**Dissertationes Forestales 105**

# The role of pollen in the changing environmental conditions of Scots pine

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Academic dissertation

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## ABSTRACT

Rapid climate warming poses a challenge to forest trees adapted to cooler conditions. However, a genetic safety net may exist for wind pollinated tree species in the form of long distance gene flow mediated by pollen containing alleles that are (pre)adapted to a warmer climate.

In order to examine long distance gene flow in Scots pine (*Pinus sylvestris* L.), we monitored flowering phenology in central, northern, and northernmost Finland and measured the amount and viability of airborne pollen over four years. Viable airborne pollen grains were detected during female flowering periods and prior to local pollen shedding in all study sites. Depending on the year and study site, female flowers were exposed only to pollen originating elsewhere for between one and four days. Foreign airborne pollen represented 2.3% of all viable grains detected and varied between years and study sites.

Artificial pollination trials were conducted to evaluate the effect of pollen origin on seed siring success. Pollen genotypes originating from southern Finland sired 76% and 48% of seeds in competition experiments where pollen from both populations was introduced simultaneously into the female strobilus. Furthermore, we investigated the importance of pollen arrival order by introducing pollen of northern and southern genotypes in to the strobilus at two-hourly intervals. Northern genotypes sired 76% of the analysed seeds when injected first but when southern genotypes were supplied first, both pollen types enjoyed equal success. The first pollen grain in the pollen chamber did not always fertilize the ovum. Instead there is likely a more subtle and complex form of competition between genotypes and their pollen grains.

To examine chemically-mediated pollen interactions we conducted an *in vitro* germination experiment where different genotypes were in chemical but not physical contact. Both positive and negative interactions were found. We found highly negative effects in the germinability of northern pollen grains when in contact with southern pollen, and an increase in the germinability of southern pollen when in contact with northern pollen.

There was no variation in the size of dry pollen grains between sources, but after hydration and germination, northern pollen grains were larger than southern pollen. Genotypes with high hydration rates had low germinability and slow pollen tube growth rates. This suggests that early germination and growth of the pollen tube is more dependent on pollen storage materials and less dependent on water intake.

Pollen source affected the outcome of competition experiments and therefore is an important factor in long distance gene flow. However, genotype also plays a role and must be taken into account.

**Keywords:** pollen transport, gene flow, pollen competition, pollen performance

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Vantaa, May 2010, Saila Varis

## LIST OF ORIGINAL ARTICLES

The thesis is based on the following articles, which are referred to by their Roman numerals. The articles I-IV are reprinted with the kind permission of the publishers while the study V is the author version of the submitted manuscript.

**I** Varis, S., Pakkanen, A., Galofre, A. & Pulkkinen, P. 2009. The extent of south-north pollen transfer in Finnish Scots pine. Silva Fennica 43: 717–726.

**II** Pulkkinen, P., Varis, S., Pakkanen, A., Koivuranta, L., Vakkari, P. and Parantainen, A. 2009 Southern pollen sires more seeds than northern pollen in southern seed orchards established with northern clones of *Pinus sylvestris*. Scandinavian Journal of Forest Research 24: 8–14.

**III** Varis, S., Santanen, A., Pakkanen, A. and Pulkkinen, P. 2008. The importance of being the first pollen in the strobili of Scots pine. Canadian Journal of Forest Research 38: 2976–2980.

**IV** Varis, S., Reininharju, J., Santanen, A., Ranta, H. and Pulkkinen, P. Interactions in vitro germination of Scots pine pollen. Trees - Structure and Function 24: 99–104.

**V** Varis, S., Reininharju, J., Santanen, A., Ranta, H. and Pulkkinen, P. The size and pollen germinability in different temperatures. Manuscript.

## AUTHOR’S CONTRIBUTION

Saila Varis participated the planning of experiments in papers IV and V and performed part of the measurements in paper V. She made most of the laboratory analyzing in paper III, most of the statistical analyzing in papers I and III-V, and part of the statistical analyzing in paper II. She was the corresponding author and responsible for writing the manuscripts I and III-V.

# CONTENTS

# 1 INTRODUCTION

## 1.1 The challenge of climate change

During the next century, climate change is going to challenge the adaptability of long-living forest tree species such as Scots pine (*Pinus sylvestris* L.). Climate change is predicted to be so rapid that the extent to which boreal forest trees can respond to the new conditions has become a serious concern (Davis and Shaw 2001, Savolainen et al. 2007, Aitken et al. 2008). By 2100, annual mean temperature is expected to increase 1.4–5.8 °C globally, and 2.0–6.3 °C in Europe 2100 (European Environment Agency 2004, IPCC 2007). In Finland, the latest estimates vary from +2 to +7 °C by the year 2080 (Jylhä et al. 2004). As such, climate modelers predict a greater increase in winter temperature and in northern latitudes (Ruosteenoja et al. 2005). Warming and other predicted changes in environmental conditions will impact the growth, phenology and distribution of plant species (Matala et al. 2006, Menzel 2006, Parmesan 2006, Scholze et al. 2006).

Boreal tree species have experienced radical changes in climate through their history, e.g., Pleistocene glacial-interglacial cycles. Fossil pollen records and genetic data show rapid postglacial species migration (Huntley and Birks 1983, Hewitt 1999, Heuertz et al. 2006, Petit et al. 2003, Willis and van Andel 2004). These findings, coupled with the analyses of tree-rings (Briffa et al. 1998, Vaganov et al. 1999, Wilmking et al. 2004, Driscoll et al. 2005), soil or air warming experiments (Peltola et al. 2002, Strömgren and Linder 2002, Kilpeläinen et al. 2004) and common garden experiments (Hänninen 1996, Persson and Beuker 1997, Rehfeldt et al. 1999, 2002, Salminen and Jalkanen 2005) have led to the conclusion that trees can adjust to a warmer climate and that growth will increase, especially in the northern parts of the distribution (Jacobson and Dieffenbacher-Krall 1995, Mátyás 1996, Rehfeldt et al. 1999, 2002, 2003, Davis and Shaw 2001, Peltola et al. 2002, Strömgren and Linder 2002, Kilpeläinen et al. 2004, Norby and Luo 2004, Reich and Oleksyn 2008). However, negative effects of rising temperature have also been documented in northern populations (Wilmking et al. 2004) and more attention has been focused on the effects of extreme climate events such as drought (rev. Adams et al. 2009).

Scots pine has the widest distribution of all pine species, from Portugal in the southwest, east to the Caucasus and north to Lapland. The environmental gradient covered by this species is diverse even when comparing populations in Finland, where the thermal growing season is about 170 days in southern Finland and hardly 120 in the north. Evidently, Scots pine is either tolerant of dynamic environmental conditions or a highly mutable species. Across the distribution, populations appear to be locally adapted, especially with respect to growth-related traits (rev. Savolainen et al. 2007). Reciprocal transplant experiments have shown that adaptation in growth has a genetic basis (e.g., Kylmänen 1980, Nikkanen 1982, Rousi 1983, Pulkkinen et al. 1995). However, reproductive traits appear to be less flexible in northern populations where the rate of reproduction is much lower than further south (Sarvas 1962, Koski and Tallqvist 1978, Henttonen et al. 1986, Persson 1994, Pessi and Pulkkinen 1994, Parantainen and Pulkkinen 2002). Luomajoki (1993) quoted the principle of Linsser (1867) which states that the heat sum needed for a given stage should, in well-adapted populations, remain proportionally the same at any locality compared to the total heat sum of the site. Luomajoki (1993) suggested that on the basis of phenology, the limit of adaptive capacity for Scots pine is currently at 63° N.

Because of strong seasonal variation in environmental conditions, boreal forest trees have developed an annual growth rhythm. The annual cycle includes an active period during warm conditions, followed by dormancy during autumn and winter (Weiser 1970, Sarvas 1972, 1974, Fuchigami at el. 1971, Hänninen 1986, 1990). The start of the active period is strongly connected to an increase in air temperature (Sarvas 1972, Fuchigami at el. 1971, Hänninen 1990), although photoperiod and local environment also have an influence on growth (Heide 1974ab, Myking and Heide 1995, Häkkinen et al. *1998, Partanen et al. 1998*).

Changes in temperatures may have important implications for the reproductive cycle and survival of the tree species, especially during winter and early spring temperature extremes (Cannel 1985). Hänninen (2006) suggests that flower buds are more vulnerable to extreme temperature fluctuations than vegetative buds. The development of Scots pine female and male strobili initially starts in late spring or early summer when the cone initials are laid down in the resting buds (Wareing 1958, Kupila-Ahvenniemi 1980). In the autumn, development is arrested until the following spring (Wareing 1958, Kupila-Ahvenniemi 1980, Kupila-Ahvenniemi 1984). In their first autumn of development, male strobili have visible pollen sacs (microsporangios), but the meiotic division of sex cells through which pollen grains are formed (microsporogenesis) takes place the following spring (Wareing 1958, Kupila-Ahvenniemi 1980, Kupila-Ahvenniemi 1984). Male strobili mature within a month the following spring and pollen shedding starts when the weather is favourable (Kupila-Ahvenniemi 1984). The development of female initials is slower than male strobili in the first summer and strobili only develop during the following spring (Kupila-Ahvenniemi 1984).

### 1.2 Pollen mediated gene flow

One adaptive mechanism may be in the form of long distance gene flow through which resident populations receive pollen from southern populations adapted to a warmer climate (Davis and Shaw 2001). Given its importance to the genetic structure of populations, gene flow has been a focus of evolutionary biology for decades. In recent years, interest in gene flow has increased due to issues surrounding genetically modified crops and predicting the consequences of climate change.

Pollen-mediated gene flow increases variation, which is the basis of evolutionary change (Davis and Shaw 2001), but at the same time it can limit the extent to which peripheral populations can become locally adapted (Garcia-Ramos and Kirkpatrick 1997). Gene flow from central to peripheral areas is based on a decrease in plant density towards the edge of a species distribution. This phenomenon can be observed in the Scots pine towards the north of its range. A lower level of local adaptation of reproduction in northern populations of Scots pine may be explained by pollen-mediated gene flow from southern areas (e.g., Koski 1970). In the future, gene flow from the south may provide the alleles that help northern Scots pine adapt to a new and warmer climate (Davis and Shaw 2001, Savolainen et al. 2007).

While pollen-mediated gene flow can have a generalizing effect on northern Scots pine populations, it can also create problems in seed orchards. Background pollination, i.e., seeds fertilized by pollen originating from outside the seed orchard, may have negative effects on tree improvement programs. Estimates of the proportion of seeds fertilized by background pollination in Finnish and Swedish Scots pine seed orchards vary between 21 and 76% (El-Kassaby et al. 1989, Harju and Muona 1989, Pakkanen et al. 1991). High levels of background pollination have been documented in southern seed orchards consisting of trees from more northern locations (Pakkanen et al. 1991).

Genetic markers have been used to estimate gene flow in wind pollinated forest trees (rev. Burczyk and Koralewski 2005, Burczyk et al. 2006, Robledo-Arnuncio et al. 2006). Estimates of the mean distances of pollen dispersal in tree species have been on the meter rather than kilometer scale (rev. Savolainen et al. 2007). However, in observation studies pollen grains of wind pollinated tree species have been reported to rise high above the trees and travel more long distances, usually tens or hundreds of kilometers (rev. Koski 1970, Williams 2009). Hesselman (1919) was among the first to observe spruce, pine and birch pollen 55 km off the coast in the Gulf of Bothnia (rev. Koski 1970). Since then, several observations have been made concerning long-distance pollen dispersal (e.g., Di-Giovanni et al. 1996, Rogers and Levetin 1998, Williams 2010). In recent modeling studies based on phenological data and measurements of airborne pollen concentrations, the dispersal of birch pollen was estimated as taking place over hundreds of kilometers (Sofiev et al. 2006, Siljamo et al. 2008). Large quantities of pollen from several species have been reported to rise over a kilometer in altitude and travel over 1000 km per day (Koski 1970, Checci et al. 2003, Sofiev et al. 2006, Skjoth et al. 2007, Siljamo et al. 2008).

Even though pollen flow is considerable, asynchrony in reproductive phenology may limit the extent to which far-dispersing pollen can contribute to gene flow (Aitken et al. 2008). The flowering of Scots pine starts at lower degree-day sums in the northern parts of Finland (Luomajoki 1993), and in calendar time flowering starts earlier in the south (Koski 1970). The difference in mean start date of flowering between southern and northern Finland can been separated by three weeks (Pessi and Pulkkinen 1994). For each study site, the mean duration of local pollen shedding was 11 days and the shedding periods overlapped even though sites were separated by over 500 km (Pessi and Pulkkinen 1994). In populations of Scots pine, female strobili become receptive half to one day earlier than the male strobili begin shedding pollen (Sarvas 1962, Jonsson et al. 1976, Chung 1981).

After long distance transport, pollen grains must remain viable in order for fertilization to take place. After 24 hours exposure to air, Lindgren and Lindgren (1996) found high germination percentages of Scots pine pollen. Earlier workers (Lindgren et al. 1995, Pulkkinen and Rantio-Lehtimäki 1995) had shown that although the germinability of airborne Scots pine pollen at the beginning of the season was lower than pollen collected later, it was high enough to fertilize the majority of seeds and that early airborne pollen is more likely from southern populations (Lindgren et al. 1995). Recent study of Williams (2010) shows that the pollen of *Pinus taeda* still germinates after 41 km dispersal.

Long distance pollen mediated gene flow requires efficient pollen dispersal and synchrony with female flowering periods in recipient populations. The efficiency of pollen flow may be questioned as successful fertilization depends on pollen viability after long distance transport. Also, many environmental and genetic factors may suppress foreign genotypes if pollen competition arises (Dyer and Sork 2001).

## 1.3 Sexual selection

When airborne pollen entering the strobilus originates from several sources, there is a possibility for pollen competition, non-random mating and sexual selection. Darwin (1871) realized that heritable animal traits that had negative or no effect on survival could not be explained by natural selection. He suggested that the number of offspring which the bearer contributed to the next generation was important and that traits like colourful tail increased the number of mates acquired (Darwin 1871). Since then, Darwin’s (1871) idea has been developed and applied to plants and pollination (e.g., Haldane 1932, Baker 1948, Bateman 1948, rev Willson 1994, Skogsmyr and Lankinen 2002). Huxley (1938) distinguished two aspects of sexual selection: 1) sexual competition among the members of one sex, usually male, to mate with individuals of the other sex, and 2) preferences of one sex, usually female, for particular mates. Stephenson and Bertin (1983) defined sexual selection as the differential reproductive success of individuals of the same sex and species that survive to reproductive age and are physically capable of reproduction.

In plants, relative reproductive success involves pre-pollination mechanisms that influence pollen movement, and post-pollination mechanisms such as pre-zygotic pollen competition and post-zygotic selection (Stephenson and Bertin 1983). Intrinsic prepollination factors are dependent on environmental conditions and local adaptation of the plants, e.g., flowering phenology and pollen production. Extrinsic prepollination factors such as air currents are important but not under the plants control. Thus, it has been suggested that differential reproductive success is mostly based on postpollination factors (Charnov 1979, Stephenson and Bertin 1983, Willson and Burley 1983).

Pre-zygotic pollen competition can take place when the amount of pollen grains in the stigma exceeds the amount of egg cells to be fertilized (Haldane 1932, Stephenson and Bertin 1983). When pollen grains germinate and grow tubes to fertilise the egg cells, genotypes express the traits that affect their seed siring ability and, if this trait varies among genotypes from different sources, nonrandom mating is possible. Post-zygotic selection may occur when developing embryos compete for resources (Kress 1981, Queller 1983) or the interests of the maternal plant and offspring do not coincide (Westoby and Rice 1982, Law and Cannings 1984, Mazer 1987). In many cases, plants produce more ovules than they can support to maturity (Lee 1984) and since the production of seed is costly, maternal selection cannot be excluded (Skogsmyr and Lankinen 2002). However, the full understanding of mechanisms of nonrandom mating are still lacking and the existence and possible role of maternal selection is debated (Skogsmyr and Lankinen 2002).

Nonrandom mating has been demonstrated in several angiosperm species (e.g., Bookman 1984, Marshall and Ellstrand 1986, Snow and Mazer 1988, Bertin 1990, Snow and Spira 1991, Björkman et al. 1995, Havens and Delph 1996, Pasonen et al. 1999, Steiner and Gregorius 1999, Skogsmyr and Lankinen 2000a,b, Krauss 2000) but also equal mating success among pollen donors has been recorded (e.g., Mazer 1987, Fenster and Sork 1988, Cruzan1990a). An increasing number of studies have detected nonrandom mating in gymnosperm species, for example in Norway spruce (*Picea abies* (L.) Karst.) (Cheliak et al. 1987, Schoen and Cheliak 1987, Nakamura and Wheeler 1992a, Xie and Knowles 1992, Skrøppa and Lindgren 1994, Aronen et al. 2002), Douglas-fir (*Pseudotsuga menziesii* (Mirb.) Franco) (Apsit et al. 1989, El-Kassaby and Ritland 1992), and radiata pine (*Pinus radiata* D. Don) (Moran and Griffin 1985, Kumar et al. 2007). However, Wiselogel and van Buijtenen (1988) found only equal mating success in loblolly pine (*Pinus taeda* L.).

## 1.4 Pollen competition

The outcome of pollen competition may be influenced by several external and internal pollen traits like pollen size, germinability, germination rate, and tube growth rate (Bertin 1988). These pollen traits are variable and appear to correspond to genotype (Skogsmyr and Lankinen 2002), although environmental conditions during development have a strong influence on pollen quality and performance (Young and Stanton, 1990, Quesada et al. 1995, Delph et al.1997, Travers 1999). These differences may represent adaptations and may affect the competitive ability of pollen and, consequently, long distance gene flow. Unfortunately, there is little evidence due to a lack of suitable study techniques.

Huxley (1942, rev. Stephenson and Bertin 1983) suggested that competition should select for rapid growth of pollen tubes, and in several studies of angiosperm species, pollen tube growth rate was found to be the most important trait (Snow and Spira 1991, Walsh and Charlesworth 1992, Pasonen et al. 1999, Skogsmyr and Lankinen 1999, 2002). However, Melser et al. (1997) found no correlation between pollen tube growth and seed siring success.

Pollen competition in Scots pine can begin when pollen grains first come into contact in the pollination drop between the micropylar arms (Doyle and O'Leary 1935, Doyle 1945, Sarvas 1962, Owens et al. 1998, Gelbart and von Aderkas 2002, Fernando et al. 2005). Pollen grains of coniferous species are relatively dry when released (12.6% moisture content in *Cypressus arizonica*: Chichiriccó et al. 2009) and in the pollination drop they absorb moisture and expand, i.e., hydrate (Dawkins and Owens 1993, Fernando et al. 2005). The exine of hydrating pollen either swells or splits open and the growing pollen tube emerges through a thin section, usually between the sacci (Fernando et al. 2005). During hydration, proteins are released that may affect the germination of other pollen arriving later in the drop (Pettitt 1985, Suarez-Cervera et al. 2003). Interactions typically take place between pollen grains that are germinating synchronously (Cruzan 1990a, Marshall et al. 1996, Steiner and Gregorius 1999, Marshall and Diggle 2001) when actively growing pollen tubes come into close contact (d'Eeckenbrugge 1990). Pollen-pollen interactions are predicted to have a chemical basis (Brewbaker and Majumder 1961, Cruzan 1990, d'Eeckenbrugge 1990, Marshall et al. 1996, Niesenbaum 1999) and the compounds secreted by germinating pollen grains can have either a positive or negative effect on pollen performance (Brewbaker and Kwack 1963, Taylor and Hepler 1997). Most studies on this subject concern the effect of pollen load on germinability or pollen performance (e.g., Brink 1924, Cruzan 1986, Nakamura and Wheeler 1992a, Nakamura and Wheeler 1992b, Niesenbaum 1999) and were carried out *in vivo* where maternal tissue can play a role.

After arriving in the pollination drop, pollen grains pass through the micropylar canal to the pollen chamber and on to the receptive surface of the nucellus (Fig. 1) (Ferguson 1901, rev. McWilliam 1958, Doyle and O'Leary 1935, Doyle 1945, Sarvas 1962). Doyle and O'Leary (1935) and Doyle (1945) suggested that the pollination drop is withdrawn to the rim of the micropylar canal and that winged pollen subsequently rely on their buoyancy to progress further. Often, several Scots pine pollen grains are attached together and there is more than one grain in the pollen chamber near the nucellus (Sarvas 1962). According to Sarvas (1962), the pollen chamber of Scots pine has room for up to six grains but on average contains only two. Sarvas (1962) suggested that the first pollen grain to enter the chamber automatically occupies the position closest to the nucellus and typically succeeds in fertilizing the ovum. However, experimental evidence in support of this hypothesis is rather scarce and experiments were made with species having a different pollination system to Scots pine (Marshall and Ellstrand 1985, Webber and Yeh 1987, Owens and Simpson 1981).

The size of pollen grains is considered to be an indication of pollen viability and the proportion of large grains has been used to estimate pollen performance (Kelly et al. 2002, Dufaÿ et al. 2008). Variation in pollen grain size has been documented in several species (e.g. Bell 1959, Bragg 1969). In a study of *Erythronium grandiflorum* Pursh., Cruzan (1990b) found that plants producing larger pollen grains sired more seeds than plants with smaller pollen grains. Correlations between pollen size and tube growth rate have been either positive (Van Breukelen 1982, Lord and Eckard 1984, Perez and Moore 1985, Gore et al. 1990, Manicacci and Barrett 1995) or not significantly correlated (Cruzan 1990b, Pietarinen and Pasonen 2004).

Germinability and pollen tube growth rate are often considered the most important traits affecting pollen competition (e.g. Skogsmyr and Lankinen 2002). Few days after arriving to the pollen chamber hydrated Scots pine pollen grains germinate and the pollen tube begins to grow through the nucellar tissue (Fig. 1) (e.g. Ferguson 1901, rev. Sarvas 1962). Win et al. (1996) germinated Scots pine pollen *in vitro* and found that grains began to germinate approximately 16 h after the onset of culture. Pollen dryness may be the reason for the relatively slow germination rate (Dawkins and Owens 1993, Fernando 2005). During growth, pollen tubes often ramify and the branches may fill the tip of the nucellus (Sarvas 1962, de Win et al. 1996). After a few weeks of growth, pollen tubes fall into dormancy when they are almost halfway through the nucellus and they remain so until the following spring when growth is resumed (Sarvas 1962).

Temperature is the most important factor controlling reproduction in plants. The effect of temperature on pollen germinability and tube growth rate has been documented both in angiosperms (e.g., Bucholz and Blakeslee 1927, Lewis 1942, Elgersma et al. 1989, Stephenson et al. 1992, Lankinen 2001, Hedhly et al. 2005a, 2005b) and gymnosperms (Parantainen and Pulkkinen 2002). In several studies, pollen grains have shown thermal optima for germinability (e.g., Smith 1942, Vara Prasad et al. 2003). There is also evidence for variation in the seed siring success of different genotypes at different temperatures, i.e., genotype-temperature interactions (Pasonen et al. 2002, Lankinen 2001, Hedhly et al. 2005a). The effect of temperature on early germination processes, such as hydration rate, is unknown.

### 1.5 Aims of the study

The aims of this study were to determine the feasibility of, and investigate any factors that might consequently affect, pollen-mediated longdistance gene flow among northern populations of Scots pine.

The specific objectives of this thesis were:

1. To examine the occurrence of viable airborne pollen grains in synchrony with receptive female strobili in target populations in order to evaluate long distance pollen movements in Finland (I).

2. To examine the effect of pollen origin on seed siring ability (II).

3. To examine the importance of pollen grain arrival order in the strobilus (III).

4. To investigate the existence and effect of chemically-mediated pollen-pollen interactions (IV).

5. To examine the role of pollen grain size, hydration rate and germination temperature on pollen germinability and tube growth (V).

**Fig. 1.** Scots pine pollen grains in the pollen chamber. Paraffin-embedded ovule stained by Alcian blue. Bar 50 µm. (Photo: © Arja Santanen)

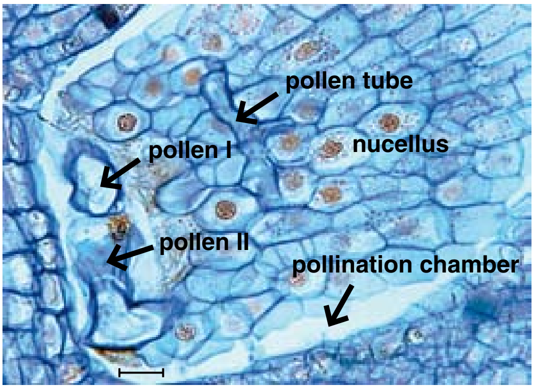

**Fig. 2.** Grafted Scots pines in the pots, strobili covered with pollination bags (Photo: © Aulis Leppänen)

**Fig. 3.** Germinated Scots pine pollen grains. Microscopic image taken after 72 h germination. Bar 50 µm. (Photo: © Saila Varis)

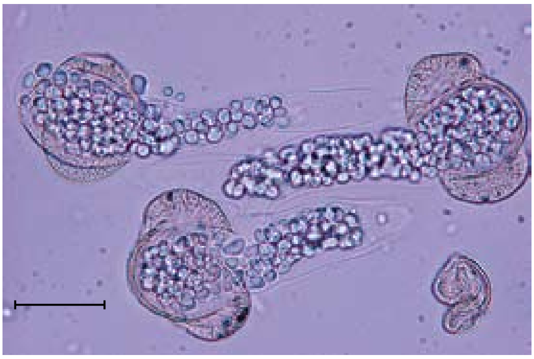

**Fig. 4.** Germinated Scots pine seeds in petri dish. (Photo: © Metla/Erkki Oksanen)

# 2 MATERIALS AND METHODS

## 2.1 Field work

### *2.1.1 Phenological data and airborne pollen collections*

Phenological observations and collection of airborne pollen (**I**) were carried out in natural Scots pine forest sites in central (Korpilahti, 62°15'N), northern (Rovaniemi, 66°23'N), and northernmost (Kevo, 69°45'N) Finland. Study sites were situated 460–850 km apart. The northernmost study site is an isolated forest approximately 50 km north of the Scots pine tree line. Each of the sites contained three sample locations. Airborne pollen for viability testing was collected in an open area near each site(s).

The developmental stage of 10765 female and 6786 male strobili was recorded for approximately 45 trees in each of three study sites. Observations were performed using the same trees each year if they were flowering. Strobili were chosen equally from south- and north-facing branches and located at eye level (ca. 1.6–1.8 m). Male strobili were classified as mature if pollen was released when gently snapping the strobilus. Female strobili were compared to a standard set of photographs of the strobilus in different receptive stages (Jonsson et al 1976, Parantainen and Pulkkinen 2003).

Quantitative samples of airborne pollen (I) were obtained with Rotorod samplers (Perkins and Leighton 1957) from the beginning of May to the end of June. Pollen grains stick to petroleum-jelly-covered tapes attached to two rotating metal rods. The rods rotated around a fixed circumference at constant speed in 5-minute periods between 1200 and 1500 hrs each day. The number of pollen grains in a cubic meter of air was estimated based on the tape area and the volume of air the rod had passed through during its 5-minute rotation. Pollen grain identification and quantification was carried out with a stereomicroscope.

Airborne pollen for viability testing (**I**) was collected using a high-volume air sampler (Hi-Vol SA2000, Graseby Andersen, GMW Inc., Village of Cleves, OH, USA) fitted with fiberglass filters (20.3 x 25.4 cm, Whatman EPM-2000). Filters were changed twice a day at 0800 and 2000 hrs. Used filters were folded and sealed in a clean envelope.

### *2.1.2 Pollen collection and artificial pollinations*

Pollen for artificial pollinations and *in vitro* germinations were chosen from numerous genotypes identified prior to and during the course of this study. Selection criteria included similar performance in germination trials. Genotypes used in studies **II-V** were from populations in southern (61°21'N–62°50'N) and northern (68°33'N–68°36'N) Finland. Four genotypes originating from northern Finland and three from southern Finland were used in study **II**. Maternal genotypes in study **II** were of northern origin and in study **III** of southern origin. Although three northern maternal genotypes used in study **II** were identical to northern pollen genotypes, all pollinations were made between different genotypes. One of those genotypes (northern P595) was also used in study **III**. Three southern and three northern pollen genotypes used in study **III** were also used in temperature (**IV**) and pollen-pollen interaction (**V**) studies. Trees were grafted and planted in order to clone collections and seed orchards. Southern pollen used in study (**II**) was collected from a clone collection (60°37'N) and northern pollen (**II-V**) from seed orchards (62°04'N–62°15'N). Thus, the pollen of northern genotypes developed in southern conditions, but there is evidence that differences in temperature and photoperiod during male meiosis and microsprogenesis have no effect on Scots pine pollen performance

(Varis et al. unpublished) and that in *Picea abies* phenotypic plasticity in seeds occurs during embryogenesis (Skrøppa and Johnsen 2000, Webber et al. 2005).

Pollen of northern origin was collected in 1994 (**II**) and 2000 (**III-V**), and southern pollen in 1991, 1992 (**II**) and 2000 (**III-V**). Male strobili were cut from several grafts of same genotype into paper bags. Fresh cut strobili were taken to the lab in insulated boxes where pollen was dried, vacuumed, bottled, and stored at -20°C until used in germination or pollination experiments.

Pollen germinability was tested *in vitro* before and after artificial pollinations (**II, III**). Artificial pollinations (**II-III**) were performed at the Haapastensyrjä breeding station (60°37'N) (**III**) or in seed orchards of southern Finland (62°04'N) (**II**). At Haapastensyrjä, grafts used as maternal plants were planted in pots (Fig. 2) and kept outside. In the seed orchard, trees were 8–12 m high. Pollinations were performed by using a syringe to spray female strobili with 1.5 µl pollen. Pollinations were performed soon after the first scales had opened. To prevent background pollination, male strobili were removed and female strobili were enclosed in pollination bags before their scales had opened (Fig. 2). Pollination bags were removed after five to ten days. Mature cones and seeds were collected for paternity analysis.

In study **II**, competition crosses were made with pollen mixtures of one northern and one southern genotype. Control pollinations were performed with a single, pure genotype. All crosses were made between parents of different genotypes. In study **III**, pure northern or southern pollen was used to perform "northern first" or "southern first" experiments. In the "northern first" experiment, northern pollen was introduced into the pollination bag 2 h before the southern pollen, and vice versa in "southern first". Pollen mixtures of one northern and one southern genotype were used in "simultaneous" (control) pollinations.

## 2.2 Laboratory work

### *2.2.1 In vitro pollen germination experiments*

Particles from filters of the high-volume air sampler were washed with B&K medium (Brewbaker and Kwack 1963) onto germination filters (4.7 cm diameter) (**I**). Germination experiments (**II-V**) used autoclaved B&K medium supplemented with thiamine (50 mg/l), riboflavin (25 mg/l) and ascorbic acid (50 mg/l). Samples were incubated at 20 °C for four to five days. In study **V,** identical genotypes were germinated at 15 °C. All samples were germinated in the dark and on a constant-motion shaker to prevent grains from sticking together. Microscope slides were prepared by placing a drop of sample onto an object glass and adding a drop of lactic acid glycerol-water solution to arrest germination. A cover slip was fixed with colourless nail varnish to conserve samples.

To study the possibility of chemical pollen-pollen interactions (**IV**), we used 12-well ThinCert™ Multiwell plates (Greiner). Each well contained a cup-like insert perforated by 8.0 µm pores that permitted chemical but not physical interactions between germinating pollen in the inserts and wells. In competition experiments, pollen of a southern genotype was germinated in the insert and pollen of a northern genotype in the well, or vice versa. In control experiments, the same pollen genotypes were germinated in the insert and well. Samples were taken only from inserts to standardize the germination conditions. Each well and insert received 1.5 mg of a pure pollen genotype added simultaneously, resulting in 3.0 mg of pollen in 2 ml of medium per well-insert unit.

The percentage of germinated pollen was estimated from 200 pollen grains per genotype under 20x magnification (Fig. 3). A pollen grain was considered germinated if there was a visible pollen tube with a length greater than the grain width. Pollen diameters and tube lengths (**V**) were estimated using Qwin (Leica) from digital photographs (Sony Color DXC-S500). Pollen diameter was measured as the maximum width of each grain, and tube length from the back of the pollen grain to the tip of the tube.

### *2.2.2 Paternity assignment*

Mature Scots pine seeds from competition crosses were germinated in petri dishes using sand and paper filters as substrate (Fig. 4). Seeds were kept close each other to enhance the germination. Seeds were kept under a grow lamp and irrigated with tap water. Enzyme and DNA extraction was performed when the seedling was 2 cm tall.

The outcome of competition crosses described in study II was determined according to variation in enzyme-coding alleles, i.e., allozymes. Loci used in the analysis and parental alleles are shown in Table 2 of study II. To study the importance of pollen arrival order in the female strobilus, DNA-based microsatellite analyses were used. Technical details of DNA extraction, PCR amplification, and gel electrophoresis are described in study III.

Paternity was analysed for 2376 seeds in study II and 1545 seeds in study III. Paternity assignment was conducted by simple exclusion: the maternal allele (common to all experiments) was subtracted from genotypes and the remaining sequence was interpreted as paternal. The DNA for analysing maternal alleles was extracted from needles collected from seed orchard trees.

## 2.3 Statistical analysis

In study **I**, the amount of viable pollen grains $m^{-3}$ air was compared among different stages of strobili development. The first flowering stage was when female strobili were not receptive and male strobili in the same study site were not shedding pollen. Second stage females were receptive but males were not shedding pollen, and in the third stage male strobili were shedding pollen at the same time as female strobili were receptive.

Differences in the number of viable pollen grains $m^{-3}$ air and the proportion of receptive female and pollen-shedding male strobili were analyzed using one-way ANOVA and Tukey's HSD post hoc test. The number of pollen grains $m^{-3}$ air was natural log (ln) transformed and germination percentages were arcsin transformed because of unequal population variances. The Kruskal-Wallis test was applied when population variances were not normalized by transformation. Day and night pollen germination percentages were analyzed using one-way ANOVA.

In competition studies (**II, III**), equal contributions of seeds sired by southern and northern fathers were evaluated with the $\chi^2$-test. Student's *t*-test was used when the seed siring success of northern genotypes (the mean ratio of seeds sired by northern pollen) was compared between "simultaneous" and "southern first" or "northern first" pollinations. A one-way ANOVA was used to explore the differences in seed siring success between genotypes. The number of full seeds per cone, percentage of empty seeds per cone, and percentages of aborted strobili and conelets were counted to estimate the seed productivity.

A one-way ANOVA was used to explore the differences in seed productivity estimates between pollen origins (**II**) or pollination experiments (**III**). In study **II**, differences in mean germination percentage before and after pollination, between mixtures and separately germinated pollen, and between pollen origins were explored with one-way ANOVA. Pearson correlation matrix was used to examine the correlation between pollen germinability and the seed-siring success of northern pollen in study **III**. In study **II,** correlation between the germinability of pollen mixtures and seed siring success of respective genotypes were examined with the Spearman correlation coefficient test.

In study **IV**, the Mann-Whitney U-test was used to compare the mean germination percentage of different genotypes between competition and control experiments. Differences in the mean germination percentages of pollen origins and all genotypes combined were explored with Student's *t*-test. Differences in germinability between competition and control experiments were explored with a Pearson correlation matrix.

In study **V**, pollen size, rehydration rate, germinability and pollen tube growth rate were dependent variables. The effect of germination temperature and pollen origin was examined with two-way ANOVA. The effect of pollen genotype and interaction of temperature and genotype was examined with a nested two-way ANOVA where pollen genotype was nested within pollen origin. Student's *t*-test was used to evaluate the influence of temperature on germinability of each genotype.

Statistical analyses were carried out in SPSS (version 15) (**I-II, IV-V**) or SYSTAT (version 11) (**III**). Differences were considered significant at the 5% risk level ($p$<0.05).

# 3 RESULTS

## 3.1 Pollen flow (I)

Viable airborne pollen grains were detected during female flowering and before local pollen shedding in all study sites in almost every year (Fig. 1 in **I**). Periods when foreign pollen was detected in the air lasted from one to four days depending on year and study site. There were also cases when viable pollen grains were detected before female strobili in the study site became receptive. Foreign airborne pollen represented 2.3% of the total amount of viable pollen detected. In most years and sites, the amount of foreign pollen was small but in 2000 there were more viable airborne pollen grains in the central study site before local pollen shedding than after it (Table 2 in **I**). However, there were no significant difference in the amount of foreign pollen with respect to year or site.

From all studied female strobili, 7.5% became receptive before local male strobili started pollen shedding. Those female strobili became receptive from two to five days prior to local pollen shedding The proportion of female strobili that were receptive prior to local pollen shedding was highest in the most northern site and lowest in the central site ($F=3.600, p<0.05$). There was no significant difference in the number of female strobili among years.

In 1997, 1999 and 2000, viable foreign pollen grains were detected in the northern study site simultaneously with active pollen shedding in the central site (Fig.1 in **I**). In 2000, foreign pollen grains were also in the air in the northernmost site when northern male strobili were active. The amount of viable pollen was highest in the northern site in 1997 (Table 3 in **I**). However, there were no receptive female strobili prior to local pollen shedding.

## 3.2 Pollen competition in vivo (II, III)

Southern pollen sired the majority (76%) of seeds in competition study **II** and 48% of seeds in study **III** where competing genotypes were introduced simultaneously into the pollination bag (Fig. 1 in **III**). In both studies, the result was the same irrespective of maternal genotype ($\chi^2$=NS in **III**). Northern genotypes varied significantly with respect to seed siring success: northern genotype N579 sired fewer seeds than other northern fathers in study **II**, and P622 sired more seeds than other northern fathers in study **III** (Table 1). No significant differences were found between southern genotypes (Table 2 in **II**, Table 1 in **III**).

When genotypes of different origins were injected into the pollination bags across a 2-hour interval (**III**), northern genotypes sired 76% of seeds when they were injected first. All northern genotypes sired an equal number of seeds in the “northern first” experiment, and more seeds than in the “simultanous” experiment. In the “southern first” experiment, both pollen types sired an equal number of seeds. Southern genotype E709 sired more seeds than other southern genotypes in the “southern first” experiment.

The strobili abortion rate in study **II** was higher in crosses performed with pure northern pollen genotypes than in crosses with southern or mixed pollen, but there was no variation in other seed productivity variables. In study **III**, there were more full seeds per cone and less empty seeds in the “northern first” experiment than in the “southern first” or “simultaneous” experiments. In both studies, the germinability of southern and northern pollen was equal and there were no significant correlations between germination percentage and seed siring success among pollen sources.

**Table 1.** Pollen genotypes used in studies **III-V** (E = southern, P = northern), seed siring success (**III**), mean germination percentage† (**III-V**), mean tube length (**V**), size of dry and germinated pollen grains (**V**), and *in vitro* competition success‡ (**IV**).

| Genotype | Seed siring success % | Mean germination % | Tube length µm | Size: dry | Size: germinated | Competition success *in vitro* |
|---|---|---|---|---|---|---|
| E2 | 53.5 | 44.9 ± 25.6 | 17.4 ± 5.8 | 28.3 ± 4.7 | 37.5 ± 4.1 | 8.5 |
| E88 | 46.5 | 61.4 ± 9.8 | 24.6 ± 8.1 | 29.8 ± 2.7 | 35.5 ± 3.2 | 1.3 |
| E709 | 45.7 | 57.8 ± 11.5 | 25.1 ± 12.1 | 31.7 ± 2.7 | 36.1 ± 3.4 | -0.4 |
| P594 | 48.0 | 33.0 ± 17.4 | 19.9 ± 9.1 | 30.3 ± 2.5 | 42.3 ± 3.8 | -1.0 |
| P595 | 45.3 | 67.5 ± 23.9 | 26.7 ± 16.5 | 29.9 ± 2.7 | 40.3 ± 3.2 | -32.8 |
| P622 | 64.3 | 63.7 ± 19.8 | 18.8 ± 9.7 | 29.7 ± 2.3 | 41.5 ± 3.9 | -9.1 |

† The germination percentages used for the calculations are from study **III**, control experiments in study **IV**, and from germinations at 20 °C in study **V**. ‡ Difference in germination percentages between control and competition experiments.

### 3.3 Pollen interactions and characteristics (IV, V)

The pollen-pollen interaction experiments revealed both positive and negative effects when mixed genotypes were compared to single genotype germinations (**IV**). The mean germination percentage of northern pollen was lower in the interaction experiment (34.5%) than in the control experiment (48.8%). Even though all northern genotypes had lower germinability in the interaction experiment than in the control, the difference in mean germination percentage of northern genotype P595 was the only statistically significant difference (Table 1).

The mean germination percentage of southern pollen genotypes was equal in the interaction (45.3%) and control (42.1%) experiments. Southern genotype E2 had significantly higher germinability during the interaction (18.5%) than the control (10%) (Table 1). When E2 was in the well as a competitor, the germination percentages of northern genotypes in the insert were lower than in the control (Table 1 in **IV**).

The mean diameter of dry pollen was 29.9±3.2 µm and germinated 38.9 ± 4.5 µm (t = 27.672, $p<0.005$) (**V**). When dry, there was no significant variation in the size of pollen grains among origins, but after hydration and germination northern pollen grains were larger than southern pollen (Table 1 in **V**). The size difference between dry and germinated pollen was significant in all pollen genotypes ($p<0.005$) but hydration rates varied among genotypes. Northern genotype P594 expanded more than other genotypes and southern E709 less (Table 1 in **V**). Genotypes that had the highest hydration rates were not associated with higher germinability or tube growth rate (Table 1 in **V**).

The mean diameter of ungerminated pollen grains was 35.4±3.9 µm, which was smaller than that of germinated grains (t = -13.040, $p<0.005$) (**V**). Mean pollen grain diameter germinated at 20 °C (39.0±4.6 µm) was the same as pollen germinated at 15 °C (38.8±4.4 µm).

The overall mean germination percentage was higher at 20 °C (53.3±14.0) than at 15 °C (46.1±16.8) (Table 2 in **V**). However, southern pollen germinated equally well in both temperatures whereas the germinability of northern pollen was higher at 20 °C (49.6±17.6) than at 15 °C (38.3±19.9) (t = -2.224, $p<0.05$). Germinability was variable among pollen genotypes; southern E709 had a lower mean germination percentage than other genotypes and southern E88 had the highest percentage at both temperatures (Fig. 2 in **V**).

The mean pollen tube length was longer at 20 °C (24.8±13.1 μm) than at 15°C (19.3±8.1 μm) even though tube length decreased as temperature increased in southern genotype E2 and northern P594 (Fig. 3 in **V**). Similar to germinability, the average length of southern pollen tubes was equal at both temperatures but the tubes of northern pollen were longer at 20 °C (26.4±7.2 μm) than at15 °C (17.2±15.1 μm) (U=4290.5, $p$<0.005).

# 4 DISCUSSION

## 4.1 Long distance pollen dispersal

Long distance gene flow is possible when viable pollen grains can travel between distant populations and arrive when local female strobili are receptive. Our studies confirmed the occurrence of viable airborne pollen from distant populations prior to local pollen shedding (Fig. 1 in **I**). Furthermore, foreign airborne pollen was detected when local female strobili were receptive.

Several direct and indirect methods of paternity analysis have been successfully used to quantify gene flow among isolated populations such as seed orchards (Ellstrand 1992, Wheeler et al. 1993). In our study, the distance between sites ranged from 460–850 km, which is considerable greater than earlier molecular studies and mathematical approaches have estimated for mean pollen dispersal distances (Robledo-Arnuncio and Gil 2004, 2005, Burczyk et al. 2006). At the local scale, seed paternity seems to be heavily influenced by the direction and distance that separates parental trees (Dow and Ashley 1998, Streiff at al. 1999, Kaufman et al. 1998, Robledo-Arnuncio and Gil 2004, 2005), but a considerable amount of pollen may come from distant sources (Dow and Ashley 1998, Streiff et al. 1999, Kaufman et al. 1998).

Although some recent research has shown a systematic pattern in European pollen distribution (Siljamo et al. 2008), direct measurement of long distance pollen transfer has been difficult due to the rather stochastic nature of the phenomenon (Burczyk et al. 2004). The source(s) of early season birch pollen in northern Europe can be diverse and depends on the specific meteorological conditions. Because of the vast birch forests in eastern Europe, the main source is believed to be in Russia more than 500 km from southern Finland (Siljamo et al. 2008). Our study suggests that airborne pollen can travel at least this far for Scots pine, but additional studies are necessary to establish the main source population(s). Studies combining meteorological, phenological and pollen observation data could provide a broader picture of pollen transfer and gene flow in Scots pine throughout Eurasia.

According to Davis and Shaw (2001) current climate projections for the 21$^{st}$ century necessitate population range shifts at rates of 300–500 km per century. Scots pine has a wide distribution from Spain to eastern Siberia and traditionally populations have been considered to be well adapted at the local scale (Kylmänen 1980, Pulkkinen et al. 1995, Hurme et al. 1997). However, evidence is now accumulating to suggest that local populations of wind-pollinated trees are less-than-maximally adapted to their growing sites (Mátyás 1996, Rehfeldt et al. 2002). Populations tend to inhabit areas colder than their optima and this is explained by high within-population variation caused by gene flow (Rehfeldt et al. 2002). The theory of local adaptation has been based on provenance experiments in which plants from disparate locations in the natural range were transferred to a new site. When the distance was short, transferred seedlings grew better than local ones. Long-distance transfer to northern sites had a negative effect on survival so that the increase in growth is irrelevant. Seedlings transferred south grew better but not as well as those from local populations (Eiche 1966, Eriksson et al. 1980, Beuker 1994). Changes to survival and growth due to gene flow over relatively short distances and from south to north indicate that these connections have been and will be important in the adaptation of northern Scots pine populations to a warmer climate.

The flow of foreign pollen into a study site has been estimated as 4.3% in isolated stands of Scots pine in Spain (Robledo-Arnuncio and Gil 2005) and 21–76% in seed orchards of Sweden and Finland (El-Kassaby et al. 1989, Harju and Muona 1989, Pakkanen et al. 1991, Wang et al. 1991). In our study, the amount of viable pollen in the air prior to local pollen shedding varied greatly among years (Table 3 in **I**). For instance, the amount of viable pollen was low in several years and in one year the amount was higher before than after local pollen shedding. However, observations and theoretical modeling suggest that even low pollen densities are effective in fertilization (Nilsson 1995, Sorensen and Webber 1997).

This study established the flow of foreign pollen among northern populations of Scots pine. Although adaptive traits of Scots pine are variable and have a genetic basis (Kylmänen 1980, Pulkkinen et al. 1995, Hurme et al. 1997, Savolainen et al. 2004), variation in population level molecular markers is low (Karhu et al. 1996, Dvornyk et al. 2002, García-Gil et al. 2003, Savolainen et al. 2004) which poses a significant obstacle to the elucidation of long distance gene flow. Besides studies based on meteorological and phenological data, genotype modeling would help determine the extent to which foreign airborne pollen contributes genetic material to the local population. Before expensive molecular diagnostics are performed, phenotypic differences in the offspring of northern populations may be an indicator of gene flow from southern populations. For example, comparison of easily measured traits such as the development of frost hardiness during the autumn (Andersson 1992) could be influenced by pollen-mediated long distance gene flow.

## 4.2 Factors affecting pollen performance

### *4.2.1 Pollen origin and genotype*

The significance of long distance pollen transfer may be minimal if the environmental and genetic factors hinder foreign genotypes during pollination and fertilization (Dyer and Sork 2001). Our results indicate that foreign genotypes compete rather well. In artificial pollinations using combinations of northern and southern pollen, southern pollen sired more seeds when the maternal genotype was northern (**II**). However, we stress that the circumstances of the experiment differed from the natural situation in that northern pollen was competing in southern environmental conditions (southern Finland). This fact questions the correspondence of our results to the natural state. On the other hand, the seed siring success of northern genotypes was constant when used in artificial pollinations in both southern and northern Finland (Varis et al. unpublished).

Pollen quality and performance can also be influenced by the environment in which pollen grains develop (Young and Stanton, 1990, Quesada et al. 1995, Delph et al.1997, Travers 1999). For example, *Cucurbita* pollen had longer tubes and sired disproportionately more seeds when developed in the presence of abundant nutrients (Lau and Stephenson 1993, Jóhannsson et al. 1994) and in cool temperatures (Jóhannsson and Stephenson, 1998). Some evidence exists that due to warm temperatures prolonging well into the autumn, microsporogenesis in the pollen sacs of Scots pine has already begun in the year preceding pollination and that pollen grains developed under these circumstance have shown abnormalities (Noskova et al. 2007). In studies II-IV the pollen of both southern and northern genotypes was developed in the southern part of Finland, which have to be taken into consideration. However, like in the case of experiment location, the competitive ability of northern Scots pine genotypes seems not to be affected by developmental location (Varis et al. unpublished).

The seed siring success of southern pollen was higher when the maternal genotype was from northern (**II**) rather than southern Finland (**III**). Pollen genotypes used in these two studies were different but the result was constant among all southern genotypes. An interaction with maternal tissue (post-zygotic selection) may have influenced the outcome of the pollen competition. In many gymnosperm species, including Scots pine, polyembryony may provide a means of selection among embryos during early development (Sorensen 1982). Embryo abortion can be selective when egg cells in a single ovule are fertilized by different genotypes (multigenotype fertilization) and embryos fertilized by a certain genotype are aborted more frequently than others (Buchholz 1920). There are few direct studies concerning multigenotype fertilization and selective embryo abortion, in part due to the lack of an efficient method to determine the paternity of young embryos (Rigney 1995, Korbecka et al. 2002). However, nuclear microsatellite markers (simple sequence repeats, nSSR) make paternity assignment feasible for many species, including Scots pine. The advantage of polymorphic microsatellite loci is the possibility to precisely determine the paternal genotype(s) of embryos resulting from controlled pollinations using known genotypes. When studying the paternity at the embryonic level, DNA-based methods are problematic because they require a considerably amount of embryonic tissue and, unfortunately, the culture of embryonic conifers is difficult. If this and other obstacles can be overcome, a combination of controlled crossing, effective tissue culturing and DNA-based paternity testing could be an effective way to study post-fertilization selection in plants.

### *4.2.2 Order of pollen arrival at the strobilus*

Given the suggestions of Sarvas (1962), that northern and southern genotypes sired an equal number of seeds in the "southern first" experiment was unexpected (**III**). Sarvas (1962) claimed that, in Scots pine, the first pollen grain to enter the nucellus has an advantage in subsequent pollen competition. Webber and Yeh (1987) suggested a similar advantage for the first pollen grain to arrive at the micropyle in Douglas-fir (*Pseudotsuga menziesii* (Mirb.)). However, Owens and Simpson (1981) found that pollen grains of Douglas-fir arriving in the first two days of the receptive period became more entangled in the stigmatic hairs nearest to the micropyle than grains arriving later. Marshall and Ellstrand (1985) made sequential pollinations with an angiosperm (*Raphanus sativus*) across a two-hour interval, and found that the first pollen donor sired 82% of seeds.

Our results from sequential artificial pollination in Scots pine do not unconditionally support this hypothesis. Webber and Yeh (1987) found similar differences in seed siring success of the first pollen to arrive in Douglas-fir, although (unlike Scots pine) this species has stigmatic hairs directing pollen grains to the pollen chamber instead of pollination drop (Owens et al. 1981). In Scots pine, the secretion and reabsorption of the pollination drop may influence the outcome of pollen competition. Doyle and O'Leary (1935) saw active reabsorption immediately after pollen landed on the drop and supposed that subsequent secretion does not take place. In contrast, Sarvas (1962) concluded that the pollination drop continues to be secreted even though there are pollen grains in the chamber. In our study, the time interval between pollinations was based on the observations of Doyle and O'Leary (1935) and chosen to be as short as technically possible. To fully test the hypothesis of Sarvas (1962), experiments should also be performed with longer pollination intervals. If the pollination drop is repeatedly secreted and reabsorbed, the importance of being the first pollen grain to reach the strobilus may be less than previously thought. There is lack in knowledge of exact mechanisms of secretion and reabsorption of pollination drop in conifers even though it is studied in many species (Tomlinson 1994, Gelbart and von Aderkas 2002, Mugnaini et al. 2007).

The impressive performance of northern pollen in sequential pollination trials may be due to a faster pollen tube growth rate than southern genotypes due to adaptation to a short summer in northern areas. Pollen from northern genotypes may begin to germinate at a lower temperature sum than pollen from southern populations. By starting earlier and growing faster, northern pollen tubes may have caught up southern tubes in the "southern first" experiment, but the equal proportions of seeds sired in the "simultaneous" experiment complicate this otherwise straightforward explanation. If pollen-tube growth rate is the main yardstick of pollen competition, northern genotypes should have sired significantly more seeds than southern genotypes in the "simultaneous" experiment. In addition, the same genotypes were used in the *in vitro* experiment where pollen tube lengths of southern and northern genotypes were equal (**V**).

Neither does *in vitro* germinability provide a satisfactory explanation, as this variable was equal in both pollen types (**III**), and in study **V**, germinability of southern genotypes was higher than that of northern pollen. In study **II**, germinability of southern and northern pollen was equal yet southern genotypes sired considerably more seeds than northern genotypes. It seems that, at least for Scots pine, mean *in vitro* germinability plays a minor role in pollen competition. This is in line with the findings of Snow and Spira (1991) and Skogsmyr and Lankinen (1999).

Maternal control is often related to self-incompatibility, which in conifers is considered to lead to the degeneration of self-fertilized early embryos and the formation of empty seeds (Dengler 1932; Dogra, 1967; Kärkkäinen et al. 1999; Owens et al. 2005, Williams 2007, Williams 2008). Similar mechanisms may affect incompatible pollinations (McCormick 1998), which may exist when genotypes are too similar or closely related. Genetic incompatibility may explain the unexpectedly poor siring-success of southern genotypes in the "southern first" experiment (**III**). However, this explanation is questioned by the equal success of southern and northern genotypes in the "simultaneous" experiment. If genetic incompatibility was influencing the outcome, the success of southern genotypes in the "simultaneous" experiment should have been lower than northern genotypes. Furthermore, there were no differences in seed productivity of southern and northern genotypes in experiments involving a single genotype (**II**).

### *4.2.3 Pollen grain size, hydration and germination temperature*

Southern and northern pollen grains had different sizes after hydration and germination (**V**). Pollen grains of Scots pine hydrate immediately after placement in liquid media and presumably perform similarly upon contact with the pollination drop. There is room for up to six pollen grains in the pollen chamber of Scots pine (Sarvas 1962), which may confer an advantage to large pollen grains arriving first. Hydrated pollen grains of northern genotypes were significantly larger than southern grains (Table 1) and may have occupied more of the pollen chamber in the "northern first" experiment. On the other hand, there is room for more small pollen grains in the pollen chamber, which might numerically favour southern pollen. Additionaly, smaller pollen grains might pass through the micropylar canal more easily on their way to the pollen chamber.

In addition to the size of dry and germinated pollen grains (**V**), germinability and tube length also varied among genotypes (Table 1 in **V**). However, no clear relationship among these variables and seed siring success was detected. It seems that pollen size, germinability and tube length are only slightly related to seed siring success at the genotypic level, as the order of genotypes in every category is different (Table 1). Also, the order of genotypes resulting from *in vitro* competition experiments differed from other categories. Northern genotype P622 had significantly better seed siring success than other genotypes, the second largest germinated grains, and the third best germinability. However, this genotype also had the second shortest pollen tubes and was the next to least successful during *in vitro* pollen-pollen interaction experiments. The increase in germination temperature had a positive effect on germinability and tube growth rate generally, but this effect did not assort clearly among genotypes, e.g., tube lengths of two genotypes were longer at 15 °C than at higher temperatures.

### *4.2.4 Pollen-pollen interactions*

Some of the pollen-pollen interaction experiments were performed *in vitro* in order to control for maternal effects. In traditional *in vitro* experiments, determination of individual pollen genotypes is prohibitively difficult. Our experiments took place across permeable membranes that allowed chemical but not physical contact between competing genotypes (IV). Our experiments produced highly negative effects on the germinability of northern pollen when germinating with southern pollen in comparison to the control. Also, we observed a positive effect in that the germinability of one southern genotype increased during competition trials compared to the control and suppressed the germination of other genotypes. These findings echo those of Parantainen and Pasonen (2004), who detected both an increase and a decrease in Scots pine germination percentages during mixed pollen experiments compared to single genotype trials.

Pollen-ovule interactions are well studied also in conifers (e.g. McWilliam 1958, Willson and Burley 1983, Williams 2009), but there are relatively few studies of pollen-pollen interactions and those that exist concern angiosperms (Landi and Frascaroli 1988, Marshall et al.1996, Pasonen and Käpylä 1998, Lankinen and Skogsmyr 2002). Landi and Frascaroli (1988) studied maize pollen and found evidence for suppression of tube growth between competing genotypes. Marshall et al. (1996) observed how different pollen genotypes of wild radish (*Raphanus sativus*) germinated faster when introduced separately to the stigma than when they were mixed and applied simultaneously. Germinability of mixed compared to single pollen donors *in vitro* has been higher in studies of birch (Pasonen and Käpylä 1998) and viola pollen (Lankinen and Skogsmyr 2002). Our results suggest the existence of a chemically-mediated interaction between genotypes of Scots pine pollen, but one that may play only a minor role in pollen competition *in vivo*.

# 5 CONCLUSIONS

The detection of foreign airborne pollen in a northern site during the pollen-shedding period of a population located over 500 km to the south confirms the possibility of long distance gene flow in Scots pine. The number of receptive female strobili and foreign viable airborne pollen varied between years and study sites. The simultaneous abundance of foreign pollen and receptive strobili was a rare phenomenon.

The competitive ability of pollen originating from southern Finland was superior or equal in the artificial pollination experiments where pollen from southern and northern Finland were introduced simultaneously to the strobilus. However, southern pollen gained no advantage from a 2-hour headstart over northern pollen, whereas the northern pollen performed better when it was placed in the strobilus first. In the *in vitro* interaction studies, southern pollen suppressed the germinability of northern pollen and increased its own germinability in the presence of northern pollen. Chemical suppression of northern pollen by southern pollen may have affected the outcome of the “northern first” experiment. However, southern pollen did not outcompete northern pollen in all simultaneous experiments, which suggests a more complex interaction that is influenced by genotype.

No clear relationship was found between dry pollen grain size, pollen hydration level, germinability, and tube growth among genotypes. High germinability or tube growth rate did not have a clear effect on seed siring success. Hydration rate was higher in northern pollen but the germination percentage was lower. On average, germinated pollen grains were larger in size than nongerminated. This supports the hypothesis that early germination and pollen tube growth are dependent on pollen storage materials and less dependent on water intake and hydration. An increase in germination temperature had a positive effect on germinability and tube growth in general, but there were also negative reactions among genotypes with respect to tube growth rate.

Although genotype can explain much of the variation, differences in pollen grain size, hydration rate, reaction to temperature and the effects of germination companion can also be explained in terms of pollen origin. Thus, pollen competition does exist in Scots pine and genotypes adapted to southern (warmer) climates can potentially participate in long distance gene flow.

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