for alleles of genetically linked loci in sexual populations (Thomson, 1977).

Genetic drift

Genetic drift is the random change in gene frequency due to gamete sampling in a small population (Kimura, 1983). It affects all genes with the same probability, and is a process that in short time causes major changes in gene frequencies. The term "bottleneck" is used to describe the situation facing a population that has been drastically reduced in size, for instance after a catastrophe. When a new environment is colonised by only few individuals, representing only a part of the genetic variation in the original population, this is considered to be a "founder effect". Founder effects may be common in fungi where the habitat is favourable for colonising only during a limited period, e.g. fungi specialised in colonising a recently burned forest site. Genetic drift leads to genetic differentiation and

increases the degree of homozygosity in local populations. In fungi with a patchy distribution, such as *Melampsora lini* on wild populations of *Linum marginale*, genetic drift may be large enough to counteract a significant gene flow and lead to differentiation (Burdon & Jarosz, 1992).

Fungal species concept

Although the species concept is central in biology, a universal definition of a species does not exist. Species definitions and concepts have been under a continuous debate from both biological and philosophical angles (e.g. Claridge *et al.*, 1997; Ereshefsky 1992), and they continue to evolve. For example, Mayden (1997) discusses 22 different species concepts, based on phenotypic similarity, ecological parameters, reproductive isolation and cohesion, evolutionary principles, and various combinations of the above.

The organismal diversity and variation in ecological strategies and breeding systems of fungi increases the problems of finding a unified species concept appropriate to all fungal groups. Fungal species concepts have evolved from strictly morphological descriptions, through a call for the biological species concept, towards a phylogenetically based species concepts (Harrington & Rizzo, 1999). Profound limitations of using morphological characters as sole criteria of fungal species, especially for simple structured microfungi, have been highlighted by many authors (Brasier, 1997 and references therein). The *biological species concept* (BSC) and the *phylogenetic species concept* (PSC) are similar in that they emphasise the lack of gene flow between populations as an important determinant of species status. However, the BSC concentrates on intrinsic isolation mechanisms to delimit species, i.e., reproductive isolation are the sole defining criterion of a species. In contrast, the PSC makes no assumptions about intrinsic isolating mechanisms, but rather require the fixation of diagnostic characters in a species with no substantial gene exchange with others. The BSC is most applicable with sympatric and sexually outcrossing populations, and has facilitated the delimitation of species of several genera, e.g. *Armillaria* and *Heterobasidion* (e.g. Korhonen, 1978 a,b; Anderson & Ulrich, 1979). However, BSC imply some major limitations for fungi. Obviously, it does not apply in defining asexual species. Furthermore, many fungi cannot be cultivated, and in the relatively few fungi for which mating is possible to induce in the laboratory, partial interfertility is common and difficult to interpret in this context (e.g. Perkins, 1994). Difficulties also arise when interpreting mating in distinctly allopatric populations. However, cladistic analysis and the nearly unlimited characters made available through direct sequencing of PCR amplified DNA have provided the alternative to the BSC in the form of the PSC. The original PSC defined species as the smallest monophyletic clade of organisms that share a derived character state (Avise & Ball, 1990). Deciding the limit of the species using this concept is arbitrary. For example, if the gene being used has two sequences, are they simply alleles in one population, or fixed differences in two populations? If several gene genealogies are available, their shared branches can

separate genetically isolated groups, while the terminal branches that are incongruent reveal individuals that are exchanging genes (Avise & Ball, 1990) and provides the basis for the *genealogical concordance PSC*. It might be argued that the requirement of multiple gene concordances for recognising reproductively isolated populations is overly restrictive. The isolated groups are not acknowledged until some time after reproductive isolation has occurred, directly related to both generation time and effective population size (Avise & Ball, 1990). Furthermore, there is a concern that although the interface of congruence and incongruence can be used to define a recombining species, it would not exist for a clonal species because there would be no incongruence. Fortunately, wholly clonal species appear to be rare, and phylogenies combining sexual and asexual species have not indicated exclusively asexual clades (Lobuglio *et al.*, 1993; Geiser *et al.*, 1996; Taylor *et al.*, 1999).

The species concept of fungi is still under debate. Because of the diversity of underlying purposes of classifying the nature into species, it will probably always be. However, it seems as the genealogical concordance PSC is in progress, as a successful combination of the BSC and the PSC in delimiting reproductively isolated fungal species, especially when other characters are limited or give ambiguous results.

Molecular markers

Molecular markers have revolutionised the analysis of fungal ecology and evolution, allowing relationships to be determined in a manner that was not possible with conventional morphological techniques. DNA-based molecular methods may be used to differentiate fungi in all taxonomic levels.

The rates of variability of different genetic markers vary owing to the differential action of fundamental processes such as recombination, mutation and selective constraints, and different levels of molecular change provide information at different levels of fungal ecology. Thus, selecting appropriate molecular markers is vital to the success of answering the question under study.

The most sensitive genetic signals are genotypic arrays, most commonly encountered in the form of multiple microsatellite loci scored in samples of individuals (Jarne & Lagoda, 1996; Goldstein & Schlötterer, 1999). In sexual species, these arrays are reshuffled at each generation, and, therefore, are useful in studies of the shortest- and finest-scale population processes, such as individual identification and tracking, parentage and relatedness of interacting individuals (Sunnucks, 2000 and references therein). Secondly, allele and/or haplotype frequencies can be used to measure genetic drift, founder effect, gene flow and population differentiation on a larger spatial and temporal scale. These can be measured by using a multilocus approach or by multiple, separate, nuclear or mitochondrial loci (Burke *et al.*, 1998). Slower again is the creation of new alleles

by mutation. The analysis of their evolutionary relationships is informative about the longer-term processes of phylogeography, speciation and deeper taxonomic phylogenetic construction (Avise; 1994; Burke *et al.*, 1998; Templeton, 1998)

Methods based on the analysis of Restriction Fragment Length Polymorphism, RFLP (Southern, 1975) of nuclear and mitochondrial DNA are widely used to reveal the genetic structure of fungal populations. RFLP provide highly reproducible markers that are co-dominant, meaning that heterozygotes can be distinguished from homozygotes (McDonald & McDermott, 1993). Critics of RFLP analysis note that while restriction sites in different individuals are most likely to be identical by descent, there are many ways in which a restriction endonuclease site can be lost, e.g. a substitution or a length mutation, and missing sites that have arisen by different routes confound evolutionary analysis. Furthermore, the technique is time-consuming and requires large quantities of high quality DNA and specific probes (Taylor *et al.*, 1999). The advent of PCR (Mullis & Faloona 1987) has facilitated the molecular analysis of fungal genomes and is particularly useful since it does not require either high quality or quantity of template DNA. The multilocus approaches of Amplified Fragment Length Polymorphism's, AFLP (Vos *et al.*, 1995), or Randomly Amplified Polymorphic DNA (AP-PCR/RAPD; Welsh & McClelland, 1990; Williams *et al.*, 1990) are commonly used to assess the genetic structure of fungal populations. The single primer used in the AP-PCR/RAPD analysis can be of different kinds, e.g. it can be short (ca. 10 bp) and completely random, or longer with the aim to amplify specific target regions such as microsatellites (Zietkiewitcz; 1994; Hantula *et al.*, 1996) or minisatellites (e.g. Stenlid *et al.*, 1994). AP-PCR with the core DNA sequence of the phage M13 minisatellite as a single primer has been used in several studies to assess the genetic structure of populations of wood-inhabiting fungal species (e.g. Stenlid *et al.* 1994; Vasiliauskas & Stenlid, 1998; Högberg *et al.*, 1999). Both AFLP and RAPD/AP-PCR produce dominant markers, and the same concerns about null alleles as with RFLP analysis, apply. Care must be taken to perform appropriate control reactions when using RAPD/AP-PCR markers, since they have been criticised for being unreproducible (Ellsworth *et al.* 1993; Penner *et al.*1993) and inherited in a non-mendelian manner (Riedy *et al.*, 1992). When no prior information of the genetics of a fungus is available, it is possible to develop robust molecular markers for population studies by the method of DNA Sequencing With Arbitrary Primer Pairs (SWAPP)(Burt *et al.*, 1994), or the identification of microsatellite loci and their flanking sequences (Bruford & Wayne 1993). Furthermore, when comparative sequence data of closely related species is available, as is the case with many ascomycetes, it is possible to create PCR primers to amplify polymorphic fragments of Single Copy Nuclear Genes (SCNG) from the organisms under study (Glass & Donaldson 1995; Carbone & Kohn, 1999; Paper III). In these last three mentioned methods, all alleles are recovered. If several loci are sequenced, additional opportunities for analysis become available, e.g. comparisons of gene genealogies.

Different parts of the nuclear ribosomal DNA (rDNA) have proven to be differentially conserved, and thus providing a means for analysing phylogenetic relationships over a wide range of taxonomic levels (Jorgensen & Cluster, 1989). One of the attractions of the rDNA genes and spacers is that they occur in high copy number, in tandem, and the uniformity of these copies is generally maintained through concerted evolution (Arnheim *et al.*, 1980; Dover, 1984). Specific PCR primers to amplify different parts of the rDNA have been developed by White *et al* (1990), followed by several other authors. The nuclear small-subunit rDNA sequences evolve relatively slowly and are useful for studying distantly related organisms, whereas the mitochondrial rRNA genes evolve more rapidly and can be useful at the ordinal or family level. The transcribed spacer regions in the rDNA operon (the internal transcribed spacer regions, ITS1 and ITS2) and the non-transcribed spacer regions between the tandem repeats of the rDNA operon (the intergenic spacer regions, IGS) evolve relatively fast and have been successfully used to resolve relationships between closely related species (White *et al.*, 1990; Hibbett *et al.*, 1995; Johannesson *et al.*, 2000). The ITS region has also been used successfully for identification of fungi in its natural environment, e.g. the fungal symbiont of ectomycorrhiza directly from root tips, and of wood-inhabiting fungi directly from samples of wood (Gardes *et al.*, 1991; Kårén *et al.*, 1997; Johannesson & Stenlid, 1999).

Aims of this thesis

The aims of the present theses were:

i) to make a survey of the presence and host-specificity of *Daldinia* species in northern Europe

ii) to reveal whether the observed host-specificity of the most abundant *Daldinia* species is due to increased wood-decay capability in the specific substrate on which stromata usually are found

iii) to develop reproducible molecular markers that can be used to assess the population structure of the postfire species *D. loculata*

iv) to elucidate the life cycle of *D. loculata* by using a population genetic approach

Results

To reveal the species-composition of *Daldinia* in northern Europe, 35 herbarium collections of *Daldinia* from 11 different host species were investigated by using ecological (substrate specificity), molecular (sequencing of the ITS-region of the ribosomal DNA), and morphological (stromata and ascospore size and shape) characters (Paper I). Five *Daldinia* species in northern Europe were identified: *D. concentrica*, *D. fissa*, *D. grandis*, *D. loculata* and *D. petriniae*. Both *D. petriniae* and *D. loculata* have previously been lumped under *D. concentrica* in the literature. *D. fissa* possesses unique morphological characters by being the only species included here having a gelatinous stroma. In contrast, the four latter species are morphologically very similar to each other and exhibit somewhat overlapping host ranges. *D. concentrica* was found exclusively on non-burned wood of *Fraxinus*, while *D. petriniae* was found on non-burned wood of *Alnus*, *Betula*, *Corylus*, and *Salix*. *D. loculata*, *D. fissa* and *D. grandis* were all found on burned hosts belonging to the genera *Betula*, *Fagus*, *Populus*, *Salix*, *Sorbus*, *Quercus*, *Ulex* and/or *Malus*. However, all five species have unique ITS sequences, with no intraspecific variation. Due to a technical mistake in the process of publishing Paper I, the ascospore sizes of the different species were not shown in the published article. Therefore, the complete table including all examined morphological characters presented in Paper I, apart from the gelatinous stromata of *D. fissa*, is included here (Table 1).

Table 1. Morphological characters of *Daldinia* species

Species	Stromatal size	Stromatal shape	Purple pigment	Inequilateral spore shape	epispore dehiscence	ascospore size (µm)
D. concentrica	large	sessile	+	strongly	+	12.3-16.1 x 5.7-7.3
D. cf. *fissa*	small / medium	short stipitate	+	slightly	+/-	10.9-14.4 x 5.5-7.6
D. grandis	large	sessile	+	slightly	-	16.1-18.2 x 7.6-10.2
D. loculata	medium / large	sessile	+	slightly	-	11.3–14.2 x 6.8-8.5
D. petriniae	Small / medium	sessile/short stipitate	(+)	strongly	+	12.2-16.0 x 6.6-8.3

In Paper II we investigated whether the observed substrate-specificity of the three most abundant *Daldinia* species in northern Europe, *D. concentrica*, *D. loculata* and *D. petriniae,* is caused by enhanced decay-extension ability of the fungus in the substrate on which stromata are usually found. A D-optimal experimental design including three random blocks of living, cut down, and cut down and burned stems of the tree host species, inoculated with two or three isolates each of the three fungal species, was set up, and the extension of decay was measured

after 12 months of incubation. The results showed that all three fungal species grew equally well in the different substrates, and thus did not indicate an increased wood-decaying ability in the substrates from which stromata are usually found. *D. loculata* was the only fungal species that exhibited a higher mean value of decay-extension in its specific substrate (burned wood), but the difference from the extension of decay in non-burned wood was small and not significant. An ANOVA general linear model for the following parameters was constructed: host species, host treatment, fungal species, fungal isolates and block. The result showed that two factors affected extension of decay significantly: host species and host treatment. All three *Daldinia* species grew significantly less in *F. excelsior* than in the other host species, and preferred burned and cut, to living, wood.

To investigate whether the specificity for burned wood possibly has evolved more than once in the evolutionary history of the genus *Daldinia*, a phylogenetic analysis was performed on the ITS sequence data, including the five species found in the survey of the species in northern Europe (Paper I). *Entoleuca mammata* was included as outgroup. *Daldinia* species that show specificity for burned substrates in nature were found to be polyphyletic; *D. grandis* and *D. loculata* clustered together, whereas *D. fissa* was unresolved in the phylogenetic tree. These results indicate that the specificity for burned substrates has been gained or lost more than once in the evolutionary history of the genus.

Eight single copy nuclear gene (scng) markers of *D. loculata*, which are of potential use to infer the population structure of the fungus, are presented in Paper III. The PCR-based markers were developed by using available sequence data of conserved nuclear genes from species closely related to *D. loculata*. Each locus spans over one or several introns, and can be used either as biallelic PCR-RFLP markers, or as multiallelic markers by DNA sequencing.

In an inventory of *D. loculata* stromata from *Betula* trees at burned forest sites in Orsa Finnmark (central Sweden) we found that the frequency of burned birches hosting stromata of the fungus peaked 2-3 years after the fire, when up to 40 % of the birches in the investigated area hosted stromata. During the following years, the frequency drastically decreased, and 7-8 years after the fire no stromata of the fungus were found (H. Johannesson & J. Stenlid, unpublished results; Fig 4).

An investigation of the local population structure of *D. loculata* is presented in Paper IV. By isolating mycelia from the interior of non-burned, sound-looking wood, we found that *D. loculata* may establish endophytically in living trees. Furthermore, we mapped the distribution of genets of *D. loculata* in a burned forest site in central Sweden by isolating mycelia from all the 17 burned trees present at the site hosting stromata of *D. loculata*, and subjecting them to vegetative compatibility tests and multilocus genotyping with five scng markers,

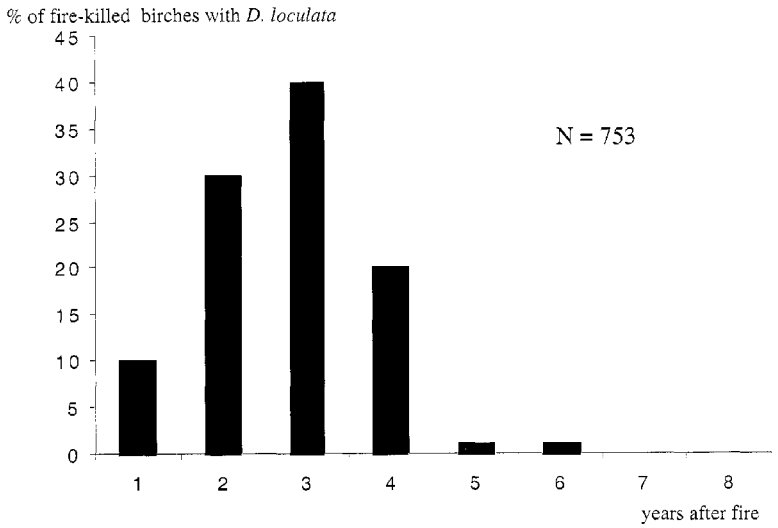

Fig. 4. The frequency of burned birches hosting stromata of *Daldinia loculata* peaks 2-3 years after the trees are killed by a forest fire. The diagram is based on an inventory of 753 burned birches from 12 forest sites, in which different time had past since the fire, in Orsa finnmark, central Sweden.

developed in Paper III, and one additional marker developed by Glass & Donaldson (1995). There was a full congruence between results from vegetative incompatibility and molecular markers; all genets of *D. loculata* inhabiting burnt wood as identified by vegetative incompatibility corresponded to a single multilocus genotype revealed by the molecular markers used. In 14 trees we found only one genet of *D. loculata* per stem, and in three of the trees we found two genets per stem. No single genet was found more than once in the forest site, suggesting that ascospore dispersal is the major way to colonise new substrates. Only one allele per locus was found in the mycelia isolated from the wood, indicating that *D. loculata* grows vegetatively as haploid mycelia. Heterothallism was detected by observed vegetative incompatibility and segregating molecular markers in the progeny of single stromata. Furthermore, more than two alleles of a highly variable multiallelic locus, described in Paper III, were detected when ascospores from numerous perithecia were sampled from single stromata, suggesting that the perithecia of a single stroma can be the result of more than one mating event. Finally, the process of mating preceding stromata formation was studied by comparing multilocus scng genotypes of vegetative mycelia within the wood with the offspring from the attached stromata. The results revealed that it is possible that the vegetative genets growing inside the stems are one of the parental strains of the attached stromata. However, most genotyped stromata of the same tree showed a distinct heterozygous genotype, suggesting multiple matings of relatively large, primary colonised mycelia in the wood by several secondary, different and small mycelia.

In Paper V the genetic population structure of *D. loculata* was studied in order to reveal any differentiation on a continental scale. Ninety-six samples of spore families, each comprising mycelia from 6-10 spores originating from single perithecia, were sampled from six Fennoscandian and one Russian (Kamchatka) forest site. The genetic structure of the subpopulations was studied by using several methods. First, we used six scng loci that were used either as co-dominant biallelic markers, by restriction enzyme or fluorescence-based DNA analysis of PCR products amplified from spore families of single perithecia, or as dominant multiallelic markers by DNA sequencing of the PCR amplified fragment of single ascospore isolates. Furthermore, we used AP-PCR with the core sequence of phage M13 as primer to analyse single ascospore isolates from three of the subpopulations. All subpopulations were found to be in Hardy-Weinberg equilibrium and gametic equilibrium was observed between all investigated loci. When analysing gene genealogies of a subset of the dataset, the consensus tree of the combined dataset had essentially no resolution. The results obtained by the different markers and the different analyses were consistent; *D. loculata* is a highly sexual species with low levels of genetic differentiation among subpopulations, even on a large geographical scale. The differentiation of the investigated subpopulations did not increase with distance; the Russian subpopulation, sampled more than 7000 km from the Fennoscandian subpopulations, was only moderately differentiated from the others (F_{ST} = 0.00 - 0.14). In contrast, one of the Swedish populations exhibited the overall highest values of F_{ST} and G_{ST} (0.10-0.16).

Discussion

Distribution and substrate specificity of *Daldinia* species

As demonstrated in Paper I, the number of *Daldinia* species in northern Europe is larger than previously reflected by the literature. At least *D. loculata* and *D. petriniae* have earlier been lumped under the name *D. concentrica*. Thus, the results of former ecological studies of the different *Daldinia* species, such as distribution and host-specificity, are difficult to interpret, as probably more than a single species were included in the analyses.

The species are apparently closely related, possessing partly overlapping ecological and morphological characters. In the four most closely related species, the ITS sequence similarity ranged from 93.6 to 99.5 %. More importantly, no variation in the ITS region was found within the species, although material of each species, except *D. grandis* of which only a single specimen was included, originated from geographically distant locations. The single studied Scottish specimen of *D. grandis* was very close to *D. loculata* as it was found on burned hosts, and shared 99.5 % of the ITS sequence of *D. loculata*. However, the two species differ in ascospore size (Table 1). Furthermore, the absence of intraspecific variation in the ITS region within *D. loculata* collections from

Kamchatka, Sweden and UK indicates that the observed difference in *D. grandis* can not be attributed to geographic distance. However, further analyses including more material of *D. grandis* have to be performed in order to claim these two species as distinct, maybe including investigations of other characters such as anamorph morphology, secondary metabolites and/or additional molecular markers. It would be interesting to analyse these two species by using the genealogical concordance species concept to reveal if they are reproductively isolated.

We interpreted the taxon commonly found on *Fraxinus* in UK as *D. concentrica.* The holotype material used in the original description by Bolton (1789) has disappeared, and in a revision of the genus, Ju *et al.* (1997) apparently described another taxon as *D. concentrica* and incorporated the species commonly accepted as *D. concentrica* on the British Isles under *D. petriniae.* However, after submitting Paper I, an article was published wherein *D. concentrica* was retypified to reflect the traditional British concept (Rogers *et al.*, 1999). In Paper I, we use "c.f." when referring to *D. petriniae* and *D. fissa.* After publication of the work we have investigated the type material of *D. petriniae* from *Alnus incana* in Switzerland (Ju *et al.*, 1997), supporting our interpretation of the species. However, we are still uncertain about the correct name of the species we refer to as *D. fissa.*

Substrate-specificity seems to be an operational character to separate at least the three most abundant species *D. concentrica, D. loculata* and *D. petriniae.* However, the underlying causes of substrate-specificity of the species remain unknown. According to the results of Paper II, in which the decay-extension of the species in different substrates were measured and analysed, the three species did not show any increased extension of decay in the substrate on which stromata are usually found. Thus, the observed substrate specificity is probably not due to differential ability of the species to cause decay within the specific substrate on which stromata are usually found. One possibility is that the observed substrate specificity is regulated in the early process of attachment and germination of spores on the specific substrate, as reported for the endophytic *Hypoxylon* spp. and *D. umbrinella* (Chapela *et al.*, 1991; Toti *et al.*, 1992). Unpublished results indicate a similar mechanism operating between *D. concentrica* and *Fraxinus* (A.J.S. Whalley, personal communication). A substrate-specific eclosion on burned substrates requires that the intact ascospores of *D. loculata* are present in a viable state on the exterior or the interior of the host until the fire. This is not impossible considering the finding that ascospores of *D. loculata* appear to be robust, as they germinate easily after many years in herbaria. Another possible explanation of the observed substrate specificity might be that the *Daldinia* species inhabit wood of a diverse range of host species, but that the physiological or ecological conditions required for the formation of the teleomorph are fulfilled only in a narrow range of host species and/or host treatments. This phenomenon has previously been suggested for species of *Daldinia* (Petrini *et al.*, 1995). A

third possible explanation of the observed substrate-specificity is that the distribution of the species confined to burned substrates are limited by the dispersal of pyrophilous insects, that are strongly attracted to burning and recently burned areas, and are well documented to feed on *Daldinia* stromata and conidia (Lundberg, 1984; Wikars, 1992).

If the species growing on burned substrates have diverged from the other species once in the evolutionary history of the genus one would expect them to form a monophyletc group in the phylogeny of the genus. However, the phylogenetic study of the informative characters obtained from the ITS-region (Paper I) shows that the species exhibiting a preference for burned substrates (*D. fissa*, *D. loculata* and *D. grandis*) have a polyphyletic origin. On the account of the xerophytic habit of *Daldinia*, it is likely that the ancestor of the genus was pyrophilous, and the adaptive traits for a xerophytic lifestyle, e.g. efficient waterholding of the stromata and high ability to grow in low water potentials, were first developed for growth in burned substrate. If so, the specificity for burned substrates has subsequently been lost more than once. An alternative interpretation of the phylogenetic study is that the ancestor of the genus was associated with non-burned hosts, and that the trait of association with burned hosts has been gained more than once. However, specimens from all species of the genus have to be included in a phylogenetic study to state whether specificity for burned hosts is a basal or derived character in the genus *Daldinia.*

Life cycle of *Daldinia loculata*

Scng loci are considered to have a moderate overall variability, and have so far mainly been used to study longer-term processes of phylogeography, speciation and deeper taxonomic phylogenetic reconstruction (Avise 1994; Slade *et al.*, 1994). In the scng loci developed for *D. loculata* (Paper III) we found a high intraspecific variability, especially in the intron sequences, and consider them to be very useful to reveal ecological questions of the kind asked in this project. The variability of the *ß-tub1* locus, when used as multiallelic, was almost too high to be informative; in a dataset of ascospore isolates of *D. loculata* from 6 Eurasian subpopulations, we found 30 alleles in 33 samples (Paper V). Interestingly, the results obtained by using the scng data to reveal the genetic structure of *D. loculata* on a continental scale (Paper V) was in accordance with the results obtained by using AP-PCR with the highly variable core sequence of the phage M13 as a single primer. The specific PCR-primers presented in Paper III have also been shown to successfully amplify the target region of four other *Daldinia* spp (*D. concentrica*, *D. fissa*, *D. grandis* and *D. petriniae*; Johannesson unpublished results), indicating a high applicability of the markers in future ecological studies of *Daldinia* spp..

The frequency of burned birches hosting stromata of *D. loculata* apparently depend on the time that has past since the fire (Fig. 4). This is an important

finding to consider when using the frequency of *D. loculata* at burned sites to indicate a high species richness of pyrophilous insects, as suggested by Wikars & Ås, 1999. If the system is used in comparative purposes, it is important to compare forest sites of which the same number of years has past since the fire.

An important finding presented in Paper IV is that *D. loculata* is able to establish in sound-looking wood of living, non-burned trees. This is in agreement with what is previously reported for several other xylariaceous fungi (Whalley, 1996). The relatively low levels of subpopulation differentiation (Paper V) indicate that prefire establishment is a predominant feature of the life cycle of *D. loculata*, since we predicted that a metapopulation structure of the fungus, resulting from a predominant postfire establishment, would have resulted in the fixation of alleles and higher subpopulation differentiation as a result from genetic drift (Burdon & Jarosz, 1992).

In spite of *D. loculata* producing numerous conidia prior to stromata formation, the absence of multiple sampling of a single geno- or haplotype highlights the importance of ascospores for dispersal and establishment in new substrata (Paper IV and V). Furthermore, the very low resolution revealed when performing combined genealogical analyses on a subset of the data confirms this result since the signature of recombination is incongruency in the genealogies of different genomic regions of DNA (Anderson & Kohn, 1998). The relative importance of ascospores for spreading of *D. loculata* to new substrata is probably a result of them being actively discharged in the open air through most of the growing season, while conidia are produced in shelter of the bark during a relatively short period.

How the prefire established ascospores of *D. loculata* survive during the fire-free intervals remains unrevealed. The fungus could possibly be present as latent propagules, such as yeast spores or hyphal fragments, in the sapstream of the tree, remaining passive until a forest fire kills the host. This phenomenon has been suggested as a possible explanation for the rapid development of extensive columns of decay, occupied by single fungal genets, in the sapwood and trunks of angiosperm trees with no sign of exterior wounds (Boddy, 1994 and references therein). However, the finding of two, spatially separated, genets of *D. loculata* within several of the stems (Paper IV) contradict this suggestion, since the presence of latent propagules of two genets in the sapstream of a tree most probably would result in a mixture of the two genets in the wood of the dead tree. Moreover, the result from Paper II indicates that the genets of the size found in Paper IV could very well be the result of growth from a single focus during a single growing season. A third indication of the presence of *D. loculata* as small propagules in the non-burned hosts, is that we found *D. loculata* in only one of the small wooden pieces when isolating *D. loculata* from sound-looking wood by the method of Griffith & Boddy (1990) (Paper IV). Although we have at several occasions found *D. loculata* in sound-looking wood (Paper IV), the results from

Paper II indicate that it possesses an as high wood-decay capability in burned as in non-burned wood. Accordingly, an alternative possibility of survival of *D. loculata* during fire-free intervals is that the decay is developed in the non-burned hosts and that it is solely the stromata formation that is dependent on the occurrence of fire. We have not searched for *D. loculata* in non-burned decayed wood to reveal if this may be a way of the fungus to survive fire-free intervals.

Irrespectively of the mechanism of establishment and the way of survival of *D. loculata* in the host during the fire-free interval, the ascospores grow into relatively large, haploid, genets in the wood of the fire-killed trees (Paper IV). In the early spring the first year after the fire, numerous conidia are produced under the loosening bark of the trees and are rapidly consumed by pyrophilous insects. Mycelia of *D. loculata* emits a fruity, aromatic odour, possibly aiding in attracting the insects, as is suggested for the attraction of nitidulid beetles to the oak wilt fungus, *C. fagacearum* (Webber & Gibbs, 1989).

In May-June, perithecia embedded in stromata are formed, probably as a result of mating between the large genet in the wood and several small genets. The results of study IV reveal that different perithecia of a single stromata can have different male parents, a pattern in accordance with findings for perithecia of *Epichloë typhina* stromata (Chung & Schardl, 1997). However, no indications of multiple male parents in ascospore families from single perithecia of *D. loculata* were found when performing segregation analyses of the loci (Paper III and V). This result is in accordance with studies of perithecia of the chestnut blight fungus, *Cryphonectria parasitica* (Milgroom *et al.*, 1993), but is in contrast to *Crumenolopsis sororia* in which single apothecia showed evidence of being the result of multiple matings (Ennos & Swales, 1987).

A tentative life cycle of *D. loculata* is presented based on the results revealed in Paper IV and V (Fig. 5). We suggest that a coherent latent population of *D. loculata* is established in deciduous trees in the non-burned forest, by a continuous influx of windspread ascospores from burned forest sites scattered in the surrounding landscape. Thus, established mycelia in an area may originate from ascospores released from several different burned forest sites during the whole fire-free interval. When the forest is subjected to wildfire, after on average 50-150 years, most of the deciduous trees dies and numerous pyrophilous insects are attracted to the forest site. The active decay of the latent mycelia of *D. loculata* in the dead trees is triggered. In the early spring the year after the fire, pyrophilous insects feed on conidia produced under the loosening bark, and mediate the sexual cycle of *D. loculata* by spreading the existent genotypes between the trees within a forest site. It is possible that conidia of *D. loculata* act as spermatia, as is demonstrated for microconidia of *Neurospora crassa* together with several other ascomycetes (Maheshwari, 1999 and references therein). However, presence of microconidia has to my knowledge never been reported for any species of *Daldinia*. As a result of the fertilisation, annual stromata are

produced, containing sexual perithecia. From May-June until September, numerous ascospores are actively released from the stromata and spread by the wind to surrounding non-burned forests that are continuously receiving ascospores from scattered burned forest site during the whole fire-free interval. This life cycle would result in an overall high genetic diversity of *D. loculata* but with local differentiation of small populations, and in populations in areas with a low level of fire continuity, due to founder effects. Furthermore, a pattern of inbreeding would be found in areas where the pyrophilous insects feeding on *D. loculata* are scarce.

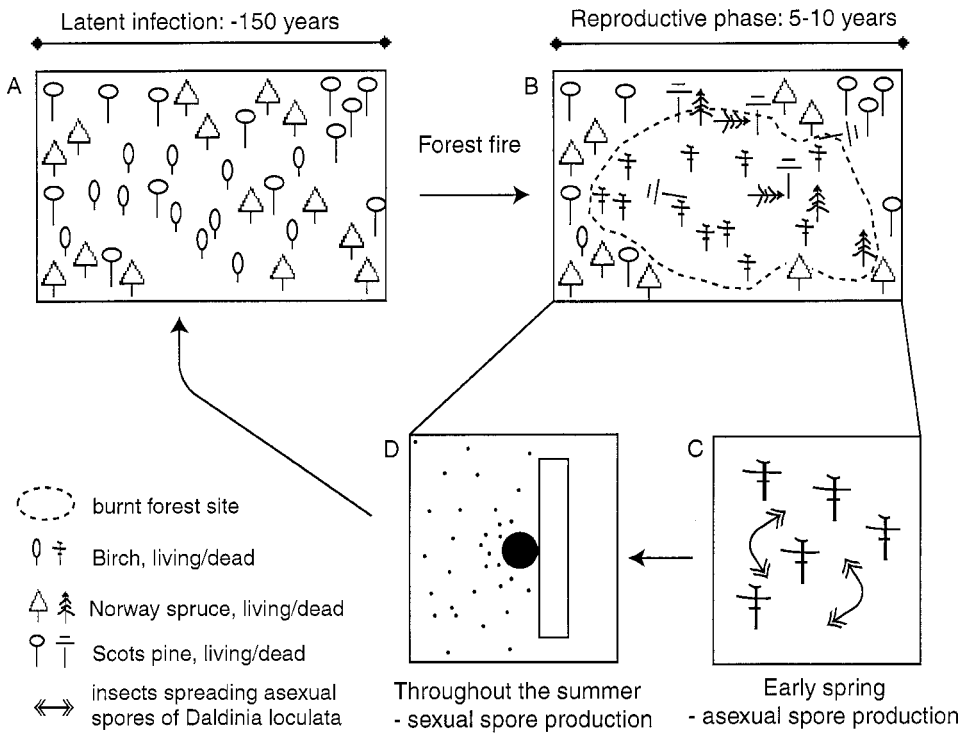

Fig. 5. Schematic presentation of the tentative life cycle of *Daldinia loculata*. A. A coherent population of latent mycelia is continuously established in deciduous trees in the unburned forest during the whole fire-free interval, by ascospores released from stromata of scattered burned forest sites. B. After on average 50-150 years, a forest stand is subjected to a wildfire, leaving a lot of dead standing and falling trees surrounded by the unburned forest. Several pyrophilous insect species are attracted to the forest site. The active decay of *D. loculata* is developed within the deciduous trees killed by the fire. C. A large amount of conidia are produced under the loosening bark of the burned birches in the early spring the years after the fire. Numerous insects are feeding on the conidia and are mediating the sexual cycle of the fungus by transferring haploid genets between the birches. D. In May-June, sexual ascospores are produced in perithecia embedded in annual stromata. The ascospores are actively released to the surrounding forest throughout the growing season.

The results of this thesis suggest that the overall genetic variation of *D. loculata* subpopulations has not diminished drastically as a result of the efficient fire-prevention of the last century. However, one of the Swedish subpopulations investigated in Paper V were apparently more differentiated than the others and showed indications of inbreeding, most probably as a direct or indirect effect of a reduced fire-continuity of forest sites including deciduous tress in the area. This indicates that the areal continuity of burned forest sites including deciduous trees is important for the fungus. As mentioned above, a high frequency of *D. loculata* stromata indicates the presence of a rich fauna of pyrophilous insects (Wikars & Ås, 1999). Thus, when performing prescribed burnings with the aim to create the specific habitat for these organisms, it is of importance to frequently burn different forest sites with still standing deciduous trees (e.g. if clear-cut or thinned forests are used) and that the fire is of sufficient intensity to kill the trees.

Conclusions

i) In northern Europe at least five *Daldinia* taxa are present: *D. fissa*, *D. grandis*, *D. concentrica*, *D. loculata* and *D. petriniae*. The last two have erroneously been referred to as *D. concentrica* in the literature.

ii) The underlying causes behind the distinct stromatal host-specificity of the three most abundant species: *D. concentrica*, *D. loculata* and *D. petriniae* is still unknown. However, it is most probably not a result of enhanced wood-decay ability of the fungi in the substrata on which stromata usually are found.

iii) Three *Daldinia* taxa are found on burned hosts: *D. fissa*, *D. grandis* and *D. loculata*. The specificity for burned host has been gained or lost more than once in the evolutionary history of the genus. The xerophytic habit of *Daldinia* spp., suggests that the ancestor of the genus was pyrophilic.

iv) Reproducible, co-dominant, PCR-based molecular markers were developed for *D. loculata* by using available sequence data from closely related species. The markers showed relatively high intraspecific variability and were found useful in studies of the population structure of *D. loculata*.

v) Based on studies of the genetic structure of *D. loculata* populations, a tentative life cycle of the fungus is presented: A coherent latent population of *D. loculata* is established in deciduous trees in non-burned forests, by a continuous influx of windspread ascospores from burned forest sites scattered in the surrounding landscape during the whole fire-free interval. When the deciduous trees die from a forest fire, the active decay by the fungus is triggered and conidia are produced under the loosening bark. Pyrophilous insects attracted to the forest site by the fire, feed on the conidia and mediate the sexual cycle of *D. loculata* by spreading the existent genotypes between the trees within a forest site. Ascospores are actively released from the sexual stromata and spread by the wind to the surrounding unburned forests during 5-10 years after the fire, resulting in a low genetic differentiation of subpopulations even on a continental scale.

Future prospects

The taxonomy of the genus *Daldinia* remains to be clarified. Not until operational keys are developed that enable accurate species determinations of anamorphs, and different authors use the species names consistently, it is possible to map the distribution of the species and compare data from ecological surveys of different species. Additional work, including comprehensive morphological and molecular studies of type material is needed. Moreover, careful investigation and description of the anamorphs have to be performed by studying cultural, biochemical and/or molecular characteristics of single ascospore isolates from freshly collected teleomorphs.

There are still many unexplored ecological questions of *Daldinia* species. One central question is the species distribution and the causes behind the stromatal host-specificity. Does any specific recognition mechanism mediate the host specificity or is it only the teleomorphic stage that are confined to a specific host-range?

The ecological knowledge of postfire species, such as *D. loculata*, is of significant importance from a nature conservation perspective. More comprehensive ecological knowledge about other postfire species is needed to optimise the use of prescribed burnings to manage biodiversity.

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