ESTABLISHING POLLEN COLLECTION FOR TAMK

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ABSTRACT

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This thesis was focused on preparation of microscopic samples for further scientific and educational use. The work for the thesis included collecting pollen samples from different species during blossom seasons, preserving the pollen samples, making microscopic samples, and observing the pollen samples through microscope. Purpose of the thesis is to serve as handout for laboratory work on pollen samples and other work continuing with the pollen collection. Additionally, there was also seasonal work in helping identify airborne pollen samples for allergic monitoring in Finland. The work was proceeded from beginning of May till beginning of September in Tampere, when there were allergenic pollen grains suspending in the atmosphere, and meanwhile pollen collection from the plants was carried out as well.

To better perform the pollen sample preparation project and the seasonal assisting work, some scientific theories and information were studied and researched as well, such as allergic rhinitis which is caused by the allergens such as pollen, animal dander, etc.; blossom times (starting from April and ending in September generally) and appearances of the species (by previewing pictures of the species of various angles); general information about plant (Classified as bryophytes, ferns, and seed plants) and pollination through which seed plants are self-pollinated or cross-pollinated; importance of pollen to human being (such as being used in historic research and pollinator research, etc.); etc. Both dry and moist (frozen) samples were tested for the preparation, in which moist ones were found more efficient and convenient for this purpose. The glue used for stabilizing the pollen grains onto the microscopic slides was prepared in the laboratory successfully as well, after several different approaches.

Preparation of the microscopic samples was the main and most challenging experimental part of the whole project, which was quite interesting at the same time. The recipe used for preparing the gel was useful for the microscopic samples; more recipes could be approached and tested though. The microscopic samples prepared in the laboratory were from species included *Alnus*, *Artemisia vulgaris, Betula, Chenopodium album, Corylus, Epilobium*, and *Rosa majalis*, for which the microscopic photos were taken as well. The checklist and instruction of entire procedure of preparing the microscopic samples from plant pollen sampling to microscopic photography are included in the thesis as well.

Key words: Pollen collection, pollen identification, allergic rhinitis, microscopic sample, pollination, palynology

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1 INTRODUCTION

Tampere University of Applied Sciences (Tampereen Ammattikorkeakoulu in Finnish, abbreviated as TAMK) is a part of the aerobiological monitoring network of Finland, which mainly studies biological particles in the atmosphere, such as seeds, spores, pollen grains, etc., especially concentrating on their health impacts. During the pollen season (possibly starting in March or April and lasting till September), the Aerobiology Unit of University of Turku (abbreviated as UTU) usually collects information and data about airborne pollen and spores within Finland, and produces broadcasting accordingly. The monitoring task performed in Tampere site (by TAMK) during the pollen season is sampling the pollen grains from the collector, delivering them to the aerobiology unit of UTU for analysis and airborne pollen grains broadcasting nationwide.

Monitoring airborne pollen is important as the pollen of some species cause allergic rhinitis commonly called hay fever, because its symptoms may feel like having a fever, such as running nose, sneezing, and so on (University of Maryland … 2011). Since the pollen exists only when the flowers blossom during the specific season or period, such allergic rhinitis occur only during a specific time in the year. And since the hay fever is pollen related disease, the times when it occurs in different regions, depend solely on the allergen content contained in the air.

TAMK seeks to have a more independent role in pollen monitoring and the microscopic pollen samples can provide resources for education and research in this field. This thesis describes the process of pollen sampling, pollen preservation and preparation of microscopic samples. Additionally it discusses about plant reproductive biology and the relations between humans and pollen. Pollen grains of different allergenic species were collected inside Tampere city and used to make certain number of microscopic samples in the microbiology laboratory of TAMK in autumn of the year.

2 POLLEN AND ITS ROLE IN PLANT REPRODUCTION

Pollen as a male gamete-producing structure in the plant has its significant role in reproduction and continuation of the species. Generally pollen exists in seed plants either inside the flowers or cones, depending on category of the species. Pollen grains move to the ovules through certain path to enable combination of sperms (in the pollen grains) and eggs (in the ovules) to form embryos which grow into seeds gradually, thus the pollination and fertilization complete to realize the reproduction. (Kaul, 2009)

2.1 Plant classification

The plant kingdom contains three parts, which are bryophytes, ferns, and seed plants. The bryophytes are the simple, small plants which are least abundant in both species numbers and population, and they are quite dependant on water availability during the sexual phase which facilitates fertilization, for producing diploid zygotes. The bryophytes don't have true roots, leaves, hard tissues, or vascular system, and they are usually about or lower than 2cm, the example species is mosses. The ferns are the spore bearing vascular plants which have true roots, leaves, vascular tissues, and hard supporting tissues as well, but they are still dependant on water for sperm to move to the egg. (Kaul, 2009)

The seed plants are more successful comparing the two kinds of plants mentioned above, due to the seed feature that protects and nourishes the embryo to survive in unfavourable condition such as lack of water, and the seed dispersal also enables the embryos to move away from the parent plant. The fertilization of seed plants is proceeded through pollen (the male gamete-producing structure as mentioned above) transfer by corresponding medium such as wind, to ovule (the female sexual structure), which is also known as pollination. The pollination makes the sexual phase independent of external water, which accounts a great advantage for the seed plant to live in the terrestrial environment (Kaul, 2009).

2.2 Structure of reproductive organs

The seed plants consist of two categories that are gymnosperms and angiosperms. The gymnosperms are the cone bearing plants which contains male cones (producing pollen), and female cones (carrying ovules). Fertilization of gymnosperms is realized through pollination by wind, when the sperms enter an egg through a pollen tube, and the egg then develops into an embryo, an example of this kind of plants is spruce. The angiosperms are the seed plants dominating the landscape currently, which contain flowers where male and female sexual structures exist (Kaul, 2009).

A flower of an angiosperm plant consists of several structures, such as sepals and petals connected with a portion of stem called ´receptacle´; the stamen including anther (with pollen grains inside) and filament that are the male sexual part of the flower, and the pistil including stigma, style, and ovary (with ovule inside) which build up the female sexual part of the flower. The movement of pollen - male reproductive structure of seed plants - starts from the petal shaped to attract insect to come and perform pollination, which lands the pollen on the stigma or tip of the pistil, where the pollen germinates and then forms a long pollen tube, which enters the cavity of the ovary through the tissues of the style. Furthermore, the pollen tube goes into the ovule and releases two haploid sperm cells into the embryo sac, one of which combines with the egg and produces zygote, and the other combines with two polar nuclei and produces a specific tissue named endosperm to supply nutrients to the embryo to grow. An idealized flower structure is illustrated in Figure 1 below. (Kaul 2009)

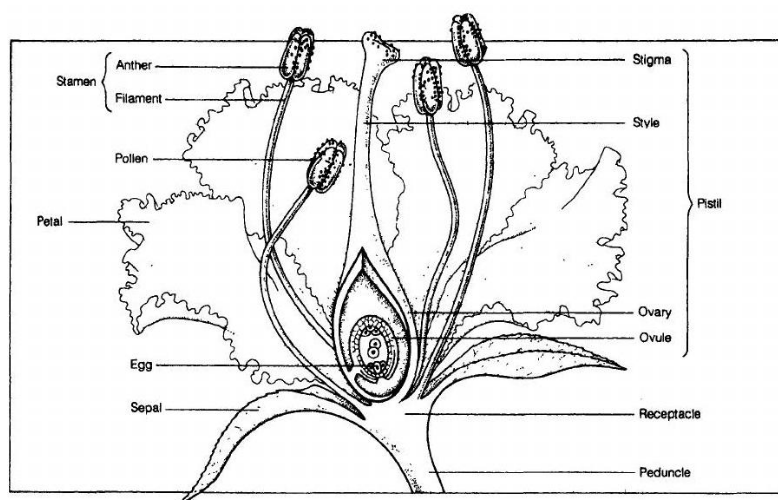


FIGURE 1. Schematic representation of an idealized flower of the angiosperms (Kaul, 2009).

The reproductive structure of gymnosperm plants are manifested in male cones (pollen bearing cones) and female cones (ovulate cones), and in many of the trees within this group, the ovules are exposed on the bracts of cones or some berry-like structures instead of being covered by a surrounding structure like ovary in angiosperm trees (Cocks, 2000). A good example of gymnosperms is pine, which has microsporophylls (pollen bearing cones) with microsporangia that contains microspore mother cells which will be developed into pollen; and megasporophylls (ovulate cones) where the fertilization of egg cell in the ovules takes place (Cocks, 2000). The arrangement of male and female cones in a pine tree is shown in Figure 2 below.

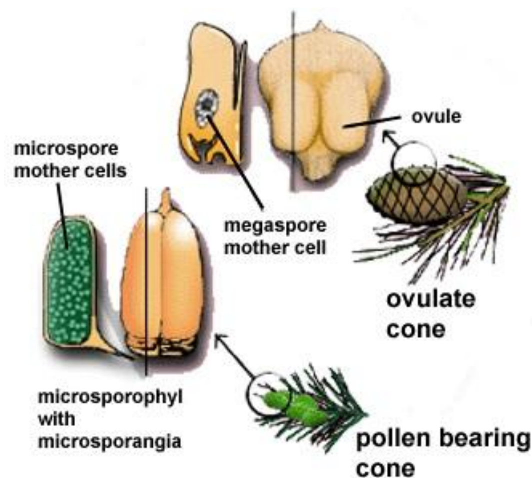


Figure 2. Arrangement of male and female cones in a pine tree (Cocks, 2000)

2.3 Pollination

The process of transfer of pollen grains from anthers to the stigma is called pollination, which is needed for germination, when the male gametes inside the pollen grains are to reach the female gametes lodged in the embryo sac (Singh, Pande & Jain 2010, 52). Generally pollination is divided into two categories which are self-pollination and cross-pollination (Singh, Pande & Jain 2010, 53).

2.3.1 Self-pollination

Transfer of pollen grains from an anther to the stigma of the same flower or to a flower on the same plant is known as self-pollination, which can occur in both bisexual flowers

and unisexual flowers existing on the same plant. According to the paths of movement of the pollen grains, the processes of self-pollination are divided into two types, which are autogamy through which pollen grains are transferred from an anther to the stigma inside the same flower; and geitonogamy through which the pollen grains are transferred among different flowers on the same plant. (Singh et al. 2010, 53)

2.3.2 Cross-pollination

According to Singh et al., transfer of pollen grains from the anther of the flower on one plant to the stigma of the flower on another plant is called cross-pollination. This kind of pollinations can occur in both dioecious and monoecious species, but is quite necessary for the dioecious species. The cross-pollination leads to cross-fertilization accordingly, which has advantage of genetic recombination. (Singh et al. 2010, 54)

2.3.3 Types of pollination agents

Since there is need of external agencies to enable transfer of the pollen grains, categorization of pollination can be realized through variety of the agencies, including biotic and abiotic types. There are also various types of pollination through abiotic agencies, such as Anemophily, in which the pollen grains are transferred through wind, and Hydrophily, in which aquatic plants realize the pollination through water. For species depending on biotic agencies for the pollination, there are also several types of pollination, including Entomophily, when the pollination is realized by insects; Ornithophily through which birds are the medium for the pollination; and Cheiropteriphily, through which bats are the pollinator for the plants. Besides all the methods above, sometimes there can be artificial pollination as well, by the plant breeders for various purposes such as hybridization. (Singh et al. 2010, 56-62)

3 REASONS TO STUDY POLLEN

Pollen plays an important role in plants and also has its specific significances for human being scientifically and healthily. Pollen grains of some plant species cause allergic rhinitis to those whose breathing systems are hypersensitive to them. Therefore in some regions airborne pollen monitoring is carried out during pollen propagation season in a year. Moreover, pollen is also used in archaeology as a historical record for researching history of plant lives.

3.1 Allergic rhinitis

Allergic rhinitis (commonly known as hay fever) is a kind of rhinitis caused by some indoor or outdoor allergens, such as pollen, mould spores, animal dander, etc. The symptoms vary in different individuals, e.g. runny nose, nasal congestion, nasal itching, or sneezing (U.S. National Library … 2012). Some people may encounter such kind of rhinitis during summer and early autumn, - or maybe even in spring, depending on the species -, which is mostly caused by the pollen spread in the atmosphere that they breathe into their nasal passages. Some people allergic to the allergenic pollen may take some pre-treatment to avoid the rhinitis to take place before the pollen accumulating in the air grows to the effective amount, some people may just tolerate the symptoms without any treatment. (University of Maryland … 2011)

3.2 Pollen monitoring

In some countries or regions of the world, the governments may arrange some broadcasting program to inform people about the pollen species and quantities existing in the air. This helps them to take some preparation before going outdoors, especially into the nature. In Finland pollen grains existing in the air are monitored and broadcasted during blossoming seasons. The whole project is carried out in nine regions through cooperation among several units including UTU, TAMK, etc. UTU is in charge of the monitoring, which receives samples from the other regions and analyzes the pollen grain content in the atmosphere through its aerobiology unit. The monitoring task carried out in TAMK includes gathering pollen grain samples in the atmosphere through a device

named Hirst Burkard collector, collecting the samples and delivering to UTU for analysis and broadcasting. (Aerobiology Unit, 2012)

3.3 Airborne pollen sampling

The airborne pollen sampling is part of the nationwide pollen broadcasting within Finland, which is carried out from spring to autumn, according to the allergenic pollen´s existing times. In 2012 the sampling was started in the beginning of May, and the sampling procedure mainly includes three steps: preparing the drum for the collector, collecting tapes attached by pollen grains, and delivering the tapes to UTU.

A drum was used for carrying the film which adsorbs the pollen grains in the air, and the film would be sent to UTU for analyzing the pollen content in the atmosphere. In the physics laboratory of main campus of TAMK, the drum was attached with appropriate length of tape covering the entire surface, and then a specific kind of glue pre-warmed was brushed onto the tape, afterwards the tape was marked with a colorful pen according to the instruction by the Aerobiology Unit, hence the drum was ready for the exchange.

The prepared drum was taken to the roof of E building where the pollen collector was located. The drum inside the collector was taken out, and the newly prepared drum was placed instead. Both before and after exchanging the drums, the tape was marked for providing information about the starting and finishing points. The collector contained a fan which turned along with wind direction, so that the pollen grains would be brought by the wind and adsorbed by the sticky tape.

The exchanged drum was brought to the physics laboratory and the tape was removed and kept into a circular box which would be sent to UTU by mail immediately. Usually the tape would be collected and delivered on Monday and Thursday, and reach to Aerobiology Unit of UTU on the following days that were Tuesday and Friday. The exact steps of the airborne pollen sampling are illustrated in Appendix 1.

Chemicals used during the work included xylene and the solution to be used as glue branded by Burkard of which the chemical name or formula was not provided. Accord-

ing to U. S. Environmental Protection Agent, Commercial or mixed xylene (C_8H_{10}) usually contains about 40-65% *m*-xylene and up to 20% each of *o*-xylene and *p*-xylene and ethylbenzene. Generally mixed xylenes are colorless liquids insoluble in water and have a sweet odor, and are used in production of ethylbenzene, as solvents in products such as paints and coatings, and are blended into gasoline as well.

Exposure to mixed xylene could cause effects such as headache, dizziness, fatigue, tremors, incoordination, etc. chronically, and nausea, vomiting, and gastric discomfort, etc. acutely. Therefore during the process, the bottle containing the xylene was treated carefully and kept sealed properly before and after rinsing the brush immediately. (U. S. Environmental Protection …, 2000)

3.4 Palynology

Palynology is the study of plant pollen, spores and certain microscopic plankton organisms in both living and fossil forms. The living pollen and spores are used for studying plant relationships and evolution, whereas fossil pollen and spores could be used to study past environments, strata or layered rock, historical geology and paleontology. The researched plant pollen, spores and microscopic plankton organisms are collectively called palynomorphs. Based on the term geologist, those who research about palynology are also called palynologists, (Jarzen, 2000))

Since palynomorphs are resistant to decomposition and are usually produced in large quantities, their recovery from rocks and sediments through special and careful chemical treatments is useful for scientists to describe plant life of past ages. Palynomorphs recovered from some organs of early humans and those associated with objects remained from early ages are useful in understanding the diets and hunting practices of these people. Study of pollen in honey (termed as melissopalynology) could help identifying the source plants from which the bees produce the honey, and this is quite important for honey producers. Moreover, palynology is also useful in many applications, such as aerobiology, insect feeding and migratory habits, detailed analysis of animal diets, etc. (Jarzen, 2000))

4 POLLEN SAMPLING

Procedure of pollen preparation included several steps: collecting the pollen samples, making gelvatol solution for stabilizing and dyeing the pollen grains on the microscopic slides, preparation of the microscopic samples and observing them through the microscope. The pollen samples were collected in Tampere, Finland, Northern Europe, which is located in a boreal zone in the northern hemisphere, where birch, pine, and spruce are the main tree species, along with various kinds of bushes and grasses, from which pollen samples were collected (Marietta College, 2000). This chapter describes the actual collection of samples and methods used for different species.

The pollen samples were collected from April to August, 2012, since some species blossom early in summer or even spring, and some species blossom late in summer or even beginning of autumn. The first collected samples were *Alnus* (commonly known as alder) and *Corylus (*hazelnut*)* in April, and the entire collection ended with grasses in August. Some pollen samples were collected in April, such as *Corylus* (commonly known as Hazelnut), some in May like *Betula* (Birch); and some could still have pollen in September like *Phleum pretense* (Timothy-grass). Almost all common names of the species mentioned in the thesis are referred from Natural Resources Conservation Service of United States Department of Agriculture (2012).

4.1 Collection of pollen samples

The pollen samples were collected from different locations inside Tampere city, including the bushes aligned at the edges of the street, trees in the quiet land such as school yard, bushes by the lake, and grasses along the small road inside the residential area. Identification of the species were realized through the study of image of the flowers, trunks, branches, and the leaves, with important guidance from the web site provided by Jyväskylä open university which shows natural photos of various species (Salonen & Heinonen, 2007) . The samples were collected into some properly sealed plastic bags, and paper bags, and marked with the date and name of the species on the surface. The plastic bags were preserved in a freezer as frozen samples, and the paper bags were preserved in the room temperature on the table of the laboratory as dry samples. The samples were preserved in both freezer and room temperature, in order to prepare the mi-

croscopic samples later on from both the frozen and dry samples and to find the better way of preservation.

Samples of different species of plants were collected gradually, according to the time when the flowers blossomed or when the pollen started appearing; samples of each species were collected by hand, and put into two or three small plastic bag one by one, and sealed before being placed into the freezer. Species from which the pollen samples were collected are, *Betula* (Birch), *Rosa* (Rose), *Urtica dioica* (Stinging nettle), *Chenopodium album* (Goosefoot), *Barbarea vulgaris* (Herb Barbara), *Artemisia vulgaris* (common wormwood), *Epilobium angustifolium* (fireweed), *Phleum pratense* (timothy grass), and *Lolium perenne* (English ryegrass). The plant species involved and methods of collecting their pollen will be described in detail in chronological order according to the time when samples were collected. Photos of the sampled plants and the sample bags are shown in Appendix 2 and Appendix 3.

1) *Alnus* (alder) and *Corylus* (hazelnut)

Alnus and *Corylus* samples were collected in April. Both of the species are wind pollinated plants (Davis 2000, Fryer 2007), and belong to *Betulaceae* (birch family) along with *Betula*, which makes them quite similar with each other in many aspects or characteristics. Pollen grains of all these three species look quite similar with each other, and the way of making microscopic samples for them were similar as well. Species of *Betulaceae* family generally appear in trees or shrubs with alternate, simple and stipulate leaves and unisexual flowers (Mohlenbrock, Robert & Paul, 2009).

2) *Betula* (birch)

The *Betula* samples were collected in the beginning of May, since they blossom quite early during a year generally. Samples were collected by removing the branch along with flower clusters from the tree and putting into a plastic bag which can be sealed properly, placing into a freezer to preserve freshly. Samples would also be kept into a big paper bag and preserved at room temperature to be used as dry samples. This kind of species generally grows unisexual flowers, with staminates in elongated, pendulous catkins, which are subtended by a bract with no petals; and sessile ovary with 2 styles (Mohlenbrock et al. 2009).

3) *Rosa* (rose)

There were various species of *Rosa* in Tampere city, majority of which *was Rosa majalis* (Cinnamon rose) that were blossoming in June, most of which were red. Species belonging to *Rosaceae* (Rosa family) are generally insect-pollinated, and bees could be seen easily while collecting the samples (Laura, Kirk & John, 2006). Another species of *Rosa* was also found, in a garden in front of the city library, which was *Rosa acicularis*, but since these two species of same family have pollen grains quite similar to each other, only *Rosa majalis* samples were used for preparing the microscopic samples.

The samples were collected through removing the flowers along with a small portion of the branches, which was quite easy, although it is well known that rose comes with sting. There were spines visible in the branches, but it was possible to avoid them while breaking the branches. Two or three flowers were kept in one plastic bag, and placed into the freezer within a short while such as one hour.

4) Chenopodium album (Goosefoot)

Chenopodium album is considered as a weed, and its flowers are wind pollinated (Navazio, Morton, Colley, Brewer & Stone, 2011). It was found in the bushes and grass clumps distributing at the sides of the streets, such as Teiskontie road in the main urban area of Tampere city. The pollen samples were collected in the same way used for *Betula*: removing the whole branch along with some flower clusters and putting into a plastic bag.

5) Barbarea vulgaris (Herb Barbara),

The *Barbarea vulgaris* is also considered as a weed, which is generally pollinated by insects (Hansen, Hansen, Osgood, 1991). It was found in some open field inside Tampere city, such as the land near the construction site, or roadside of some overpass. The weeds were not standing as clumps, but staying alone of their own. The samples were collected in different locations in the beginning of July, when it was possible to find the flowers. The flowers of *Barbarea vulgaris* are much smaller than those from *Rosa majalis*, but bigger than the ones in *Betula*, since it is still easy to recognize the flowers by eyes. The way of sampling was same as the one used for *Chenopodium album*.

6) *Artemisia vulgaris* (common wormwood)

Artemisia vulgaris is generally known as wind pollinated species, but it also is visited by some insects sometimes (Barney & Ditommaso, 2002). The pollen samples were collected twice in July. The first time was in the beginning of the month, but the flowers were not blossoming. Hence the pollen samples in the bags could not be used for pollen collection, because there were no pollen grains developed inside the petals, and it had not reached to the allergenic season either. I was told that it was not the right time to collect pollen sample for this kind of species yet. So those samples were not quite useful for the microscopic purpose.

In the late middle of July, *Artemisia vulgaris* was checked once again, and seemed to be ready for pollen collection, since the flowers were open wider and in bigger quantity within one stem or trunk. The pollen samples collected into the plastic bags seemed to be more useful and usable than the first collection, although the flowers were not quite widely open; I collected the samples, but was still planning to check this species after some days to see if it would open even wider. However, later on, the flowers were opened widely but seemed not to have any pollen grains already. Fortunately, the samples from second collection were quite useful and some microscopic samples were made from them successfully.

7) *Urtica dioica* (stinging nettle)

Urtica dioica samples were also collected twice, because of its stingy property, and flowers of this species are wind-pollinated (Cardina, Herms, Koch & Webster, 2009). I was told that this species would cause feeling of itchy to human skin, but I did not pay enough attention to that and went to collect the samples just by my hands. While touching the tiny flowers of *Urtica dioica*, skin of my fingers and hand felt itchy, and it turned red as well, so I gave up the collection at that day. The redness on the skin disappeared in two days, and everything in my hand was all right.

A few days after the unsuccessful collection, I brought a pair of scissors and went to the site again, and collected some samples fortunately. Flowers of the plant were quite small, and could not be easily recognized by eyes. Since the pollen samples were collected with scissors, some leaves were also included, although normally I would remove those leaves beside the flowers, since they were not useful for the laboratory work. For

my own occupational health and safety sake, I used the scissors to cut the stem and avoided touching the plant by any part of my body.

8) *Epilobium angustifolium* (fireweed)

Epilobium angustifolium blossomed in late July, and the plants were quite easy to find, mainly because of conspicuous color of the flowers, which was purple, although they were not big in size. This species of plants is insect- pollinated (Grainger & Turkington, 2011). The plants could be found at roadsides, hassocks, and also some small gardens. The *Epilobium angustifolium* clumps sampled were standing in the small decorative garden outside of one grocery store. The pollen samples of this species were easy to collect, simply through removing the flowers from the stem one by one, and putting into the plastic and paper bags. The whole flower set was collected, just to keep as many pollen grains as possible.

9) Grasses

Two species of grass were also collected mainly in autumn of the year, which were *Phleum pratense* (Timothy) and *Lolium perenne* (English ryegrass, Repo & Pulli, 1996). *Phleum pratense* is wind-pollinated species (National Museum of … 2012), as well as *Lolium perenne* (Department of Health … 2008). These two species were collected quite late in summer, since I decided to search for some grass species in the end of August, and it was not very easy to find the exact species either. At the time of collection, some of the *Phleum pratense* had turned yellow already. Grass of these two species were standing much lower than the other species that had been collected previously, and also harder to find the parts containing pollen inside. Since the grass was quite thin, it was not quite easy to sample when the wind was blowing, because it was moving quickly towards direction of the wind.

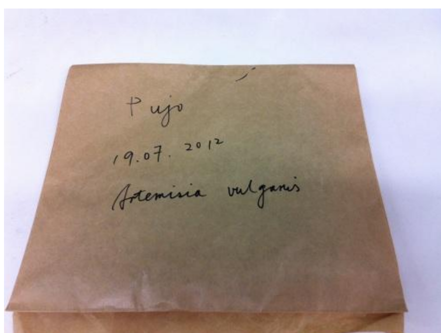
Differing from the *Phleum pratense* grass, *Lolium perenne* was a little green while collecting the pollen samples. It did not appear with blossomed flowers, so I just removed some of the top parts of the grass and put into the bags as samples. Since the time of sampling was a little late, the samples were not quite suitable for preparing the microscopic samples, because it was not easy to find pollen grains in the samples

4.2 Preservation of pollen samples

The pollen samples sealed in the plastic bags were placed into the freezer in the microbiology laboratory in main campus of TAMK (Picture 1). Samples were collected into plastic bags in two different sizes, as seen in the picture, and actually there was no difference in use of the bags, reason of which was that bigger bags were found in the laboratory later than the small bags and used afterwards.

PICTURE 1. Pollen samples preserved in the freezer (2012)

For examining how preservation affects the suitability of pollen samples for microscopic study, each of the species sampled were also collected into a big paper bag and preserved in room temperature to be used as dry samples later on. All the bags were placed on the table of the same laboratory, and all of them appeared same, except the content written on the cover for providing information (Picture 2).



PICTURE 2. *Artemisia vulgaris* dry samples (2012)

5 Pollen microscopy

The microscopic process of pollen sample preparation started when all the planned species were collected and preserved in the proper places. The procedure started from preparing the gel used for stabilizing and dyeing the pollen grains on the microscopic slides. Preparation of the gel lasted for a few days, since there was deep and slow stirring process required for the solution. After that the samples were unfrozen and used for preparing the microscopic samples, which lasted for a few months intermittently. And the whole project completed when the microscopic photos of the samples were captured through a specific device connected with the computer (Appendix 4).

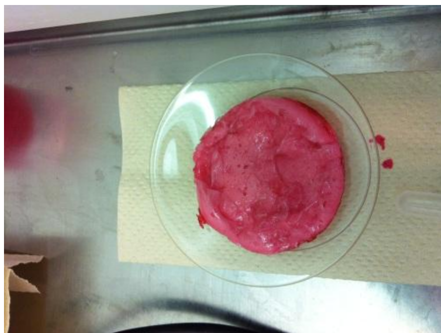
5.1 Preparation of the stabilizing and dyeing medium

The pollen samples preserved during the summer were used for making microscopic samples in the autumn, and both of the dry and frozen samples were tested to see which are more suitable for the microscopic samples. Stabilizing gel named Gelvatol was also prepared manually in the laboratory. To obtain the useful gel, various approaches were applied, including melting ready-made gel from the previous year, and preparing new solutions with some ingredients.

The Gelvatol gel was prepared in the microbiology laboratory in nursing campus of TAMK, where the previously prepared gel had been preserved for about one year. The gel preserved in a glass bottle had been placed in a freezer, which was taken out for melting. Picture 3 shows the bottle of gelvatol with a tag attached that explains the ingredients of the solution.

Picture 3. Ready-made gelvatol previously from the freezer (2012)

The gel was in a form like a block of pudding, and transferred into a beaker which was then placed on a hot plate stirrer for mixing. Temperature of the hot plate started from 50°C, and gradually increased to about 150°C, meanwhile the magnetic mixing rod was also placed into the beaker to accelerate the melting process, from 250 rpm to 500rpm gradually. The melting process was carried out intermittently for about 15 hours totally within 5 days, but the result was not quite successful, since the gel was too hard to flow, and was almost in solid form (Picture 4), therefore it was not possible to apply it for the microscopic sample preparation.



Picture 4. Unsuccessful hard gel (2012)

While melting the ready-made gel, a new combination of solution was prepared as well. The ingredients used here were:

-- PVA (Polyvinyl alcohol) 15g,

-- distilled water 50ml,

-- glycerol 50ml

-- one drop of safranin

All the ingredients were mixed in a beaker, and placed on a hot plate stirrer for further mixing. Mixing of the solution was processed simultaneously with melting of the ready-made gel, with same change of temperature and magnetic rod moving speed, in same time interval. The result of the mixed solution was not so successful either, although the product was softer than the first approach, but still did not meet the requirement of stabilizer for the microscopic samples. The gel for the stabilizing purpose needed to be at least in liquid form so that it would be transferred easily from the container onto the microscopic slide for making the samples, would combine with the pollen grains properly, and would be sticky to some extent so that it would stabilize the pollen grains on the slide as well.

Since neither of the above mentioned approaches was successful, a new recipe was introduced and tested, including:
-- PVA 20g,
-- glycerol 40ml
-- PBS (Phosphate buffered saline) 1* (at pH 7,4) 80ml.

The beaker of the solution was placed in a bigger beaker, with some ice and water present to maintain the surrounding temperature for about 4°C. A magnetic rod was used for the mixing process as well, and the mixing speed was 200 rpm constantly. The mixing process was carried out for about 20 hours continuously, during which there was automatic overnight operation inside the hood, but the temperature in the beaker maybe was only room temperature, since I was not in the laboratory to maintain 4°C.

The mixture then went through the centrifugation, by being transferred into two tubes equally and sealed properly, then placed into the centrifugation machine. The tubes were centrifugated in the machine at 21°C, with 1500rpm, in 10 minutes, and then in another 10 minutes at same temperature but with 3000rpm. Thus the undissolved solid was centrifugated from the solution at the bottom, whereas upper part of the solution was transferred into a bottle in dark color (to avoid sunlight penetration) to be used as the gel.

Three drops of safranin were added into the bottle, which was then sealed properly and shaken up and down, to mix the safranin with the solution. The safranin here was used for dyeing the solution into red color for better observation under the compound light microscope. Afterwards the bottle was packed with a piece of silver paper, and preserved at room temperature in a draw away from sunlight. The recipes mentioned above were instructed by Ms.Laaksonen in microbiology laboratory of nursing campus in TAMK, there could be other recipes yet to be searched and researched.

5.2 Chemicals used for the preparation

1) Gelvatol

Gelvatol is a kind of homemade mounting media for fluorescent microscopy, which appears as gel. Theoretically gelvatol can be made in large quantity and stored well for a long time. (Northwestern University, 2009) However, the gelvatol used in the project was made in small quantity which was less than 50ml, and previously preserved gelvatol from one year earlier was not usable even after the melting. There are various recipes for preparing the gelvatol, two of which were tested in the laboratory with one successful result.

2) PVA

Polyvinyl alcohol is produced commercially from polyvinyl acetate through continuous processes. The acetate groups used for the production are hydrolyzed by ester interchange with methanol with presence of anhydrous sodium methylate or aqueous sodium hydroxide. PVA is categorized into two types according to levels of the hydrolysis, one of which is partially hydrolyzed, and the other is fully hydrolyzed. PVA is an odorless and tasteless, translucent, white granular powder. (Saxena, 2004)

During the experiment in the laboratory, the PVA powder was not dissolving in water quite easily, and it was the main part that required the mixing process in long term. However, while testing the first recipe for preparing gelvatol, even after many hours of stirring and heating, the powder still did not integrate with other ingredients properly; therefore the experiment was not successful. As mentioned in Material Safety Data Sheet by Sigma-Aldrich, PVA is not a hazardous substance or mixture according to Regulation (EC) No. 1272/2008, and is not classified as dangerous according to Directive 67/548/EEC, and the experiments involving PVA were performed quite safely

3) Glycerol

Glycerol ($C_3H_5(OH)_3$) is a colorless, odorless, viscous liquid with a sweet taste that can be derived from both natural and petrochemical feedstocks. Generally crude glycerol is 70-80% pure and often concentrated and purified to 95, 5-99% purity before the commercial sale. Because of its unique combination of physical and chemical properties, glycerol is utilized in quite many commercial products, such as application as an ingredient or processing aid in cosmetics, toiletries, personal care products, etc. Additionally, glycerol is highly stable under normal storage conditions, compatible with many other chemical materials, non-irritating in its various uses, and has no known negative environmental effects so far. (Pagliaro & Rossi, 2008)

As mentioned in Material Safety Datasheet by Sigma-Aldrich, glycerol is not a hazardous substance or mixture according to Regulation (EC) No. 1272/2008, and this sub-

stance is not classified as dangerous according to Directive 67/548/EEC. The glycerol used in the experiment was of 87% purity and stored in a nontransparent plastic bottle. The glycerol was applied in both of the recipes tested in the laboratory, which showed that this material is important for making gelvatol to some extent.

4) PBS

Phosphate buffered saline is a buffer solution generally used in biological research. The buffer property helps maintain a constant pH which is usually 7.4. PBS is applied in various uses because of its isotonicity and non-toxicity to cells such as diluting substances, rinsing containers containing cells, drying biomolecules in a way of diluent since the water molecules within it will be structured around the substance to be ´dried´ and immobilized to a solid surface, etc. (Indian institute of … 2009)

According to Material Safety Data Sheet by Sigma-Aldrich, PBS solution of pH7.4 is not a hazardous substance or mixture according to Regulation (EC) No. 1272/2008, and it is substance is not classified as dangerous according to Directive 67/548/EEC. The PBS solution used in the experiment was contained in a transparent glass bottle closed properly by a plastic cover. The ingredient required by the recipe was of 1* PBS solution, but the material available was for 10* concentration, so it was diluted in the laboratory with distilled water in corresponding volume.

5) Safranin

Safranin ($C_{20}H_{19}ClN_4$)is a kind of dyeing material which is in dark red color. It generally can be used in staining various materials such as bacteria, cellulose, mitochondria, etc. Biologically safranin can be used for measuring membrane potential, detecting micro-organisms, food packaging, etc. Moreover, there are also industrial applications related with safranin, such as polymeric electro-mechanic devices, recording material, inks, textiles, etc. (Sabnis, 2010)

As mentioned in Material Safety Data Sheet by Sigma-Aldrich, safranin is considered Serious eye damage (Category 1) according to Classification according to Regulation (EC) No 1272/2008 [EU-GHS/CLP], and Risk of serious damage to eyes according to Classification according to EU Directives 67/548/EEC or 1999/45/EC. Fortunately, the experiments involving safranin in the laboratory were carried out quite safely, and only several drops of the material were used.

5.3 Preparation of the microscopic pollen samples

Through practices and tests, suitable methods for different kinds of plants were explored, and the processes have been developed over time. Generally, each of the samples used for the preparation went through several steps, including picking pollen grains from the whole flowers, spreading the pollen grains on the microscopic slides, dropping gelvatol onto the pollen grains, covering mixture of the gelvatol and samples, and examining the samples under the compound light microscope.

Dry samples of *Urtica dioica* were used first to make the microscopic samples, which were removed from the branches and leaves and placed into a microbiology petri. The petri was then placed under a stereo microscope, for separating the flowers from the leaves and unrelated elements which were quite tiny. The flowers were then ground by a flat ferric spoon, and transferred to a microscopic slide through a small cotton stick and a ferric stick. The fine grains were then covered by two or three drops of gel solution prepared earlier as mentioned previously. The rectangle glass cover was placed on the mixture of gel and pollen grains, to complete the preparation of one microscopic sample. Placement of the cover started from one edge, at angle of about 45° above the microscopic slide, and then covered the whole mixture quickly, in order to minimize formation of the air bubbles. After that, nail polish was used to seal the edges of the cover, so that the mixture would remain stable inside.

A small bowl was also applied for grinding the dry flower samples, but since the pollen grains were mixed with all other items such as petals, this method was tested not so suitable for the microscopic samples. Dry samples of *Betula* was also tested for making the microscopic samples, but all parts of the flowers were dried, it was not easy to separate the pollen grains from other materials. And the pollen grains did not have their true shapes, but appeared distorted under the compound light microscope, so finally the frozen samples of *Urtica dioica* and *Betula* were used for the preparation.

Frozen samples were quite moist and clear under the stereo microscope during the preparation, and it was quite easy to pick the anthers from the flowers. In the preparation of the first sample (from *Betula*) the whole anthers were transferred from the original samples and placed on the sample plate, and made into microscopic samples. However, the

big and dense fibers of the anthers disturbed the observation remarkably under the compound light microscope.

So more effort was put into picking the pure pollen grains from the samples and transferring onto the sample plates. Also practicing helped identify the pollen grains from the anthers, and better methods were found out. Under the stereo microscope, the pollen grains appeared like rows inside and surrounding the anthers. The anthers were transferred to the microscopic slide, and the pollen grains were taken out from the anthers and placed on the slide directly, with small tweezers. Sometimes the anthers were held up through the tweezers and knocked at the slide, so that the pollen grains would drop onto the slide by themselves, such kind of methods was used for preparing microscopic samples for species like *Chenopodium album,* which has quite tiny anthers and pollen grains.

The pollen grains were concentrated at center of the microscopic slide, so that it would be easier to place the cover and observe in the microscope. The more grains collected at the slide, the denser they would appear under the compound light microscope, the easier the observation would be. And during the practices, a better treatment after placing the cover was also found out, which was absorbing the gel solution leaking out of the cover by some pieces of tissue paper, so that it would be easier to brush the nail polish for the sealing. Ready-made microscopic samples are shown in Picture 6 below.

Thus the microscopic sample was prepared and placed under the microscopic lens to test the results. The magnifying size was started from the smallest one that was 10*, the focus was adjusted during observing through the mirror. Afterwards the magnifying size was moved to 20*, and adjusted properly as well. Then the magnifying size was moved to 40* that is the best size for observing pollen grains. If the pollen grains were found easily and appearing in complete shapes, the samples could be considered successful and useful.

So the samples would be marked with name of the species, type of the samples (dry or moist) and date of the preparation. The ready-made microscopic samples would be placed in the box specifically used for microscopic samples and preserved in room temperature. Two kinds of microscopic slides were used, in which the slides made of pure glass was used first, to test the preparation methods, and a piece of rectangle white

sticker would be pasted beside the samples for writing the preparation information. The slides with white writing area already available was used when the proper method of preparing samples for specific species was achieved. The ready-made microscopic samples of this kind of slides would be marked with preparation information directly.

For better visualizing the pollen grains in the microscopic samples, microscopic photos were captured for each of the species from which microscopic samples were prepared. The microscopic photography was performed through a device branded Nikon with microscopic function, which connects with the computer aside. The software used for the photography in the computer is named as Infinity Capture.

The microscopic slide was placed under the microscopic lens of the device, for which the focus was adjusted while observing from the mirror. When the pollen grains were found under the microscope, the rotary knob was turned to ´photo´, and the image would appear in the computer screen through a window of Infinity Capture application. The picture could be captured by clicking the camera button in the main window of the application and saved in the format chosen from the list in the pop-up window after processing the ´save´ command. The detailed instruction of operating the device and the software is available in the book besides the device in the laboratory.

5.4 Results of the preparation

Various samples of the species were tested and used for making the microscopic samples, most of which were the frozen samples. The prepared microscopic samples were from species included *Alnus*, *Artemisia vulgaris, Betula, Chenopodium album, Corylus, Epilobium*, and *Rosa majalis*. *Urtica dioica* samples were tested as well, but because they had been collected a little late during the blossoming season, and there were not quite many pollen grains remaining, therefore they were not used further. Some of the microscopic samples made can be seen in Picture 6 below, in which some anthers could even be realized since they were quite big comparing with the pollen grains.

Picture 6. Some microscopic samples made in the laboratory (2012)

Alnus, Betula and *Corylus* had quite more pollen grains than the other species within each of the flowers; therefore it was quite easy to make microscopic samples for these three species. *Chenopodium album* and *Epilobium angustifolium* had quite smaller pollen grains than the other species, therefore the microscopic samples made from these two species were not appearing so clear under the compound light microscope, and this can be seen in the microscopic photos in Appendix 3. *Rosa majalis* had quite less pollen grains in each of the flowers than the other species, so they were not so densely distributed in the microscopic samples, therefore it was not so easy to find them while observing through the compound light microscope.

6 DISCUSSION

To prepare the microscopic pollen samples, from sampling from the plants, through preparing the gel, to making the microscopic samples, there are various items and contents to include, apply, and pay some attention, which can be concluded into the checklists described as follows. And the entire procedure is explained as an instruction in the content below as well.

6.1 Checklist of preparing pollen samples.

During the practising and groping, various essentials were noticed and learnt for easier and better performance for the various tasks. Key elements of collecting, preserving and preparing microscopic pollen samples successfully are summarized in the checklists below which are divided into groups according to the phases of the entire process for preparing the microscopic samples.

1) Collection of pollen samples along with the whole flowers or other containers accordingly.
   a. To be taken into consideration.
      - Blossoming times of different species.
      - Avoid rainy weather.
      - Avoid insects in the petals of the flowers, especially while sampling from *Rosa*.
   b. Equipment needed.
      - Plastic bags with sealing lines to collect pollen samples from the plants.
      - Big paper bags to contain pollen samples to preserve dry samples.
      - Special tools for sampling from special species such as scissors used for *Artemisia vulgaris* which causes itchy skin.
      - A marker to write about the species and date of sampling on the bags.

2) Preservation of the samples.
   a. To be taken into consideration.

- Preserve the samples directly after sampling.
- Maintain same condition for preserving the samples.

b. Equipment needed.

- A freezer to preserve frozen samples.
- A big box to preserve dry samples.

3) Gel preparation.

a. To be taken into consideration.

- Safety instruction of the laboratory.
- One or more recipes for making the gel.
- Material Safety Data Sheet information for all the chemicals used.

b. Equipment needed.

- Laboratory clothes and accessories, such as coat, goggles, gloves, etc.
- Equipment and glassware for preparing the gel, such as: beaker, hot plate stirrer, magnetic stirring rod, etc.
- A glass bottle of dark color and a piece of silver paper for preserving the gel.

4) Preparation of microscopic samples.

a. To be taken into consideration.

- Avoid air bubbles while covering the mixture of gel and pollen grains.
- Observe the microscopic samples from smallest magnification number such as 10*, and gradually move to 40*, which is the best size for observing pollen grains.

c. Equipment needed.

- Stereo microscope for preparing the samples, and compound light microscope for observing the samples.
- At least two small tweezers for picking the anthers and pollen grains.
- Big petri dishes for containing the samples.
- Microscopic slides and covers for making the microscopic samples.
- Small plastic pipettes for dropping the gel onto the microscopic slides.
- Tissue paper nearby to dry the mixed solution out of the glass cover.
- Nail polish to seal edges of the cover.
- A pen to write name of the species and date of preparing the samples.

- Suitable boxes for preserving the ready-made microscopic samples.

6.2 Instruction for the entire process of pollen sample preparation.

1) Before starting the sampling.
   Read the materials about blossoming times of the species, look for some photos about the species, of the whole plant bodies, flowers, trunks, branches, and the leaves to identify the species easily.
2) While collecting pollen samples.
   Remove the flower parts of the plant, put them into the plastic bags, and seal the bag. Write the species name and date that the sample is collected on the bag which can be seen easily, and place the bags into the freezer. Collect some samples in the same way and put into a big paper bag and preserved at room temperature.
3) While preparing the gel.
   - Decide the recipe to be used for preparing the gel; prepare the equipment and glassware for the experiments. Mix the ingredients and stir with the equipment for sufficient time, till softness and density of the gel are suitable to use.
   - Transfer the gel solution into two centrifugation tubes equally, which will be placed into a centrifugation machine. Centrifugate the tubes for 10 minutes with 1500 rpm at 21°C, if the result is not so satisfying, centrifugation can be operated for 10 more minutes with higher speed such as 3000 rpm at same temperature.
   - Transfer the clearer solution at upper parts into a dark glass bottle, add two or three drops of safranin and cover the bottle properly. Shake the bottle, till the safranin is mixed well with the solution.
   - Place a piece of sticker onto the bottle, write name of the solution, name of the owner, and date of preparation. For further protection, the bottle could be packed by a piece of silver paper, and another sticker is to be pasted with same content written down. The bottle then is to be placed somewhere away from sunlight, e.g. draw.
4) While preparing microscopic samples.

- Unfreeze one bag of the targeted pollen samples, approximately half hour before preparing the microscopic samples. Or it is also possible to take out half of the bags for unfreezing, and put the rest back to the freezer to be used next time. The samples can be spread into a big petri, which will be placed under a stereo microscope when they are unfrozen.
- Under the stereo microscope, use two small tweezers to pick the anthers from the flowers and place them onto the microscopic slide, number of which can be 9 or 10 at one time. Use the tweezers to open the anthers and spread the pollen grains on the microscopic slide, or tweeze the anthers and knock it on the glass, so that the pollen grains will drop on the glass by themselves. For the samples from species in which the anthers and pollen grains are quite tiny, the pollen grains can be forced out through squeezing the anthers through the tweezers. More anthers can be transferred to the slide again to bring more pollen grains, until there are quite dense pollen grains appearing on the slide.
- Remove the anther crumbs and leave the pure pollen grains on the microscopic slide, use the small pipette to pour two or three drops of gel onto the samples. Place one edge of the glass cover onto the slide at angle of about 45° above the slide, and quickly cover the whole area.
- When there is liquid coming out from the cover, tear the tissue paper into small pieces, and place them around the cover to absorb the liquid. When the surrounding of the cover is almost dry, brush the nail polish at four edges of the cover, to stabilize the samples inside, thus the microscopic sample is ready to use. In order to avoid unneeded movement of the cover while adding the nail polish, it is better to brush the polish slightly.
- Place the microscopic sample under a compound light microscope to observe the result. The viewing magnitude is started from 10*, meanwhile the focus is also adjusted to find the best distance for the view. Gradually the magnitude can be increased to 20*, and then 40*, which is the best size theoretically for observing pollen grains.
- When the pollen grains can be found easily in the microscopic sample, it can be considered as a successful product. Write name of the species from which the sample is collected, and date that the microscopic sample is prepared onto the white area of the slide if there is. Sometimes there is

no white area prepared in the microscopic slide; a piece of white sticker can be pasted instead.

7 Final remarks

In a word, process of establishing the microscopic pollen samples requires patience and lots of practices and effort in improving the methods simultaneously. Since pollen appears only in specific seasons, there is need of paying attention to the blossoming times of the species planned for sampling, and collection of the samples at right time, when there are plenty of pollen grains available in the flowers.

Through processing the whole project, various knowledge, information, and experiences were gained, which are quite precious and useful. From the project I have learned specific and scientific knowledge about allergic rhinitis, governmental reaction against allergenic pollen in Finland, plant classification, pollen and its importance for human being, pollination, blossoming times and identification of various species, and various chemicals used for specific purposes. During the preparation of the microscopic samples I also have practised operating different types of microscopes, preparing microscopic slides for pollen grains, and operating the camera device for capturing microscopic photos. Moreover, I have also learned and practised preparing gelvatol solution which is used for stabilizing the samples in microscopic slide in different recipes. All that are mentioned above are quite valuable knowledge, information and experiences for my future academic and working lives, and quite helpful in my personal life as well.

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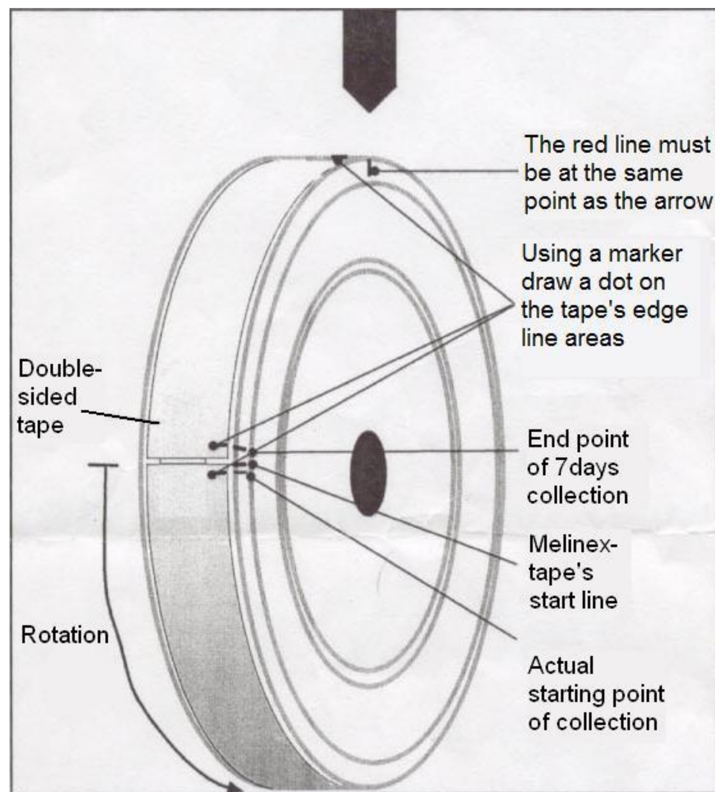
Appendices

Appendix 1. Illustration for airborne pollen sampling.

Picture 7. Procedure of airborne pollen sampling (2012)

1. Warm the glue bottle in some vessel or sink with water at 35°C - 40°C.
2. Place the drum onto the stand, paste a segment of double-sized adhesive tape against the three closely separated lines of the coating drum. Coat a transparent plastic tape around the drum, starting from the middle of the line area, without leaving any empty space between two edges of the tape.
3. Brush the warmed glue onto the tape twice with the brush dipped into the glue. Rinse the brush with xylene and then put back on the beaker or some other vessel to dry naturally. Mark 3 dots to the edge of the plastic tape line. Detailed illustration is shown in the figure below.

Appendix 1



Marking dots illustration (Aerobiology Unit of UTU, 2012)

4. Place the prepared drum into a box, along with other tools needed such as needle, key, air flow meter, etc., and visit the roof where the collector is located.
5. Stabilize the fan by placing the small rod into the hole at the thinnest part of the collector.
6. Measure the air intake, which usually is between 9-11 l/min.
7. Mark the end point of the tape by inserting the needle to scratch the tape through the orifice.
8. Open the upper cover and take out the drum inside, turn the clock with the key anti-clockwise, till it reaches to the end. Place the prepared drum and put the cover back properly, and the red line of the drum should meet the arrow of the collector.
9. Again measure the air intake and mark the tape for a new start. Release the fan by taking out the small rod from the hole.
10. Take the drum back to the laboratory, and place it onto the stand, usually the tape on the drum is colored by the pollen grains.
11. Paste two segments of adhesive tape into a sending box provided by Aerobiology unit of UTU.
12. Remove the tape from the drum and place into the box inwards (side stuck with pollen grains facing center of the box) so that the pollen sample is preserved without touching external materials. Send the box to the Aerobiology unit of UTU by mail for the analysis.

Appendix 2 Photos of the species from which pollen samples were collected

Betula

Rosa acicularis

Barbarea vulgaris

Artemisia vulgaris

Urtica dioica

Chenopodium album

Epilobium angustifolium

Picture 8. Some of the species from which pollen were sampled (2012)

Appendix 3 Pollen sample bags

Alnus

Corylus

Betula

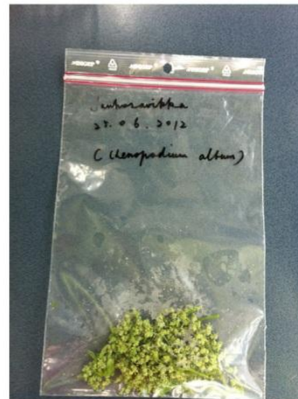
Rosa acicularis

Rosa majalis

Picture 9. Pollen sample bags (2012)

Appendix 3

Barbarea vulgaris



Chenopodium album

Artemisia vulgaris

Epilobium angustifolium

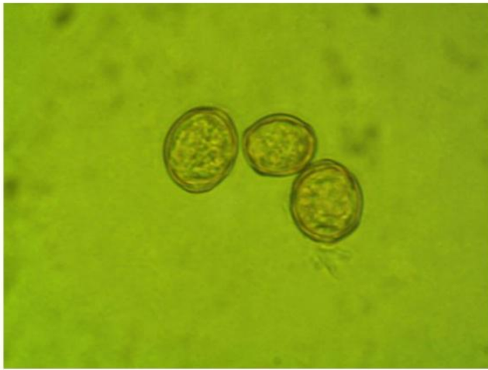
Urtica dioica

Lolium perenne

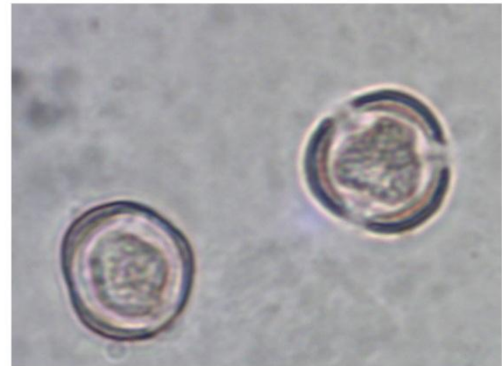
Phleum pratense

Picture 10. Pollen sample bags (2012)

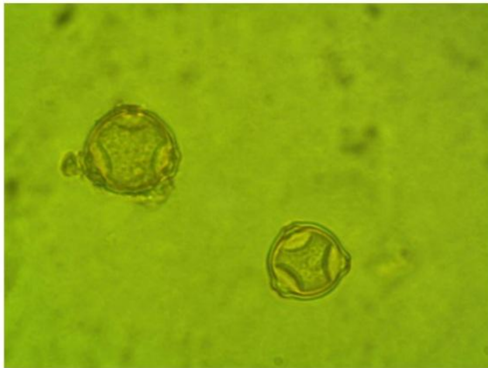
Appendix 4 Microscopic photos for the pollen preparates



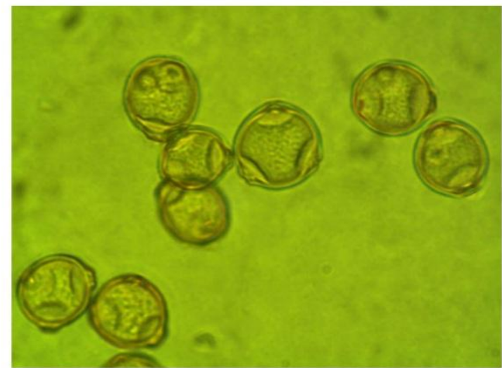
Alnus (frozen)



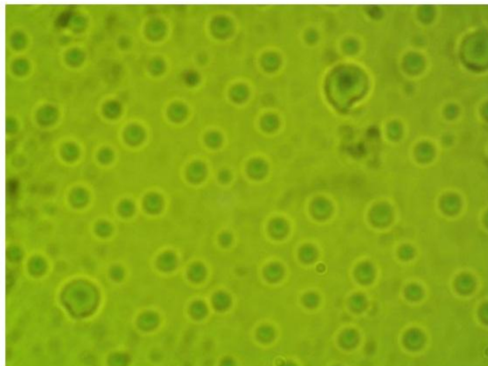
Artemisia vulgaris (frozen)



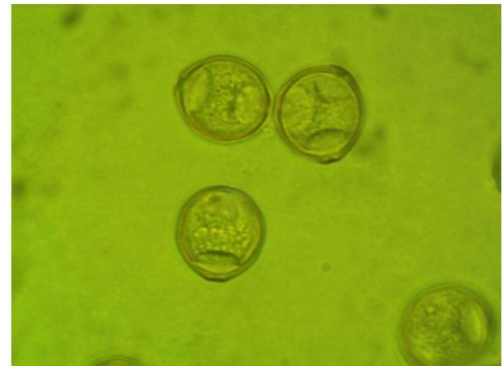
Betula (dry)



Betula (frozen)



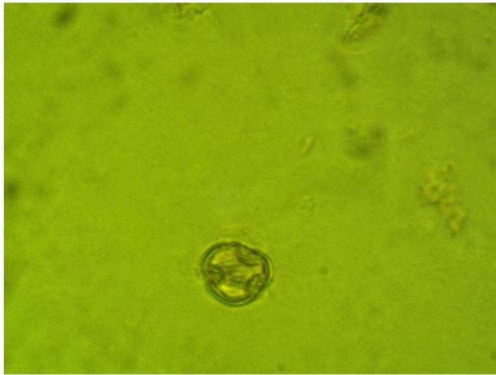
Chenopodium album (frozen)

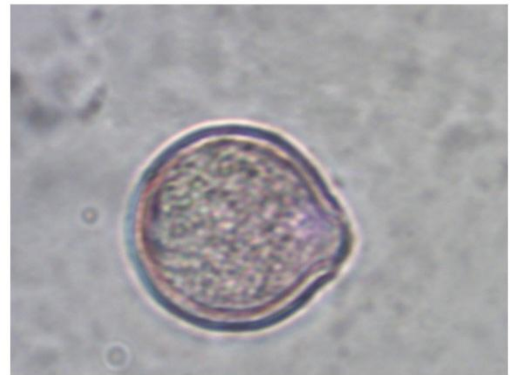


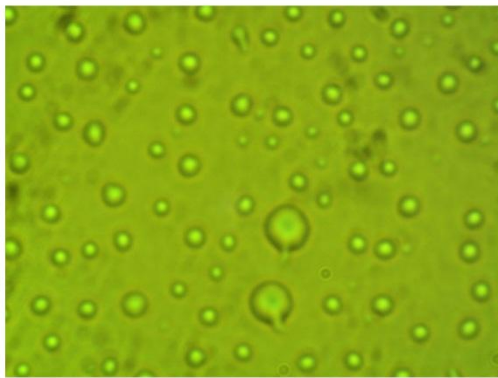
Corylus (frozen)

Picture 11. Microscopic photos for the pollen samples under scale 40* (2012)

Appendix 4


Rosa majalis (dry)


Rosa majalis (frozen)


Epilobium angustifolium (frozen)

Picture 12. Microscopic photos for the pollen samples under scale 40* (2012)