AEROBIOLOGY, IMAGE ANALYSIS AND ALLERGENICITY OF POLLEN AND SPORES IN SINGAPORE

ONG TAN CHING

NATIONAL UNIVERSITY OF SINGAPORE

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List of Abbreviations

Chemicals and Reagents

AP buffer	buffer for alkaline phosphatase
BCIP	5-bromo-4-chloro-3-indolyl phosphate
BSA	bovine serum albumin
NBT	nitroblue tetrazolium
PBS	phosphate-buffered saline
PBS-BSA	phosphate-buffered saline with 1% bovine serum albumin (w/v)
PBS-milk	phosphate-buffered saline with 4% skim milk (w/v)
PBS-T	phosphate-buffered saline with 0.05% Tween 20 (v/v)

Units and Measurements

cm	centimeter
m	meter
μl	microliter
μm	micrometer
μm^2	micrometers square
μm^3	micrometers cube
mm	milimeter
pollen $cm^{-3}day^{-1}$	pollen grains per cubic meters per day
spores $cm^{-3}day^{-1}$	spores per cubic meters per day
OD	optical density

Others

ELISA	Enzyme-linked immunosorbent assay
FAST	Fluorescent allergosorbent test
IgE	Immunoglobulin E
MicroImage	Olympus MicroImage™
OD	Optical density
RAST	Radio allergosorbent test
SD	Standard deviation
SPT	Skin prick test

Summary

An optimal method for screening Singapore's outdoor airspora samples captured by the Burkard 7-day volumetric spore trap was developed and consists of screening slide mounted tapes via three longitudinal traverses, 3 mm apart starting from the middle of the slide viewed with 400 magnification but 12 vertical traverses can be employed when diurnal patterns are of interest. Peak fungal spore counts were observed annually from February to March and October to November. The majority of the pollen count peaks are in November to March but a mid-year peak was observed for Acacia auriculiformis and Casuarina equisetifolia. The major peak period for fern spore counts was found to be from May to August. Diurnal patterns were also observed in our local airspora. High levels of ascospores were found during the night while Deuteromycetes spore counts were high during the late morning to early evening. Pollen and fern spore counts were high during the middle of the day. Correlations with meteorological parameters were also observed for daily and diurnal patterns. During the screening process, a previously unidentified fungal spore (with affinities to Dothideomycetes and Chaetothyriomycetes based on DNA information) that made up a large proportion of the outdoor and even indoor airspora, was discovered.

It has been demonstrated that image analysis coupled with light microscopy is a feasible and useful approach for developing an automated airspora quantification system. Local airspora and closely related pollen types that are quite similar morphologically can be satisfactorily differentiated.

An immunoarray has been developed to simultaneously screen specific IgEs to a large panel of allergens ranging from those of mites, pollen, fungi, epithelial tissues/dander, venom and even food. It has been shown that the immunoarray is a useful semiquantative tool to be used for mass screening purposes.

The development and subsequent use of the immunoarray to screen for the prevalence of airspora allergens has provided us with important information. Results obtained from screening studies seem to suggest an under recognition of pollen and spore allergens in Singapore because a large panel of foreign spores and pollen were also included in the screens and some reactions to the foreign airspora were higher than those found locally. This has demonstrated to us the existence of other allergenic local airspora that were not captured in the sampling trap. Possible cross-reactivity patterns were observed in more pollen types than fungal allergens. However, for pollen types the patterns were partly confounded by the absence of a candidate primary local sensitizer.

In conclusion, new and useful information has been obtained from the work done. The study has provided useful solutions and answers and suggested much future work that can be done to understand allergy better in Singapore.

CHAPTER 1: INTRODUCTION

1.1 ALLERGY

1.1.1 Hypersensitivity

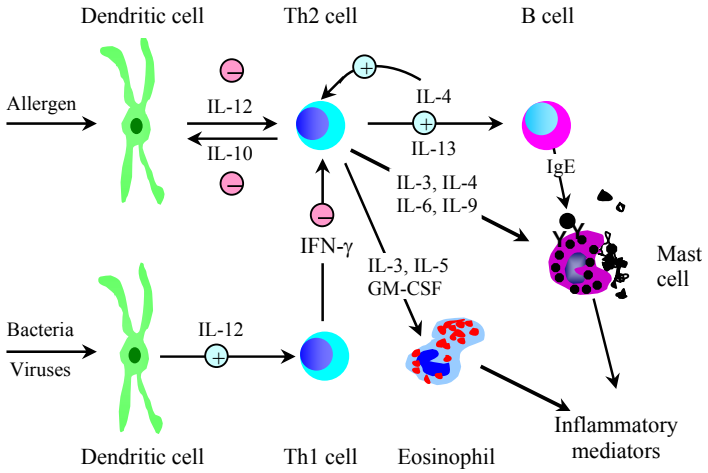
When an adaptive immune response is mounted excessively or in an exaggerated form, the term "hypersensitivity" is applied (Roitt *et al*., 2001). A normal immune system is beneficial to the body. However, in the case of hypersensitivity the immune system behaves inappropriately and can result in inflammation and cellular damage. Hypersensitivity can be divided into four categories viz., types I, II, III and IV (Coombs and Gell, 1975). Type V hypersensitivity, termed "stimulatory" was later added. Type I, II, III and V are mediated by antibodies. Type IV hypersensitivity is a delayed reaction involving a cell-mediated immune response rather than a humoral response.

1.1.1.1 *Allergy – Type I hypersensitivity*

The term "allergy" is basically used to refer to a type I immediate hypersensitivity reaction (Roitt *et al*., 2001). Allergic individuals will produce immunoglobulin E (IgE) upon contact (with prior sensitization) with an antigen, termed as an allergen (Figure 1.1). IgE binds to the IgE-specific Fcε receptors of mucosal and cutaneous mast cells and circulating basophils (von Bubnoff *et al*., 2003; Kay, 2000). This reaction occurs within minutes upon re-exposure to the allergen in an allergic individual. Cross-linking occurs when an allergen binds to an IgE variable region of two adjacent antibodies on mast cells or basophils. This causes rapid uptake of

calcium ions into the mast cells resulting in degranulation and release of proinflammatory mediators like histamine, leukotrienes, prostaglandins and tryptase. These, in turn, result in the symptoms of immediate allergic reactions. The mast cells can also contribute to delayed reactions four to eight hours after the immediate response. Interleukin-4 (IL-4) has autocrine effects and provides positive feedback to the T helper 2 (Th2) lymphocytes resulting in the production of more IgEs.

Figure 1.1: Proposed cellular and molecular mechanism of allergy. Adapted from Holgate (1999).



At the same time, mast cell-derived mediators cause endothelial cells to upregulate their expression of adhesion molecules for eosinophils, basophils and lymphocytes (Platts-Mills, 2001, Kay, 2000). Pro-inflammatory mediators like tryptases may activate the proteinase-activated receptor-2 on endothelial cells resulting in increased vascular permeability. The recruitment of lymphocytes occurs during the symptom-free period. They then release cytokines and proteases causing damage in tissues and finally contributing to what is termed the late-phase reaction. This is expressed as

congestion in allergic rhinitis and bronchial obstruction or inflammation in asthma. Chronic inflammation eventually causes airway hyperresponsiveness.

1.2 Allergens

Allergens are antigens that stimulate the allergic reaction (Blumenthal and Rosenberg, 1999). Commonly inhaled allergens that result in the manifestation of allergic disease can be divided into indoor and outdoor allergens (Kerkhof *et al*., 2003; Burge and Rogers, 2000; Boulet *et al*., 1997; Sporik *et al.*, 1996). Mites, fungi and endothelial tissues/dander from pets are common indoor allergens (Kerkhof *et al*., 2003; Boulet *et al*., 1997; Sporik *et al.*, 1996). For outdoor allergens, pollen and fungal spores dominate (Burge and Rogers, 2000; Boulet *et al*., 1997; Sporik *et al.*, 1996). The work involved in this thesis focuses on outdoor allergens.

1.2.1 Fungal allergenicity

Fungi constitute a very large group of organisms virtually found in every ecological niche (Hawksworth, 2001). It is estimated that 1.5 million species of fungi exist worldwide (Alexopoulos *et al*., 1996). Fungi are heterotrophic organisms devoid of chlorophyll, have cell walls made of chitin, are non-motile and reproduce by spores. Fungi are usually filamentous and multicellular. The filaments termed hyphae constitute the body (soma) of a fungus which elongates by apical growth. The reproductive structures differentiate from somatic structures. Most fungi reproduce sexually by meiosis, producing spores in or on a specialized structure like the

basidium or ascus, respectively. These types of fungi are referred as *Fungi Perfecti*. Until recently, fungi that lack sexual reproductive structures altogether and make only mitospores or no spores at all were segregated in the *Fungi Imperfecti* or Deuteromycota (Hawksworth 2001; Taylor *et al*., 1999; Reynolds and Taylor, 1993). However, the analysis of nucleic acid variation has enabled the classification of mitosporic fungi with their meiosporic relatives (Agerer, 2003; Taylor, 1995). To avoid confusion and for the ease of review and discussion in this thesis, the older taxonomic classification which includes Deuteromycota will be used since a large number of past and current literature on fungi, especially those in relation to allergy, still refer to the older classification system.

Fungal spores have long been identified as one of the sources of indoor or outdoor allergies (Perzanowski *et al*., 1998; Platts-Mills *et al*., 1996). Fungal spores owing to their smaller size can penetrate into the lower respiratory tract resulting in allergies (Reponen *et al*., 2001; Lehrer *et al*., 1983). The manifestation of a fungal allergy ranges from the common conjunctivitis, rhinitis and rhinoconjunctivitis to the more detrimental in ascending order of severity, i.e., sinusitis, asthma, bronchopulmonary mycoses, hypersensitivity pneumonitis and allergic alveolitis (Fink, 1998; O'Hollaren *et al*., 1991; Lehrer *et al*., 1983; O'Brien *et al*., 1978). Fungi have also been demonstrated to affect human lives by producing metabolites that are toxic to humans and animals. The allergenic fungi and their major grouping is shown in Table 1.1.

Table 1.1: Allergenic fungi. Adapted from Vijay and Kurup (2004).

Phycomycetes	Deuteromycetes (*Fungi Imperfecti*)
Mucor	*Acremonium*
Phytophtora	*Alternaria*
Plasmophora	*Aspergillus*
Rhizopus	*Aureobasidium*
Ascomycetes	*Botryotrichum*
Chaetomium	*Botrytis*
Claviceps	*Cephalosporium*
Daldinia	*Chrysosporium*
Didymella	*Coniosporium*
Erysiphe	*Curvularia*
Eurotium	*Cylindrocarpon*
Microsphaera	*Drechslera*
Zylaria	*Epicoccum*
Yeasts	*Fusarium*
Candida	*Gliocladium*
Rhodotorula	*Helminthosporium*
Saccharomyces	*Monilia*
Basidiomycetes	*Neurospora*
Agaricus	*Paecilomyces*
Calvatia	*Penicillium*
Cantharellus	*Phoma*
Cyanthus	*Pyrenochaeta*
Ganoderma	*Scopulariopsis*
Geastrum	*Sporotrichum*
Lentinus	*Stachybotrys*
Merulius	*Stemphylium*
Phollogaster	*Torula*
Pleurotus	*Tricoderma*
Polyporus	*Trichophyton*
Psilocybe	*Ulocladium*
Puccinia	*Wallemia*
Tilletia	
Urocystis	
Ustilago	
Xylobolus	

1.2.2 Pollen allergenicity

The Oxford English Dictionary (2004) defines pollen as the fine granular or powdery substance, produced by and discharged from the anther of a flower, constituting the male element destined for the fertilization of the ovules. It is rich in proteins and enzymes (Roulston *et al*., 2000; Baraniuk *et al*., 1992). The transfer of pollen to a receptive surface, the stigma, will result in the production of a pollen tube which finally leads to the fertilization of the ovule, which develops into the seed, and the ovary, into a fruit (Nemeth and Smith-Huerta, 2003). Pollination happens mainly by two routes: wind or animals (van der Pijl, 1982). It is also these properties (light weight and rich in proteins and enzymes) that have resulted in the deposition of pollen onto the mucosal surfaces of human, and animals, subsequently resulting in allergies (Ciprandi *et al*., 1994).

The term "hay fever" was coined by a Dr. John Bostock in 1828 when he noticed that his allergy symptoms worsened during the haying season in spring (Coca and Cooke, 1923). Today, "hay fever" or seasonal allergic rhinitis, describes nasal congestion, coughing, runny nose, sneezing, and breathing difficulties caused by seasonal allergies mainly to pollen. Common symptoms elicited in allergic patients are rhinitis, conjunctivitis, rhinoconjunctivitis, sinusitis and asthma (Traidl-Hoffmann *et al*., 2003; Varela *et al*., 1997; Bousquet *et al*., 1993). Anaphylaxis rarely occurs because of pollen exposure but may be induced by ingestion of food such as peach, apple, plum and cherry in food allergic individuals due to cross reactivity with tree pollen allergens namely to birch pollen which is also known as oral allergy syndrome (OAS) (Lopez *et al*., 2002; Valenta and Kraft, 1996).

More than 250,000 well-described pollen producing plants exist but fewer than 100

represent potent sources of allergens (D'Amato *et al*., 1998; D'Amato and Spieksma FTM; 1990; Lewis *et al*., 1985). The known types of pollen allergens can be divided into three main categories, viz., tree, weed and grass pollen (Lockey *et al*., 2004).

1.2.2.1 *Tree pollen allergenicity*

The most allergenic tree pollen types are from the order Fagales, especially from the family Betulaceae (Mothes *et al.*, 2004). They are a major source of springtime allergies in temperate climates of the northern hemisphere (D'Amato *et al*., 1998; Jarolim *et al*., 1989; Lewis *et al*., 1985). The Fagales are found in Europe, Northwest Africa, East Asia, North America (Zomlefer, 1994) and locally namely the Casuarina (Tan, 1997). Pollen allergens from *Betula verrucosa*, like Bet v 1, have been identified to be major allergens and similar allergens can be found across many plant species. Other pollen types from *Cupressus* (Pinales) and *Olea* (Lamiales) are also important allergens (Iacovacci *et al*., 2002; Rodriguez *et al*., 2001; Aceituno *et al*., 2000). The olive (*Olea europea*) is an important commercial crop in regions with a Mediterranean climate, and is currently cultivated in North and South America, South Africa and Australia, resulting in the increase of allergies to olive pollen. The members of the Cupressaceae grow in the Mediterranean region, Australia, New Zealand and South America (Zomlefer, 1994). The Taxodiaceae (Pinales) produce important allergens in Japan (Sado and Takeshita, 1991). Allergens, especially from tree pollen, produce important allergens that have been shown to cross-react with plant-based food, e.g., Bet v 1 and fruits of the Rosaceae like peach, apple, plum and cherry (Vieths *et al*., 2002; Breiteneder and Ebner, 2001; Gall *et al*., 1994).

1.2.2.2 *Dicotyledonous weed pollen allergenicity*

Pollen of the Asteraceae is the main source of allergenic weed pollen types (Lewis *et al*., 1985). The short ragweed (*Ambrosia artemisiifolia*) has been well studied due to its role as a source of allergens from the wind-pollinated weeds in many parts of Europe and United States. It is one of the main sources of hay fever in late summer in countries such as Austria, Hungary, Italy, France, Switzerland and United States (D'Amato and Spieksma, 1990; Lewis *et al*., 1985; King, 1976). Many allergens of the short ragweed have been identified (Amb a1, Amb a 2, Amb a 3, Amb a 5, Amb a 6 and Amb a 7) (I.U.I.S. Allergen Nomenclature Sub-Committee, 2004). Ragweed allergy is a major problem in the United States and increase in the spread of ragweed in Europe is alarming due to the highly allergenic properties of its pollen (D'Amato *et al*., 1998; D'Amato and Spieksma, 1990; Lewis *et al*., 1985). Other weed pollen like that of *Artemisia* (Garcia-Stelles *et al*., 2002; Pasterello *et al*., 2002; Diaz-Peralez *et al*., 2000), *Helianthus* (Asturias *et al*. 1998; Fernandez *et al*., 1993), *Parietaria* (Ford *et al*., 1986; Corbi and Carreira, 1984), *Plantago* (Calabazo *et al*., 2001; Asero *et al*., 2000) and *Parthenium* (Sriramarao and Rao, 1993) have also been shown to be allergenic. Cross reactivities between pollen from different weed families have been reported (Hirschwehr *et al*., 1998; Fernandez *et al*., 1993; Sriramarao and Rao, 1993). Allergens from tree and grass pollen, and also food, have been shown to cross react with pollen from weed pollen (Barral *et al*., 2004; Pham and Baldo, 1995; Valenta *et al*., 1992).

1.2.2.3 *Grass pollen allergenicity*

Early work on hay fever was conducted on grasses, such as Charles Blackley's experiments on the etiology of hay fever (Taylor and Walker, 1973) and description of immunotheraphy by Noon (1911). Grasses can be commonly found worldwide (Zomlefer, 1994) and have been found to be the most common airborne pollen in Southeast Asia, viz., in Malaysia, the Phillipines and Thailand except in Singapore (Zomlefer, 1994; Ho *et al*., 1995; Phanichyakarn *et al*., 1989; Cua-Lim *et al*., 1978). The grass family can be divided into five subfamilies with the Chloridoideae, Panicoideae and Pooideae being the most well studied for their allergenic properties (Table 1.2) (Esch, 2004). The Chloridoideae, Panicoideae and Pooideae are well studied owing to their wide distributions in the temperate zone and their ability to elicit allergies in the humans. The allergens in grasses have been classified into nine groups according to the International Union of Immunological Societies (IUIS) Allergen Nomenclature (I.U.I.S. Allergen Nomenclature Sub-Committee, 2004). *Cynodon dactylon, Holcus lanatus, Lolium perenne, Phleum pratense* and *Sorghum halepense* are grasses which have been considerably well studied in terms of their allergen components. Grasses are also cultivated as crops and contribute to the main food staple in the daily diet. Pollen of grasses planted as commercial crops have also been found to be allergenic, in descending order of importance, like rye (*Secale cereale*), wheat (*Triticum aestivum*), rice (*Oryza sativa*), maize (*Zea mays*), sorghum (*Sorghum vulgare*) and sugarcane (*Saccharum officinarum*). Allergens from grasses have also been demonstrated to cross-react with other allergens from tree or weed pollen and even food (Grote *et al*. 2002; Boccafogli *et al*. 1994).

Table 1.2: Grasses and their subfamilies. Adapted from Esch (2004) and Soreng *et al*. (2004).

Subfamily	Tribe	Genus
Arundinoideae	Aristideae	*Aristida*
	Arundineae	*Arundo, Cortaderia, Phragmites*
	Centotheceae	*Chasmanthium,*
	Danthonieae	*Danthonia, Schismus*
	Pappophoreae	*Cottea, Enneapogon, Pappophorum*
Bambusoideae	Bambuseae	*Thuarea*
	Brachyelytreae	*Brachyelytrum*
	Diarrheneae	*Diarrhena*
	Oryzeae	*Leersia, Luziola, Oryza, Zizania, Zizaniopsis*
Chloridoideae	Aeluropodeae	*Allolepis, Distichlis, Monanthochloe*
	Chlorideae	*Bouteloua, Buchloe, Cathestecum, Chloris, Cynodon, Enteropogon, Eustachys, Gymnopogon, Hilaria, Microchloa, Schedonnardus, Spartina, Trichloris, Willkommia*
	Eragrosteae	*Blepharidachne, Blepharoneuron, Clamovilfa, Dactyloctenium, Dasyochloa, Eleusine, Eragrostis, Erioneuron, Leptochloa, Lycurus, Monroa, Muhlenbergia, Redfieldia, Scleropogon, Sporobolus, Trichoneura, Tridens, Triplasis, Tripogon, Triraphis, Vaseyochloa*
	Unioleae	*Uniola*
	Zoysieae	*Tragus, Zoysia*
Panicoideae	**Andropogoneae**	*Andropogon, Arthraxon, Bothriochloa, Chrysopogon, Dichanthium, Elionurus, Eremochloa, Hemarthria, Heteropogon, Imperata, Ischaemum, Microstegium, Mnesithea, Rottboellia, Saccharum, Schizachyrium, Sorghastrum, Sorghum, Themeda, Trachypogon, Tripsacum, Zea*
	Paniceae	*Anthaenantia, Axonopus, Brachiaria, Cenchrus, Dichanthelium, Digitaria, Echinochloa, Eriochloa, Melinis, Oplismenus, Panicum, Paspalidium, Paspalum, Pennisetum, Sacciolepis, Setaria, Stenotaphrum, Urochloa*
Pooideae	**Aveneae**	*Agrostis, Aira, Alopecurus, Anthoxanthum, Avena, Cinna, Holcus, Koeleria, Limnodea, Phalaris, Phleum, Polypogon, Rostraria, Sclerochloa, Sphenopholis, Vulpia*
	Bromeae	*Bromus*
	Hainardieae	*Hainardia, Parapholis*
	Meliceae	*Glyceria, Melica*
	Poeae	*Briza, Dactylis, Desmazeria, Festuca, Gastridium, Lamarckia, Lolium, Poa*
	Stipeae	*Hesperostipa, Nassella, Oryzopsis, Piptochaetium, Stipa*
	Triticeae	*Agropyron, Brachypodium, Elymus, Hordeum, Leymus, Psathyrostachys, Secale, Triticum*

Tribes that are important allergenically are highlighted in **bold**.

1.2.3 Fern spore allergenicity

Ferns are plants which do not produce flowers or fruits and reproduce by means of spores which are small and light and easily transported by wind to far away places (Holttum, 1968). Spores are normally found on the under surface of fronds (sporophylls — leaves bearing sporangia) contained in club-shaped structures called the sporangia. Because of its small size, the spore carries very limited food reserves compared to the seeds in angiosperms and gymnosperms and is highly dependent on the nutrients from the medium they germinate on for survival. The sporangium, when ripe, breaks open because of the shrinkage of cells on drying and flicks back to disperse the spores into the air. In the tropics, ferns are found in abundance because of the hot and humid climate. The allergenicity of fern spores is not well studied partly due to their main distribution in the tropics partly where allergenicity research is not as intensive. Reports of fern spore allergies are based on the exposure to them as indoor plants (Geller-Bernstein *et al*., 1987; Kofler, 2000; Paulsen *et al*., 1998; Wuthrich and Johansson, 1997). The presence of allergenic outdoor fern spores has been reported in Malaysia (Ho *et al*., 1995), Singapore (Chew *et al*., 2000) and Thailand (Bunnag *et al*., 1989). Countries outside Southeast Asia which have reported fern spores as part of their outdoor airspora are Taiwan (Yang and Chen, 1998) and the United Kingdom (Lacey and McCartney, 1994). However, the allergenic properties of the spores were not reported.

1.3 TRENDS IN ALLERGIC DISEASES

Allergy is a major health problem in most countries (Gruchalla *et al*., 2003; Arshad *et al*., 2001; Leung *et al*., 1997). Even though allergic diseases are not new, consensus is that there has been an increasing trend of allergy prevalence (Wang *et al*., 2004; Maziak *et al*., 2003; Pearce *et al*., 2000). The International Study of Asthma and Allergies in Childhood (ISAAC) was designed to allow for comparison of prevalence of allergic disorders between different populations across the world. A large number of participating countries reported an increase in the prevalence in allergic diseases.

In Singapore, Wang *et al*. (2004) reported opposing trends in the prevalence of current wheeze between 6 to 7 and 12 to 15 year age groups. A decrease was seen (16.6 to 10.2%) in the younger age group while an increase in the older age group (9.9 to 11.9%). An increase was however, observed in the current eczema symptoms of both age groups. Similar results were obtained in Hong Kong where there was an increase in allergic rhinitis (35.1 to 37.4%) and eczema (28.1 to 30.7%) (Lee *et al*., 2004). In Australia, a reduction in the 12-month period prevalence of reported wheeze from 27.2 to 20.0% was reported (Robertson *et al*., 2004). However, an increased prevalence was reported for eczema (11.1 to 17.2%) and rhinitis (9.7 to 12.7%). In Germany, it was observed that there was a general increase for all symptoms (asthma, eczema and hay fever) (Maziak *et al*., 2003). In Thailand, studies in Bangkok (Vichyanond *et al*., 2002) and Khon Kaen (Teeratakulpisarn *et al*., 2000), saw increasing asthma among children and university students. Similar increasing trends have also been seen in Eastern Europe (Heinrich *et al*., 2002), Central America (Soto-Quiros *et al*., 2002), Japan (Tanihara *et al*., 2002) and the United States (Sly, 1999).

The main causes that have been suggested for the increase in allergic diseases reported including: 1) Raised awareness of allergic diseases (Ng *et al*., 2001), 2) improvement of diagnostic techniques (Ng *et al*., 2001), 3) changes in lifestyles resulting in decreasing birth rates as a result of the increase in mean age of marriage, increasing exposure to allergens through the easy availability to food from all over the world, improved hygiene (decreased infections) leading to an unchallenged immune system resulting in no training of the immune system for handling allergens (Maziak, 2002; Maziak, 2002a; Huovinen *et al*., 2001; Alm *et al*., 1999; Huazi *et al*., 1998) and 4) the increase in use of paracetamol (Shaheen *et al*., 2002; Newson *et al.*, 2000; Raghuram and Archer, 2000; Shaheen *et al*., 2000).

However, there are recent studies from schoolchildren in Italy (Ronchetti *et al.,* 2001), and United States (Akinbami and Schoendorf, 2002) and adolescent in Switzerland (Braun-Fahrlander *et al.,* 2004) which suggest that the upward trend in allergic diseases seems to have slowed down or plateau. It was suggested that the maximum effects of the changing environmental exposure on individuals with susceptible genetic background could have been reached and is postulated that genetic-environmental interaction studies may shed more light on the mechanism of susceptibility (Novak *et al.,* 2004).

CHAPTER 2: AEROBIOLOGY IN SINGAPORE

2.1 INTRODUCTION

2.1.1 Singapore

Singapore is an island city-state located at the southern tip of the Malay Peninsula at 1º 19' North and 103 º 31' East. It has a relatively uniform temperature throughout the year coupled with abundant rainfall and high humidity (Foo, 2002; Chia and Foong, 1991). December to January is generally cooler with May to July being hotter (Chia and Foong, 1991). Rainfall tends to be more abundant from November to January with July receiving the least rain. Humidity often exceeds 90 percent at night till dawn with average daily humidity at 84.3%.

2.1.2 Airspora

The term “airspora” was first used by Gregory and Hirst (1957) to describe the population of airborne particles of biological origin. This meaning of the term has evolved and is now commonly being used to describe airborne pollen and fungal spores (Burge, 1986; Mandrioli and Comtois, 1998).

2.1.2.1 *Fungal spores*

Fungal spores can be found year round (Tan *et al*., 1992; Lim *et al*., 1998). They make up between 86.0 to 88.1% of the total airspora (Lim *et al*., 1998). The average

fungal spore load in the air is 1688 spores m^{-3} day^{-1} and can reach as high as 19,075 spores m^{-3} day^{-1}. The latest survey by Lim *et al*. (1998) found the major fungal spore components consist, in descending order of percentages, of *Cladosporium* (33.5 to 41.0%), *Didymosphaeria* (21.9 to 28.6%), *Pithomyces* (10.2 to 14.7%), *Curvularia* (4.1 to 10.6%) and *Drechslera*-like spores (1.4 to 2.3%). Other identified or unidentified fungal spores make up less then 1% of the total airspora.

The seasonality pattern for the major fungal airspora has also been described with a peak starting in February stretching through March and a second peak in October to November. Both patterns were also found to coincide with those of *Cladosporium* and *Didymosphaeria*.

2.1.2.2 *Fern spores*

Ferns are found in abundance in the tropics, including Singapore, due to the high humidity and moderate even temperatures providing and ideal habitats for the growth of ferns (Johnson, 1977; Piggott and Piggott, 1959). A study of the airspora composition in Singapore demonstrated fern spores make up 6.2 to 8.6% of the total airspora (Lim et al., unpublished). Average densities of fern spores range from 114 to 173 fern spores m^{-3} day^{-1}. The major components of fern spores found using volumetric traps were, in descending order of percentages, *Nephrolepis auriculata* (50.9 to 55.8%), *Dicranopteris linearis* (24.4 to 27.1%), *Stenochlaena palustris* (5.2 to 6.2%), *Dicranopteris curranii* (3.4 to 4.4%), *Pteridium aquilinum* (2.8 to 3.6%) and *Asplenium nidus* (2.0 to 3.8%).

2.1.2.3 *Pollen*

No distinct definable major flowering season for plants was found in Singapore (Rao and Wee, 1989) as compared to temperate countries. The airspora composition study previously done (Lim et al., unpublished) showed that pollen grains make up 4.4 to 5.4% of the total airspora. Average levels of pollen were between 92 to 109 pollen grains m^{-3} day^{-1}. For all pollen types, oil palm pollen (*Elaeis guineensis*) (23.7 to 45.3%), was found to be the most abundant pollen type followed by that of ru (*Casuarina equisetifolia*) (7.2 to 28.0%), greater kyllinga (*Kyllingia polyphylla*) (5.3 to 23.2%) and white pine/pine pollen (*Podocarpus/Pinus*) (2.3 to 15.6%). Grass pollen, the major pollen type in other countries in Southeast Asia (Ho *et al*., 1995; Dhorranintra *et al*., 1990; Phanichyakarn *et al*., 1989; Cua-Lim *et al*., 1978) was found in lower concentrations and make up only 2.2 to 3.5% of total airspora.

2.1.3 Technical factors influencing airspora quantification

Currently, the Hirst spore trap is the most popular method for sampling airspora (Mandrioli and Comtois, 1998). The trap sucks in air at a constant rate of 10 l min^{-1}. A tape coated with adhesive is wound around a drum, which rotates at a constant rate of 2 mm hr^{-1} and changed weekly. Airborne particles are collected upon impactation on the tape's adhesive. The tape will then be removed and cut into 48 mm long strips to represent each day of the week. The tape is then mounted on a glass slide.

Although the principles of the sampling and equipment used are similar, the method for counting individual types of pollen and spores used by aerobiologists around the world still varies. Essentially, three methods are used — horizontal traverses along

the length of the slide, vertical traverses along the width of the slide and random or systematically located microscopic fields (Mandrioli and Comtois, 1998). Pilot work to study the Singapore airspora composition was done using a 250× screening magnification on three horizontal traverses evenly positioned along the length of the slide (Tan *et al*., 1992).

2.1.4 Effects of airspora counts on health

The concentrations of airborne allergens and durations of exposure to these allergens have been found to be important factors influencing the exacerbation of allergic diseases. Studies to date have demonstrated the components found in our local airspora to be allergenic (Kimura *et al*., 2003; Chew *et al*., 2000; Baratawidjaja *et al*., 1999; Lim *et al*., 1995). The association of airspora, such as fungal spores and pollen, have long been associated with increase of symptoms of allergic disease and asthma in Finland (Rossi *et al*., 1993), Austria (Zwick *et al*., 1991) and United States (Salvaggio *et al*., 1971). Leuschner and Boehm in Switzerland (1979) also showed that symptoms can be induced by pollen grains remaining in the mucosal membrane and be continually active for some time even when concentrations of pollen are not high in the air.

Donovan *et al*. (1996) in Canada and Fontana *et al*. (1974) in France demonstrated the influence of the duration of exposure in a controlled environment using ragweed pollen. Results suggested that when levels of ragweed ranged between 7 to 20 pollen grains, an outdoor exposure of just 30 minutes (Donovan *et al*., 1996) is sufficient to elicit symptoms in sensitive patients. Creticos *et al*. (1984) in United States, showed

an increasing trend in leukotriene release in patients challenged with pollen intranasally while Lebel (1988) in France demonstrated the threshold levels for release of mediators when challenged with grass pollen.

Even though seasonal allergic rhinitis has never been reported in Singapore, a study by Chew et al. (1998) found distinct seasonal peaks in the cases of Ambulatory and Emergency asthma cases in the local hospitals. An increase of 33% above the norm was found. This finding demonstrates the presence of seasonal pattern of clinical allergic symptoms even though it is less distinct than those of temperate countries. The difference of exposure levels and the intensity of the exposure could be factors differentiating the intensity of seasonal patterns seen when compared. In temperate countries, exposure is high within a short period of defined flowering period or season (Laaidi, 2001; Weber, 1995; D'Amato G and Spieksma, 1990) where else the local flowering season has been found to be spread out through the year for different plant types studied (Rao and Wee, 1989) and was further supported by the airspora seasonality results.

However, by staying indoors, exposure to airspora allergens can be reduced. Levels of airspora are relatively low compare to outdoor levels (Shelton *et al*., 2002; Sterling and Lewis, 1998). Nevertheless, indoor airspora sources such as fungal spores should be first removed. Vacuum cleaning (Fahlbusch *et al*., 2001) and the use of air cleaners (Mahieu *et al*., 2000) can reduce the amount of airspora in homes.

2.1.5 Aims

This study aimed to evaluate the effects of various factors such as 1) screening magnification, 2) number of traverses (one to five traverses), 3) position of traverses along

the width of the slide and 4) orientation of traverses (horizontal or vertical) on the airspora counts. This will enable us to establish the optimal method to be employed in the airspora counting process. Seasonal and diurnal patterns of the major airspora components were studied together with the effects of meteorological factors on airspora levels. With the availability of these patterns, individuals allergic to airspora can better plan their activities to minimize unnecessary exposure to high levels of airspora.

2.2 MATERIALS AND METHODS

2.2.1 Airspora sampling and meteorological data

Air sampling was carried out using the Burkard seven-day volumetric spore trap (Burkard Manufacturing Co. Ltd., UK). The traps were set up on the rooftops in three locations at Clementi (1° 18' 55.8" N, 103° 46' 5.9" E), Hougang (1° 21' 28.4" N, 103° 53' 18.1" E) and Kent Ridge (1° 17' 44.8" N, 103° 46' 44.2" E), in Singapore (Figure 2.1). The traps were located 61 m, 28 m and 45 m above sea level or 57 m, 27 m and 44 m above ground respectively. The Kent Ridge location is at the fringe of a secondary forest located on a ridge while Clementi and Hougang are urban townships.

The trap consists of a drum wound with silicon grease- (Beckman Instruments Inc., USA) coated tape. The drum rotates at 2 mm per hour and was changed weekly. The tapes were than cut at the 12 am line, corresponding to the 7 days of the week. Simultaneous meteorological data were recorded at the Kent Ridge station site using an En-

virondata Automatic Weather Station (Queensland, Australia). The same meterological data obtained from the Kent Ridge station was used for Clementi because of their close proximity (2.4 km). For the Hougang station, the data were interpolated from those of Paya Lebar and Seletar meteorological stations maintained by the Singapore Meteorological Services, since the airspora sampling station was situated between these two stations.

Figure 2.1: Locations of the sampling and meteorological stations.

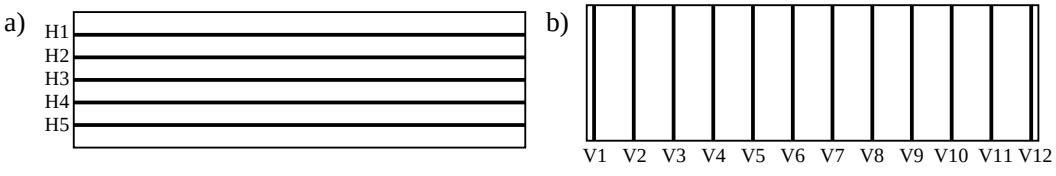
2.2.2 Evaluation and optimisation of screening factors

Each slide consisted of the appropriate length and corresponding position of the tape which was wound around the clockwork drum in the spore trap. In total, 14 continuous days (2 to 16 June 1995) worth of slides were used. The slides were screened using continuous sweeps for the major airspora which have been identified in an earlier study (Tan *et al*. 1992; Lim *et al*. 1998).

Magnifications of 400× (field area diameter 0.45 mm) and 250× (field area diameter 0.70 mm) were used to screen five horizontal traverses (H1–H5) 3 mm apart (Figure 2.2a). Airspora were categorized into all airspora, airspora <200 μm^2 in image area (*Cladosporium* spp., *Curvularia* spp., *Didymosphaeria* sp. and *Pithomyces* sp.) and airspora >200 μm^2 in image area (*Acacia* spp., *Asplenium nidus*, *Casuarina equisetifolia*, *Dicranopteris curranii*, *Dicranopteris linearis*, *Drechslera*-like spore, *Kyllingia polyphylla*, *Nephrolepis auriculata*, Poaceae, *Pteridium aquilinum* and *Stenochlaena palustris*) for analysis. Area size for airspora was measured using the Olympus MicroImage^{TM} software (Media Cybernetics, USA). The number of traverses and magnifications used in screening determine the area of the slide screened thus influencing the time spent in obtaining the daily airspora counts.

After the screening magnification, the number and position of traverses were evaluated. Screening by means of 12 vertical traverses (V1 to V12) was also performed. A 400× magnification was used for screening as it was found to be better early in this study. Twelve vertical traverses provided diurnal information but require more screening time. This allowed us to study the feasibility of changing the current method to 12 vertical traverses to obtain satisfactorily accurate counts. All counts were expressed in number of pollen grains / spores m^{-3} day^{-1}.

Figure 2.2: Slide screening methods used: (A) five horizontal traverses (3 mm apart) and (B) 12 vertical traverses (4 mm apart). Orientation of traverses: Horizontal = H and vertical = V.



Calculation for the horizontal traverse method:

Number of horizontal traverses counted = 3

Field diameter = 0.45 mm

Total sampled area = width of tape × length of tape = 14 × 48 = 672 mm^2

Total screened area = number of traverses counted × field diameter × length of tape

= 3 × 0.45 × 48 = 64.8 mm^2

Amount of air sucked in per day = 24 hours × 0.600 m^3 h^{-1} = 14.4 m^3

Number of pollen grains/ spores daily if, for example, 300 fungal spores were counted

= numbers counted × sampled area / (screened area × amount of air sucked in daily)

= 300 × 672 / (64.8 × 14.4)

= 216 fungal spores m^3 day^{-1}

2.2.3 Seasonal patterns

Counts were made from 1st June 1995 to 31st May 1996. The method using three horizontal traverses under 400× magnification was adopted. The major airspora taxa, *Acacia* spp., *Asplenium nidus*, *Casuarina equisetifolia*, *Cladosporium* spp., *Curvularia* spp., *Dicranopteris curranii*, *Dicranopteris linearis*, *Didymosphaeria* sp., *Drechslera*-like spore, *Kyllingia polyphylla*, *Nephrolepis auriculata*, *Pithomyces* sp., Poaceae spp., *Podocarpus/Pinus* spp., *Pteridium aquilinum* and *Stenochlaena palustris* were counted. The rest of the airspora were classified as "other fungal spores", "other fern spores" or "other pollen". Counts were then combined with available airspora data dating from 5th May 1990 to 31st May 1995 counted by Madam Siti Dahlia Mohd Dali.

2.2.4 Diurnal patterns

Tapes collected during the months of March, April and June from years 1995, 1996 and 1997, respectively, were examined. These months were chosen based on overlapping seasonal peak periods for most of the airspora types coupled with the availability of the sample slides (samples from certain periods of the year were missing owing to problems encountered with power supply when the Burkard traps were running). The 12 vertical traverses method was adopted for the diurnal pattern study. Only the major airspora taxa, *Acacia* spp., *Asplenium nidus, Casuarina equisetifolia*, *Cladosporium* spp., *Curvularia* spp., *Dicranopteris curranii, Dicranopteris linearis*, *Didymosphaeria* sp., *Drechslera*-like spore, *Kyllingia polyphylla*, *Nephrolepis auriculata, Pithomyces* sp., Poaceae spp., *Pteridium aquilinum* and *Stenochlaena palustris* were identified.

2.2.5 Statistical analyses

To determine the better magnification (250× or 400×) to use and effects of the position (H1 to H5) or number (one to five) of traverses along the length of the slide that were screened, Spearman's Correlation Test and the Wilcoxon Rank Test were performed. Counts using 12 vertical traverses were compared to those for three horizontal traverses by using the Spearman's Correlation Test and the Wilcoxon Rank Test .

Yearly variations and seasonal patterns were determined using the 12-point centered moving average of the weekly means. The on-site meteorological data were used for correlation studies between airspora load and meteorological factors. Spearman's rank

correlation test was used for this study. Comparison of counts between stations were also made by Spearman's Correlation Test and the Wilcoxon Rank Test.

Individual diurnal patterns were obtained by using the 2-hourly means from the counts. Individual diurnal patterns were obtained by using the means from the counts. Only the days with the desired pollen and spores present were used. Influences of meteorological factors on airspora diurnal patterns were investigated by the Spearman's Correlation Test.

All statistical analyses were performed using SAS software version 8 statistical package for Microsoft Windows (SAS Institute Inc., USA). Non-parametric statistic like the Spearman's Correlation Test and the Wilcoxon Rank Test was use for the analysis skewed distribution of the data. The spore counts itself is inferred by counting only partial area of the slide and not actual or absolute counts. Thus, analyses were also based on ranked scores of the data and not absolute counts.

2.3 RESULTS

2.3.1 Evaluation and optimisation of screening factors

2.3.1.1 *Screening magnifications*

All counts using 250× and 400× magnifications were significantly correlated ($r = 0.3570$ to 0.7453, $p<0.001$). However, counts for all airspora ($p = 0.0464$) and smaller airspora with an image area less than 200 μm^2 ($p<0.0001$) were significantly

higher at 400× magnification (Figure 2.3). Such airspora species were *Cladosporium* spp., *Curvularia* spp., *Didymosphaeria* sp. and *Pithomyces* sp. (Figure 2.4). Even though the correlation for airspora counts with image size more than 200 μm^2 were relatively weak, no significant differences were observed between the counts made using 250× or 400× magnification.

2.3.1.2 *Position of traverses*

The distribution of pollen grains and spores along the width of the tape was analysed. Significant positive correlations were observed for all counts ($r = 0.26674$ to 0.86417, $p<0.05$). No significant difference was obtained when the Wilcoxon Rank Test was performed to check for the differences between the counts from different traverse positions on the tape (Figure 2.5). This indicated pollen grains and spores were evenly distributed along the width of the whole slide.

2.3.1.3 *Number of traverses*

There was strong positive correlation between counts done on one, three and five traverses (Figures 2.6, 2.7 and 2.8). Strength of correlation between counts in descending order are as follows, five and three traverses ($r = 0.7213$ to 0.9857, $p<0.001$), three and one ($r = 0.6479$ to 0.9304, $p<0.001$), and lastly, five and one ($r = 0.5855$ to 0.9301, $p<0.001$). Stronger correlations were observed between counts screened using three traverses with either five or single traverses, thus making three the best number of traverses to use. The counts made on one, three and five traverses were also found not to be significantly different ($p = 0.2990$ to 0.9673).

2.3.1.4 *Orientation of traverses*

After studying the magnification, position and number of traverses used, the influences of the orientation of the traverses were also evaluated. Counts obtained using 12 vertical traverses (75.6 mm^2) were compared to those of three horizontal traverses (64.8 mm^2). Even though the correlation between airspora types with area size more than 200 μm^2 were relatively weak, there was also no significant differences between the airspora counts. All counts were correlated ($r = 0.3862$ to 0.7224, $p<0.001$) and found not to be significantly different with the p values ranging from 0.0534 to 0.1337 (Figure 2.9).

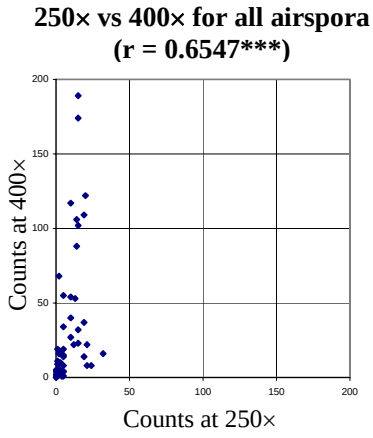
2.3.2 Seasonal patterns

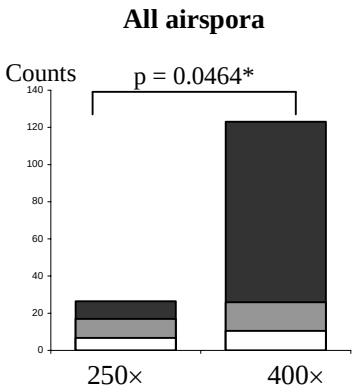
2.3.2.1 *Fungal spores*

Seasonality patterns were observed in fungal spores (Figure 2.10, p. 41). Correlations between counts of different years are shown in Table 2.1 (p. 39). A major peak stretching from June to September and a shorter minor peak period from February to March was observed for *Cladosporium* spp. Spore counts for 1992 were negatively correlated to those of 1991, 1994 and 1995. Trends for 1991, 1993 and 1995 were similar to each other. For *Curvularia* spp., peaks were obtained in February, July and October. Counts for 1992 were negatively correlated to those of the other four years studied. Counts from 1994 and 1995 were also uncorrelated. *Didymosphaeria* sp. showed broad double peaks in March and November and counts for all five years were significantly correlated.

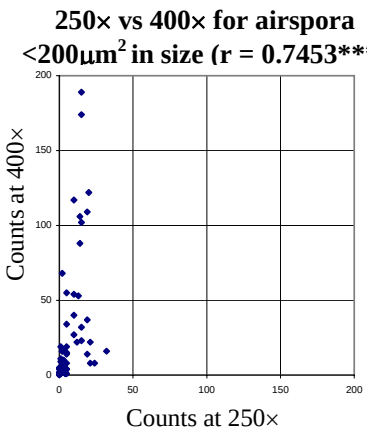
Figure 2.3: Scatter plots and comparisons of counts made at 250× and 400× magnifications for a) all airspora types, b) airspora <200µm^2 in size and c) airspora >200µm^2 in size using Spearman's Correlation Test (Correlation coefficient = r) and the Wilcoxon Rank Test. p-value: p<0.001***, p<0.01**, p<0.05* .

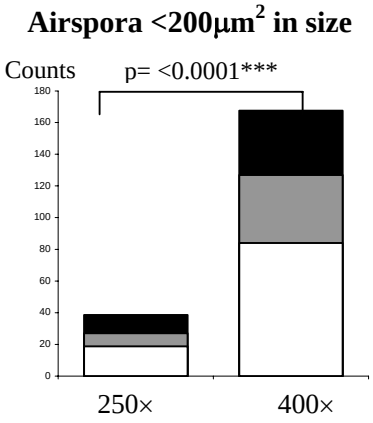
a)



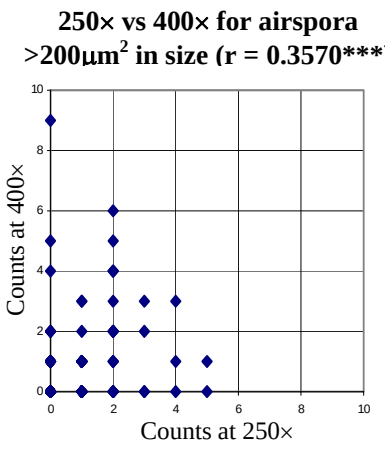


b)





c)



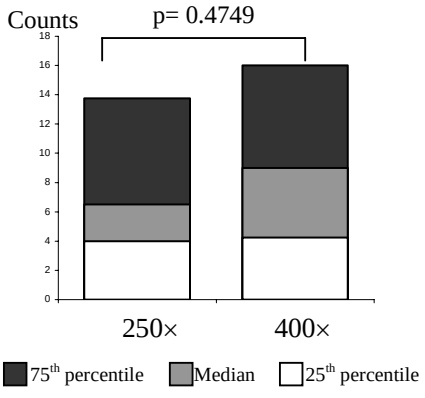


Figure 2.4: Examples of fungal spore counts <200 μm^2 in area screened at 250× and 400× magnifications.

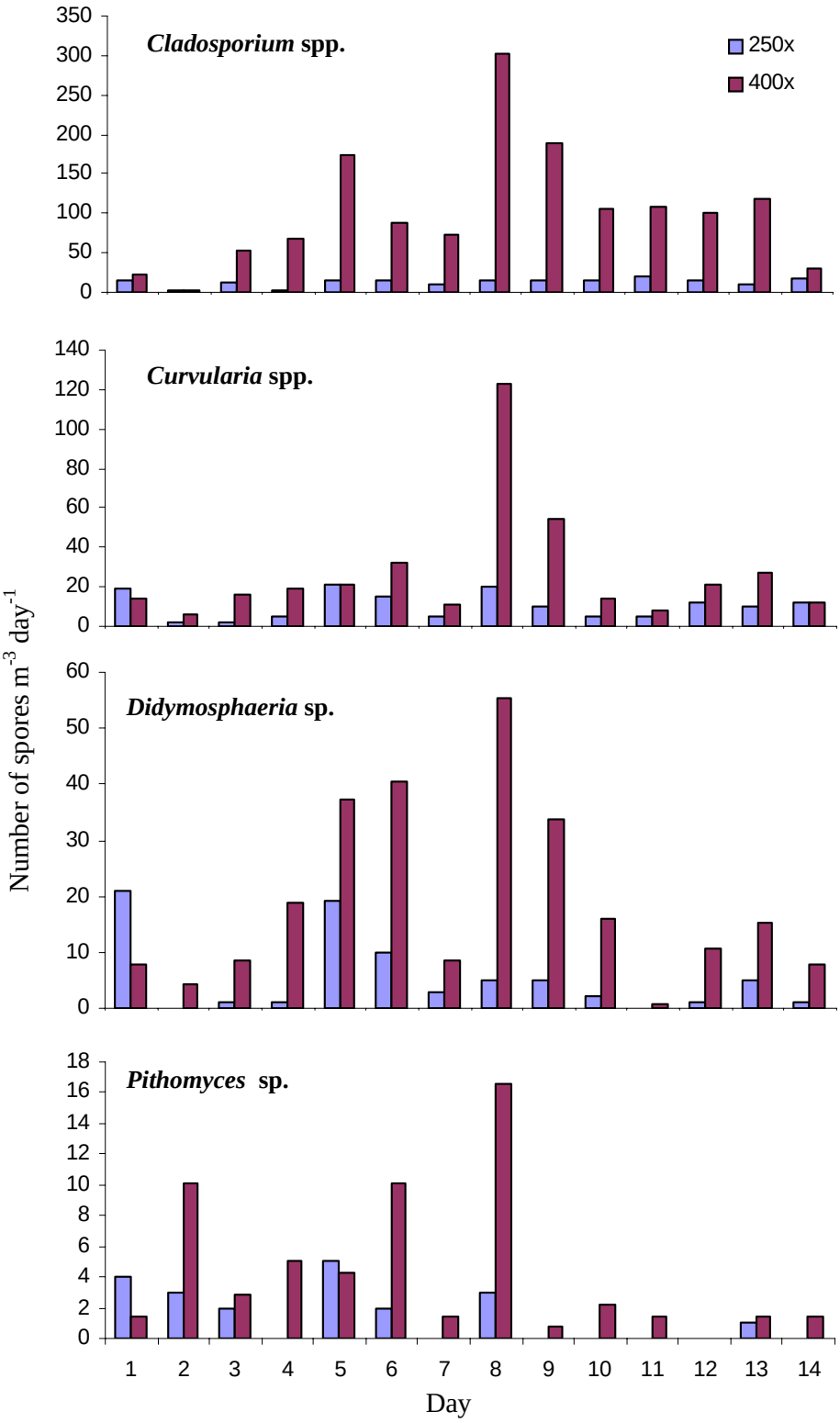


Figure 2.5: Comparisons of airspora counts at different horizontally positioned traverses (H1 to H5) along the length of the slide using the Wilcoxon Rank Test. a) all airspora types, b) airspora <200µm^2 and c) airspora >200µm^2. p-value: p<0.001***, p<0.01** and p<0.05*.

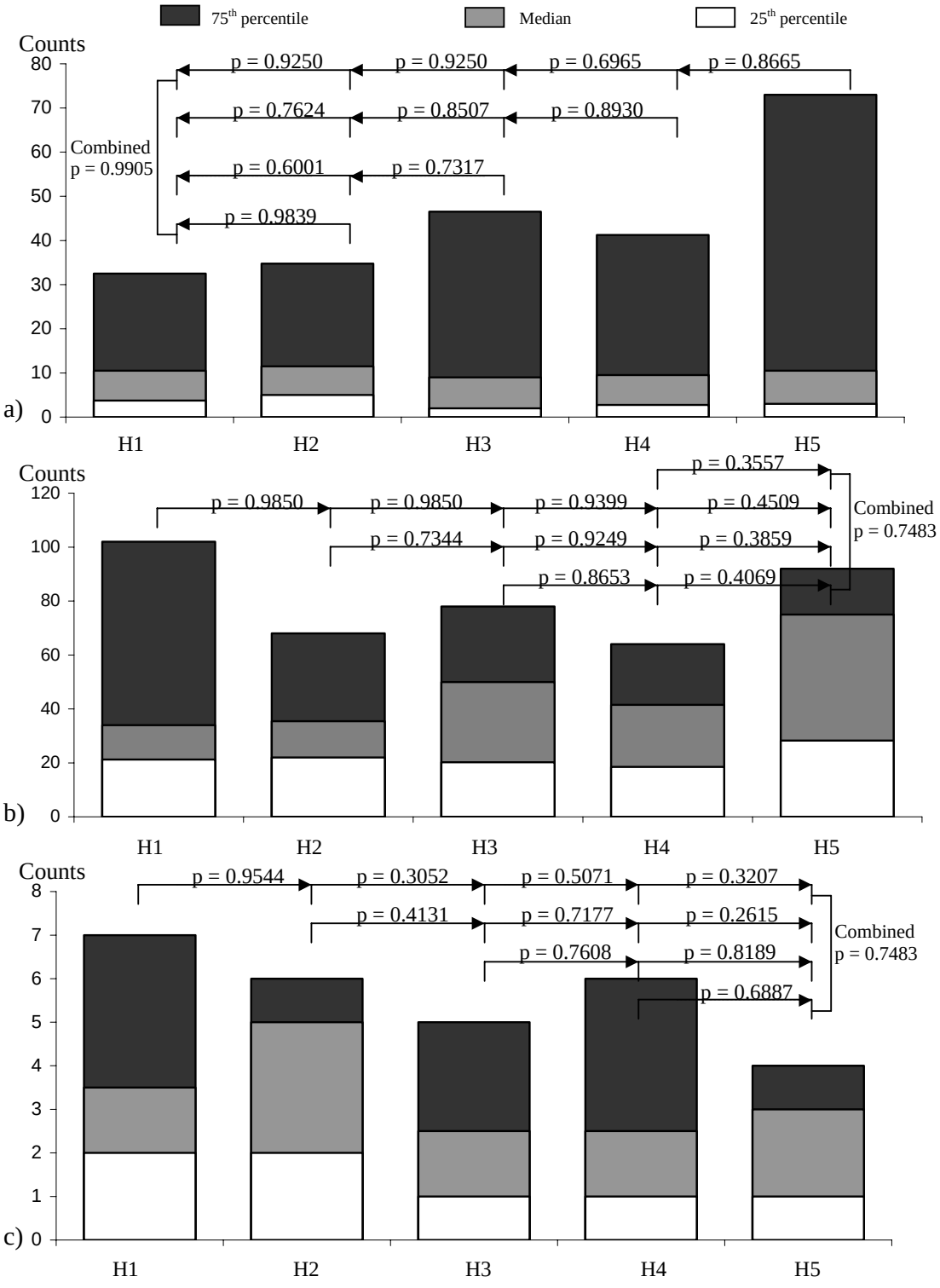


Figure 2.6: Scatter plots and count comparisons for different numbers of screening traverses for all airspora types using Spearman's Correlation Test (correlation coefficient = r) and the Wilcoxon Rank Test. p-value: p<0.001***, p<0.01** and p<0.05*.

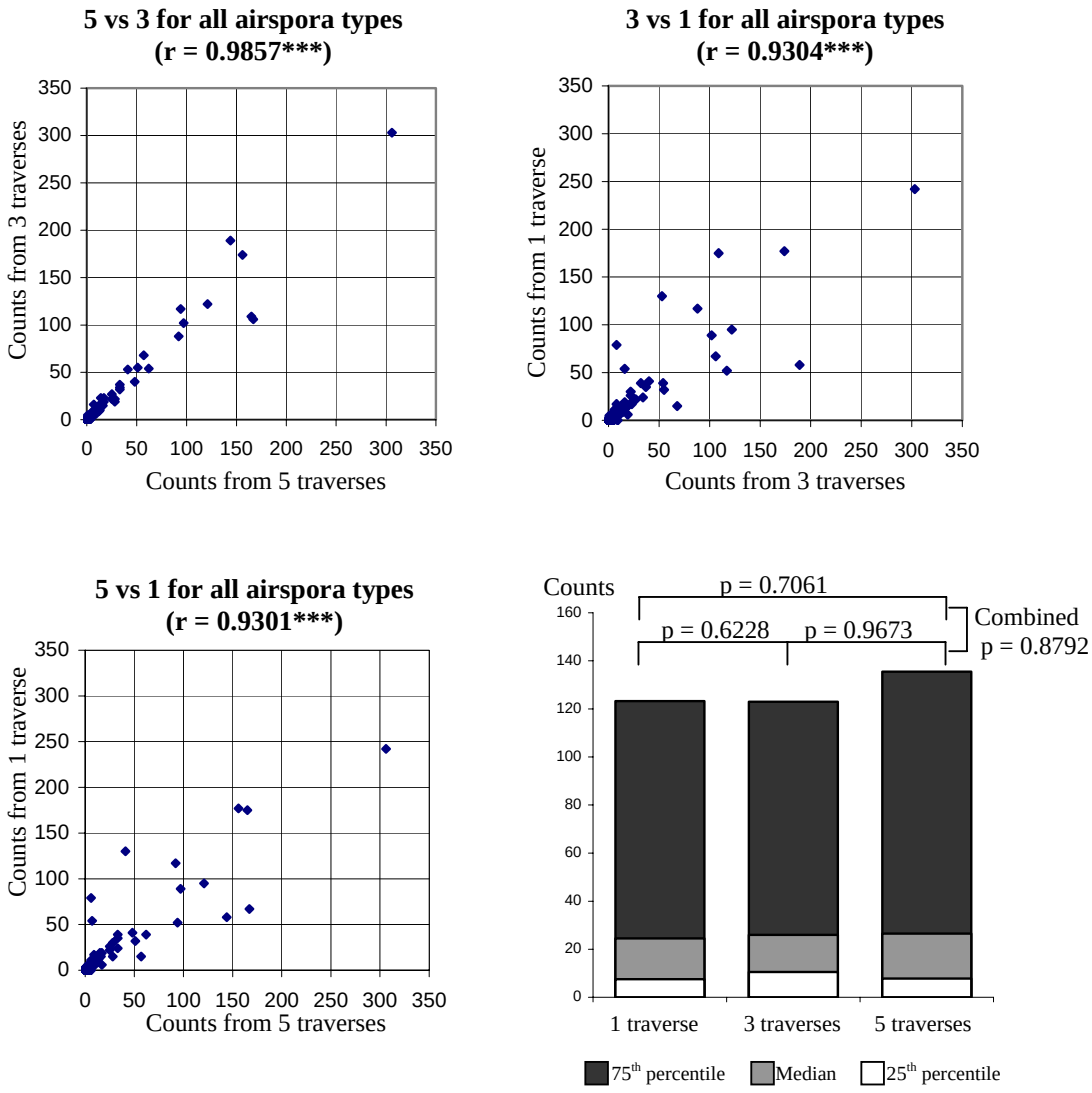


Figure 2.7: Scatter plots and count comparisons using different numbers of screening traverses for airspora <200 μm^2 in area size using Spearman's Correlation Test (correlation coefficient = r) and the Wilcoxon Rank Test. p-value: p<0.001***, p<0.01** and p<0.05*.

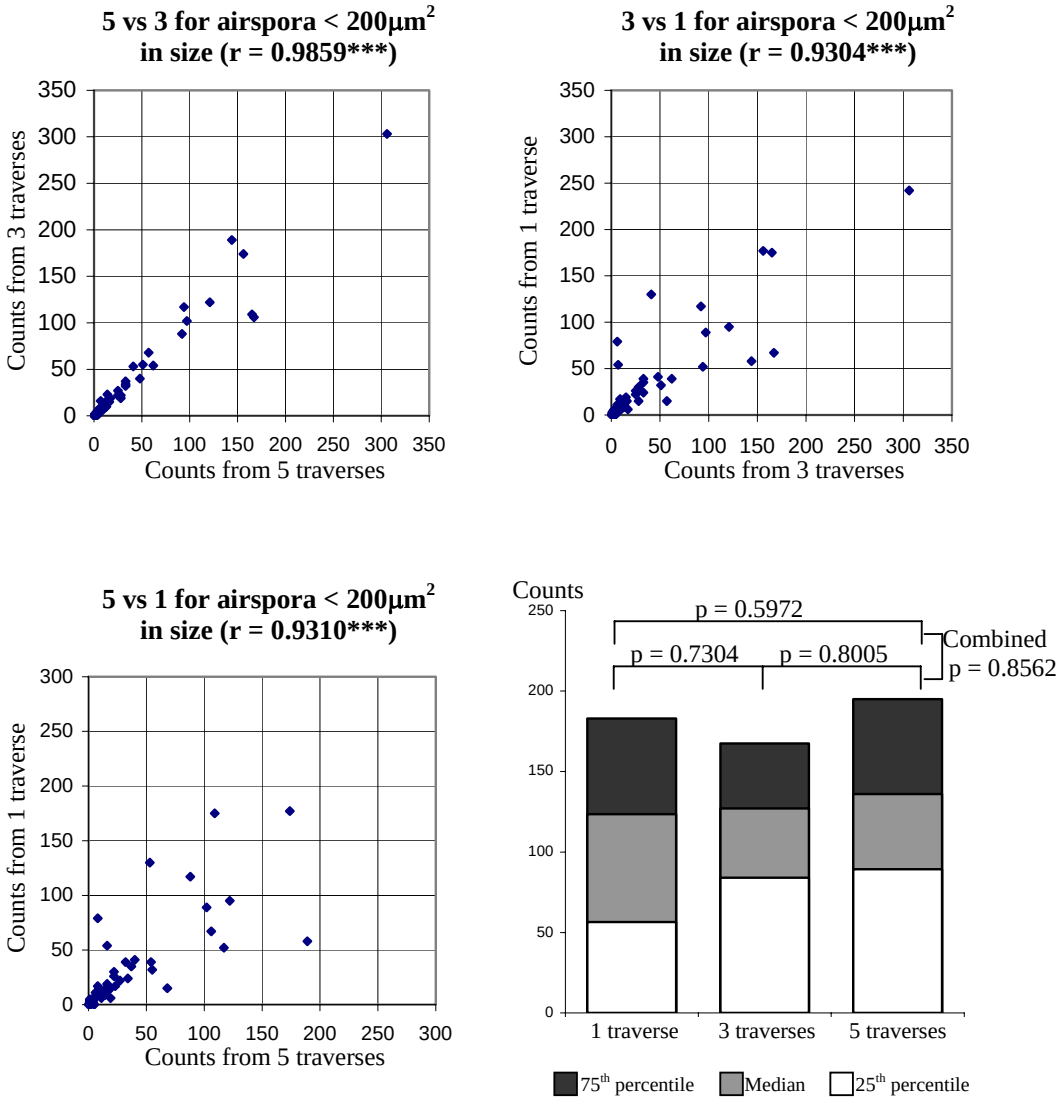


Figure 2.8: Scatter plots and count comparisons using different numbers of screening traverses for airspora >200µm^2 in area size using the Spearman's Correlation Test (correlation coefficient = r) and the Wilcoxon Rank Test. p-value: p<0.001***; p<0.01** and p<0.05*.

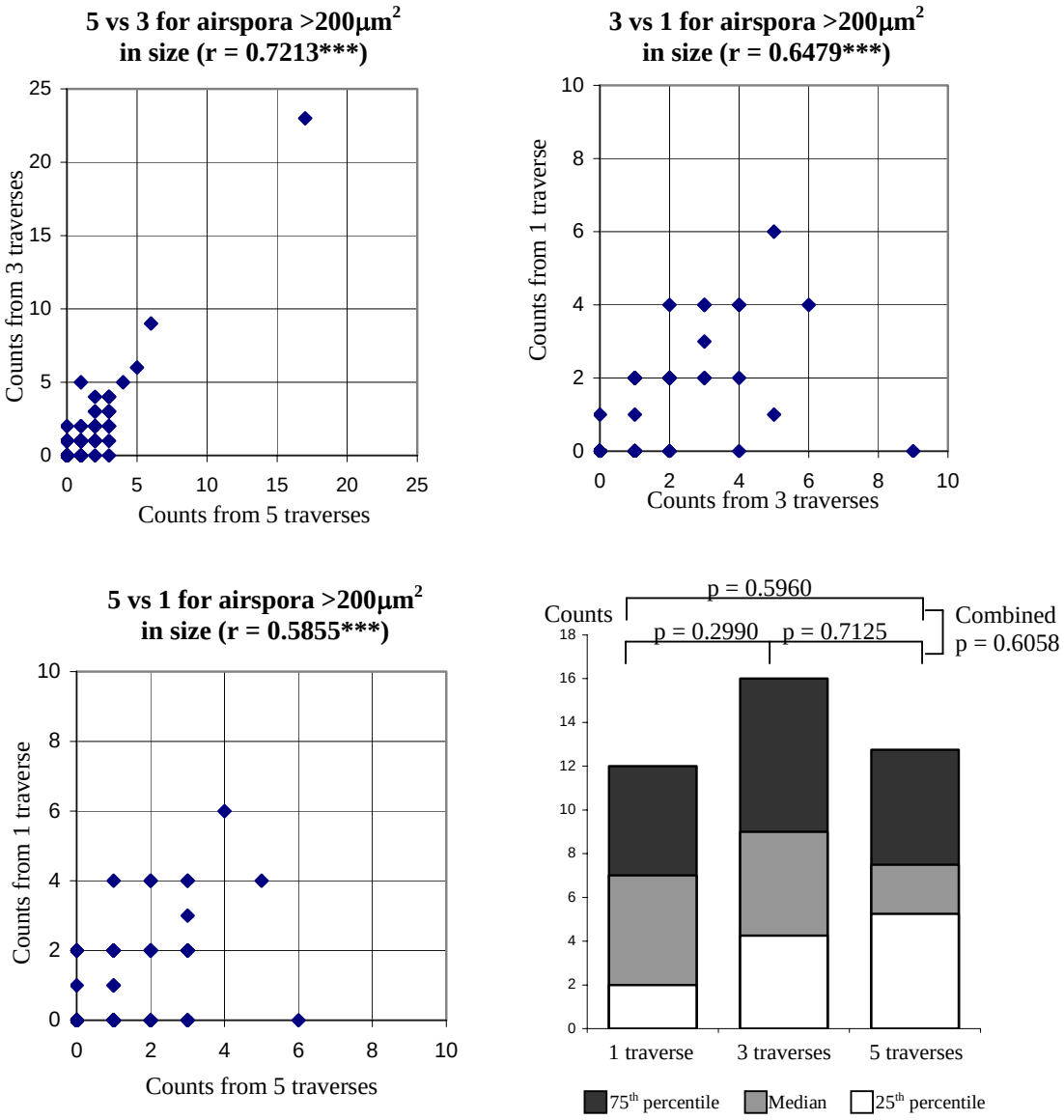
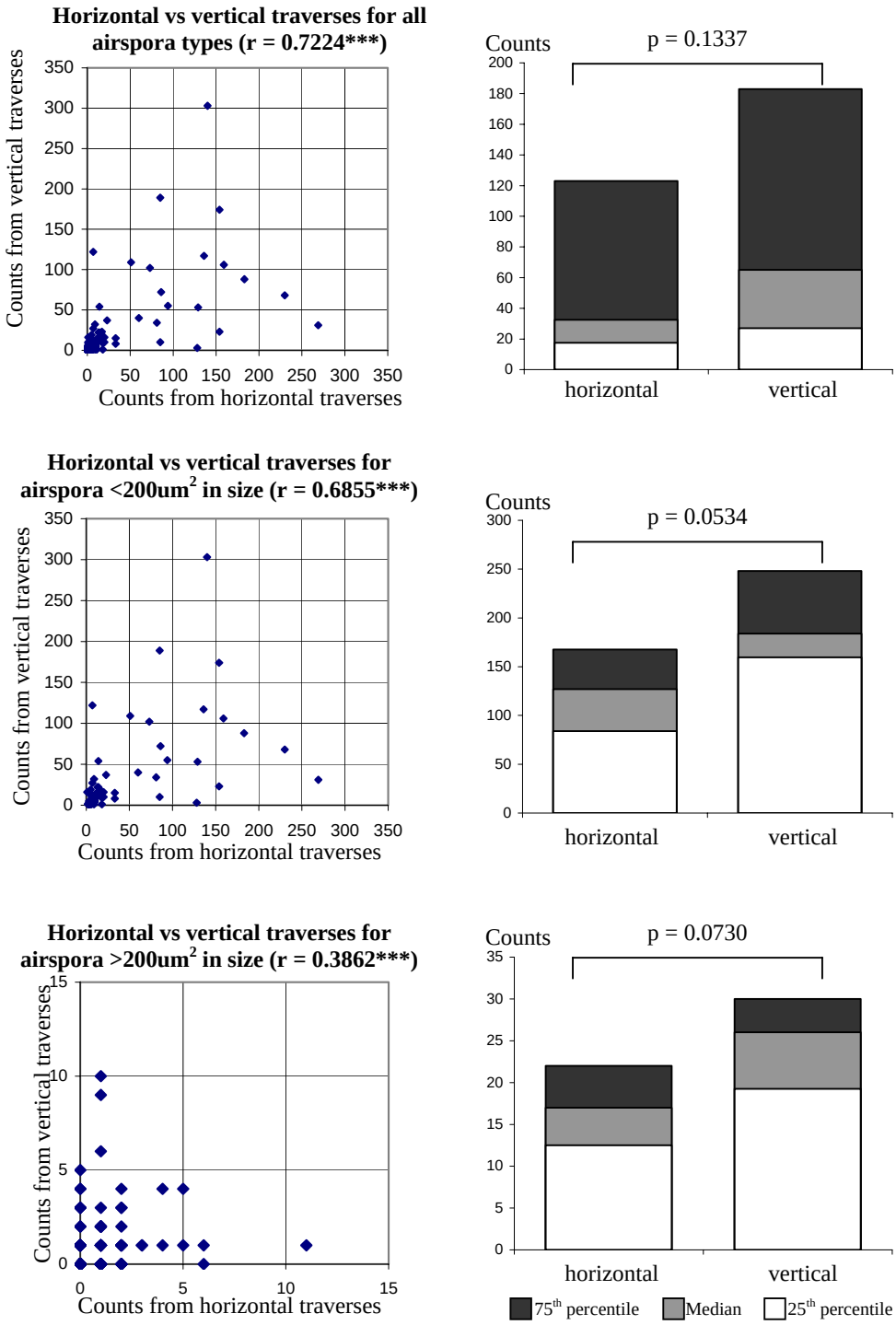


Figure 2.9: Scatter plots and count comparisons from horizontal and vertical traverses using the Spearman's Correlation Test (correlation coefficient = r) and the Wilcoxon Rank Test. p-value: p<0.001***, p<0.01** and p<0.05*.



The *Drechslera*-like spore counts peak in December. Similar trends were observed in 1992 and 1995 which are inversed to those seen in 1991, 1993 and 1994. *Pithomyces* sp. demonstrated a major peak in February followed by peaks with lower spore densities in June to July. Patterns were similar between 1991 and 1994 which were also inversed to those seen in 1992, 1993 and 1995.

2.3.2.2 *Unidentified fungal spore*

A fungal spore (Figure 2.11), approximately 10 μm in diameter and shaped like a melon seed with a single scar at the narrower end of the spore was observed to be present frequently in the June 1995 to May 1996 slides that were screened. The spore was named 'kuaci', which means "melon seed" in Cantonese. This spore was previously not identified by Lim *et al.* (1998) in their fungal airspora study.

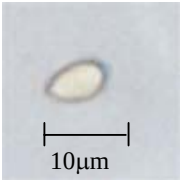
The identification of this spore as a major component in the airspora changes the distribution within the fungal components. 'Kuaci' was found to occur in all slides screened, ranging between 18 to 972 spores m^{-3} day^{-1}. The average numbers of airborne spores was at 213 m^{-3} day^{-1}. Using the average numbers of the different major fungal spore types, 'kuaci' was found to make up 21.4% of the total fungal composition, even higher than *Cladosporium* spp. (21.3%), previously identified as the most major fungal component. 'Kuaci' was later also found to be present in all samples collected from homes for an indoor allergen study by another postgraduate student (Saurabh JSK, personal communication, 2003). In indoor samples, it ranked second in percentage, making up 17.3% of total fungal composition after *Penicillium* and *Aspergillus* spp. combined (44.6%).

Attempts to try to identify the spores by inducing sporulation in culture from germinating spores obtained from old and fresh sampled slides, exposed agar plates or the cyclone sampler (collects airborne particles into an Eppendorf tube) failed. Different culture media (plain, malt, potato dextrose and oat meal agar with of without rice leaf), levels of humidity, temperature and lighting conditions were tested but failed to induce sporulation which is essential for fungal identification work.

An attempt to identify the spore by phylogenetic analysis was carried out by another graduate student using parsimony to compare with ITS (Internal Transcribed Spacer) and 18S rDNA sequences of other available fungal sequences from Genbank. It was found to fall in a clade with members from the Dothideomycetes and the Chaetothyriomycetes (Saurabh JSK, personal communication, 2003). Subsequently, the spores were found to yield positive skin prick test results in ten out of 58 patients (48 previously tested positive to pollen and or fungal allergens and 10 non-atopic) at the National Skin Center (Chew FT, personal communication, 2003). Results from non-atopic were all negative. Results were considered positive when the wheal size is 3 mm or more than the saline prick. The extracts were prepared from fungal mats.

Thus it is important monitor the concentration of 'kuaci' in the air. In the seasonality study 'kuaci' was found to peak in November followed by lower peaks in January, April and June (Figure 2.12, p.42).

Figure 2.11: Photomicrograph of unknown spore "kuaci"



2.3.2.3 *Pollen*

Seasonality was also observed in the pollen types studied (Figure 2.13, p.43). Correlations between counts from different years are shown in Table 2.1 (p.39 and 40). A peak from June to August was observed for *Acacia* spp. All counts were correlated except those in 1992 and 1995. For *Casuarina equisetifolia*, a lower peak was obtained from mid-February till April followed by a broad and high peak from August to October. Counts were positively correlated between 1991 with 1992 and 1994, 1992 with 1994 and 1995, 1993 with 1995 while an inverse correlation was obtained for 1993 with 1991 and 1992. Slightly higher counts were obtained from December to March for *Kyllingia polyphylla*. Counts in general were positively correlated except in 1992 with 1991 and 1995. The pollen season for *Elaeis guineensis* starts in October rising rapidly to peak in February. Counts obtained from all five years were correlated. High levels of *Podocarpus/Pinus* spp. pollen grains were observed in mid-January to mid-March. Counts from the different years were generally correlated except for those obtained in 1993.

2.3.2.4 *Fern spores*

Seasonality was also observed in fern spores (Figure 2.14, p.44). Correlations between counts of different years are shown in Table 2.1. The season for *Asplenium nidus* starts in October and ends in November. No correlation was obtained except for counts from 1991 with 1994 and counts from 1992 with 1995. Similar seasonal patterns were seen for both *Dicranopteris curranii* and *Pteridium aquilinum* where broad peaks from June to October were observed. Counts for *Dicranopteris curranii* for 1991 and 1995 were not correlated with those of 1992 to 1994 while all counts

were significant correlated for *Pteridium aquilinum* except for those between 1991 and 1995.

For *Dicranopteris linearis*, the season starts in May with spore counts becoming highest in August and ending in October. Counts between all years studied were correlated. Meanwhile, the *Nephrolepis auriculata* season starts in mid-March reaching its peak in May till June. Counts from 1995 were found to be either uncorrelated or inversely correlated to those from other years. For *Stenochlaena palustris* peaks were observed in May and November while counts between the different years were significantly correlated.

2.3.2.5 *Comparisons of counts between different stations*

The counts between the three stations at Kent Ridge, Clementi and Hougang were significantly correlated (Table 2.2, p. 45). However, comparison of airspora count densities were found to be significantly different for all airspora when analysed by the Wilcoxon Rank Test with p value less than 0.01 for all except for *Cladosporium* spp. ($p = 0.026$).

Only grass pollen and *Cladosporium* spp. counts were similar for all three stations. *Dicranopteris linearis* counts for Kent Ridge and Clementi were also found to be similar. Mostly fern spore (*Asplenium nidus, Pteridium aquilinum* and *Stenochlaena palustris*) and weed pollen (*Kyllingia polyphylla*) counts were similar for the Clementi and Hougang stations. *Dicranopteris curranii* counts for Kent Ridge were found to be similar to counts from Hougang. Highest correlations between the three stations were obtained for *Elaeis guineensis*.

2.3.2.6 *Association with meteorological parameters*

Correlation results for the different airspora types are shown in Table 2.3 (p. 46). *Cladosporium* spp. were found to be positively correlated to temperature and negatively to relative humidity. *Curvularia* spp. and *Pithomyces* sp. counts increased with wind speed and reduced with relative humidity. Both *Didymosphaeria* sp. and 'kuaci' counts increased with relative humidity but decreased with temperature. The *Drechslera*–like spore counts were positively correlated to relative humidity and wind speed but negatively with temperature.

In general, pollen counts increased with temperature except for those of *Podocarpus/Pinus* spp., and are negatively correlated to humidity, rainfall and wind speed. Exceptionally, *Elaeis guineensis* was positively correlated to wind speed. Correlation patterns with meteorological factors for fern spores were quite similar to those obtained for pollen. Fern spores increase with temperature but decrease with wind speed and relative humidity.

Table 2.1: Spearman's Correlation Coefficients for airspora counts between 1991 to 1995 for the Kent Ridge Station.

		Spearman's correlation coefficient				
Airspora type	Year	1991	1992	1993	1994	1995
Cladosporium spp.	1991	1	-0.4143**	0.413**	ns	0.8884***
	1992	-0.4143**	1	ns	-0.6060***	-0.4164**
	1993	0.4130**	ns	1	ns	0.5444***
	1994	ns	-0.606***	ns	1	0.4727***
	1995	0.8884***	-0.4164**	0.5444***	0.4727***	1
Curvularia spp.	1991	1	-0.6851***	0.5429***	0.9266***	ns
	1992	-0.6851***	1	-0.3433*	-0.6249***	-0.4551***
	1993	0.5429***	-0.3433*	1	0.6615***	-0.3371*
	1994	0.9266***	-0.6249***	0.6615***	1	ns
	1995	ns	-0.4551***	-0.3371*	ns	1
Didymosphaeria sp.	1991	1	0.7154***	0.3774**	0.6058***	0.8146***
	1992	0.7154***	1	0.8507***	0.5747***	0.5266***
	1993	0.3774**	0.8507***	1	0.4889***	0.2978*
	1994	0.6058***	0.5747***	0.4889***	1	0.8041***
	1995	0.8146***	0.5266***	0.2978*	0.8041***	1
Drechslera-like spores	1991	1	0.6748***	ns	0.9579***	ns
	1992	0.6748***	1	-0.5936***	0.6649***	-0.5816***
	1993	ns	-0.5936***	1	ns	0.733***
	1994	0.9579***	0.6649***	ns	1	ns
	1995	ns	-0.5816***	0.733***	ns	1
Pithomyces sp.	1991	1	-0.5957***	-0.8044***	0.413**	-0.4257**
	1992	-0.5957***	1	0.6973***	ns	ns
	1993	-0.8044***	0.6973***	1	-0.4402**	0.4815***
	1994	0.413**	ns	-0.4402**	1	-0.7589***
	1995	-0.4257**	ns	0.4815***	-0.7589***	1
Acacia spp.	1991	1	0.5911***	0.7588***	0.8423***	0.3735**
	1992	0.5911***	1	0.7505***	0.4918***	ns
	1993	0.7588***	0.7505***	1	0.5608***	0.5573***
	1994	0.8423***	0.4918***	0.5608***	1	ns
	1995	0.3735**	ns	0.5573***	ns	1
Casuarina equisetifolia	1991	1	0.5797***	-0.4067**	0.5085***	ns
	1992	0.5797***	1	-0.4346**	0.5231***	0.281*
	1993	-0.4067**	-0.4346**	1	0.261ns	0.6095***
	1994	0.5085***	0.5231***	ns	1	0.838***
	1995	ns	0.281*	0.6095***	0.838***	1
Kyllingia polyphylla	1991	1	-0.2842*	0.8852***	0.3586**	0.2913*
	1992	-0.2842*	1	ns	0.2894*	-0.5863***
	1993	0.8852***	ns	1	0.4724***	ns
	1994	0.3586**	0.2894*	0.4724***	1	ns
	1995	0.2913*	-0.5863***	ns	ns	1
Elaeis guineensis	1991	1	0.9117***	0.9125***	0.9577***	0.9711***
	1992	0.9117***	1	0.9549***	0.8497***	0.9168***
	1993	0.9125***	0.9549***	1	0.8942***	0.9434***
	1994	0.9577***	0.8497***	0.8942***	1	0.9597***
	1995	0.9711***	0.9168***	0.9434***	0.9597***	1

Spearman's correlation p-values: $p<0.05$*, $p<0.01$**, $p<0.001$***.

Table 2.1 (continued): Spearman's Correlation Coefficients for airspora counts between 1991 to 1995 for the Kent Ridge Station.

		Spearman's Correlation Coefficient				
Airspora type		1991	1992	1993	1994	1995
Podocarpus/ Pinus spp.	1991	1	0.3897**	ns	0.8382***	0.5919***
	1992	0.3897**	1	ns	0.2772*	0.3459*
	1993	ns	ns	1	ns	0.5026***
	1994	0.8382***	0.2772*	ns	1	0.5538***
	1995	0.5919***	0.3459*	0.5026***	0.5538***	1
Asplenium nidus	1991	1	ns	ns	0.7997***	ns
	1992	ns	1	ns	ns	0.7077***
	1993	ns	ns	1	ns	ns
	1994	0.7997***	ns	ns	1	ns
	1995	ns	0.7077***	ns	ns	1
Dicranopteris curranii	1991	1	ns	ns	ns	0.8025***
	1992	ns	1	0.4332**	0.5223***	ns
	1993	ns	0.4332**	1	0.8647***	ns
	1994	ns	0.5223***	0.8647***	1	-0.4158**
	1995	0.8025***	ns	ns	-0.4158**	1
Dicranopteris linearis	1991	1	0.6151***	0.8418***	0.8169***	0.7533***
	1992	0.6151***	1	0.8016***	0.8812***	0.7752***
	1993	0.8418***	0.8016***	1	0.96***	0.8798***
	1994	0.8169***	0.8812***	0.96***	1	0.8808***
	1995	0.7533***	0.7752***	0.8798***	0.8808***	1
Nephrolepis auriculata	1991	1	0.6694***	0.9285***	0.7335***	-0.2837*
	1992	0.6694***	1	0.6613***	ns	-0.4294**
	1993	0.9285***	0.6613***	1	0.7404***	ns
	1994	0.7335***	ns	0.7404***	1	ns
	1995	-0.2837*	-0.4294**	ns	ns	1
Pteridium aquilinum	1991	1	0.5722***	0.7117***	0.8562***	ns
	1992	0.5722***	1	0.3465*	0.2997*	-0.3208*
	1993	0.7117***	0.3465*	1	0.6264***	0.4686***
	1994	0.8562***	0.2997*	0.6264***	1	0.3334*
	1995	ns	-0.3208*	0.4686***	0.3334*	1
Stenochlaena palustris	1991	1	0.6326***	0.4306**	0.6819***	0.3909*
	1992	0.6326***	1	0.6198***	0.6516***	0.6623***
	1993	0.4306**	0.6198***	1	0.4551***	0.5383***
	1994	0.6819***	0.6516***	0.4551***	1	0.5859***
	1995	0.3909**	0.6623***	0.5383***	0.5859***	1

Spearman's correlation p-values: $p<0.05$*, $p<0.01$**, $p<0.001$***.

Figure 2.10: Seasonal patterns of major fungal spores from the Kent Ridge Station. Fungal spore counts are in number of fungal spores m^3 day^{-1}.

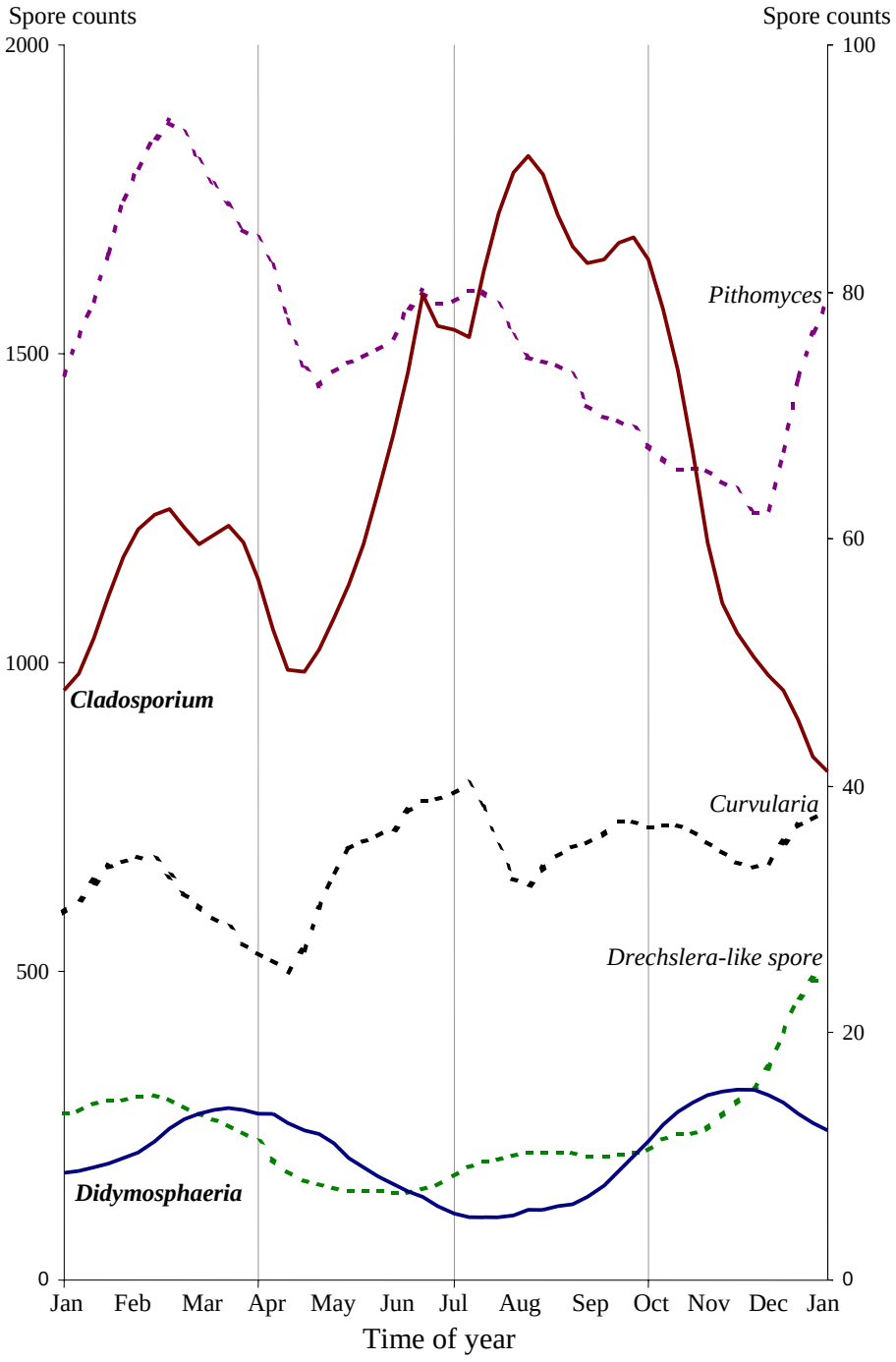


Figure 2.12: Seasonal patterns of the ascospore 'kuaci' from the Kent Ridge Station.

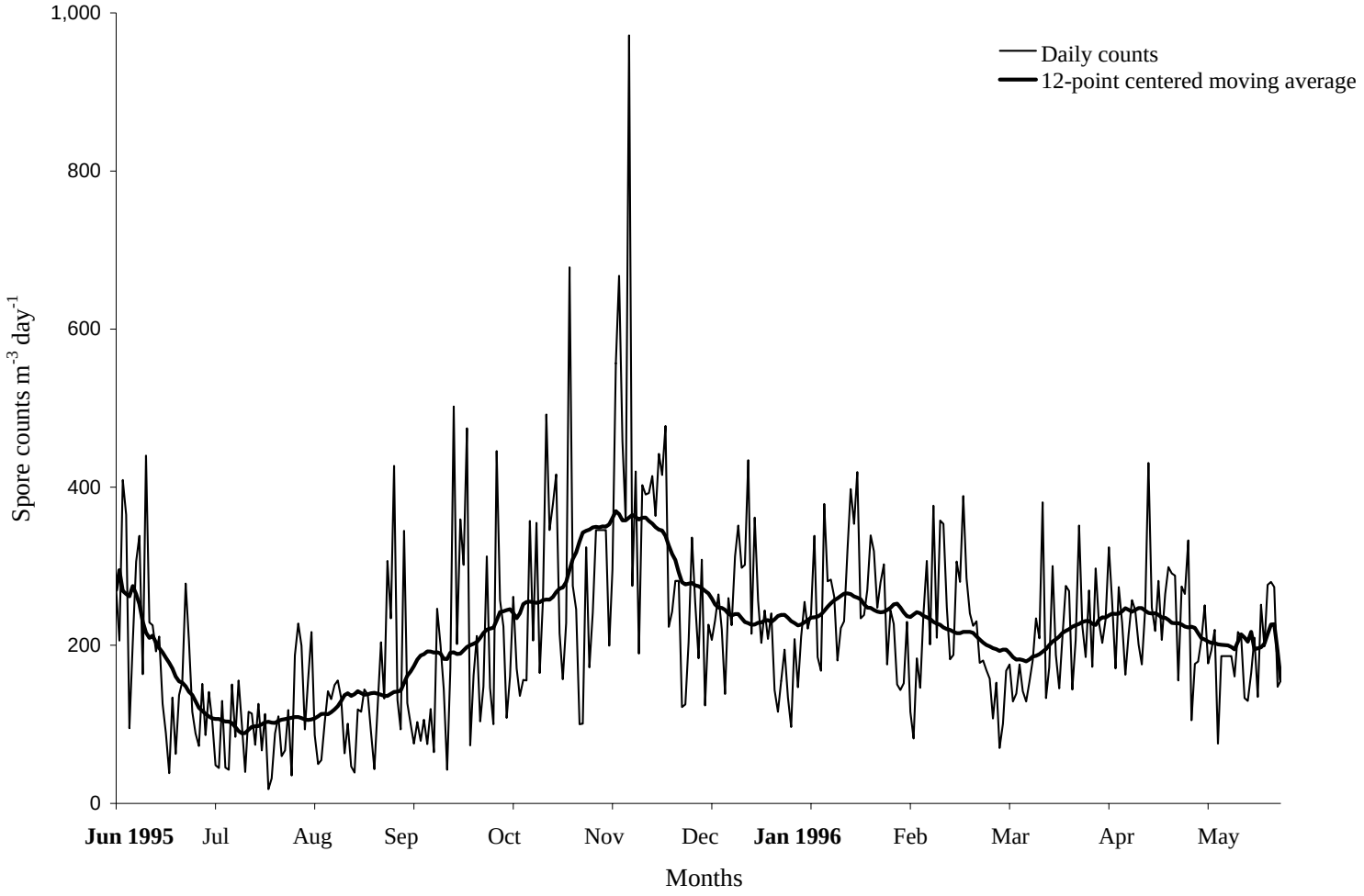


Figure 2.13: Seasonal patterns of pollen types and airspora from the Kent Ridge Station. Pollen counts are in number of pollen grains m^3 day^{-1}.

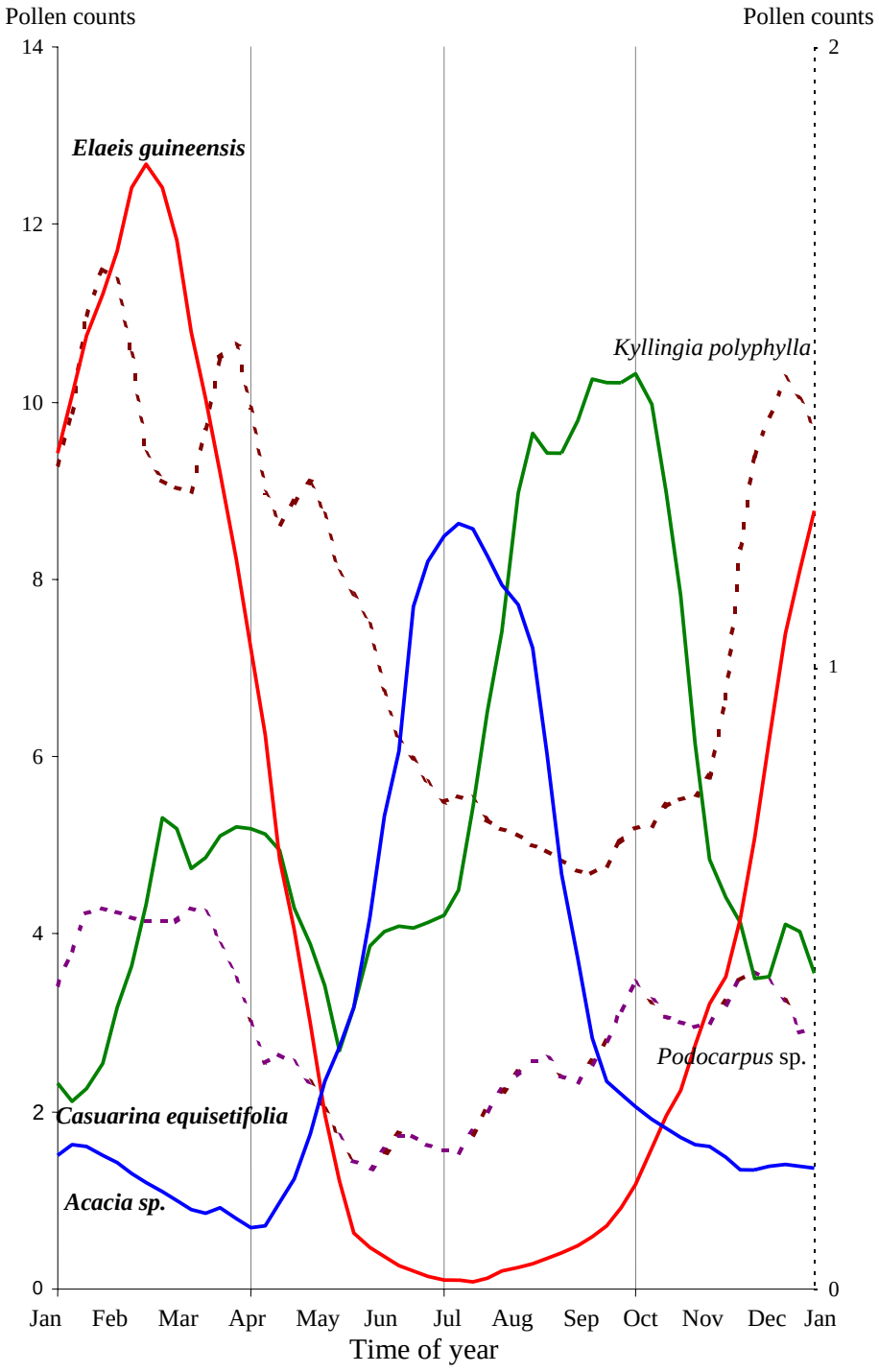


Figure 2.14: Seasonal patterns of fern spores from 1991 to 1995 at the Kent Ridge Station. Fern spore counts are in number of fern spores m^{-3} day^{-1}.

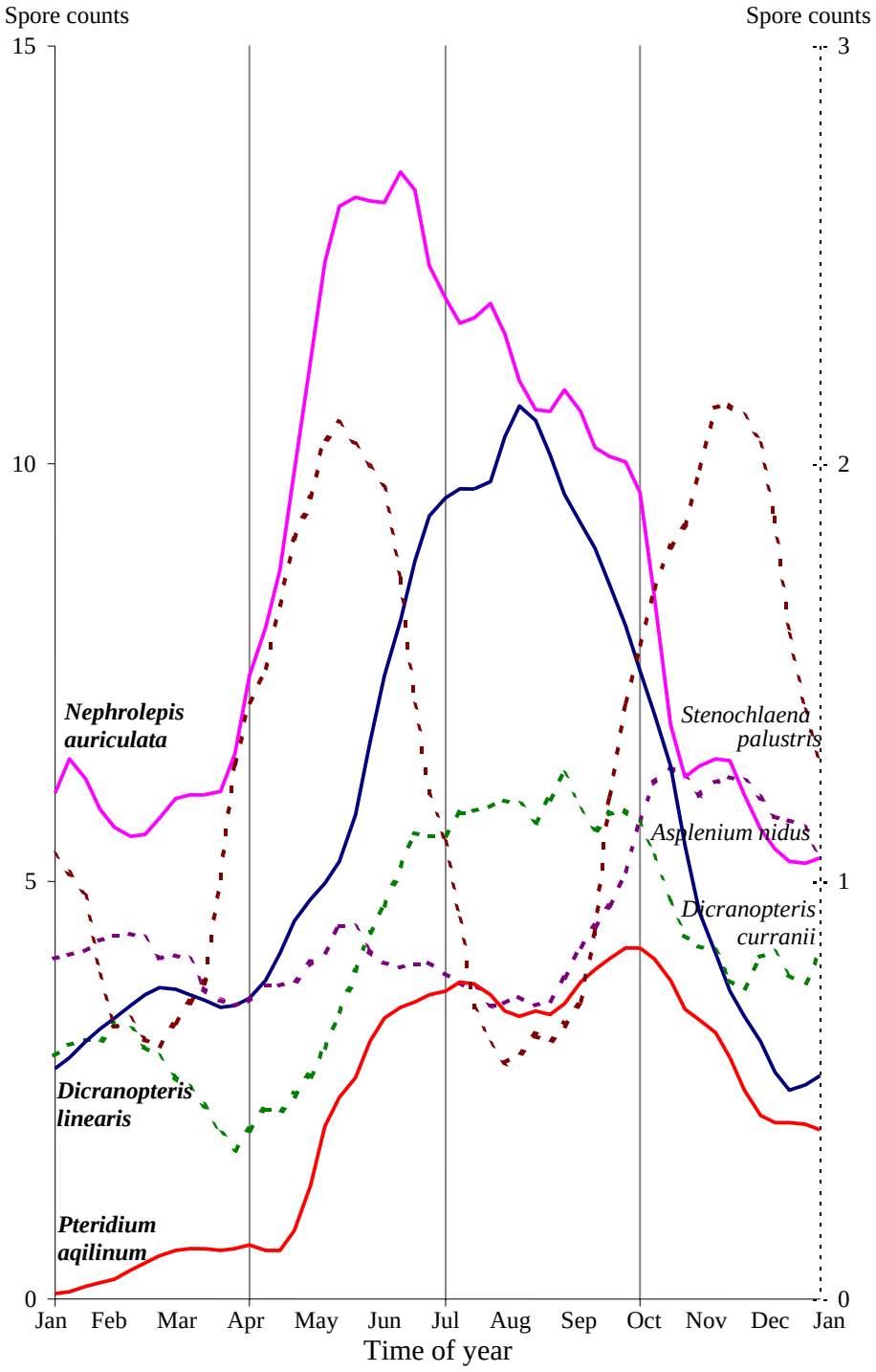


Table 2.2: Spearman's correlations coefficients of airspora counts between the sampling stations at Clementi, Hougang and Kent Ridge.

Airspora type	Kent Ridge vs Clementi	Kent Ridge vs Hougang	Clementi vs Hougang	Chi square	Wilcoxon rank result
Cladosporium spp.	0.2657***	0.2255***	0.2374***	7.26*	
Curvularia spp.	0.4673***	0.3666***	0.2063***	627.4***	
Didymosphaeria sp.	ns	0.5416***	0.1178**	75.1***	KR=HO
Drechslera-like spores	0.2162***	ns	ns	97.0***	
Pithomyces sp.	0.3035***	ns	0.1311***	165.3***	KR=HO
Asplenium nidus	0.1631***	0.1031**	0.0860*	190.6	KR=HO
Dicranopteris curranii	0.3024***	0.1197***	0.2100***	21.8***	KR=HO
Dicranopteris linearis	0.4447***	0.4065***	0.4440***	18.3***	
Nephrolepis auriculata	0.4596***	0.3519***	0.4292***	18.7***	
Pteridium aquilinum	0.5204***	0.4291***	0.4900***	8.17*	KR=HO
Stenochlaena palustris	0.3270***	0.3301***	0.3361***	13.6**	KR=HO,CL=HO
Acacia spp.	0.2273***	0.2610***	0.1080**	18.6***	
Casuarina equisetifolia	0.3332***	0.1332***	0.1705***	11.0***	KR=CL
Elaeis guineensis	0.6873***	0.7080***	0.6972***	55.5***	
Kyllingia polyphylla	ns	ns	0.0941*	64.4***	
Podocarpus/ Pinus sp.	0.0842*	ns	ns	37.7***	KR=HO
Poaceae	ns	0.0816*	ns	17.3***	KR=HO

Abbreviations used for the stations: CL = Clementi, HO = Hougang, KR = Kent Ridge. Correlations that were not significant are indicated as "ns". Correlation p-values: $p<0.05$*, $p<0.01$**, and $p<0.001$***.

Table 2.3: Spearman's correlation coefficients between airspora counts and meteorological factors from 1991 to 1996 at the Kent Ridge Station.

	Spearman's Correlation Coefficient			
Pollen or spore type	**Temperature (°C)**	**Relative humidity (%)**	**Rainfall (mm)**	**Total wind speed (ms^{-1})**
Fungus				
Cladosporium spp.	0.0651**	-0.0954***	ns	ns
Curvularia spp.	ns	-0.1778***	-0.1761***	0.046*
Didymosphaeria sp.	-0.2469***	0.1832***	0.2524***	ns
Pithomyces sp.	0.1427***	ns	-0.1077***	0.1976***
Drechslera-like spore	0.1481***	-0.2469***	ns	-0.0796***
Ascospore 'kuaci'	-0.2680***	0.1638**	ns	ns
Fern spore				
Asplenium nidus	ns	ns	ns	ns
Dicranopteris curranii	0.1138***	-0.1206***	-0.0974***	-0.0580**
Dicranopteris linearis	0.1067***	-0.0988***	-0.2008***	-0.1202***
Nephrolepis auriculata	0.2307***	-0.2372***	-0.0550*	0.1364***
Pteridium aquilinum	0.2361***	-0.2619***	-0.0446*	-0.3393***
Stenochaelena palustris	ns	ns	ns	-0.0511*
Pollen				
Podocarpus/ Pinus spp.	-0.0755***	ns	-0.0541*	0.1260***
Acacia spp.	0.1883***	-0.1140***	-0.2055***	-0.0582*
Elaeis guineensis	0.1717***	-0.1329***	-0.1895***	0.6064***
Casuarina equisetifolia	0.0646**	-0.1605***	ns	-0.1197***
Poaceae	0.2309***	-0.2132***	ns	-0.2215***
Kyllingia polyphylla	ns	-0.0893***	ns	-0.0453*

Correlations that were not significant are indicated as "ns". Correlation p-values: $p<0.05$*, $p<0.01$**, and $p<0.001$***.

2.3.3 Diurnal patterns

2.3.3.1 *Fungal spores*

On average spores of *Cladosporium* spp. decreased from 12 midnight (Figure 2.15). Counts at the peak hour ranged from an average of 396 spores m^{-3} h^{-1} to a maximum of 9830 spores m^{-3} h^{-1}. However, the pattern in 1995 was different from those obtained in 1996 and 1997. In 1995, *Cladosporium* spp. counts peak at 2 am as opposed to noon. *Didymosphaeria* sp. spores were found to peak at 7 pm followed by high but gradually decreasing levels till 6 am in the morning. The mean density at the peak hour is 246 spores m^{-3} h^{-1} while the maximum reaches as high as 1296 spores m^{-3} h^{-1}. The ascospore 'kuaci' peaks at 2 am to 6 am. Peak densities range from an average of 403 to a maximum of 2496 spores m^{-3} h^{-1}.

Counts of *Curvularia* spp. peak at 10 am (Figure 2.16). High levels persist till 4 pm. Peak density averaged 32 spores m^{-3} h^{-1} with a maximum of 852 spores m^{-3} h^{-1}. *Pithomyces* sp. counts peak at 12 pm with an average of 31 and a maximum of 622 spores m^{-3} h^{-1}. The *Drechslera*-like spore counts peak at 12 pm with a second peak at 6 pm. Peak density averaged 2 spores m^{-3} h^{-1} with a maximum of 252 spores m^{-3} h^{-1}.

Figure 2.15: Diurnal calendars for *Cladosporium* spp., *Didymosphaeria* sp. and the ascospore 'kuaci' for 1995, 1996, 1997 and average of all 3 years.

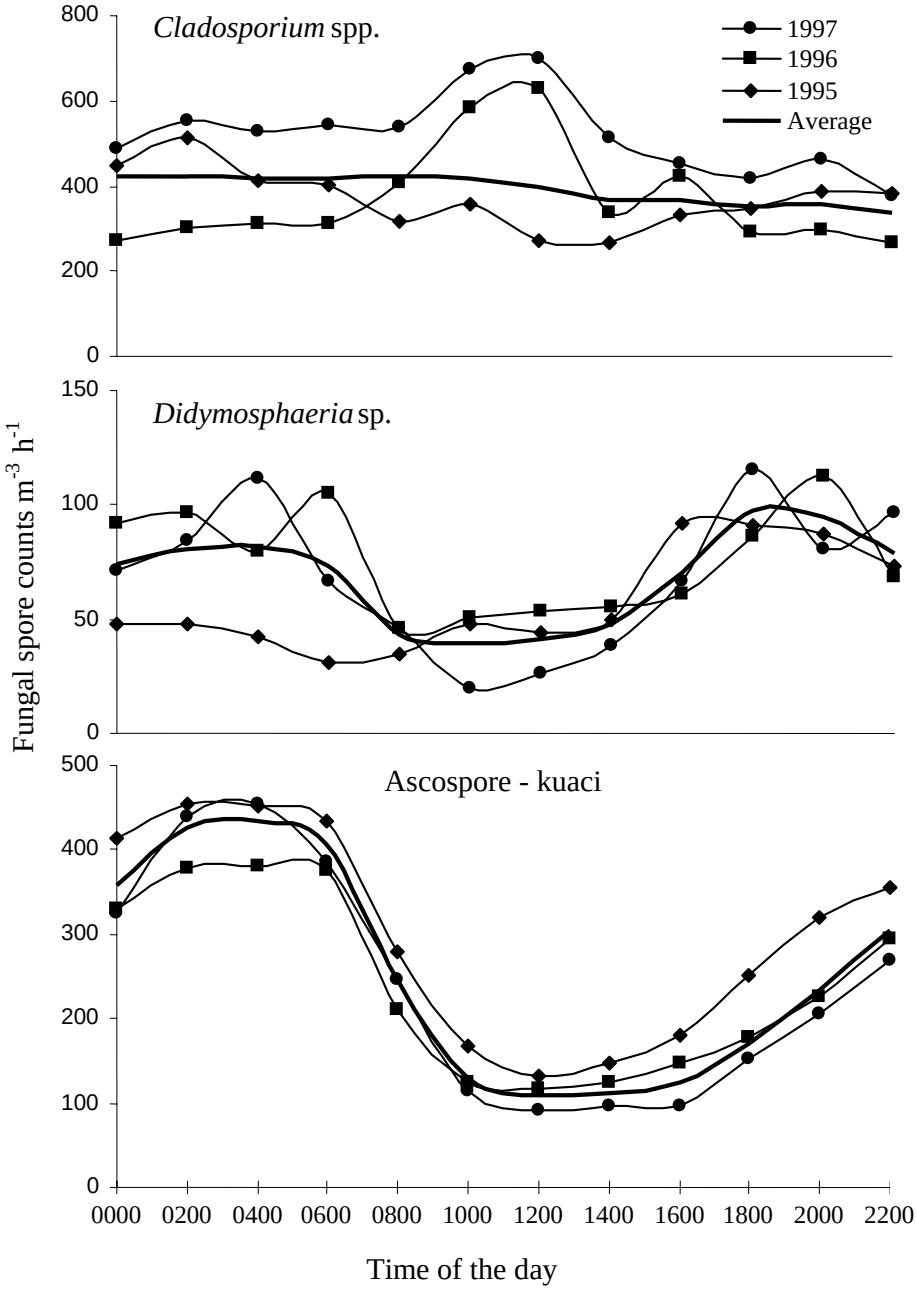
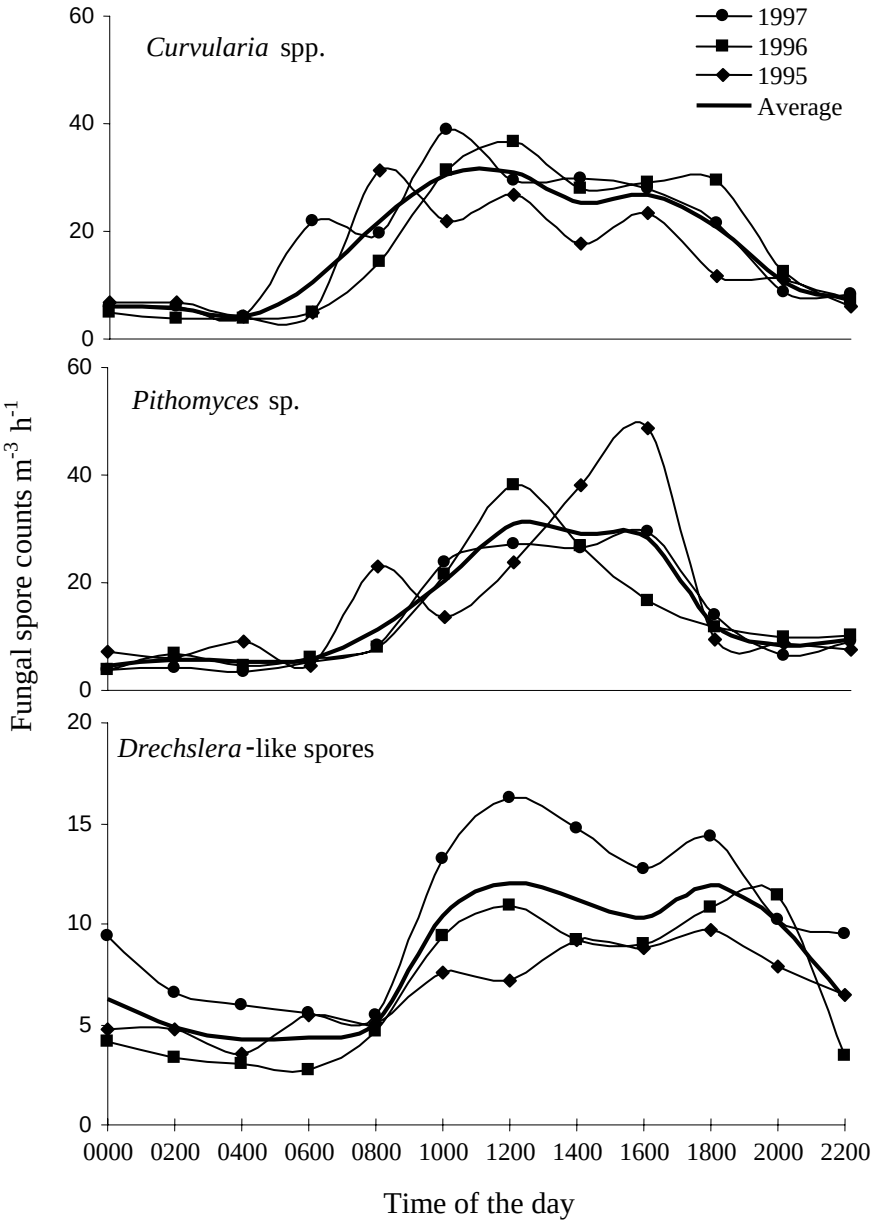


Figure 2.16: Diurnal calendars for *Curvularia* spp., *Pithomyces* sp. and *Drechslera*-like spores for 1995, 1996, 1997 and average of all 3 years.



2.3.3.2 *Pollen*

A broad peak was observed for *Casuarina equisetifolia* pollen counts from 8 am to 2 pm (Figure 2.17, p. 52). The peak average density is 4 pollen grains m^{-3} h^{-1} with the maximum reaching 133 pollen grains m^{-3} h^{-1}. *Kyllingia polyphylla* pollen density is highest at 10 am with an average of 4 pollen grains m^{-3} h^{-1} and maximum density of 44 pollen grains m^{-3} h^{-1}. Distinct diurnal patterns were observed only in 1995 and 1996 for the Poaceae pollen counts. In 1995, grass counts peaked at 6 am while in 1996 a later peak at 10 am was observed. The average Poaceae pollen counts at the peak hour are 3 pollen grains m^{-3} h^{-1} with the maximum density reaching as high as 89 pollen grains m^{-3} h^{-1} in 1996.

Variable peak hours were observed for *Acacia* spp. pollen counts which normally fall between 6 am to 4 pm (Figure 2.18, p. 53). Two peaks were observed for all years studied with the first recording higher counts than the second except in 1995. Average counts at the peak hour were 6 pollen grains m^{-3} with maximum reaching 52 pollen grains m^{-3} h^{-1}. The pollen type with the latest peak hour is *Elaeis guineensis*. Gradually increasing densities were observed from 10 am to peak at 2 pm with average densities of 6 pollen grains m^{-3} h^{-1} with maximum counts reaching 44 grains m^{-3} h^{-1}. The diurnal pattern for *Podocarpus/Pinus* spp. pollen counts was not obtained due to the low counts during the period studied.

Figure 2.17: Diurnal calendars for *Casuarina equisetifolia*, *Kyllingia polyphylla* and Poaceae for 1995, 1996, 1997 and average of all 3 years at the Kent Ridge Station.

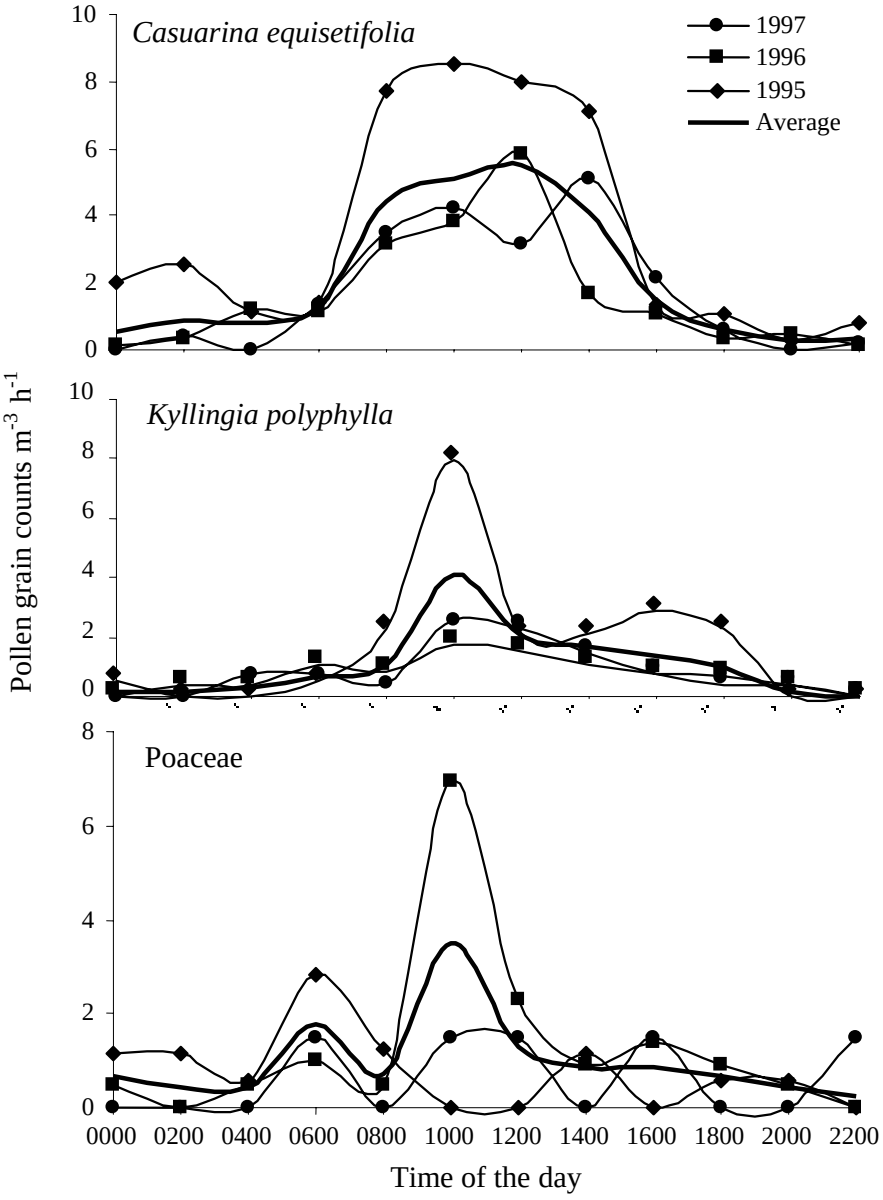


Figure 2.18: Diurnal calendars for *Acacia* spp. and *Elaeis guineensis* for 1995, 1996, 1997 and average of all 3 years at the Kent Ridge Station.

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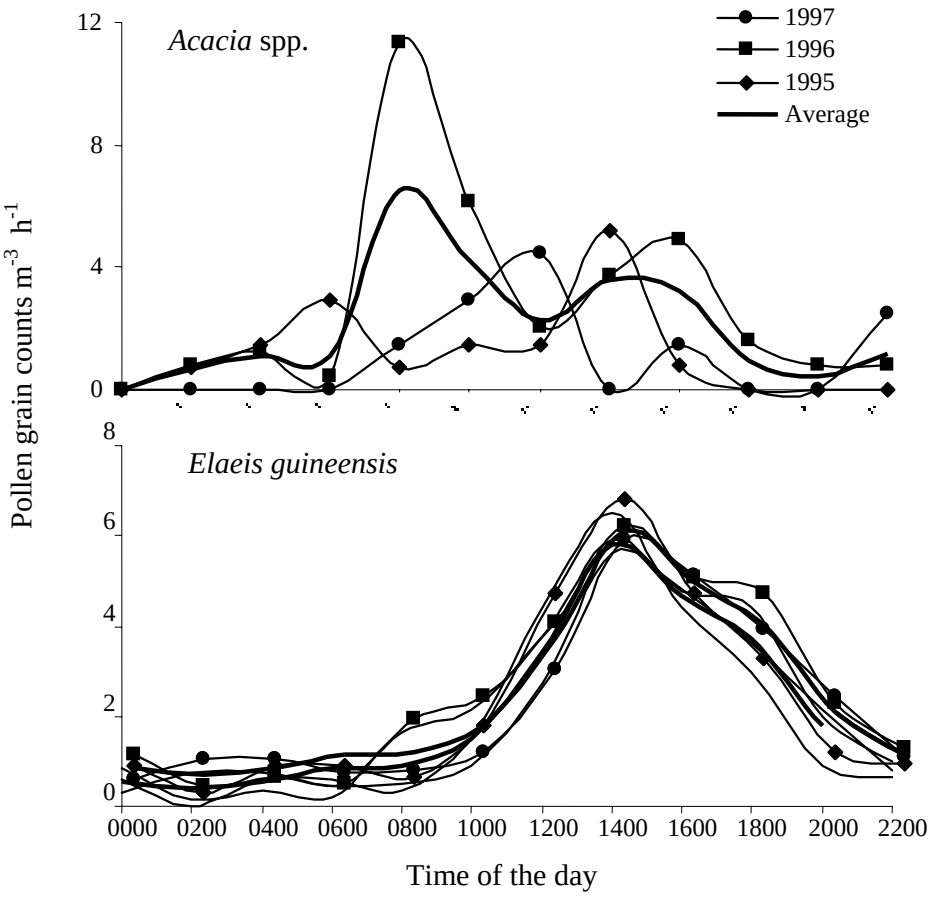

2.3.3.3 *Fern spores*

Diurnal fern spores counts are seen in Figure 2.19. *Dicranopteris curranii* spore counts peak sharply at 2 pm while *Dicranopteris linearis* demonstrated a broader peak from 12 to 4 pm. Average counts for the former is 3 spores m^{-3} h^{-1} with the maximum totaling 15 spores m^{-3} h^{-1} and the latter averaging at 2 spores m^{-3} h^{-1} with a maximum of 37 spores m^{-3} h^{-1}. An exception was observed in 1995 for *Dicranopteris linearis* where three peaks were observed — at 2 am, 12 pm and 4 pm. *Nephrolepis auriculata* spore counts peaked at 10 am except in 1995 (4 pm). Average peak hour densities were 4 spores m^{-3} h^{-1} with maximum reaching 133 spores m^{-3} h^{-1}.

Asplenium nidus peaks earlier at around 8 am except in 1996 (4 pm) (Figure 2.20). Average peak counts were 3 spores m^{-3} h^{-1} with the maximum reaching 30 spores m^{-3} h^{-1}. Broad peaks were observed for *Pteridium aquilinum* from 2 to 6 pm. On average, 4 spores m^{-3} h^{-1} was recorded at peak hours with the maximum reaching 74 spores m^{-3} h^{-1}. A variable diurnal pattern was observed for *Stenochlaena palustris.* Double peaks were observed at 10 am and 4 pm in 1995 followed by continuous high counts from 6 am to 4 pm in 1996 and a single peak at 1 pm in 1997. *Stenochlaena palustris* peak counts averaged at 2 spores m^{-3} h^{-1} with maximum of 30 spores m^{-3} h^{-1}.

2.3.2.2 *Association with meteorological variables*

Correlations between airspora counts and meteorological variables are shown in Table 2.4. Fungal spores *Cladosporium* spp., *Didymosphaeria* sp. and 'kuaci' were negatively correlated to temperature, evaporation, solar radiation and wind speed except for *Cladosporium* spp. but positively for relative humidity. A positive correlation

with wind direction was also observed for *Cladosporium* spp. between 110° to 150° and rainfall in *Didymosphaeria* sp. but negative correlation for the ascospore 'kuaci'. A positive correlation was observed for temperature, evaporation, wind speed and solar radiation while a negative correlation was observed with relative humidity for *Curvularia* spp., *Pithomyces* sp. and the *Drechslera*-like spore.

High pollen hourly counts were correlated with for high temperatures, evaporation and wind speed coupled with low humidity. A negative correlation with rainfall was also observed for *Elaeis guineensis*. In general, fern spores showed similar associations to meteorological factors with those found in pollen. A negative association with rainfall was obtained for *Nephrolepis auriculata*. For *Stenochlaena palustris* counts were negatively correlated with wind direction.

Figure 2.19: Diurnal calendars for *Nephrolepis auriculata*, *Dicranopteris curranii* and *Dicranopteris linearis* for 1995, 1996, 1997 and average of all 3 years at the Kent Ridge Station.

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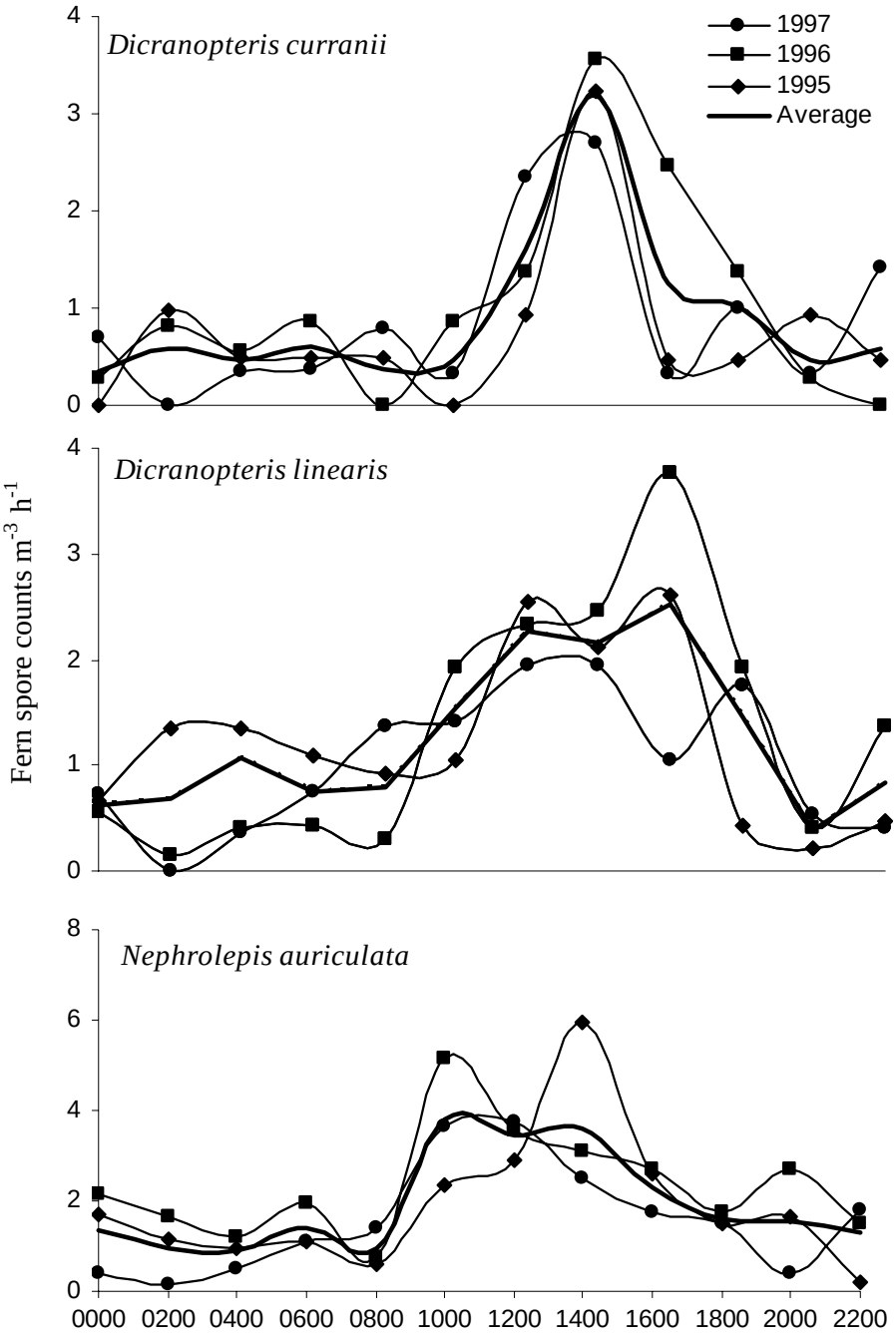

Figure 2.20: Diurnal calendars for *Asplenium nidus*, *Pteridium aquilinum* and *Stenochlaena palustris* for 1995, 1996, 1997 and average of all 3 years at the Kent Ridge Station.

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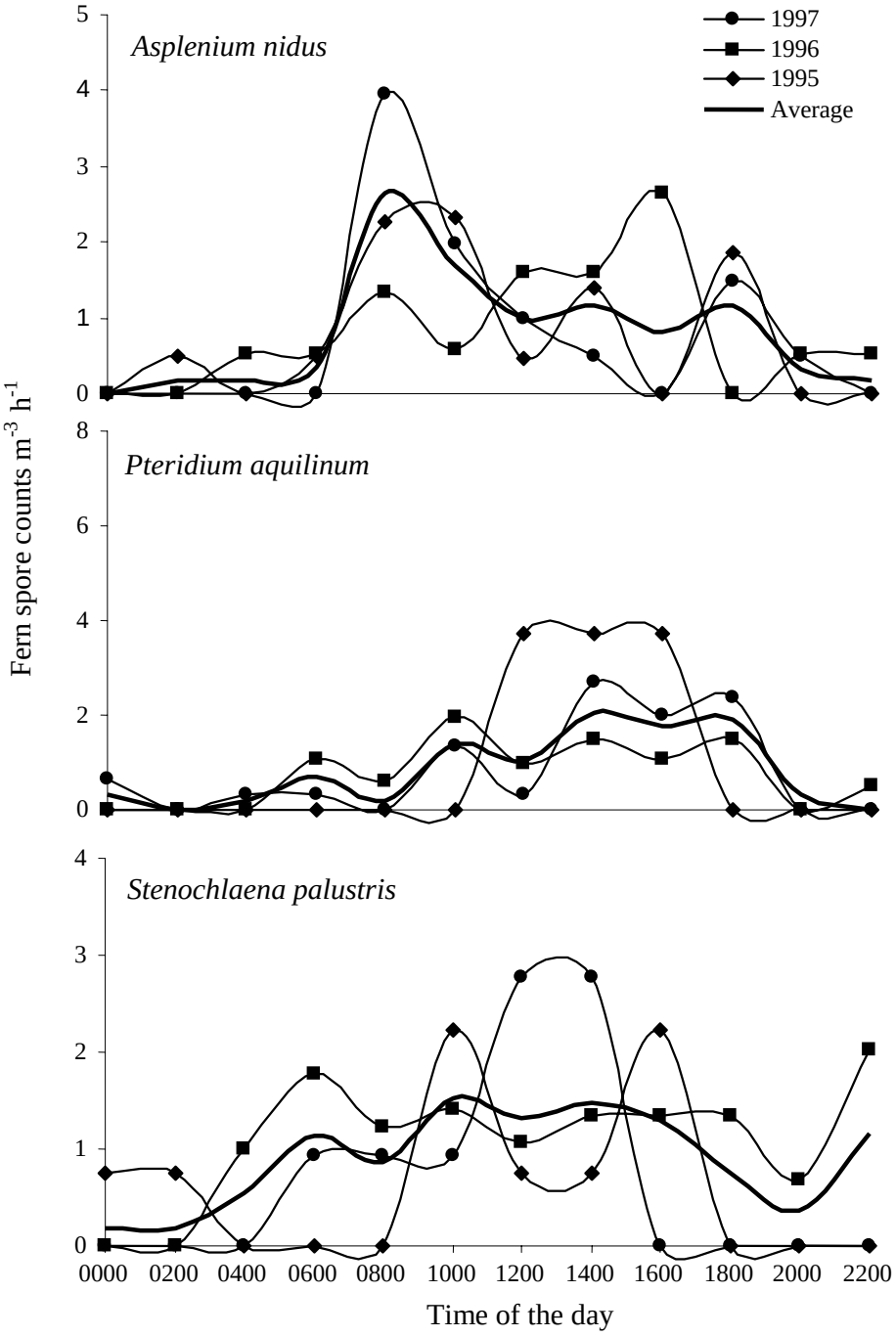


Table 2.4: Correlations of diurnal counts of airspora with meteorological factors.

Spore or pollen type	Spearman's Correlation Coefficient (r)						
	Temperature (°C)	Rainfall (mm)	Relative humidity (%)	Evaporation (MHW/cm^2)	Total wind speed (ms^{-1})	Wind direction (°)	Total solar radiation (MJm^{-2})
Fungal spore							
Cladosporium spp.	-0.134***	ns	0.205***	-0.167***	ns	0.075***	-0.042*
Curvularia spp.	0.374***	-0.057**	-0.364***	0.392***	0.295***	ns	0.394***
Didymosphaeria sp.	-0.292***	0.212***	0.389***	-0.359***	-0.207***	0.146***	-0.148***
Pithomyces sp.	0.214***	0.063**	-0.210***	0.237***	0.190***	ns	0.258***
Drechslera-like spore	0.095***	ns	-0.117***	0.142***	0.210***	ns	0.153***
Ascospore 'kuaci'	-0.427***	-0.090***	0.425****	-0.450****	-0.290***	ns	-0.461***
Pollen							
Acacia spp.	0.108***	ns	-0.052*	0.069**	ns	ns	0.084***
Casuarina equisetifolia	0.125***	ns	-0.086***	0.121***	0.072***	ns	0.159***
Elaeis guineensis	0.164***	-0.045*	-0.247***	0.231***	0.258***	ns	0.186***
Kyllingia polyphylla	0.113***	ns	-0.083***	0.114***	0.085***	ns	0.150***
Poaceae	ns	ns	-0.052*	0.052*	ns	ns	ns
Fern							
Asplenium nidus	ns	ns	ns	ns	ns	ns	0.068**
Dicranopteris curranii	0.119***	ns	-0.127***	0.120***	ns	ns	0.096***
Dicranopteris linearis	0.153***	ns	-0.145***	0.149***	ns	ns	0.137***
Nephrolepis auriculata	0.207***	-0.045*	-0.138***	0.173***	ns	ns	0.186***
Pteridium aquilinum	0.062**	ns	-0.045*	0.056*	0.051*	ns	0.077***
Stenochaelena palustris	0.064**	ns	-0.071**	0.079***	0.043*	-0.044*	0.072***

Correlations that were not significant are indicates as "ns". Correlation p-values: $p<0.05$*, $p<0.01$**, and $p<0.001$***.

2.4 DISCUSSION

2.4.1 Evaluation and optimisation of screening factors

The more suitable magnification was determined to be 400× even though a correspondingly smaller area of the slide would be screened. This is due to the high error rate of up to more than three times (data not shown) for smaller particles such as fungal spores which make up more than 80% of the total airspora composition when screened at 250×. The higher magnification also allows an increase in identification accuracy and perception, both of which contribute to the higher counts at 400×. Even though the Burkard spore trap operating manual recommends screening for pollen and fern spores at a magnification of 250× instead of 400×, to obtain a larger depth of field for the larger objects, the objective can be switched to the 25× objective lens on the turret (for a 250× observation, i.e. 25× objective and 10× eyepiece) when a large but blurred object is encountered while screening at 400× (using the 40× lens with a 10× eyepiece). Screening at 400× is also a common practice recommended by the Europe Aerobiology Advisory Committee, Pan American Aerobiology Association and most aerobiology network researchers (Irdi *et al*., 2002; Comtois *et al*., 1999; Molina *et al*., 1996; Kapylae *et al*., 1981). The significant difference in counts observed for all airspora is also contributed by the significantly higher counts of the smaller airspora because when counts for airspora components with an image area of more than 200 μm^2 were analysed ($p = 0.4749$), no significant difference was obtained.

The Wilcoxon Rank Test showed that the distribution of the pollen and spores to be uniform throughout the width of the slide. Work done by Irdi *et al*. (2002) using pol-

len counts from 10 horizontal traverses (along the length) on a single slide, showed similar results. However, fungal spore counts were significantly different indicating a difference in spores distribution along the width of the slide. This could be because of the smaller area examined (0.187 mm^2 area size per field) that was used compared to the 0.70 mm^2 area size per field that was used in this study. Uniform large airspora distribution (of particles >200μm^2, and which mostly include pollen grains and fern spores) was an interesting result because previous workers reported that pollen deposition along the width of the slide does differ, with loss of pollen when the traverse moves away from the middle of the tape (Comtois *et al.*, 1999; Molina *et al.*, 1996; Kapylae *et al*., 1981). The difference in result may be because of the low number of pollen grains and fern spores in the study. However, counts from horizontal traverses and vertical traverses (covering the whole width of the slide) were weakly but significant correlated. Furthermore, counts obtained from differently oriented traverses were also not significant different indicating the absence of differences in the distribution of airspora along the slide.

Stronger correlation was obtained for counts of five to three, and three to one traverses compared to five to one traverses. A stronger correlation when a higher number of traverses were screened is because of the increase in the screening area. Using just three traverses is optimal owing to stronger significant correlations of airspora counts coupled with the larger screening area compared to just a single traverse.

Results showed that counts using vertical versus horizontal traverses were comparable. This made us decide to continue with the current screening method, i.e., using horizontal traverses, to save time by screening a smaller area. Using horizontal traverses will also account for the variation occurring over time, which could be missed

by vertical traverses. Vertical traverses screening would only be employed when the diurnal data are needed.

2.4.2 Seasonal and diurnal patterns

2.4.2.1 *Fungal spores*

Seasonal and diurnal patterns were observed in the major airspora types in Singapore. The variations in spores or pollen concentration in the environment exist despite the uniform tropical climate, thus disputing the notion that tropical phenomena are uniform.

Seasonal patterns for all fungal spores correspond to those reported by Lim *et al*. (1998) and Dhorranintra *et al*. (1990). Similar patterns were also seen by Singh *et al*. (1987) in tropical Dehra Dun, India. In general, common peak periods from October to February were observed. Two peaks were observed in Malaysia (Ho *et al*., 1995), one in February and another in October, while in a broad peak was observed in December to February in Bangkok, Thailand (Phanichyakarn *et al.*, 1989) which overlaps with our seasonal patterns.

Cladosporium spp. are probably the commonest airbone fungus: it has been found whenever systematic air sampling has been undertaken (Pady *et al., al*., 1969). A similar pattern for *Cladosporium* spp. was observed in Malaysia (Ho *et al*., 1995). However, in Bangkok (Phanichyakarn *et al*., 1989) *Cladosporium* spp. spore counts peak in January while Vittal and Krishnamoorthi (1989) observed a different pattern with a minor peak in December and another higher peak in June, in Madras. In Egypt, total spore counts for *Cladosporium* spp. peak in February while that for

Cladosporium cladosporioides has another lower peak in October. The single peak in February was observed for *Cladosporium herbarum* which was five times higher in concentration (Sobhy *et al.*, 1989).

Variable diurnal trends have been observed for *Cladosporium* spp. spore counts ranging from an early morning peak from 7 and 8 am (Pady *et al*. al, 1969), daytime double maxima at 8 am, 4 pm and 10 pm (Rich and Waggoner, 1962), a 12 noon peak (Vittal and Krishnamoorthi, 1989 and Burch and Levetin, 2002) and a 10 pm peak (Molina *et al*., 1997). However, our study shows the presence of two trends. The mid-day peak corresponds with finding by Burch and Levetin (2002) and Vittal and Krishnamoorthi (1989). The second showed a 2 am peak in 1995 has never been observed in other studies.

Cladosporium spp. showed increasing spore counts with increasing temperature and decreasing humidity in the seasonal pattern. The same correlation patterns were also reported by Lim *et al*. (1998), Molina *et al*. (1997), Herrero *et al*. (1996) and Ho *et al*. (1995). An inverse seasonal correlation pattern, negative to temperature and positive to relative humidity was seen in the diurnal counts which differ to those results reported by Nayak *et al*. (1998), Molina *et al*. (1997) and Pady *et al*. (1969). However, a direct correlation with relative humidity was also obtained by Katial *et al*. (1997). Further analysis showed that the diurnal pattern for *Cladosporium* spp. differs not only from year to year but between different months in the same year. Meteorological factors found to be significantly correlated with *Cladosporium* spp. were further investigated. Correlation ($r = 0.0928$ to 0.2379) with wind direction was observed for months showing 10 pm to 4 am peak in spore counts. Counts were found to be high when the wind direction blows from northwest between 110° to 150°. A negative cor-

relation to temperature and/or positive correlation to relative humidity were also obtained. This unique profile maybe influenced by the source. *Cladosporium* spp. thrive in soil and high humidity promotes excellent growth of the fungus. Northwest from the trap is mainly the reservoir area, made up of dense secondary forests with mangroves at the water edge. Near mid-day peaks were found to be positively correlated to temperature and negatively correlated to relative humidity. Pady *et al.* (1969) demonstrated using *Cladosporium herbarum* that spores are released only when there is a sharp drop of relative humidity from 91% to 29% whereas a small drop from 95% to 70% failed to release spores even in high airflow situations. This is contrary to findings by Meredith (1962) who observed the twisting and collapse of the conidial apparatus of *Cladosporium musac* causing the conidial chains to break and releasing the spores when the culture on banana leaves was removed from a damp chamber to a microscope stage. However, similar hygroscopic movements were not seen when carried out by Pady *et al*. (1969) in *Cladosporium herbarum*. This led them to believe *Cladosporium herbarum* spores are released passively.

Reports of the occurrence of *Didymosphaeria* sp. are rare. The seasonal patterns for *Didymosphaeria* and the ascospore 'kuaci' are similar with both having spore count peaks in April and November. The seasonal pattern observed was similar to that found for *Didymosphaeria* sp. by Lim *et al*. (1998), ascospores by Phanichyakarin *et al*. (1989) and interestingly, by Ho *et al*. (1995) to a two-celled spore. *Didymosphaeria* sp. is also a two-celled spore.

Diurnal periodicities of *Didymosphaeria* sp. and 'kuaci' spore counts were found in late evenings and the night when the temperature is low, relative humidity, high and wind speed, low. Burge (1986) reported a similar diurnal pattern for ascospores.

Similar correlations were observed between daily and diurnal counts to meterological factors. High spore counts were made during the night, possibly in response to increasing relative humidity and falling temperatures. Lyon *et al.* (1984) reported a direct positive correlation of airborne ascospore counts with higher minimum wind velocities but decreasing spore counts with increasing maximum wind velocities. Waggoner (1973) found that a low wind velocity (3 m s^{-1}) for 15 s could remove 20% of *Helminthosporium maydis* spores. Aylor and Lukens (1974) have shown a low wind speed of 1 m s^{-1} releases 60 to 75% of similar spores even when the leaf was not swaying. Our studies also show diurnal counts for *Didymosphaeria* sp. increase with rainfall. Hirst (1953) also reported brief increases of ascospores numbers after rain and during periods of high relative humidity at night. Royes (1987) observed high counts of ascospores during months with the highest rainfall suggesting the dispersal mechanism maybe aided by the mechanical effects of rain.

Seasonal patterns for the spore counts of *Curvularia* spp., *Drecshlera*-like spore and *Pithomyces* sp. were similar to those reported by Lim *et al*. (1998). For *Curvularia* spp., Ho *et al.* (1995) observed peaks in January, June and September while Panichyakarn *et al*. (1989) observed a single peak in November. The *Drecshlera*-like spore counts peak in November and March locally but only a single peak in May was obtained by Panichyakarn *et al*. (1989). For *Pithomyces* sp., a major season was observed in February and two lower peaks in June and July (Lim *et al*. 1998). Panichyakarn *et al*. (1989) observed a single broad peak in November to December. For *Curvularia* spp. and *Drechslera*-like spore counts, similar 'mid-day pattern' diurnal patterns were observed by Vittal and Krishnamoorthy (1989) and Nayak *et al.* (1998). Atluri *et al*. (1988) also demonstrated a similar 'mid-day pattern' but with double peaks for *Drechslera*-like spores with sharper peaks compared to our observations

while Couture and Sutton (1978) observed a broad single peak at 4 pm. Troutt and Levetin (2001) found high counts of basidiospores including *Pithomyces* during high temperature periods. However, the diurnal pattern for *Pithomyces* was not shown. The positive effects of wind direction and wind speed were also observed.

2.4.2.2 *Pollen*

Seasonality and diurnal patterns were distinctly observed for the major pollen types studied. The seasonality of tropical pollen types has also been observed in Malaysia (Ho *et al*., 1995) and Panichyakarn *et al*. (1989). Seasonality patterns of *Acacia* spp., *Elaeis guineensis* and *Podocarpus/Pinus* spp. did not correspond to the flowering periods noted in the trees in Singapore (Rao and Wee, 1989). *Casuarina equisetifolia* has high counts in March to April, which coincide with the reported flowering season, also has a major peak in August to October, which does not coincide with the reported flowering season. This maybe due to the changes in climate since the observations were made in the 1980s by Rao and Wee (1989) but the airspora were sampled from 1990 to 1996. The correlation between pollen counts and increasing temperature and decreasing relative humidity may be a result of dehydration which causes pollen anthesis for the pollen release into the air as in corn (Keijzer *et al*., 1996), 35 plant taxa in West Bengal, India (Bhattacharya and Datta, 1992) and ragweed (Bianchi *et al*., 1959).

The diurnal pattern for *Casuarina equisetifolia* pollen counts was similar to that obtained for the species in southern Spain by Garcia *et al.* (1997). Pollen counts in our study were also positively correlated with temperature and sunshine.

Kyllingia polyphylla pollen counts demonstrated a diurnal pattern similar to all the other pollen types with a mid-day peak. The study by Perez *et al*. (2001) on Cyperaceae showed higher percentages of pollen during the night than in daylight hours with two periods of high concentrations observed from 6 am to 4 pm and from 10 pm to 4 am. The Singapore pattern also has a major peak at 11 am but does not exhibit a second smaller peak but also showed increasing counts with increasing temperature and wind speed. Perez *et al*. (2001) also demonstrated that intermediate relative humidity values of 48 to 74% showed stronger positive effects on counts compared to values above 74%. Our results showed an inverse effect of relative humidity to pollen counts. This can be explained by the difference in relative humidity levels. The times of the day with high temperatures and wind speed as well as relative humidity levels below 74% occurs from 10 am to 6 pm after which there increasing relative humidity values were observed. A similar mid-day pattern for all major pollen types studied indicated low relative humidity during the mid-day period are suitable to the pollen release. Thus, it is the optimal humidity levels that influence the pollen release.

For the Poaceae pollen counts, the only distinct pattern was observed in 1995 with higher levels in 1996. An early peak at 7 am was seen in 1995 and an 11 am mid-day peak in 1996. The early peak in 1995 is quite similar to the one observed by Singh and Babu (1980) at 6 am in Delhi, India. Our local profiles were different from diurnal profiles obtained in other studies (Ong *et al.,* 1995 and Norris-Hill and Emberlin, 1991; Mullins *et al*., 1986). Different patterns were observed by Mullins *et al*. (1986) at Cleppa Park and Cardiff in United Kingdom. Grass pollen peaked at 3 pm at Cleppa Park coinciding with high temperatures, wind speeds and sunshine and low relative humidity while Cardiff demonstrated a later peak at 7 pm caused by rising air in the city preventing pollen deposition. Our data also showed a positive correlation

of the pollen counts to evaporation and a negative correlation to temperature. Ong *et al.* (1995) showed three diurnal patterns in Melbourne with one peaking at 5 to 9 pm, 7 am to 1 pm and 3 to 9 pm. A 5 to 9 pm peak was also observed by Norris-Hill and Emberlin (1991) in London. Our pattern coincides with the 7 am to 1 pm peak noted by Ong *et al.* (1995). The difference in diurnal periodicity is greatly influenced by the grasses present at the site. Reddi *et al.* (1988) studied the circadian patterns of 54 grass species in Visakhapatnam, India by studying anther dehiscence during the height of the flowering season of each taxon. In general, the grass release patterns were categorized into 10 patterns, viz., 24-hour, bimodal frequency, pre-middle night, middle night, post-middle night, early morning, fore-noon, middle-day, afternoon and evening patterns. From the different anthesis patterns, Reddi *et al.* (1988) concluded that the relationship with environmental factors is an inherent property, probably controlled through genetic make-up.

Acacia auriculiformis was reported to flower year round in Singapore (Rao and Chin, 1989). The variable peak hour for pollen counts observed may be influenced by the sources of pollen. The trap at Kent Ridge is located at the edge of a secondary forest. An early peak observed at 8 am is most probably contributed by pollen released by the abundant trees nearby while later peaks by pollen that is transported from trees further away.

The *Elaeis guineensis* peak period is the latest in the day among all the pollen types studied. A similar influence of meteorological factors, as observed for other pollen types was seen for *Elaeis guineensis*. It was observed that pollen counts increased with high wind speed while rainfall causes the counts to decrease. This could be due to the source of the pollen itself. *Elaeis guineensis* is commonly cultivated in Singa-

pore but is a very major crop in neighbouring Indonesia and Malaysia and very likely, pollen is brought over by wind.

2.4.3.3 *Fern spores*

Even though Singapore is a highly urbanized city-state, ferns are found in abundance in fringes at the mangroves or as large patches of vegetation in open abandoned areas or secondary forest (Johnson, 1977). Since ferns are found in low abundance in the temperate environment, not many studies have reported the seasonal and diurnal periodicity of fern spores except for *Pteridium aquilinum* which is a possible health hazard (Taylor, 1990; Trotter, 1990) as it has carcinogenic properties (Smith and Seawright, 1995). *Pteridium aquilinum* spore counts were found to be high in late August and September in the United Kingdom (McCartney and Lacey, 1990) while a broad peak was observed from June to October in Singapore. The diurnal profiles of all the fern spores studied generally overlapped between 10 am to 5 pm. Similar correlation patterns with meteorological factors were obtained for all fern species studied. The study of Lacey and McCartney 1994) on *Pteridium aquilinum* showed a diurnal profile with spore counts reaching the peak at 9 am. However, the influences of meteorological factors on spore counts were not studied with the spore trap placed next to the source area. Locally, high spore counts were observed from 1 to 5 pm and a positive correlation to wind speed was also observed. The current sources of known *Pteridium aquilinum* spores in Singapore's airpora are most likely Indonesia or Peninsular Malaysia as this species is extremely rare, being categorized as locally endangered (Wee, 1995).

2.4.3.4 *Comparison of counts between different stations*

Most counts between the three stations were significantly correlated indicating similarity in the trends. High and strongly significant correlations ($r = 0.6873$ to 0.7080; $p<0.001$) for counts between the three stations for *Elaeis guineensis* was observed. This further supports the suggestion of a one or more common foreign source(s) for this pollen type. However, levels of the airspora counted did differ between sites and these have been shown to occur in other studies (Trigo *et al.*, 2000; Palmas and Cosentino, 1989; Long and Kramer, 1972). If marked differences were observed between sites, a local source of allergens is indicated. Counts from the Kent Ridge station were the lowest for 10 out of the 16 airspora types studied. This is also the station that is positioned at the greatest height (61 m above sea level) compared to Clementi (28 m) and Hougang (45 m).

2.5 CONCLUSIONS

Results have shown that screening magnification, number and orientation of traverses do influence the airspora counts. The screening method using 400× magnification, three horizontal traverses 3 mm apart from the middle of the slide for daily counts was found to be optimal after taking into account the accuracy of results and time spent.

Seasonal patterns were observed for all airspora types even with the relative low variation in the Singapore climate. The peak season for different airspora types oc-

curred during different times of the year. An ascospore termed as 'kuaci', identified by means of phylogenetic analysis to be in the clade with members from the Dothideomycetes and Chaetothyriomycetes, was found to occur frequently in the samples. Diurnal variations of the individual major airspora components were established. High levels of ascospores were found during the night while Deuteromycetes counts were high during the late morning to early evening. Pollen and fern spore counts were high during the middle of the day. Meteorological factors were also found to influence the both the seasonal and diurnal profiles of the airspora.

All the information gathered from these studies has provided us with the basis for future forecasting work and studies on the effects of airspora on exacerbation of allergic disease. Future work on the effects of airspora counts and the presentation of allergic symptoms will be important. Studies on the relationships between the levels of allergens and airspora counts will also further strengthen the point that airspora is an important source of allergens locally.

CHAPTER 3: DIFFERENTIATION OF AIRSPORA COMPONENTS BY IMAGE ANALYSIS

3.1 INTRODUCTION

3.1.1 Shortcomings of current airspora quantification methods

Current methods in identifying and quantifying airspora components are tedious and labour intensive. Total variances of 50% (Pedersen and Moseholm, 1993) and 23% (Comtois *et al*., 1999), respectively, were reported in these two studies for pollen counts which were much higher than the 5 to 10% human counters themselves expected. Accuracy of airspora counts is also highly dependent on the expertise of the human counter. Human error in the process of counting and identifying the individual airspora components probably constitutes the largest proportion of non-biased errors. The reported average error between experienced and trained counters ranged from 2 to 13% (Mandrioli and Comtois, 1998; Pedersen and Moseholm, 1993). The average error associated with the four longitudinal lines screening protocol was 23%. These errors are largely operator-related because the counting process requires not only good training but also a clear and alert mind to detect and identify the airspora captured on the tapes and which are mixed together with large amounts of debris.

Results of all current screening methods are obtained by extrapolation of that achieved by counting more than the 10% recommended of the tape surface (Irdi *et al*., 2002; Mandrioli and Comtois, 1998; Molina *et al*. 1996; Pedersen and Moseholm, 1993; Kapyla *et al*. 1981). This suggests that when a higher percentage of the tape

area was screened, the more accurate the counts will be (Irdi *et al*., 2002; Comtois *et al*., 1999; Molina *et al*., 1996; Kapyla *et al*., 1981). However, the more number of screening traverses needed, the longer the time spent. If the whole tape area for one day's capture is screened, 31 longitudinal traverses under 400× magnification will be needed. In this study, an average of about 30 minutes was spent to screen one longitudinal traverse for pollen as well as fern and fungal spores, amounting to almost 15 hours spent screening just one slide!

All these shortcomings in the current method prompted us to consider the feasibility of developing an automated airspora identification and quantification system. To achieve this goal, we initiated the work on using image analysis tools to differentiate the airspora in Singapore and further expanded the work to include allergenic pollen types found worldwide. Quantitative morphological data were used in this study instead of the conventional qualitative data used in most of current airspora identification work.

The need for an automated system was partly spurred on by the labour-intensive nature of this work. We currently have samples collected from 1990 to present but counts data that are available only date back to June 1996. The huge backlog, coupled by the difficulty in finding interested and willing airspora counters, further elevated the need for a solution to these problems.

3.1.2 Pollen grain identification

For pollen grain identification, six main types of morphological characters were used (Huang, 1972; Erdtman and Sorsa, 1971; Echlin, 1968; Erdtman, 1943), i.e., 1) The type of pollen grain units, i.e., monad (a single solitary grain), dyad (in a pair), tetrad (four coherent grains) or polyad (more than four grains), 2) Polarity resulting from separation of the pollen grains, i.e., apolar (with no polarity), polar (with distinct poles), heteropolar (with two different polar faces) and isopolar (with an equatorial plane separating the grain into two identical halves), 3) Type of symmetry, i.e., bilateral (with two vertical planes of symmetry) or radiosymmetry (with more than two vertical planes of symmetry), 4) Shape of the grains for the polar or equatorial view (e.g., circular, elliptical, ovate, lanceolate, rectangular, rhombic, triangular or quadrangular), 5) Size or grains (length of the equatorial or polar diameter), 6) Number, type, shape and location of the germinal aperture, a preformed thinning or absence of a part of the exine (e.g., inaperture, colpate, colporate, operculate or syncolpate), 7) Sculpturing of the external exine wall (e.g., baculate, clavate, echinate, foveolate, luminate, psilate, reticulate or striate) from the surface or lateral views.

3.1.3 Fern spore identification

Fern spore identification is quite similar to pollen grain identification (Lellinger and Taylor, 1997; Tyron and Lugardon, 1991; Huang, 1981). It also involves using the shape, size, symmetry, fissure aperture (type and shape) and sculpturing of the exine, and in fern spores, also the perine. Terminologies used are also similar to those used in pollen identification. The differences between identification of pollen grains and

fern spores are the additional use of the dehiscence aperture called the laesura (at the margin or ridges), instead of the germinal aperture, and also the proximal ridges.

3.1.4 Fungal spore identification

Fungal spores are also identified from their size, shape and texture (Sivanesan, 1984; Ellis 1976; Funder 1953). Shapes of the spores can vary dramatically from globose, oval, short-cylindrical, elongated, fusoid, filamentous, coiled, stellated or irregular. Colour of the spores is also used, i.e., hyaline, bright or dark coloured. The presence or absence of septa indicates the number of cells in a spore, i.e., from one to multi-celled. The thickness of the cell wall is also an additional feature that is important in fungal spore identification. The type and location of the fissure scar is also important. Spores can also present themselves in a single conidium and/or in chains like those in *Cladosporium, Aspergillus* and *Penicillium*.

3.1.5 Aims

In this study we aimed to determine the feasibility of identifying the local airspora components and foreign pollen types that are quite similar morphologically using image analysis. Characters that are important in identifying the airspora were delineated. The usefullness of quantitative morphological characters instead of the usual qualitative characters in taxonomic classification was also studied. Results from this study will allow us to study the feasibility of automating the airspora identification and quantification process.

3.2 MATERIALS AND METHODS

3.2.1 Sample preparation

Our study started with the collection of local airspora and but later expanded to include more than 150 allergenic pollen types found worldwide. The airspora taxa studied and their sources are listed in Table 3.1.

Local pollen types and fern spores were obtained from multiple locations in the Republic of Singapore by collecting inflorescences and fern sporophylls. Fungal spores were obtained by knocking the sporulating culture plates upside down onto a clean sheet of paper. Potato dextrose agar was use to culture *Cladosporium cladosporioides, Curvularia lunata* and *Pithomyces maydicus*. Oatmeal agar with a piece of rice leaf (*Oryza sativa*)on it was used for culturing *Didymosphaeria donacina*. Spores were only seen in the agar plates after 10 days for *Cladosporium cladosporioides,* 15 days for *Curvularia lunata* and 21 days for *Didymosphearia donacina*. As for *Pithomyces maydicus,* spores were obtained only after 30 days of culture.

Pollen types not found locally were purchased from Greer Laboratories Incorporated (USA) in the form of pure pollen (mostly certified to be 97% pure).

All the spore and pollen identities used in this study were reconfirmed with available literature and reference slides available from our airspora slide collection. Slides of the various spore and pollen types were mounted using glycerine jelly (15% gelatine, 46% glycerol and 2% phenol) (Erdtman, 1943) and sealed with low temperature melting wax. No staining was carried out.

Table 3.1: Airspora studied.

Classification	Airspora types
[l]Common local pollen types	*Acacia auriculiformis, Casuarina equisetifolia, Elaeis guineensis, Kyllinga polyphylla, Podocarpus polystachyus*
[l]Common local fern types	A*splenium nidus, Dicranopteris curranii, Dicranopteris linearis, Nephrolepis auriculata, Stenochlaena palustris*
[l]Common local fungi	*Cladosporium cladosporioides, Curvularia lunata, Curvularia brachyspora, Didymosphaeria donacina, Exserohilum* spp., *Pithomyces maydicus*
[g]Poales (Poaceae) pollen	*Agropyron repens, Agropyron smithi, Agrostis alba, Anthoxanthum odoratum, Avena sativa, Bromus inermis, Cynodon dactylon, Dactylis glomerata, Elymus condensatus, Festuca elatior, Holcus lanatus, Lolium multiflorum, Lolium perenne, Paspalum notatum, Phalaris arundinacea, Phleum pratense, Poa compressa, Poa pratensis, Secale cereale,* S*orghum halepensis, Triticum aestivum, Zea mays*
[g]Asterales (Asteraceae) pollen	*Ambrosia acanthicarpa, Ambrosia artemisiifolia, Ambrosia psilostachya, Ambrosia trifida, Artemisia californica, Artemisia frigida, Artemisia vulgaris, Baccharis halimifolia, Baccharis sarothroides, Chrysanthemum leucanthemum, Eupatorium capillifolium, Helianthus annus, Hymenoclea salsola, Iva axillaries, Iva xanthifolia, Solidago* sp., *Taraxacum commune* and *Xanthium commune*
[g]Caryophyllales pollen	*Rumex acetosella, Rumex crispus, Allenrolfea occidentalis, Amaranthus hybridus, Amaranthus palmerii, Amaranthus retroflexus, Atriplex canescens, Atriplex confertifolia, Atriplex lentiformis, Atriplex polycarpa, Atriplex wrightii, Chenopodium album, Chenopodium ambrosioides, Chenopodium botrys, Tamarix gallica*
[g]Fabales pollen	*Acacia* spp., *Prosopis juliflora, Trifolium pratense*
[g]Fagales pollen	*Alnus glutinosa, Alnus rhombifolia, Alnus rubra, Alnus rugosa, Betula lenta, Betula nigra, Betula verrucosa, Betula populifolia, Corylus americana, Corylus avellana, Casuarina equisetifolia, Fagus americana, Quercus agrifolia, Quercus alba, Quercus dumosa, Quercus garryana, Quercus ilex, Quercus kelloggii, Quercus lobata, Quercus macrocarpa, Quercus nigra, Quercus robur, Quercus rubra, Quercus stellata, Quercus velutina, Quercus virginiana, Carya alba, Carya glabra, Carya illinoensis, Carya laciniosa, Carya ovata, Juglans californica, Juglans nigra , Juglans regia, Myrica cerifera*

Unlike the local airspora, the foreign pollen types are grouped according to their respective order. Source of materials: [l]local and [g]Greer Laboratories (USA). The airspora types were classified according to The Angiosperm Phylogeny Group (2003). Changes made to the classification as recommended by The Angiosperm Phylogeny Group for the pollen types studied are listed in Appendix 1.

Table 3.1 (continued): Airspora studied.

Classification	Airspora types
[g]Lamiales pollen	*Fraxinus americana, Fraxinus latifolia, Fraxinus pennsylvanica, Fraxinus velutina, Ligustrum vulgare, Olea europea, Plantago lanceolata*
[g]Malpighiales pollen	*Populus alba, Populus deltoides, Populus nigra. Populus sargentii. Populus tremuloides. Populus trichocarpa. Populus wislizenii. Salix discolor. Salix lasiolepis*
[g]Pinales pollen	*Cupressus arizonica, Cupressus sempervirens, Juniperus ashei, Juniperus monosperma, Juniperus occidentalis, Juniperus osteosperma, Juniperus scopulorum, Juniperus virginiana, Pinus echinata, Pinus elliotti, Pinus monticola, Pinus palustris, Pinus ponderosa, Pinus strobus, Pinus virginiana, Cryptomeria japonica, Taxodium distichum*
[g]Proteales pollen	*Platanus acerifolia, Platanus occidentalis, Platanus orientalis, Platanus racemosa*
[g]Rosales pollen	*Elaeagnus angustifolia, Broussonetia papyrifera, Morus alba, Morus rubra, Rosa* spp., *Celtis occidentalis, Ulmus americana, Ulmus crassifolia, Ulmus pumila, Urtica* spp.
[g]Sapindales pollen	*Acer macrophyllum, Acer negundo, Acer saccharinum, Acer saccharum, Schinus molle, Schinus terebinthifolius, Citrus sinensis*
[g]Other pollen types	*Cocos plumosa* (Arecales), *Eucalyptus globulus* (Myrtales), *Liquidambar styraciflua* (Hamamelidales), *Melaleuca quinquenervia* (Myrtales), *Ricinus communis* (Euphorbiales),

Unlike the local airspora, the foreign pollen types are grouped according to their respective order. Source of materials: [l]local and [g]Greer Laboratories (USA). The airspora types were classified according to The Angiosperm Phylogeny Group (2003). Changes made to the classification as recommended by The Angiosperm Phylogeny Group for the pollen types studied are listed in Appendix 1.

3.2.2 Image capture

Reference slide images were captured using a KY F-50E 3-chip CCD camera with a resolution of 400 000 pixels (JVC Limited, Japan) on a BX50 microscope (Olympus Corporation, Japan) at 400× magnification. An apochromat lense with 0.95 numerical aperture size was used. A high intensity 12V, 100W halogen lamp light source Images were standardised instead of equipment setting to ensure the images properties do not change with equipment used. A single image size was 760 × 570 pixels. A calibration scale of 3.36 pixels, equivalent to 1 μm was used for all images. A uniform background was obtained by presetting the background pixels within 190 to 210 optical densities (OD) for all three RGB (red, green blue) colour channels prior to image capture. Approximately 1000 spores or pollen grains were scanned for each type of airspora to ensure sufficient samples were available for discrimination (Barcikowski and Stevens, 1975). All the scanning work was manually done because of the highly variable of the spores and pollen grains density found on each slide.

3.2.3 Feature measurement

Segmentation was performed on the images to obtain a clear and accurate outline before feature measurements were taken. MicroImage™ image analysis software (Olympus Europe, Germany) was used to obtain the feature measurements. The automated histogram equalization for colour thresholding method was employed to differentiate the airspora from the background. Spores and pollen grains that were not well segmented were re-segmented manually prior to feature measurement. In total, 46

primary and secondary parameters were measured except for the local airspora, whereby only 39 parameters were measured in the initial work (Table 3.2).

3.2.4 Statistical analyses

Step-wise canonical discriminate analysis was used to differentiate and subsequently identify all the airspora (Currie *et al*., 1997; Cruz-Castillo *et al*., 1994, White *et al*., 1988). SPSS version 12 for Windows (SPSS Incorporated, New York) was used for all statistical analyses.

The canonical discriminate analysis uses the variations from the differences between airspora types to classify and subsequently identify the airspora (Tabachnick and Fidell, 1996). Canonical coefficients given in the results were used to assess the relative importance of individual parameters' contributions to a given canonical function. The canonical coefficients are the standardized weights in the linear equation of variables. The magnitude of the canonical coefficients indicates the contribution of the parameter in each canonical factor used in discrimination.

Identifications, using image analysis, of 1) local airspora, 2) Poaceae, 3) Asteraceae weed and 4) *Olea* look-alike pollen types were studied. These groups were chosen based on airspora that occur together temporally and spatially and to test the feasibility of differentiating similar looking airspora. Differentiation work on a total of 153 pollen types was also carried out.

Table 3.2: Primary and secondary morphological parameters measured using the Olympus MicroImage™ software (Media Cybernetics, 1999).

Feature/ measurement	Formula
[2]Angle	Angle between the vertical axis and major axis
[1]Area	Number of pixels
[1]Area (polygon)	Number of pixels in the polygon defining object
[1]Area/ Box	Number of pixels/ Bounding rectangle
[2]Aspect	Major axis/Minor axis
[1]Axis (major)	Length of main axis of the ellipse equivalent to object
[1]Axis (minor)	Length of minor axis of the ellipse equivalent to object
[1]Box height	Height of smallest rectangle encompassing whole object
[1]Box width	Width of smallest rectangle encompassing whole object
[2]Box ratio	Ratio of area to area of the bounding box
[1]Box X/Y*	Box width/Box height
[3]Clumpiness*	Fraction of pixel deviating from the average remaining pixel after dilation, reflecting texture variation
[3]Compactness	Form factor/Size (length)
[2]Concavity	Area (polygon) - Area
[1]Dendritic length	Total length of all dendrites
[4]Density (max)	Maximum intensity or density inside the object
[4]Density (mean)	Mean value of red, green and blue channels (RGB)
[4]Density (min)	Minimum intensity or density inside the object
[3]Density (standard deviation)	Standard deviation on intensity or density inside object
[4]Density (blue)*	Object mean blue value
[4]Density (red)*	Object mean red value
[4]Density (green)*	Object mean green value
[1]Diameter (max)	Length of longest line joining 2 outline points passing the centroid
[1]Diameter (mean)	Average length of diameters at 5° intervals around centroid
[1]Diameter (min)	Length of shortest line joining 2 outline points passing the centroid
[2]End points	Number of end points
[1]Equivalent circular diameter (ECD)	$\sqrt{(4 \times \text{Area}/\pi)}$
[1]Feret (mean)	Average caliper (feret) length
[1]Feret (max)	Maximum caliper (feret) length
[1]Feret (min)	Minimum caliper (feret) length
[2]Form factor	$4 \times \text{Area}/\text{Perimeter}^2$
[1]Fractal dimension	Fractal dimension of the object's outline
[3]Heterogeneity*	Fraction of pixel that deviate more than 10% from the average intensity
[4]Integrated optical density (IOD)*	Area × Density mean
[3]Margination*	Relative distribution of object between center and margin with larger values from the brighter centers (0.33 = homogenous object)
[2]Modification ratio (MODR)	Radius (min)/Size (length)
[1]Perimeter	Length of outline of object
[1]Perimeter (convex)	Perimeter of the convex outline of object
[1]Perimeter (ellipse)	Perimeter of the equivalent ellipse surrounding the outline of object
[2]Perimeter (ratio)	Ration of convex perimeter and perimeter of the object outline
[1]Radius (max)	Maximum distance between centroid and object perimeter
[1]Radius (min)	Minimum distance between centroid and object perimeter
[2]Radius ratio	Ratio of maximum to minimum distance between centroid and object's perimeter
[2]Roundness	$(\text{Perimeter}^2)/(4 \times p \times \text{Area})$
[1]Size (length)	Feret diameter along major axis of object
[1]Size (width)	Feret diameter along minor axis of object

*Indicates the parameters that were not measured for local airspora. Types of parameters: [1]size, [2]shape, [3]texture and [4]colour.

Analysis of variance (ANOVA) to check for variance between the different groups studied was carried out (Currie *et al*., 1997). When significant differences were obtained, Tukey's Honest Significance Determinant (HSD) test was subsequently performed. Hierarchical cluster analysis was performed on the different pollen types to study the feasibility of using pollen morphology in taxonomic classification (Felsenstein, 1983). The data were normalized by variables using the z score. This normalization step will allow the parameters that were measured to be compared on an equal scale.

3.3 RESULTS

3.3.1 Local airspora

The major local airspora were measured for their primary and secondary parameters as outlined in Table 3.2 (Figure 3.2). In total, 16 pollen and spore types were studied. Correct identifications were obtained for 85.3% of the airspora in the original group (Table 3.3). Accuracies of more than 90% were obtained for *Acacia auriculiformis* (96.8%), *Cladosporium cladosporioides* (98.8%), *Curvularia* spp. (96.0%), *Cynodon dactylon* (93.6%), *Didymosphearia donacina* (99.1%), the *Drechslera*-like spore (98.0%), *Kyllingia polyphylla* (93.9%) and *Podocarpus polystachyus* (92.7%). *Asplenium nidus* (correctly identified 88.0% of the time) and *Pithomyces maydicus* (84.9%) had accuracies between 80 to 90%. *Casuarina equisetifolia* (72.1%), *Dicranopteris linearis* (78.2%), *Elaeis guineensis* (79.4%) and *Stenochlaena palustris* (71.6%) had accuracies between 70 to 80%.

Lower accuracies were obtained for *Dicranopteris curranii* (63.5%) and *Nephrolepis auriculata* (52.6%). *Nephrolepis auriculata* (16.2%) was often misidentified as *Kyllingia polyphylla* while *Dicranopteris curranii* misidentified as *Kyllingia polyphylla* (27.7% of the time) and *Nephrolepis auriculata* (18.5%).

Misidentification rates of more than 10% for a single airspora type were observed for the *Casuarina equisetifolia* pollen grain (23.5%) which was sometimes confused with that of *Cynodon dactylon*, the *Elaeis guineensis* pollen grains (13.9%) which was sometimes confused with that of *Dicranopteris linearis*, the *Pithomyces maydicus* spore (14.8%) which was sometimes confused with the *Curvularia* spp., and the *Stenochlaena palustris* spore (11.7%) which was sometimes confused with the pollen grain of *Podocarpus polystachyus*.

Most airspora that were misidentified were mistaken as *Kyllingia polyphylla*. In total, 4.55% of airspora samples were misidentified as *Kyllingia polyphylla*.

A total combination of 36 parameters was used for identification (Table 3.4). Fourteen canonical functions were needed to differentiate 100% of the samples. Area, ellipse perimeter, equivalent circular diameter (ECD), length size, maximum diameter, minor axis and polygon area are important parameters in differentiating the local airspora.

The mean values for all the parameters used were not equal when ANOVA ($p < 0.001$) was performed. Subsequently, post-hoc Tukey's analysis was performed. Tukey's HSD results for discriminating parameters are shown in Table 3.5.

Table 3.3: Identification accuracies of the local airspora using step-wise canonical discriminate analysis.

Airspora types / **Actual identity**	**Identified as**	[p]*Acacia auriculiformis*	[fe]*Asplenium nidus*	[p]*Casuarina equisetifolia*	[fu]*Cladosporium cladosporioides*	[fu]*Curvularia* spp.	[fu]*Didymosphaeria* donacina	[fe]*Dicranopteris linearis*	[p]*Elaeis guineensis*	[fe]*Nephrolepis auriculata*	[fu]*Pithomyces maydicus*	[p]*Podocarpus polystachyus*	[fe]*Stenochlaena palustris*	[p]*Kyllingia polyphylla*	[fe]*Dicranopteris curranii*	[p]*Cynodon dactylon*	[fu]*Drechslera*-like spore
[p]*Acacia auriculiformis*		**96.8**	0.0	0.2	0.2	0.0	0.0	0.0	0.0	0.0	0.2	2.2	0.2	0.0	0.0	0.0	0.0
[fe]*Asplenium nidus*		0.0	**88.0**	0.0	0.0	0.0	0.0	0.0	0.2	0.0	0.0	2.2	9.5	0.0	0.0	0.0	0.0
[p]*Casuarina equisetifolia*		0.0	0.2	**72.1**	0.0	0.0	0.0	0.5	1.2	0.2	0.0	0.5	1.5	0.2	0.0	**23.5**	0.0
[fu]*Cladosporium cladosporioides*		0.0	0.0	0.0	**98.8**	0.0	1.2	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
[fu]*Curvularia* spp.		0.0	0.0	0.0	0.0	**96.0**	0.9	0.0	0.0	0.0	2.4	0.0	0.0	0.0	0.0	0.0	0.7
[fu]*Didymosphaeria donacina*		0.0	0.0	0.0	0.2	0.6	**99.1**	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.2
[fe]*Dicranopteris linearis*		0.0	0.0	0.0	0.0	0.0	0.0	**78.2**	1.7	0.0	0.0	1.0	1.0	15.2	2.7	0.2	0.0
[p]*Elaeis guineensis*		0.0	0.5	0.2	0.0	0.0	0.0	**13.9**	**79.4**	0.7	0.0	0.0	0.0	4.2	0.5	0.0	0.5
[fe]*Nephrolepis auriculata*		0.0	0.0	0.0	0.0	0.0	0.0	0.2	1.7	**52.6**	0.0	0.0	0.7	**26.2**	**18.5**	0.0	0.0
[fu]*Pithomyces maydicus*		0.0	0.0	0.0	0.0	**14.8**	0.2	0.0	0.0	0.0	**84.9**	0.0	0.0	0.0	0.0	0.0	0.0
[p]*Podocarpus* polystachyus		3.6	1.0	0.0	0.0	0.0	0.0	0.0	0.2	0.0	0.0	**92.7**	1.5	0.0	1.0	0.0	0.0
[fe]*Stenochlaena palustris*		0.2	9.0	0.5	0.2	0.0	0.0	1.7	0.0	0.0	0.0	**11.7**	**71.6**	0.2	4.7	0.0	0.0
[p]*Kyllingia polyphylla*		0.0	0.0	0.5	0.0	0.0	0.0	1.2	0.7	0.5	0.0	0.0	0.5	**93.9**	2.7	0.0	0.0
[fe]*Dicranopteris curranii*		0.9	0.0	1.3	0.0	0.0	0.0	1.8	0.2	3.3	0.0	0.7	0.2	**27.7**	**63.5**	0.2	0.2
[p]*Cynodon dactylon*		0.0	0.0	5.3	0.0	0.0	0.0	0.5	0.0	0.2	0.0	0.0	0.3	0.2	0.0	**93.6**	0.0
[fu]*Drechslera*-like spore		0.0	0.0	0.0	0.0	2.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	**98.0**
Total misidentification (%)		**0.29**	**0.63**	**0.63**	**0.04**	**1.04**	**0.15**	**1.20**	**0.36**	**0.32**	**0.16**	**1.08**	**0.93**	**4.55**	**1.76**	**1.41**	**0.10**

All values are given as a percentages. Types of airspora: [fe]fern spores, [fu]fungal spores and [p]pollen.

Table 3.4: Canonical discrimination coefficients for the local airspora.

	Function (Percentage of the total sample dispersion accounted for by the canonical variables in brackets)														
	1 (54.2%)	2 (71.3%)	3 (85.0%)	4 (89.1%)	5 (93.1%)	6 (95.4%)	7 (97.3%)	8 (98.4%)	9 (99.1%)	10 (99.5%)	11 (99.7%)	12 (99.8%)	13 (99.9%)	14 (100%)	15 (100%)
Area	**-2.292**	**2.187**	**2.370**	**2.165**	-0.538	**-1.347**	0.046	-0.432	-0.442	**-1.333**	-0.472	-0.110	0.615	0.606	**-2.260**
Aspect	0.378	0.115	-0.341	0.774	**1.169**	0.649	**1.118**	-0.231	-0.357	0.024	0.071	0.564	-0.263	-0.159	0.433
Area/Box	-0.038	0.104	0.084	-0.129	-0.014	0.041	0.084	0.307	0.526	-0.487	-0.411	-0.533	-0.392	-0.208	-0.498
Box X/Y	0.031	-0.087	0.052	-0.093	-0.022	0.021	-0.112	-0.253	-0.345	0.256	0.133	0.419	0.384	-0.105	0.194
Density (mean)	0.108	-0.127	-0.240	-0.212	0.228	0.701	-0.600	0.651	**-1.070**	**-1.886**	0.934	-0.681	0.016	-0.697	-0.121
Angle	0.006	-0.001	-0.007	-0.029	-0.012	-0.024	0.000	-0.007	-0.034	0.003	0.144	-0.024	-0.005	0.131	-0.192
Axis (major)	-0.709	-0.720	-0.820	**-1.201**	**-3.132**	**-2.322**	**-2.965**	**1.991**	**3.954**	0.897	-0.270	**-1.690**	**10.444**	**-8.005**	**-4.478**
Axis (minor)	**1.129**	-0.088	-0.424	**1.162**	-0.556	**-1.138**	**1.775**	0.622	0.471	-0.512	-0.656	-0.336	**2.791**	**-3.486**	**-2.338**
Diameter (max)	**1.475**	0.419	-0.123	**-1.375**	-0.631	**1.313**	**2.327**	0.318	**-1.346**	**-1.040**	**-1.403**	**1.045**	**-4.983**	**1.347**	**2.486**
Diameter (min)	-0.005	-0.236	0.347	0.428	0.074	0.236	**-1.261**	0.072	0.762	0.265	0.787	0.494	-0.414	-0.692	0.279
Diameter (mean)	0.090	0.449	-0.502	-0.482	-0.029	0.344	**2.199**	-0.322	**-1.092**	0.305	-0.247	-0.060	0.117	-0.113	**-1.378**
Radius (max)	-0.495	-0.586	-0.691	0.262	**-1.185**	**-1.401**	-0.155	**0.206**	-0.113	0.702	0.508	-0.651	**-1.539**	**-3.134**	**-1.020**
Radius (min)	-0.755	0.534	0.656	0.069	**-1.779**	**1.709**	**-2.099**	**-0.262**	**2.379**	-0.824	-0.003	0.603	**1.282**	0.698	0.625
Perimeter	0.389	0.179	-0.133	-0.877	0.552	**1.209**	-0.047	-0.482	0.306	**1.243**	**1.121**	-0.297	-0.371	0.367	**1.578**
Radius Ratio	0.166	0.027	0.140	-0.040	-0.058	0.210	0.091	-0.185	-0.057	0.018	0.000	0.146	0.110	0.079	-0.026
Roundness	-0.048	0.026	0.087	-0.002	-0.213	0.200	0.033	-0.125	0.106	-0.056	-0.096	0.078	0.009	0.014	0.125
Size (length)	**1.788**	**-1.947**	**3.545**	**1.606**	-0.532	**5.687**	**4.344**	**-3.410**	**-1.564**	0.547	0.897	-0.125	0.723	-0.720	**-1.626**
Size (width)	-0.335	0.000	-0.262	0.272	-0.682	-0.767	-0.882	0.171	-0.708	0.174	0.660	-0.455	**-2.469**	**1.129**	0.553
Perimeter convex	-0.230	0.130	0.056	0.130	0.609	-0.743	-0.139	0.222	0.279	**-1.379**	**-1.270**	**1.283**	**2.326**	**-1.939**	-0.534
Perimeter ellipse	**-1.800**	-0.986	0.151	**3.335**	**-2.888**	**-3.091**	**-2.037**	-0.138	**-3.638**	0.261	**1.168**	**-1.994**	**-10.234**	**5.569**	**1.339**
Area (polygon)	**1.068**	0.648	-0.937	**-2.564**	-0.379	**1.492**	0.113	0.352	-0.143	**1.638**	0.524	-0.211	-0.513	-0.590	**2.097**
Box Width	-0.215	-0.120	0.076	-0.003	0.744	0.608	-0.137	0.189	0.150	-0.126	-0.305	0.234	0.544	-0.510	**1.195**
Box Height	-0.145	-0.030	0.268	-0.356	0.519	0.430	-0.345	0.256	0.252	-0.270	-0.555	0.099	0.698	-0.848	**1.074**
Feret (min)	0.148	-0.140	0.370	0.115	0.639	-0.519	-0.586	0.324	-0.008	-0.070	-0.975	-0.175	**1.349**	**1.620**	-0.069
Feret (max)	**-1.695**	**1.907**	**-1.296**	-0.711	**2.574**	**-3.490**	**-4.522**	**2.074**	**2.404**	-0.956	-0.302	**1.395**	**3.111**	**6.932**	0.508
Feret (mean)	-0.054	0.176	-0.336	-0.229	-0.053	0.123	-0.347	0.114	-0.219	-0.343	0.117	0.558	-0.299	-0.047	-0.016

The magnitude of the canonical discrimination function coefficient indicates the weightage of the parameter in each factor in differentiating airspora. Parameters with high magnitudes in an earlier factor also carries a higher weightage in differentiating the airspora.

Table 3.4 (continued): Canonical discrimination coefficients for the local airspora.

	Function (Percentage of the total sample dispersion accounted for by the canonical variables in brackets)														
	1 (54.2%)	2 (71.3%)	3 (85.0%)	4 (89.1%)	5 (93.1%)	6 (95.4%)	7 (97.3%)	8 (98.4%)	9 (99.1%)	10 (99.5%)	11 (99.7%)	12 (99.8%)	13 (99.9%)	14 (100%)	15 (100%)
Density (min)	-0.174	0.158	0.348	0.332	-0.438	-0.856	**1.128**	0.083	**1.052**	0.817	-0.500	0.384	0.418	**1.050**	**1.004**
Density (max)	0.141	-0.239	-0.026	-0.199	-0.498	-0.765	0.978	0.212	**1.659**	**3.185**	-0.791	**2.974**	-0.561	**1.039**	**-1.923**
Density (std.dev.)	-0.846	0.756	0.761	0.792	0.450	-0.150	0.321	0.102	-0.131	-0.865	-0.252	**-2.362**	0.812	-0.083	2.853
Dendritic length	-0.121	-0.030	0.093	0.263	-0.097	-0.057	0.231	-0.066	-0.317	-0.354	0.195	-0.309	0.404	-0.182	0.324
End points	0.135	-0.033	0.025	-0.269	0.168	0.012	-0.227	0.080	0.080	0.279	0.419	0.186	-0.373	0.168	-0.667
Form Factor	0.204	0.138	0.002	-0.784	0.822	**-1.566**	0.115	-0.081	-0.400	0.000	0.511	0.928	-0.838	**-1.187**	0.695
ECD	**2.643**	**-1.159**	**-2.381**	**-2.236**	**4.535**	**2.600**	**1.936**	**-1.268**	0.357	-0.321	0.391	0.893	**-1.975**	**1.710**	**2.240**
Compactness	0.336	-0.167	0.980	-0.598	-0.060	0.518	0.121	-0.448	-0.306	0.298	0.284	**1.164**	0.264	-0.166	0.986
Box ratio	0.011	-0.194	-0.121	0.106	0.169	0.179	0.026	-0.195	-0.399	0.468	0.361	0.320	0.328	0.237	0.677
MODR	0.521	-0.679	-0.689	0.713	0.748	0.409	2.026	0.209	**-1.919**	**1.016**	-0.526	**-1.773**	-0.244	0.688	**-1.304**

The magnitude of the canonical discrimination function coefficient indicates the weightage of the parameter in each factor in differentiating airspora. Parameter with high magnitudes in an earlier factor also carries a higher weightage in differentiating the airspora. Abbreviated parameters: ECD = equivalent circular diameter, MODR = modification ratio.

Table 3.5: Means of important parameters used in local airspora identification.

Airspora types	Area	Minor axis	Maximum diameter	Length size	Area polygon	ECD
Acacia auriculiformis	1873.68	45.95	52.32e	51.89	1848.83	6.93
Asplenium nidus	1242.72	33.21	48.41	48.92	1217.54	5.67a
Casuarina equisetifolia	633.61	27.1	29.86	29.71ab	618.53b	4.05
Cladosporium cladosporioides	9.12	2.31a	4.9abcd	5.04	7.03	0.48
Curvularia spp.	137.14	9.24	18.9	19.23	130.22	1.87
Didymosphaeria donacina	300.32	8.51	31.99cd	32.81c	265.16	2.3
Dicranopteris linearis	649.72	26.99a	31.49bcd	31.63bc	633.54b	4.09
Elaeis guineensis	634.15	26.79a	31.41bcd	33.2c	615.45b	4.05b
Nephrolepis auriculata	514.14a	20.75	31.42bc	31.54bc	498.94a	3.65ab
Pithomyces maydicus	80.55	7.09	14.13	14.25	74.78	1.44
Podocarpus polystachyus	1385.79	33.65	52.28e	52.28	1356.28	5.98a
Stenochlaena palustris	994.73	29.73	42.78	43.6	976.79c	5.06b
Kyllingia polyphylla	498.02a	22.16	28.96ad	28.97a	483.54a	3.59
Dicranopteris curranii	632.83	24.32	32.49d	32.5c	616.32b	4.02
Cynodon dactylon	647.14	27.99	29.34ab	29.14ab	630.99b	4.09
Drechslera-like spore	809.88	15.70	74.66	75.33	945.31c	4.43

All the means for the parameters were statistically unequal. Post-hoc Tukey's HSD results are shown. Airspora types with the same letter are statistically not significantly different from each other with a minimum p = 0.05. Abbreviated parameter: ECD = equivalent circular diameter.

3.3.2 Grass (Poaceae) pollen

In this study, a total of 22 types of grass pollen were studied (Figure 3.3). One hundred percent accuracy was achieved for the pollen grain of *Zea mays* (Table 3.6). Above 90% accuracy was obtained for those of *Avena sativa* (90.0%), *Agrostis alba* (92.8%), *Cynodon dactylon* (97.8%), *Lolium perenne* (91.3%), *Phleum pratense* (92.6%), S*orghum halepensis* (90.4%), *Secale cereale* (90.8%) and *Triticum aestivum* (98.8%). More then 80% accuracy were obtained for the pollen grains of *Paspalum notatum* (85.0%), *Poa compressa* (85.0%), *Bromus inermis* (86.0%) and *Festuca elatoir* (83.0%). An accuracy above 50% was obtained for those of *Agropyron repens* (65.5%), *Agropyron smith* (59.6%), *Anthoxanthum odoratum* (72.5%), *Dactylis glomerata* (79.6%) and *Elymus condensatus* (61.2%).

Only the pollen grains of *Lolium multiflorum* (43.0%) and *Phalaris arundinacea* (24.8%) were misidentified more then 50% of the time. Pollen grains of *Lolium multiflorum* tend to be misidentified as those of *Anthoxanthum odoratum* (17.6% of the time) and *Festuca elatoir* (12.4%) while *Phalaris arundinaceae* tends to be misidentified as *Paspalum notatum* (16.5% of the time) or *Poa compressa* (27.6%).

Misidentification rates of more then 10% were observed in pollen types being classified within the Pooideae except for those of *Phalaris arundinaceae* (16.5%) and *Dactylis glomerata* (14.2%) which was misidentified as *Paspalum notatum* belonging to the Panicoideae.

Most grasses that were misidentified were mistaken as *Poa compressa*. In total, 2.79% of grass pollen grains were misidentified as those of *Poa compressa*.

Forty measured parameters were used for this classification (Table 3.7). Eighteen canonical functions were needed to differentiate 100% of the samples. Integrated optical density (IOD), mean density, minor axis, perimeter and red density were parameters found to be important in differentiating the grass pollen types.

The mean values for all the parameters used were not equal when ANOVA ($p < 0.001$) was performed. Subsequently, post-hoc Tukey's analysis was performed. Tukey's HSD results for discriminating parameters are shown in Table 3.8.

Table 3.6: Identification accuracies of grass pollen types by step-wise canonical discriminate analysis.

Poaceae types / Identified as	[pa]*Paspalum notatum*	[c]*Cynodon dactylon*	[po]*Poa compressa*	[po]*Bromus inermis*	[po]*Phalaris arundinacea*	[pa]*Zea mays*	[po]*Agropyron repens*	[pa]*Sorghum halepensis*	[po]*Poa pratensis*	[po]*Festuca elatior*	[po]*Avena sativa*	[po]*Dactylis glomerata*	[po]*Agrostis alba*	[po]*Secale cereale*	[po]*Elymus condensatus*	[po]*Lolium multiflorum*	[po]*Lolium perenne*	[po]*Anthoxanthum odoratum*	[po]*Phleum pratense*	[po]*Holcus lanatus*	[po]*Triticum aestivum*	[po]*Agropyron smithi*
Actual identity																						
[pa]***Paspalum notatum***	**85.0**	1.1	8.9	0.5	0.9	0.0	0.1	0.0	0.0	0.0	0.0	1.4	0.3	0.0	0.0	0.0	0.1	1.0	0.1	0.6	0.0	0.1
[c]***Cynodon dactylon***	0.2	**97.8**	0.8	0.0	0.2	0.0	0.0	0.0	0.0	0.0	0.0	0.0	1.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
[po]***Poa compressa***	3.4	0.3	**85.0**	0.2	2.1	0.0	0.5	0.1	0.2	0.3	0.0	0.6	2.6	0.0	0.2	0.3	0.1	3.5	0.0	0.6	0.0	0.1
[po]***Bromus inermis***	0.9	0.0	1.5	**86.0**	0.4	0.2	1.1	3.1	0.0	0.4	0.0	0.4	0.0	0.2	3.4	0.2	0.0	0.2	0.0	0.0	0.0	2.2
[po]***Phalaris arundinacea***	16.5	0.3	27.6	0.2	24.8	0.0	2.7	0.5	0.8	3.7	0.0	2.0	4.9	0.0	0.3	0.5	0.3	8.4	0.0	5.7	0.0	0.9
[pa]***Zea mays***	0.0	0.0	0.0	0.0	0.0	**100.0**	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
[po]***Agropyron repens***	0.1	0.0	1.1	0.6	0.4	0.0	65.5	10.0	0.0	7.1	1.3	0.3	0.0	1.4	0.8	2.0	0.0	3.3	0.0	0.2	0.0	5.9
[pa]***Sorghum halepensis***	0.0	0.0	1.6	0.8	0.1	0.1	2.3	**90.4**	0.0	0.0	0.0	0.0	0.0	0.1	2.5	0.2	0.0	0.5	0.0	0.0	0.0	1.3
[po]***Poa pratensis***	0.2	0.1	0.0	0.0	0.3	0.0	0.0	0.0	**93.0**	0.4	0.0	0.2	0.6	0.0	0.0	0.0	0.1	0.1	0.0	5.0	0.0	0.0
[po]***Festuca elatior***	1.3	0.0	1.3	0.2	0.7	0.0	3.1	0.0	0.2	**83.0**	0.0	1.2	0.0	0.0	0.1	1.0	0.0	4.8	0.0	2.1	0.0	1.0
[po]***Avena sativa***	0.0	0.0	0.0	0.0	0.0	0.0	1.5	0.0	0.0	0.0	**90.0**	0.0	0.0	1.8	0.0	0.6	0.0	0.9	0.0	0.0	0.1	5.1
[po]***Dactylis glomerata***	14.2	0.0	1.1	0.1	0.4	0.0	0.2	0.1	0.8	0.3	0.0	79.6	0.0	0.0	0.3	0.0	0.4	2.4	0.0	0.1	0.0	0.1
[po]***Agrostis alba***	0.4	0.9	0.5	0.1	2.1	0.0	0.1	0.0	1.7	0.0	0.0	0.2	**92.8**	0.0	0.0	0.0	0.0	0.0	0.0	1.3	0.0	0.0
[po]***Secale cereale***	0.0	0.0	0.0	0.0	0.0	0.7	1.5	0.0	0.0	0.0	3.1	0.0	0.0	**90.8**	0.0	0.9	0.0	0.0	0.0	0.0	0.0	3.0
[po]***Elymus condensatus***	0.3	0.0	1.8	12.5	0.5	0.1	2.1	17.0	0.0	0.1	0.0	0.1	0.0	0.3	61.2	0.2	0.0	1.1	0.0	0.0	0.0	2.7
[po]***Lolium multiflorum***	3.2	0.0	3.6	0.2	0.5	0.0	4.2	3.6	0.7	12.4	1.4	0.8	0.1	2.3	0.0	43.0	0.5	17.6	0.0	1.2	0.0	4.8
[po]***Lolium perenne***	0.2	0.0	0.1	0.0	0.0	0.0	0.0	0.0	0.4	0.0	0.0	0.3	0.0	0.0	0.0	0.1	**91.3**	0.7	6.8	0.0	0.1	0.1
[po]***Anthoxanthum odoratum***	4.6	0.1	9.2	0.1	1.0	0.0	1.9	0.1	0.6	4.3	0.3	1.2	0.5	0.0	0.3	1.7	0.6	72.5	0.2	0.5	0.0	0.4
[po]***Phleum pratense***	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	7.0	0.0	**92.6**	0.0	0.0	0.3
[po]***Holcus lanatus***	0.6	0.0	1.1	0.0	1.1	0.0	0.4	0.0	4.9	0.5	0.0	1.5	3.5	0.0	0.0	0.1	0.5	0.5	0.0	**85.5**	0.0	0.0
[po]***Triticum aestivum***	0.0	0.0	0.0	0.0	0.0	0.4	0.0	0.0	0.0	0.0	0.1	0.0	0.0	0.1	0.0	0.0	0.4	0.0	0.2	0.0	**98.8**	0.0
[po]***Agropyron smithi***	0.0	0.0	0.0	1.4	0.1	0.0	13.9	3.0	0.0	1.5	5.1	0.0	0.0	6.4	0.5	5.9	0.0	2.5	0.1	0.0	0.0	59.6
Total misidentification (%)	**2.19**	**0.14**	**2.79**	**0.77**	**0.5**	**0.07**	**1.65**	**1.69**	**0.5**	**1.47**	**0.54**	**0.47**	**0.62**	**0.6**	**0.31**	**0.65**	**0.46**	**2.26**	**0.36**	**0.83**	**0.01**	**1.26**

All values are given in percentages. Subfamily: [c] Chloridoideae, [pa]Panicoideae and [po]Pooideae.

Table 3.7: Canonical discrimination coefficients for grass pollen types.

	Function (Percentage of the total sample dispersion accounted for by the canonical variables in brackets)									
	1(53.3%)	2(70%)	3(82%)	4(88.1%)	5(92.5%)	6(94.3%)	7(95.9%)	8(97.1%)	9(97.9%)	10(98.6%)
Area	0.270	**2.376**	**2.494**	**-1.925**	**-3.485**	**-3.657**	**-2.698**	**-2.069**	-0.516	0.514
Aspect	0.371	-0.814	0.868	0.029	0.994	0.489	0.977	**1.719**	0.139	**1.001**
Area/Box	0.032	-0.122	0.023	-0.061	-0.228	0.079	-0.220	-0.265	0.240	0.416
Box X/Y	-0.032	-0.059	-0.037	-0.046	0.010	-0.002	-0.027	0.070	-0.073	0.021
Density (mean)	**-1.782**	**2.280**	-0.580	**4.921**	**1.376**	**-2.258**	**-2.119**	**1.390**	**1.479**	-0.219
Angle	-0.004	0.011	0.001	0.003	0.013	-0.002	0.026	0.014	0.030	-0.002
Axis (major)	-0.553	**1.986**	**-2.283**	**1.015**	0.663	-0.687	3.096	0.292	0.920	**-2.014**
Axis (minor)	**-1.677**	-0.295	**-2.720**	**-2.112**	0.768	**-1.662**	**-1.257**	**1.583**	-0.198	-0.510
Diameter (max)	-0.712	**-1.041**	-0.762	0.825	0.851	0.270	0.052	**-1.397**	0.492	**-3.951**
Diameter (min)	0.217	0.398	0.185	0.900	0.208	-0.173	**1.239**	0.022	**-1.073**	**1.406**
Diameter (mean)	0.630	**1.913**	-0.923	-0.074	**-2.269**	0.915	-3.663	**-2.264**	**-6.747**	**3.821**
Radius (max)	0.290	0.188	0.628	-0.289	-0.482	-0.100	0.108	0.575	0.266	**1.958**
Radius (min)	0.819	**-1.134**	0.694	**2.043**	**3.518**	**2.537**	**2.444**	**3.400**	0.933	-0.432
Perimeter	**1.234**	**-1.003**	**1.908**	0.557	**1.158**	0.329	**2.009**	**1.737**	**2.828**	**2.272**
Radius ratio	-0.370	0.130	-0.368	-0.177	-0.399	-0.244	-0.556	-0.357	0.221	-0.206
Roundness	-0.556	0.551	-0.654	-0.264	-0.661	-0.365	-0.639	-0.643	-0.189	-0.289
Density (red)	**1.253**	**-1.533**	0.011	**-2.627**	**-1.066**	**1.490**	**1.509**	-0.593	**-1.779**	0.801
Density (green)	0.508	-0.362	0.932	**-2.535**	0.062	-0.137	0.924	**-1.063**	-0.184	-0.337
Size (length)	0.500	0.752	**1.618**	0.600	0.942	-0.175	1.350	-0.092	-2.208	**-1.149**
Size (width)	0.260	0.286	-0.006	0.191	0.557	0.583	0.217	-0.366	-0.684	-0.499
IOD	**-1.532**	**-1.963**	**-2.161**	0.768	0.145	**2.669**	0.939	-0.035	**1.014**	-0.601
Perimeter (convex)	0.303	-0.641	-0.068	-0.242	0.752	0.264	-0.125	0.035	**-1.204**	**-1.263**
Perimeter (ratio)	-0.117	0.332	0.012	0.056	-0.389	-0.155	0.020	-0.075	0.718	0.749
Fractal Dim.	0.205	-0.006	0.321	-0.005	-0.003	-0.267	0.095	-0.130	**1.099**	0.087
Box Width	0.177	-0.370	0.058	-0.331	**-1.015**	0.327	-0.913	-0.929	**1.380**	**1.369**
Box Height	0.079	-0.501	-0.006	-0.534	-0.957	0.267	**-1.071**	-0.629	**1.308**	**1.457**
Feret (min)	-0.071	-0.067	0.741	-0.202	-0.564	-0.224	-0.433	**1.250**	**1.110**	**-1.367**
Feret (max)	0.660	0.217	0.114	0.033	-0.034	-0.037	**-1.181**	-0.285	-0.148	**-1.410**
Density (min)	0.208	-0.252	-0.003	-0.101	0.262	0.361	-0.307	0.120	-0.204	-0.136
Density (max)	-0.065	0.441	-0.209	0.255	-0.311	0.142	-0.067	-0.007	-0.295	-0.224
Density (std.dev.)	-0.125	0.013	0.387	-0.620	**1.809**	0.795	**-1.174**	-0.949	-0.239	-0.189
Dendritic length	0.017	-0.023	0.000	0.046	0.039	-0.028	0.023	0.006	-0.165	0.201
Dendrites	0.091	0.090	0.063	-0.100	-0.046	-0.031	-0.063	-0.037	0.068	-0.025
Margination	-0.093	0.066	0.098	-0.333	0.128	0.272	0.195	0.044	0.214	-0.560
Heterogeneity	0.209	0.680	-0.693	0.104	**-1.411**	-0.436	**1.100**	0.997	0.287	0.345
Clumpiness	0.197	0.025	0.111	-0.020	-0.106	-0.062	-0.055	-0.028	-0.063	0.013
Form Factor	**1.606**	**-1.573**	**2.247**	0.054	**1.678**	-0.265	**1.646**	**1.403**	**3.748**	0.875
Compactness	**1.400**	-0.051	**1.785**	**1.628**	**2.334**	**1.367**	**1.602**	**1.936**	-0.768	-0.126
MODR	-0.890	0.497	-0.577	**-1.278**	**-1.691**	**-1.490**	-0.650	**-1.471**	0.069	-0.942
Concavity	0.079	0.013	0.027	-0.010	-0.016	-0.146	0.076	-0.012	0.280	0.104

The magnitude of the canonical discrimination function coefficient indicates the weightage of the parameter in each factor for differentiating airspora. Parameters with high magnitudes in an earlier factor also carry a higher weightage in differentiating the airspora components. Abbreviated parameters: IOD = integrated optical density, MODR = modification ratio.

Table 3.7 (continued): Canonical discrimination coefficients for grass pollen types.

	Function (Percentage of the total sample dispersion accounted for by the canonical variables in brackets)										
	11(99%)	12(99.3%)	13(99.6%)	14(99.7%)	15(99.8%)	16(99.9%)	17(99.9%)	18(100%)	19(100%)	20(100%)	21(100%)
Area	**-1.358**	**1.948**	0.545	0.620	**-1.322**	**1.184**	**-3.882**	**-1.192**	**-3.320**	**1.512**	0.135
Aspect	**-1.446**	0.514	0.989	**2.132**	0.980	**-1.208**	**2.406**	**-1.469**	-0.810	-0.176	**2.288**
Area/Box	-0.078	-0.094	-0.537	-0.207	-0.413	**1.167**	-0.183	0.169	-0.043	-0.185	0.599
Box X/Y	-0.109	0.106	-0.048	0.138	-0.107	-0.083	0.226	-0.248	-0.133	-0.029	0.156
Density (mean)	0.287	-0.776	0.717	1.328	-0.240	**1.513**	-0.351	-0.235	**-1.204**	-0.218	0.180
Angle	-0.029	0.000	-0.007	-0.020	0.003	-0.075	0.101	-0.180	-0.103	-0.072	-0.070
Axis (major)	**7.356**	**-2.923**	**-7.222**	**-9.365**	**-2.602**	-0.558	**-2.430**	**-1.846**	-0.665	0.659	**-3.086**
Axis (minor)	**2.535**	**-1.628**	**1.082**	**-3.615**	**5.714**	**-2.132**	**3.361**	**-2.369**	**-1.896**	**7.900**	**2.911**
Diameter (max)	**-2.754**	**2.133**	**3.776**	**-1.915**	**-1.527**	**1.031**	-1.703	-0.638	**2.084**	**-2.851**	**2.820**
Diameter (min)	0.503	**2.089**	**-1.780**	**1.207**	0.681	**2.648**	0.614	-0.048	**-1.103**	**-7.655**	**-3.162**
Diameter (mean)	**-2.591**	**1.923**	0.623	**10.031**	**2.164**	**-4.083**	**-2.893**	**3.582**	**-1.328**	-0.109	-0.250
Radius (max)	**1.163**	**-1.827**	**-2.428**	**1.707**	**1.141**	-0.375	0.654	**-1.388**	-0.218	0.532	**-2.382**
Radius (min)	-0.884	**-0.521**	0.896	**-3.103**	**-3.987**	**-2.298**	0.652	0.876	0.764	**5.559**	**-2.171**
Perimeter	**-1.910**	**3.241**	**1.867**	**3.665**	**2.837**	-0.064	**3.891**	**2.452**	-0.511	**-1.164**	**-3.361**
Radius ratio	0.221	0.058	0.072	-0.618	-0.846	0.114	-0.849	0.672	0.563	0.277	**-1.441**
Roundness	**1.287**	-0.550	**-1.168**	**-1.717**	-1.044	0.342	**-1.790**	0.730	0.159	0.536	**-1.664**
Density (red)	**1.552**	0.511	**1.000**	-0.341	-0.541	0.184	-0.165	-0.040	0.453	0.468	-0.055
Density (green)	**-1.877**	0.395	**-1.505**	-0.727	0.448	**-1.589**	0.156	0.122	0.459	-0.097	-0.139
Size (length)	0.001	**1.884**	**2.956**	**1.292**	4.184	**-1.019**	**-1.275**	0.950	**2.262**	-0.022	**3.057**
Size (width)	0.721	**-2.834**	**-1.314**	-0.498	0.802	-0.770	**2.609**	**-2.286**	**-1.524**	**2.248**	0.963
IOD	**1.658**	**-1.223**	**-1.829**	**-1.274**	0.215	0.358	**3.927**	0.423	**3.196**	**-1.110**	-0.368
Perimeter (convex)	**-1.073**	**-3.042**	**1.199**	0.945	-0.925	**-1.923**	-0.727	**-2.165**	0.657	-0.694	**4.466**
Perimeter (ratio)	0.778	**1.344**	-0.513	-0.439	0.384	0.690	0.195	**1.225**	0.059	0.113	-0.864
Fractal Dim.	0.138	-0.396	0.723	0.628	0.147	-0.302	0.123	0.833	0.646	0.211	0.113
Box Width	0.432	-0.627	**-2.440**	**-1.306**	-1.051	**3.802**	-0.390	**1.122**	-0.079	-0.028	**2.510**
Box Height	0.335	-0.408	**-2.485**	**-1.101**	-1.269	**3.657**	0.160	0.518	-0.326	-0.126	**2.956**
Feret (min)	-0.975	**3.078**	**1.338**	-0.898	-5.882	0.708	**-5.775**	0.100	**4.103**	**-7.193**	-0.039
Feret (max)	**-1.293**	-0.321	**2.300**	-0.494	0.000	**1.539**	**3.587**	0.923	**-2.470**	**2.427**	-2.778
Density (min)	-0.376	0.062	-0.644	-0.052	-0.066	0.438	-0.313	0.288	0.244	0.402	0.148
Density (max)	0.170	0.403	-0.268	0.082	-0.206	-0.530	0.003	0.036	0.139	0.140	-0.016
Density (std.dev.)	0.354	0.516	-0.763	0.219	-0.504	0.507	-0.364	0.209	0.089	0.640	0.242
Dendritic length	0.043	-0.213	-0.018	0.094	0.112	0.027	-0.118	-0.374	-0.094	0.489	-0.242
Dendrites	-0.067	-0.058	0.088	-0.077	-0.428	0.007	0.256	0.649	0.004	-0.420	0.253
Margination	0.196	0.113	-0.138	0.604	-0.212	0.098	-0.019	-0.087	-0.255	0.009	0.041
Heterogeneity	-0.540	-0.664	0.753	-0.405	0.565	-0.104	0.217	-0.133	0.000	-0.518	-0.184
Clumpiness	0.010	0.129	-0.084	0.591	0.015	0.051	0.008	-0.061	0.519	0.150	-0.037
Form Factor	**-2.217**	**-1.420**	**3.959**	**4.587**	1.472	**-2.363**	**1.063**	**1.526**	**1.134**	-0.262	**-2.687**
Compactness	0.027	-0.614	0.770	-0.006	0.766	**-1.146**	0.313	0.205	0.275	-0.440	0.088
MODR	0.361	0.362	-0.348	0.516	1.241	0.596	0.512	-0.436	-0.343	0.135	**1.070**
Concavity	-0.007	-0.076	0.001	-0.135	0.026	0.045	0.047	-0.280	0.402	-0.016	0.069

The magnitude of the canonical discrimination function coefficient indicates the weightage of the parameter in each factor for differentiating airspora. Parameters with high magnitudes in an earlier factor also carry a higher weightage in differentiating the airspora components. Abbreviated parameters: IOD = integrated optical density, MODR = modification ratio.

Table 3.8: Means of important parameters used in grass pollen identification.

Grass pollen types	Area	Aspect	Major axis	Minor axis	Max imum diameter	Mean diameter	Max imum radius	Perimeter	Roundness	IOD	Convex perimeter	Maximum feret	Form factor
Paspalum notatum	844.0bc	1.1181i	34.6c	31.0a	34.4b	32.4b	17.4b	103.6ab	1.0375ab	151109c	102.6b	34.4bc	102.1c
Cynodon dactylon	647.1a	1.0466a	29.3	28.0	29.3a	28.4a	14.9a	92.5	1.0911cd	126693a	89.6a	29.4a	87.7a
Poa compressa	902.7c	1.0546ab	34.6ac	32.9	34.6	33.5	17.5b	106.5c	1.0314ab	165613	105.7	34.7c	105.7
Bromus inermis	1557.4e	1.0779eg	46d	42.8d	46.1e	44.1d	23.4e	145.1fh	1.1171def	278750e	140.8d	46.1f	134.6e
Phalaris arundinacea	980.5	1.0962h	36.7	33.6	36.6	34.9	18.6	111.1	1.0361ab	178934	110.1	36.6	109.7
Zea mays	5728.2	1.127i	90.0	80.2	90.1	84.7	46.1	280.0	1.1201def	975701	269.1	90.3	254.9
Agropyron repens	1561.0e	1.0731cdef	45.9d	42.8d	46.1e	44.1d	23.5e	144.6ef	1.1073de	280566ef	139.9d	46.2f	134.9e
Sorghum halepensis	1592.1ef	1.0548ab	46.1d	43.7	46.1e	44.6d	23.3e	142.2e	1.037ab	277920e	140.9d	46.2f	140.1d
Poa pratensis	491.9	1.1263i	26.4	23.6	26.2cd	24.7c	13.3	78.4	1.0484ab	89142	78.0	26.2	78.3
Festuca elatior	1128.9d	1.0939gh	39.5	36.2b	39.5	37.5	20cd	121.1d	1.0648bc	203900	118.8c	39.5de	116.8
Avena sativa	1934.0	1.2068j	54.3	45.3	54.8	49.5	28.0	164.8	1.1491f	339964	157.9	54.9	147.7
Dactylis glomerata	820.5b	1.0924gh	33.7b	30.9a	33.9b	31.9b	17.4b	106.0bc	1.1282de	139016b	101.9b	34bc	97.1b
Agrostis alba	629.5a	1.0773efg	29.3a	27.2	29.2a	27.9a	14.9a	88.8	1.0303ab	114514	88.2a	29.3a	88.5a
Secale cereale	2334.5	1.2071j	59.7	49.6	59.3	54.2e	30.1	174.5	1.0642bc	425351	172.3	59.3	167.8d
Elymus condensatus	1693.9g	1.0789fg	48.0	44.6	47.9	46.0	24.3	151.3	1.1373ef	301910d	147.2e	48.0	141.3
Lolium multiflorum	1286.6	1.1245i	42.6	37.9c	42.3	39.9	21.5	126.6	1.0302ab	228743	126.0	42.4	125.8
Lolium perenne	817.2b	1.1282i	34.1bc	30.3a	33.9b	31.8b	17.2b	102.2a	1.0537abc	142292bc	100.9b	33.9b	99.8c
Anthoxanthum odoratum	1223.0	1.0646bcdef	40.3	38.0c	40.2d	38.9	20.3d	122.8d	1.0175	222708d	122.3	40.2e	123.1
Phleum pratense	1108.1d	1.0565abc	38.5	36.5b	38.8c	37.2c	19.8c	122.5d	1.1131def	191402	118.1c	38.9d	113.5
Holcus lanatus	734.2	1.062abcde	31.4	29.6	31.3	30.2	15.9	96.3	1.0377ab	134990ab	95.4	31.4	95.3b
Triticum aestivum	2961.1	1.0657bcdef	63.2	59.4	63.3	61.0	32.0	193.5	1.0256ab	516333	192.2	63.3	191.6
Agropyron smithi	1644.0fg	1.1566	49.0	42.5d	48.8	45.5e	25.0	150.0h	1.1259def	290442f	145.2e	48.9	137.6

All the means for the parameters were statistically unequal. Post-hoc Tukey's HSD results are shown. Airspora types with the same letter are statistically not significantly different from each other with a minimum p = 0.05. Abbreviated parameter: IOD=integrated optical density.

3.3.3 Asteraceae weed pollen types

A study of the pollen of the Asteraceae, a major source of weed allergens in temperate countries, was carried out (Figure 3.4). Twenty pollen types from species of *Ambrosia*, *Artemisia*, *Baccharis*, *Chrysanthemum*, *Eupatorium*, *Helianthus*, *Iva*, *Solidago*, *Taraxacum* and *Xanthium* were image analysed (Table 3.9).

Eleven pollen types could be identified with accuracies of more than 90%, viz., *Ambrosia acanthicarpa* (93.5%), *Artemisia californica* (90.3%), *Artemisia frigida* (94.3%), *Artemisia vulgaris* (95.3%), *Baccharis halimifolia* (93.3%), *Baccharis sarothroides* (91.6%), *Chrysanthemum leucanthemum* (99.4%), *Helianthus annuus* (99.0%), *Iva axillaries* (91.4%), *Solidago* sp. (95.6%) and *Xanthium commune* (93.0%). Four other pollen types, viz., *Ambrosia artemisiifolia* (85.9%), *Ambrosia psilostachya* (86.3%), *Ambrosia trifida* (87.0%) and *Iva xanthifolia* (83.9%) had accuracies between 80 to 90%. Accuracies between 50 to 80% were observed for the pollen of *Ambrosia deltoidea* (73.9%), *Iva angustifolia* (76.4%) and *Taraxacum officinale* (54.2%).

An accuracy of below 50% was obtained for two pollen types: *Eupatorium capillifolium* (49.3%) and *Hymenoclea salsola* (22.8%). *Eupatorium capillifolium* was mostly misidentified as *Baccharis halimifolia* 47.1% of the time. *Hymenoclea salsola* was also frequently misidentified as *Artemisia callifornica* (31.8% of the time), or *Ambrosia psilostachya* (16.3%) or *Iva axillaries* (13.5%).

Misidentification of more than 10% as another pollen type was also observed. *Ambrosia deltoidea* pollen grains were misidentified as those of *Ambrosia acanthicarpa* 12% of the time and *Iva angustifolia* pollen grains as *Ambrosia trifida* 12.4% of the time.

In total, 7.82% of weed pollen types studied were misidentified as those of *Baccharis halimifolia*.

Twenty-five parameters were used to differentiate the weed pollen types (Table 3.10). The first 15 canonical functions were sufficient to differentiate 100% of the samples. Box ratio, equivalent circular diameter, feret mean and integrated optical density (IOD) were important parameters in differentiating the weed pollen types.

The mean values for all the parameters used were not equal when ANOVA ($p < 0.001$) was performed. Subsequently, post-hoc Tukey's analysis was performed. Tukey's HSD results for discriminating parameters are shown in Table 3.11.

Table 3.9: Identification accuracies of the Asteraceae weed pollen types by step-wise canonical analysis.

Asteraceae / **Actual identity**	**Identified as**	[w]*Ambrosia acanthicarpa*	[w]*Ambrosia artemisiifolia*	[w]*Ambrosia del-toidea*	[w]*Ambrosia psilostachya*	[w]*Ambrosia trifida*	[w]*Artemisia californica*	[w]*Artemisia frigida*	[w]*Artemisia vulgaris*	[i]*Baccharis halimifolia*	[i]*Baccharis sarothroides*	[i]*Eupatorium capillifolium*	[i]*Chrysanthemum leucanthemum*	[i]*Helianthus annuus*	[w]*Hymenoclea salsola*	[w]*Iva angustifolia*	[w]*Iva axillaris*	[w]*Iva xanthifolia*	[i]*Solidago* spp.	[i]*Taraxacum officinale*	[w]*Xanthium commune*
[w]*Ambrosia acanthicarpa*		**93.5**	0.0	3.9	0.0	0.2	0.0	0.0	0.0	0.0	1.3	0.0	0.0	0.0	0.0	0.0	0.8	0.0	0.2	0.0	0.2
[w]*Ambrosia artemisiifolia*		0.0	**85.9**	2.6	0.0	0.1	0.2	0.0	7.5	0.4	0.7	0.0	0.6	0.0	0.0	0.0	1.1	0.6	0.1	0.3	0.0
[w]*Ambrosia deltoidea*		**12.0**	0.2	**73.9**	0.0	2.3	0.0	0.4	0.0	0.2	1.8	0.3	0.0	0.0	0.3	0.0	6.9	0.0	1.4	0.2	0.2
[w]*Ambrosia psilostachya*		0.0	0.0	0.1	**86.3**	1.3	4.1	0.0	0.0	3.5	0.1	0.2	0.2	0.0	1.1	0.2	1.2	0.0	0.0	0.0	1.8
[w]*Ambrosia trifida*		0.0	0.0	5.6	3.4	**87.0**	0.2	0.0	0.0	2.2	0.5	0.0	0.0	0.0	0.3	0.0	0.6	0.0	0.1	0.1	0.0
[w]*Artemisia californica*		0.0	0.8	0.3	1.3	0.0	**90.3**	0.2	0.6	1.1	0.0	0.7	1.0	0.0	0.8	0.0	1.0	0.1	0.0	0.1	1.8
[w]*Artemisia frigida*		0.0	0.1	1.9	0.0	0.0	0.1	**94.3**	0.0	0.1	0.2	0.3	0.0	0.0	0.0	0.0	1.8	0.0	1.1	0.1	0.0
[w]*Artemisia vulgaris*		0.0	1.7	0.0	0.0	0.0	0.8	0.1	**95.3**	0.0	0.0	0.0	0.2	0.1	0.0	0.0	0.0	0.7	0.0	1.2	0.0
[i]*Baccharis halimifolia*		0.0	0.0	0.3	2.3	0.2	1.2	0.1	0.0	**93.3**	0.4	1.7	0.0	0.0	0.0	0.1	0.0	0.0	0.2	0.2	0.0
[i]*Baccharis sarothroides*		1.7	0.0	1.8	0.2	0.3	0.0	0.0	0.0	1.6	**91.6**	0.2	0.0	0.0	0.5	0.0	0.4	0.0	1.7	0.0	0.1
[i]*Eupatorium capillifolium*		0.0	0.1	0.0	0.5	0.2	1.3	0.2	0.0	**47.1**	0.1	**49.3**	0.0	0.0	0.0	0.4	0.0	0.2	0.0	0.6	0.0
[i]*Chrysanthemum leucanthemum*		0.0	0.0	0.0	0.1	0.0	0.1	0.0	0.0	0.0	0.0	0.0	**99.4**	0.1	0.0	0.0	0.0	0.0	0.0	0.3	0.0
[i]*Helianthus annuus*		0.0	0.0	0.0	0.0	0.0	0.0	0.3	0.0	0.0	0.0	0.0	0.6	**99.0**	0.0	0.0	0.0	0.0	0.0	0.1	0.0
[w]*Hymenoclea salsola*		0.1	0.5	3.5	**16.3**	3.1	**31.8**	0.0	0.1	5.7	0.1	0.2	0.0	0.0	**22.8**	0.5	**13.5**	0.1	0.0	0.2	1.5
[w]*Iva angustifolia*		0.0	0.1	0.0	6.8	0.2	**12.4**	0.0	0.0	2.8	0.0	0.1	0.0	0.0	0.2	**76.4**	0.0	0.0	0.0	0.1	1.0
[w]*Iva axillaris*		2.5	0.3	2.6	0.0	0.2	0.7	0.3	0.3	0.0	0.2	0.0	0.1	0.0	0.3	0.0	**91.4**	0.1	0.5	0.2	0.4
[w]*Iva xanthifolia*		0.0	1.1	0.0	0.0	0.0	2.9	0.2	10.0	1.3	0.0	0.0	0.1	0.0	0.0	0.0	0.2	**83.9**	0.0	0.4	0.0
[i]*Solidago* spp.		0.0	0.0	0.4	0.0	0.0	0.0	1.0	0.0	0.3	2.2	0.0	0.0	0.0	0.0	0.0	0.5	0.0	**95.6**	0.0	0.0
[i]*Taraxacum officinale*		0.0	0.0	0.0	0.0	0.0	0.1	0.0	0.1	0.4	0.0	0.1	**39.0**	5.7	0.0	0.0	0.0	0.3	0.0	**54.2**	0.0
[w]*Xanthium commune*		0.0	0.0	0.0	1.9	0.0	1.7	0.0	0.0	0.0	0.0	0.0	0.3	0.0	0.4	0.0	2.5	0.0	0.1	0.1	**93.0**
Total misidentification (%)		**5.48**	**4.46**	**5.05**	**5.86**	**5.08**	**7.31**	**5.10**	**5.58**	**7.82**	**4.93**	**2.59**	**6.85**	**5.12**	**1.29**	**4.42**	**6.00**	**4.32**	**4.93**	**2.84**	**4.96**

Identification results are given in percentages. Route of pollination: [i]insect- or [w]wind-pollinated. Insect-pollinated pollen grains generally have longer spines than wind-pollinated ones.

Table 3.10: Canonical discrimination coefficients for the Asteraceae weed pollen types.

	Function (Percentage of the total sample dispersion accounted for by the canonical variables in brackets)									
	1(47.4%)	2(68%)	3(81.4%)	4(88.3%)	5(91.9%)	6(95.2%)	7(96.7%)	8(97.8%)	9(98.6%)	10(99.2%)
Aspect	0.092	-0.012	-0.050	0.148	0.073	-0.001	-0.093	0.459	0.134	0.142
Area/Box	-0.134	0.144	-0.169	0.156	0.392	-0.301	0.086	-0.091	-0.122	0.237
Box X/Y	-0.054	0.003	0.056	-0.123	-0.061	0.050	-0.066	-0.001	-0.001	0.023
Axis (major)	0.225	0.613	**-2.351**	**5.542**	-0.275	**-4.364**	-0.081	**-1.789**	**-2.026**	**-3.671**
Radius Ratio	-0.016	0.013	-0.046	-0.025	0.117	0.022	0.077	-0.014	-0.081	-0.046
Roundness	-0.058	0.030	-0.027	0.167	0.257	-0.173	-0.063	0.205	0.009	0.038
Density (red)	-0.240	-0.435	0.127	0.666	0.359	0.762	0.631	-0.029	0.102	0.503
Density (green)	0.170	-0.196	**1.062**	0.329	-0.223	0.009	-0.437	0.125	-0.031	-0.327
Density (blue)	0.437	0.741	-0.140	**-1.085**	-0.070	**-1.060**	-0.104	-0.183	-0.063	-0.135
IOD	**1.877**	-0.747	**-1.390**	**1.556**	0.817	-0.931	-0.378	0.315	-0.560	0.530
Perimeter (ratio)	-0.145	0.127	-0.029	0.358	0.380	-0.367	0.417	0.611	0.577	0.045
Fractal Dim.	0.036	0.072	0.044	-0.032	0.668	0.062	0.256	0.194	-0.075	0.015
Feret (mean)	**-5.449**	**2.316**	**5.711**	**-8.690**	-0.440	**6.162**	**1.648**	-0.676	**2.115**	**3.295**
Density (min)	0.105	-0.201	-0.164	0.432	0.298	0.141	-0.653	-0.003	0.229	0.567
Density (max)	-0.192	0.038	-0.304	-0.182	-0.245	0.133	-0.210	0.667	-0.078	0.446
Density (std.dev.)	0.738	0.566	0.073	0.609	0.264	0.429	-0.304	0.206	-0.460	0.275
Dendritic length	-0.022	0.008	-0.062	0.122	0.032	-0.101	-0.052	-0.018	-0.035	0.033
Dendrites	0.166	-0.115	0.132	-0.439	0.241	0.189	0.373	0.384	0.501	-0.013
Margination	0.177	0.143	0.119	-0.033	-0.460	-0.010	0.589	0.074	-0.132	0.389
Heterogeneity	0.068	-0.017	-0.201	-0.052	0.004	-0.110	0.248	-0.611	0.816	-0.016
Clumpiness	0.017	-0.006	0.077	-0.052	0.005	-0.012	-0.020	0.218	0.249	0.041
ECD	**5.300**	**-2.813**	**-3.828**	**4.999**	0.889	**-2.767**	**-1.966**	**4.022**	0.974	**-1.483**
Compactness	-0.725	0.455	0.126	0.247	-0.122	0.185	-0.313	0.824	0.578	-0.526
Box ratio	**-2.302**	0.837	**1.846**	**-2.977**	**-1.441**	**2.074**	0.468	**-1.061**	0.074	0.443
MODR	0.100	-0.028	-0.110	-0.322	0.674	0.078	0.323	-0.204	-0.524	-0.028

The magnitude of the canonical discrimination function coefficient indicates the weightage of the parameter for each factor in differentiating airspora types. Parameters with high magnitudes in an earlier factor also carry a higher weightage in differentiating the airspora. Abbreviated parameters: ECD = equivalent circular diameter, IOD = integrated optical density, MODR = modification ratio.

Table 3.10 (continued): Canonical discrimination coefficients for the Asteraceae weed pollen types.

	Function (Percentage of the total sample dispersion accounted for by the canonical variables in brackets)								
	11(99.5%)	12(99.7%)	13(99.9%)	14(99.9%)	15(100%)	16(100%)	17(100%)	18(100%)	19(100%)
Aspect	0.163	-0.158	0.276	0.475	-0.485	0.204	0.803	0.285	0.708
Area/Box	-0.193	0.096	0.636	0.084	0.145	0.932	-0.176	-0.161	-0.252
Box X/Y	-0.024	-0.118	0.015	0.085	0.270	-0.174	0.682	-0.171	-0.370
Axis (major)	0.611	0.312	**-2.677**	**2.084**	**3.029**	**1.305**	**2.936**	**11.207**	-8.416
Radius Ratio	0.162	0.130	0.043	-0.046	0.113	-0.052	-0.134	-0.249	0.064
Roundness	0.153	-0.132	0.197	-0.047	-0.725	0.148	0.105	-0.474	-0.142
Density (red)	-0.135	-0.300	**-1.392**	0.622	-0.290	0.540	-0.021	-0.275	-0.248
Density (green)	0.302	0.216	**1.640**	-0.360	0.182	-0.687	-0.009	0.205	0.203
Density (blue)	-0.083	0.035	-0.529	-0.001	0.033	0.240	-0.003	0.070	0.016
IOD	-1.588	0.604	0.696	0.991	-0.321	-0.069	0.169	**2.322**	-0.757
Perimeter (ratio)	-0.120	-0.558	-0.311	-0.545	0.479	-0.598	-0.010	0.010	0.389
Fractal Dim.	0.273	-0.209	-0.261	0.075	**1.012**	-0.262	0.076	0.151	0.591
Feret (mean)	3.838	**-2.175**	**2.952**	**-5.401**	**-1.985**	**1.107**	**-4.469**	**-14.878**	**3.187**
Density (min)	0.264	0.394	-0.172	-0.490	0.055	0.081	0.087	0.060	-0.009
Density (max)	0.302	-0.394	0.270	0.393	-0.093	-0.083	-0.145	0.000	-0.047
Density (std.dev.)	-0.135	0.358	-0.277	-0.719	0.069	0.200	0.199	-0.085	-0.030
Dendritic length	-0.081	0.046	0.083	0.162	0.134	0.316	0.059	-0.226	0.734
Dendrites	-0.135	-0.124	0.148	-0.459	-0.128	0.126	-0.050	0.313	-0.522
Margination	0.241	0.485	0.068	-0.025	0.126	0.050	0.084	0.147	0.017
Heterogeneity	0.321	-0.133	0.261	0.583	-0.058	-0.206	-0.167	0.094	-0.014
Clumpiness	-0.267	0.345	0.003	0.286	0.176	-0.173	0.064	-0.596	-0.045
ECD	-3.983	**3.028**	0.521	**4.432**	**-2.511**	0.809	**2.192**	**6.572**	**2.179**
Compactness	-0.304	0.586	-0.176	0.702	-0.511	0.224	0.437	**1.073**	0.400
Box ratio	1.366	**-1.390**	**-1.808**	**-1.725**	**1.594**	**-2.937**	-0.584	**-4.438**	**3.564**
MODR	1.028	0.086	0.061	-0.066	-0.033	-0.355	0.110	-0.445	-0.116

The magnitude of the canonical discrimination function coefficient indicates the weightage of the parameter for each factor in differentiating airspora types. Parameters with high magnitudes in an earlier factor also carry a higher weightage in differentiating the airspora. Abbreviated parameters: ECD = equivalent circular diameter, IOD = integrated optical density, MODR = modification ratio.

Table 3.11: Means of important parameters used in identification of the Asteraceae pollen types.

Asteraceae pollens	IOD	Feret mean	ECD	Box ratio
Ambrosia acanthicarpa	69486a	21.6bcd	0.7515gh	0.992abc
Ambrosia artemisiifolia	59155a	20.0abcd	0.7234cdef	1.000bcd
Ambrosia deltoidea	56854a	19.5abc	0.7152cde	0.997bcd
Ambrosia psilostachya	54632a	19.9abcd	0.7216cdef	0.996bcd
Ambrosia trifida	53081a	19.5abc	0.7149cd	1.000bcd
Artemisia californica	69401a	21.5bcd	0.7523gh	1.000bcd
Artemisia frigida	72795a	21.2bcd	0.7463fgh	1.000bcd
Artemisia vulgaris	69615a	21bcd	0.7419efgh	1.001bcd
Baccharis halimifolia	44904a	17.7ab	0.6771ab	0.999bcd
Baccharis sarothroides	52182a	19.1abc	0.7015bcd	0.993abc
Eupatorium capillifolium	40939a	16.9a	0.6656a	1.001bcd
Chrysanthemum leucanthemum	102746a	27.7e	0.8350	0.999bcd
Helianthus annuus	173771a	35.8	0.9415	1.002cd
Hymenoclea salsola	57881a	19.9abcd	0.7224cdef	1.000bcd
Iva angustifolia	53578a	19.6abc	0.718cde	0.994abcd
Iva axillaris	72180a	21.9cd	0.7581h	0.994bcd
Iva xanthifolia	53728a	18.7abc	0.6988bc	0.983a
Solidago spp.	62534a	20.6abcd	0.7274cdefg	1.003
Taraxacum officinale	3074609	88.4	1.2804	0.991ab
Xanthium commune	76159a	23.8de	0.7928	0.997bcd

All the means for the parameters were statistically unequal. Post-hoc Tukey's HSD results are shown. Airspora types with the same letter are statistically not significantly different from each other with a minimum p = 0.05. Abbreviated parameters: ECD = equivalent circular diameter, IOD=integrated optical density.

3.3.4 *Olea* look-alike pollen types

In this study, we evaluated the feasibility of using image analysis to differentiate between morphologically similar pollen grains (Figure 3.5). Eighteen similar-looking pollen types, *Brassica* spp., *Elaeagnus angustifolia*, *Fraxinus americana*, *Fraxinus latifolia*, *Fraxinus pennsylvanica*, *Fraxinus velutina*, *Ligustrum vulgare*, *Olea europea*, *Populus alba*, *Populus deltoides*, *Populus nigra*, *Populus sargentii*, *Populus tremuloides*, *Populus trichocarpa*, *Populus wislizenii*, *Salix discolor*, *Salix lasiolepis* and *Salix nigra* were evaluated.

In total, 83.5% of the pollen types studied were correctly identified (Table 3.12). Eight pollen types could be identified with accuracy rates above 90%, i.e., *Brassica* spp. (92.5%), *Elaeagnus angustifolia* (96.9%), *Fraxinus latifolia* (92.9%), *Fraxinus pennsylvanica* (95.6%), *Ligustrum vulgare* (98.2%), *Olea europea* (99.2%), *Salix lasiolepis* (91.5%) and *Salix nigra* (94.8%). Eight other pollen types, i.e., *Fraxinus americana* (89.3%), *Fraxinus velutina* (80.6%), *Populus deltoides* (84.2%), *Populus nigra* (80.7%), *Populus sargentii* (83.5%), *Populus tremuloides* (89.1%), *Populus trichocarpa* (84.1%) and *Salix discolour* (88.4%) were identified with more than 80% accuracy. An accuracy rate of 78.4% was obtained for *Populus alba*, and 62.2% was obtained for *Populus wislizenii*.

However, misidentifications of more than 10% were mainly within the *Populus* spp. *Populus alba* was misidentified as *Populus nigra* for 13.6% of the time. *Populus nigra* for 10.7% of the time was confused as *Populus alba*. *Populus wislizenii* was misidentified for 29.3% of the time as *Populus deltoides*. Most of *Olea* look-alike pollen types were often misidentified as *Salix nigra*. In total, 3.10% of pollen types tested were misidentified as that of *Salix nigra*.

Forty measured parameters were used for identification (Table 3.13). Eighteen canonical functions were needed to differentiate 100% of the samples. Convex perimeter, equivalent circular diameter, mean diameter, minor axis and polygon area are important parameters in differentiating the *Olea* look-alike pollen types.

The mean values for all the parameters used were not equal when ANOVA ($p < 0.001$) was performed. Subsequently, post-hoc Tukey's analysis was performed. Tukey's HSD results for discriminating parameters are shown in Table 3.14.

Table 3.12: Identification accuracies of the *Olea* look-alike weed pollen types by step-wise canonical analysis.

***Olea* look-alike pollen** / **Actual identity**	**Identified as**	[l]*Elaeagnus angustifolia*	[l]*Fraxinus americana*	[l]*Fraxinus latifolia*	[l]*Fraxinus pennsylvanica*	[l]*Fraxinus velutina*	[l]*Ligustrum vulgare*	[l]*Olea europea*	[m]*Populus alba*	[m]*Populus deltoides*	[m]*Populus nigra*	[m]*Populus sargentii*	[m]*Populus tremuloides*	[m]*Populus trichocarpa*	[m]*Populus wislizenii*	[m]*Salix discolor*	[m]*Salix lasiolepis*	[m]*Salix nigra*	[b]*Brassica* spp.
[l]*Elaeagnus angustifolia*		**96.9**	0.1	0.4	0.0	0.1	0.0	0.2	0.0	0.0	0.0	0.0	0.0	0.0	0.0	1.2	0.7	0.4	0.1
[l]*Fraxinus americana*		0.4	**89.3**	0.1	0.0	0.3	0.5	0.2	0.5	0.1	0.1	0.2	0.2	0.0	0.0	7.1	0.8	0.2	0.1
[l]*Fraxinus latifolia*		0.4	0.6	**92.9**	0.1	2.0	0.2	0.1	0.0	0.0	0.0	0.0	3.0	0.0	0.0	0.3	0.3	0.1	0.0
[l]*Fraxinus pennsylvanica*		0.0	0.0	0.0	**95.6**	1.6	0.0	0.0	0.0	1.0	0.0	0.0	0.2	0.0	0.4	0.0	0.9	0.3	0.0
[l]*Fraxinus velutina*		0.1	0.4	8.3	8.8	**80.6**	0.0	0.1	0.0	0.4	0.0	0.0	0.3	0.0	0.0	0.3	0.1	0.4	0.0
[l]*Ligustrum vulgare*		0.0	0.0	0.4	0.0	0.0	**98.2**	0.6	0.0	0.0	0.1	0.0	0.0	0.0	0.0	0.0	0.0	0.1	0.6
[l]*Olea europea*		0.0	0.0	0.0	0.0	0.0	0.4	**99.2**	0.1	0.1	0.1	0.0	0.0	0.0	0.1	0.0	0.0	0.0	0.0
[m]*Populus alba*		0.2	0.5	0.0	0.1	0.0	0.1	0.6	**78.5**	0.6	**13.6**	0.3	0.2	4.1	0.9	0.1	0.3	0.0	0.0
[m]*Populus deltoides*		0.0	0.1	0.1	2.0	0.0	0.0	1.0	0.8	**84.2**	0.0	0.0	5.8	0.0	5.8	0.1	0.1	0.0	0.0
[m]*Populus nigra*		0.0	0.1	0.0	0.0	0.0	0.1	0.4	**10.7**	0.0	**80.7**	0.3	0.3	6.0	0.0	1.2	0.2	0.0	0.0
[m]*Populus sargentii*		0.1	0.4	0.0	0.0	0.2	0.0	0.2	1.1	0.0	0.6	**83.5**	0.0	**12.2**	0.0	1.6	0.2	0.0	0.1
[m]*Populus tremuloides*		0.1	0.4	1.6	0.9	0.2	0.0	0.5	0.5	5.1	0.5	0.0	**89.1**	0.0	0.2	0.8	0.3	0.0	0.0
[m]*Populus trichocarpa*		0.0	0.1	0.0	0.0	0.0	0.0	0.9	3.0	0.0	4.6	6.2	0.0	**84.1**	0.0	0.8	0.4	0.0	0.0
[m]*Populus wislizenii*		0.0	0.0	0.0	2.4	0.3	0.0	1.0	3.5	**29.3**	1.0	0.0	0.3	0.1	**62.2**	0.0	0.0	0.0	0.0
[m]*Salix discolor*		1.0	4.1	0.3	0.0	0.2	0.2	0.0	0.0	0.0	0.7	0.5	0.1	0.3	0.0	**88.4**	3.8	0.3	0.2
[m]*Salix lasiolepis*		0.9	0.4	0.0	0.3	0.1	0.0	0.0	0.0	0.0	0.1	0.1	0.0	0.0	0.0	3.2	**91.5**	3.5	0.0
[m]*Salix nigra*		0.8	0.0	0.0	0.0	0.2	0.0	0.1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.9	3.2	**94.8**	0.0
[b]*Brassica* spp.		0.1	1.2	0.0	0.0	0.4	4.5	0.4	0.0	0.0	0.0	0.3	0.0	0.0	0.0	0.5	0.0	0.0	**92.5**
Total misidentification (%)		**0.2**	**0.5**	**0.7**	**0.9**	**0.3**	**0.4**	**0.4**	**1.0**	**1.9**	**1.1**	**0.4**	**0.5**	**1.3**	**0.4**	**1.1**	**2.3**	**3.1**	**0.1**

Identification rates are given in percentages. Order: [b]Brassicales, [l]Lamiales and [m]Malpighiales.

Table 3.13: Canonical discrimination coefficients for the *Olea* look-alike pollen types.

	Function (Percentage of the total sample dispersion accounted for by the canonical variables in brackets)																
	1(30.9%)	2(55%)	3(74.2%)	4(85.7%)	5(91.8%)	6(94.2%)	7(96.2%)	8(97.2%)	9(98.1%)	10(98.7%)	11(99.2%)	12(99.5%)	13(99.8%)	14(99.9%)	15(100%)	16(100%)	17(100%)
Aspect	-0.229	0.113	-0.239	0.169	0.072	-0.143	0.016	-0.145	0.379	0.261	-0.252	0.017	0.225	-0.495	-0.115	0.256	-0.307
Area/Box	0.140	-0.337	-0.895	-0.073	-0.110	-0.086	-0.180	-0.286	0.188	**1.424**	0.305	**-2.034**	**1.190**	**1.735**	-0.194	-0.787	**1.070**
Box X/Y	-0.099	-0.033	0.043	-0.186	-0.054	0.135	0.063	0.109	-0.174	0.043	0.115	0.164	-0.138	-0.021	0.112	-0.159	-0.098
Density (mean)	-0.760	-0.616	**1.275**	**-1.882**	**-1.676**	0.963	0.627	0.620	**-1.778**	**1.934**	**-1.582**	**-1.067**	**2.386**	**-1.546**	**-1.071**	**-1.220**	**-1.349**
Axis (minor)	**3.456**	-0.105	**2.474**	-0.490	**-1.647**	**2.124**	**5.023**	**1.998**	**-3.113**	**3.400**	**-1.155**	**-4.922**	**-3.517**	**6.619**	**1.912**	**-1.831**	-0.761
Diameter (max)	-0.936	0.717	0.766	1.087	-0.540	0.096	0.571	**1.406**	**-1.824**	-0.419	0.103	-0.012	**-3.655**	**-2.201**	**-1.325**	-0.348	-0.893
Diameter (min)	0.230	-0.034	-0.171	-0.266	0.158	0.152	0.486	-0.384	-0.572	**-1.081**	**-1.008**	0.322	**1.140**	0.490	0.914	-0.193	**-3.647**
Diameter (mean)	**1.399**	**-1.570**	**1.733**	-0.362	**-3.585**	-2.279	0.370	**-1.847**	0.970	**4.909**	**-4.358**	**-2.293**	0.781	**1.718**	0.173	**3.865**	**-2.123**
Radius (max)	0.563	-0.364	-0.543	0.030	0.461	-0.552	-0.797	**-1.419**	**1.054**	0.462	-0.194	-0.487	**1.523**	**1.046**	0.728	**1.354**	0.875
Radius (min)	0.569	0.291	-0.465	**1.045**	0.507	-0.215	0.156	**1.208**	**1.173**	0.414	0.761	**1.161**	**-1.423**	**-1.157**	-0.137	**-1.329**	**2.614**
Perimeter	0.679	-0.133	**1.419**	0.601	-0.450	-0.695	0.264	-0.486	**2.818**	**-2.777**	0.285	-0.735	**1.115**	**2.084**	**2.522**	**-2.247**	-0.080
Radius Ratio	0.029	-0.010	-0.032	-0.012	-0.062	0.022	0.059	-0.060	0.091	0.057	0.043	-0.131	0.043	0.094	0.128	-0.101	-0.270
Roundness	-0.076	0.019	-0.225	-0.062	0.087	0.137	-0.015	0.152	-0.254	0.360	0.324	0.115	0.261	-0.183	-0.052	-0.028	0.407
Density (red)	0.016	-0.490	**-1.085**	0.845	-0.370	-0.144	-0.558	0.002	0.655	-0.326	**1.555**	0.642	**-1.498**	0.887	0.341	0.833	0.609
Density (blue)	0.615	**1.250**	0.202	-0.132	**1.177**	**-1.007**	-0.442	-0.667	0.004	-0.975	0.723	0.276	**-1.197**	0.604	0.337	0.478	0.536
Size (length)	-0.178	0.020	-0.253	**-1.412**	**1.027**	**1.195**	0.720	-0.890	**-1.241**	-0.150	**1.147**	0.833	0.837	0.210	-0.967	0.809	**-1.730**
Size (width)	-0.086	0.147	-0.142	0.215	-0.610	-0.973	-0.234	**1.045**	0.718	0.089	-0.002	-0.732	**-1.391**	-0.158	0.937	-0.357	0.040
IOD	-0.062	0.401	0.859	**1.749**	**2.403**	**2.765**	**1.593**	**2.462**	**9.460**	**-3.852**	**-3.721**	**1.843**	**1.240**	**1.472**	**1.279**	0.630	**1.464**
Perim. (convex)	**-1.185**	**1.075**	0.040	-0.290	**1.114**	**1.872**	0.530	0.314	**-1.306**	**1.092**	0.454	**2.285**	**-3.382**	**1.087**	**-1.588**	**-1.614**	-0.459
Perim. (ellipse)	**3.086**	0.634	0.866	2.234	**2.836**	0.364	-0.180	-0.403	**-1.176**	**-3.943**	0.347	**1.602**	**2.163**	4.684	0.162	-0.102	**3.100**
Perim. (ratio)	0.404	-0.324	0.031	0.011	-0.321	-0.575	-0.173	-0.132	0.594	-0.419	0.027	-0.584	0.973	-0.241	**1.189**	0.388	0.197
Area (polygon)	**-3.358**	**-1.284**	**-3.858**	**-3.646**	**-1.907**	**-3.088**	**-4.363**	**-3.408**	**-7.400**	**2.144**	**4.133**	**-1.627**	0.711	**-3.462**	-0.965	-0.149	-0.042
Fractal Dim.	0.116	-0.052	0.012	0.153	-0.121	-0.001	-0.126	0.154	0.116	0.040	0.140	-0.136	0.338	0.028	0.330	**1.037**	0.507

The magnitude of the canonical discrimination function coefficient indicates the weightage of the parameter in each factor in differentiating airspora types. Parameters with high magnitudes in an earlier factor also carry a higher weightage in differentiating the airspora. Abbreviated parameter: IOD = integrated optical density.

Table 3.13 (continued): Canonical discrimination coefficients for the *Olea* look-alike pollen types.

	Function (Percentage of the total sample dispersion accounted for by the canonical variables in brackets)																
	1(30.9%)	2(55%)	3(74.2%)	4(85.7%)	5(91.8%)	6(94.2%)	7(96.2%)	8(97.2%)	9(98.1%)	10(98.7%)	11(99.2%)	12(99.5%)	13(99.8%)	14(99.9%)	15(100%)	16(100%)	17(100%)
Box Width	0.930	0.048	-0.335	0.180	0.490	0.512	0.146	0.635	**-1.072**	**2.102**	0.978	-0.691	**1.481**	**-2.456**	**-2.154**	0.459	**2.652**
Box Height	0.154	-0.478	-0.801	-0.195	-0.106	0.102	-0.220	-0.052	0.286	**1.639**	0.791	**-1.943**	**1.297**	**1.189**	-0.152	-0.832	**1.772**
Feret (min)	-0.047	-0.156	-0.240	0.110	0.996	**1.553**	-0.004	-2.307	**-1.361**	-1.440	-0.328	0.065	0.579	-0.962	-2.868	0.368	0.863
Feret (max)	0.284	-0.219	-0.855	-0.801	-0.727	**-1.144**	-0.591	0.400	**2.369**	0.049	0.379	**-1.602**	0.142	**1.809**	**1.209**	**-2.385**	0.276
Feret (mean)	-0.498	-0.573	-0.592	**-1.152**	**-1.009**	-0.252	**1.030**	**2.584**	**-2.499**	-0.219	**-1.156**	**3.077**	**-1.649**	**-5.345**	0.937	**2.260**	**-4.321**
Density (min)	0.111	-0.278	0.128	0.148	-0.036	0.524	-0.046	-0.831	0.114	0.440	-0.086	0.142	-0.252	-0.147	0.229	-0.233	0.213
Density (max)	-0.083	0.145	-0.356	0.165	0.639	0.040	0.584	0.241	0.043	0.756	-0.400	0.162	0.007	0.094	0.339	-0.033	-0.087
Density (std.dev.)	0.025	0.278	-0.241	-0.122	**-1.367**	0.076	**1.204**	**-1.598**	0.232	0.440	0.107	0.464	-0.294	-0.454	0.256	-0.228	0.928
Dendritic length	0.046	-0.008	-0.021	-0.061	-0.025	-0.077	0.033	0.065	-0.049	-0.033	-0.015	0.093	0.069	0.225	0.002	0.180	-0.204
Margination	0.010	0.218	0.008	-0.111	0.072	-0.198	-0.166	0.007	-0.120	0.227	0.137	-0.221	0.222	-0.073	0.364	0.006	0.555
Heterogeneity	0.484	0.436	0.004	0.062	0.728	0.394	**-1.534**	**1.354**	-0.107	-0.274	-0.044	-0.386	0.284	0.320	-0.274	0.041	-0.795
Clumpiness	0.001	-0.111	0.045	-0.197	-0.054	-0.076	-0.001	-0.029	-0.070	0.022	0.150	0.195	-0.314	0.323	0.166	0.399	-0.209
Form Factor	-0.197	0.916	**2.052**	**1.450**	0.076	0.464	0.787	0.533	**1.589**	**-2.147**	**1.519**	**1.687**	**-1.363**	**4.707**	**1.713**	**-2.716**	-0.793
ECD	**-4.232**	0.575	**-2.763**	0.753	0.750	**-2.506**	**-5.084**	**-1.724**	**4.560**	**-1.965**	0.774	**1.379**	**2.976**	**-5.843**	-0.861	**2.271**	**1.249**
Compactness	0.169	0.302	-0.542	-0.029	0.537	0.113	0.417	0.469	-0.263	0.016	0.340	1.070	-0.115	-0.333	-0.038	0.246	-0.227
Box ratio	0.775	0.556	0.837	0.086	0.616	**1.144**	0.455	**1.443**	**-2.040**	0.862	**1.086**	**2.553**	0.631	**-6.522**	**-1.153**	**1.962**	**1.040**
MODR	-0.465	-0.300	0.340	-0.897	-0.223	0.452	-0.170	-0.587	-0.452	-0.136	0.448	-0.925	0.280	0.376	0.055	0.567	-0.970
Concavity	0.522	0.714	**1.595**	**1.611**	**1.055**	**1.915**	**1.195**	**2.218**	**2.270**	**-1.308**	0.455	**1.484**	0.920	**1.982**	0.756	**-1.150**	**2.217**

The magnitude of the canonical discrimination function coefficient indicates the weightage of the parameter in each factor in differentiating airspora types. Parameters with high magnitudes in an earlier factor also carry a higher weightage in differentiating the airspora. Abbreviated parameters: ECD = equivalent circular diameter, MODR = modification ratio.

Table 3.14: Means of important parameters used in identification of the *Olea*-look alike pollen types.

***Olea* look-alike**	**Minor axis**	**Mean diameter**	**Convex-perimeter**	**Ellipse perimeter**	**Polygon area**	**ECD**
Elaeagnus angustifolia	40.1	41.1	135.7	131.1	1336.6	1.0186
Fraxinus americana	25.5	26.4a	84.4	84.1	546.2a	0.8124
Fraxinus latifolia	25.0	26.2	83.0a	83.3	539.4a	0.8052
Fraxinus pennsylvanica	23.8a	24.8	78.6	78.8	480.5	0.7852b
Fraxinus velutina	21.1	23.3	74.7	71.1	430.3	0.7376a
Ligustrum vulgare	27.4ab	28.7	92.1	91.5	645.9	0.8434cd
Olea europea	20.7	21.3	68.6	67.9	354.2	0.7327a
Populus alba	27.8b	29.4	93.7	93.7a	678.8c	0.8484c
Populus deltoides	31.0	31.6	99.9	100.3	785.9	0.8966
Populus nigra	27.6b	28.3	90.2	90.1	629.9	0.8466c
Populus sargentii	26.6	27.2	86.2	86.5	581.0	0.8300
Populus tremuloides	28.4	29.8	94.5	94.8a	700.3bc	0.8566
Populus trichocarpa	27.1b	27.9	88.7	88.6	612.1	0.8373d
Populus wislizenii	31.8	32.3	102.0	102.4	823.0	0.9071
Salix discolor	23.5a	24.2	76.6	76.9	457.3	0.7812b
Salix lasiolepis	19.8	20.1	64.4	64.1	317.2	0.7159
Salix nigra	18.0	18.3	59.2	58.4	261.8	0.6832
Brassica spp.	29.4	30.2	95.9	95.1	718.1b	0.8724

All the means for the parameters were statistically unequal. Post-hoc Tukey's HSD results are shown. Airspora types with the same letter are statistically not significantly different from each other with a minimum $p = 0.05$. Abbreviated parameter: ECD = equivalent circular diameter.

3.3.5 All pollen types

One hundred and fifty-three pollen types listed in Table 3.1 were used in this analysis. Fifty-seven pollen types had classification accuracies of more then 70% (Table 3.15). Classification accuracy between 50 to 70% were achieved for 43 pollen types, 30 to 50% for 39 pollen types and less than 30% for 14 pollen types. Most of all the pollen types studied were often misclassified as *Lolium perenne* (4.38% of the time) followed by those of *Agrotis alba* (3.62%), *Acer negundo* (3.35%), *Taxodium distichum* (1.44%), *Juniperus scopulorum* (1.38%) and *Cupressus sempervirens* (1.18%) for all the pollen types studied.

Thirty-eight canonical functions were generated (Table 3.16). However, 31 functions were needed to differentiate 100% of the samples. A combination of 38 parameters was used for discrimination. Parameters that rank high in importance for differentiating the airspora were box height, box width, clumpiness, convex perimeter, dendrites, dendritic length, density standard deviation, diameter mean, equivalent circular diameter, form factor, fractal dimension, heterogeneity, integrated optical density, length size, margination, maximum density, maximum diameter, minimum density, minimum diameter, minimum feret, minor axis, minor axis, perimeter ratio, polygon area and width size. These encompass size, shape, colour and textural characters.

Table 3.15: Pollen types and their classification accuracies in percentage ranges by step-wise canonical discriminate analysis.

Percentage of accuracy	Pollen types
70% to 100% n = 57	*Acacia* spp., *Agrostis alba, Ambrosia acanthicarpa, Ambrosia artemisiifolia, Ambrosia psilostachya, Ambrosia trifida, Atriplex canescens, Atriplex lentiformis, Baccharis hamilifolia, Baccharis sarothroides, Betula lenta, Betula nigra, Bromus inermis, Broussonetia papyrifera, Carya alba, Carya glabra, Carya ovata, Chenopodium botrys, Chenopodium album, Chrysanthemum leucanthemum, Cupressus arizonica, Cupressus sempervirens, Dactylis glomerata, Elaeagnus angustifolia, Elymus condensatus, Eucalyptus globulus Fagus americana, Fraxinus pennsylvanica, Helianthus annuus, Juglans regia, Juniperus monosperma, Ligustrum vulgare, Liquidambar styraciflua, Lolium perenne ,Paspalum notatum, Pinus echinata, Pinus elliotti, Pinus monticola, Pinus strobus, Plantago lanceolata, Platanus acerifolia, Poa compressa, Populus deltoides, Populus sargentii, Populus tremuloides, Quercus alba, Quercus lobata, Quercus nigra. Rumex crispus, Salix lasiolepis, Schinus terebinthifolius, Secale cereale, Tamarix gallica, Taxodium distichum, Triticum aestivum, Urtica* spp., *Xanthium commune*
50% to 70% n=43	*Acer negundo, Acer saccharinum, Acer saccharum, Allenrolfea occidentalis, Alnus glutinosa, Alnus rubra, Alnus rugosa, Ambrosia deltoidea, Anthoxanthum odoratum, Artemisia californica, Artemisia frigida, Artemisia vulgaris, Atriplex polycarpa, Atriplex wrightii, Avena sativa, Carya illinoensis, Carya laciniosa, Cocos plumosa, Cryptomeria japonica, Fraxinus americana, Holcus lanatus, Iva xanthifolia, Juglans nigra, Juniperus ashei, Juniperus virginiana, Melaleuca quinquenervia, Morus alba, Myrica cerifera, Olea europea, Pinus ponderosa, Pinus virginiana, Platanus occidentalis, Poa pratensis, Populus trichocarpa, Quercus dumosa, Quercus garryana, Quercus kelloggii, Rosa* spp., *Rumex acetosella, Salix discolor, Sorghum halepensis, Trifolium pratense, Ulmus pumila*
30% to 50% n=39	*Acer macrophyllum, Amaranthus hybridus, Amaranthus palmerii, Amaranthus retroflexus, Betula populifolia, Chenopodium ambrosioides, Corylus americana, Corylus avellana, Cynodon dactylon, Eupatorium capillifolium,Fraxinus latifolia, Fraxinus velutina, Iva angustifolia, Iva axillaris , Juglans californica, Juniperus occidentalis, Juniperus scopulorum, Phalaris arundinacea, Phleum pratense, Platanus orientalis, Populus alba, Populus nigra, Prosopis juliflora, Quercus agrifolia, Quercus ilex, Ricinus communis, Schinus molle, Taraxacum officinale, Ulmus americana, Ulmus crassifolia, Zea mays, Agropyron repens, Agropyron smithi, Atriplex confertifolia, Festuca elatior, Lolium multiflorum, Morus rubra, Pinus palustris, Populus wislizenii*
Less than 30% n=14	*Alnus rhombifolia, Betula verrucosa, Casuarina equisetifolia, Celtis occidentalis, Citrus sinensis, Hymenoclea salsola, Juniperus osteosperma, Platanus racemosa, Quercus macrocarpa, Quercus robur, Quercus rubra, Quercus stellata, Quercus velutina, Quercus virginiana*

Table 3.16: Canonical discrimination coefficients for all pollen types.

	Function (Percentage of the total sample dispersion accounted for by the canonical variables in brackets)									
	1(40.2%)	2(53.6%)	3(65.4%)	4(73.8%)	5(80.9%)	6(84.7%)	7(87.8%)	8(90.2%)	9(92.5%)	10(94%)
Aspect	-0.116	-0.237	0.150	0.053	0.024	-0.349	0.175	0.104	-0.035	-0.126
Area/Box	0.154	-0.192	0.163	-0.317	0.194	0.550	-0.569	0.006	-0.382	0.200
Box X/Y	-0.038	0.056	0.017	0.090	0.016	0.182	-0.149	0.041	-0.011	-0.029
Angle	0.006	-0.003	0.007	-0.004	0.001	-0.005	0.004	0.010	-0.011	0.019
Axis (minor)	**3.943**	**1.161**	-0.449	0.944	-0.453	0.810	**-2.026**	**1.329**	**-1.653**	0.978
Diameter (max)	**1.241**	-0.148	0.424	0.380	-0.826	**3.351**	**-2.252**	-0.919	0.882	**1.390**
Diameter (min)	**1.571**	**-1.540**	0.767	0.485	-0.915	**-1.900**	0.671	-0.112	**-1.799**	**-2.017**
Diameter (mean)	**-7.082**	**3.532**	**-2.248**	**1.557**	-0.849	**-5.642**	**3.304**	**8.502**	**-5.030**	**2.544**
Radius (max)	0.040	-0.566	0.004	-0.116	-0.334	**-1.072**	0.311	**1.038**	**-1.394**	**-2.208**
Radius (min)	-0.421	-0.452	0.106	-0.704	0.294	**-1.019**	**1.248**	-0.938	**1.598**	-0.084
Perimeter	0.521	**-1.336**	0.945	0.036	**-1.282**	**-1.437**	**1.315**	**-1.146**	**1.012**	0.857
Radius Ratio	0.060	-0.077	0.046	0.025	0.001	-0.032	0.007	0.056	-0.072	0.047
Roundness	0.006	0.003	0.074	0.168	0.124	0.047	-0.103	0.214	-0.176	-0.013
Density (red)	-0.138	-0.535	-0.612	**1.789**	**1.523**	-0.460	-0.454	-0.530	-0.440	0.354
Density (green)	0.182	-0.072	-0.090	-0.224	**-1.238**	0.988	**1.207**	**1.118**	0.920	-0.427
Density (blue)	0.097	0.953	**1.258**	-0.632	-0.542	-0.530	-0.535	-0.315	-0.045	0.392
Size (length)	**-1.055**	**4.274**	**-2.605**	-0.188	**2.682**	-0.993	2.171	-0.113	**1.892**	**1.028**
Size (width)	-0.024	0.793	0.045	0.300	0.493	**2.593**	-0.946	**-1.157**	**1.911**	-1.427
IOD	-0.533	0.549	0.063	0.406	0.456	**3.263**	**-2.803**	0.584	-0.648	-0.342
Perim. (convex)	**-1.172**	**2.059**	**-1.681**	-0.467	**1.044**	-0.353	0.365	0.947	-0.773	**-1.435**
Perim. (ratio)	0.163	-0.154	0.177	0.175	-0.291	0.180	-0.180	0.339	-0.286	0.178
Area (polygon)	**1.142**	**-2.881**	**1.440**	-0.357	**-1.196**	**-2.656**	**1.767**	**-1.245**	0.336	-0.317
Fractal Dim.	0.174	-0.081	0.118	0.124	-0.061	0.292	-0.119	0.005	-0.112	0.380
Box Width	**1.198**	-0.581	0.845	-0.838	0.769	**4.134**	**-3.150**	**-2.602**	**1.309**	0.555
Box Height	**1.213**	-0.404	0.975	-0.375	0.800	**5.035**	**-3.911**	**-2.427**	**1.110**	0.487
Feret (min)	**2.340**	**-3.201**	0.997	-0.383	-0.320	**-2.377**	**1.913**	**-1.069**	**1.356**	-0.365
Density (min)	-0.130	-0.023	-0.139	-0.021	0.138	-0.093	0.246	-0.213	0.131	0.126
Density (max)	0.026	0.116	0.031	0.005	0.311	-0.378	-0.329	0.082	-0.010	-0.704
Density (std.dev.)	-0.218	0.277	0.610	0.411	0.285	0.529	**1.109**	-0.751	-0.645	-0.038
Dendritic length	0.009	0.012	-0.045	0.030	-0.047	-0.043	-0.042	0.248	-0.276	-0.115
Dendrites	-0.069	-0.041	0.065	-0.064	0.038	-0.091	0.237	-0.318	0.468	0.209
Margination	0.001	0.112	0.162	0.168	0.110	-0.055	-0.020	0.080	0.010	-0.271
Heterogeneity	0.053	-0.386	0.080	-0.466	0.400	-0.216	-0.656	0.986	0.776	0.290
Clumpiness	0.004	-0.015	0.017	0.133	-0.056	-0.040	0.077	-0.002	0.096	-0.006
Form Factor	-0.427	0.346	-0.560	-0.335	-0.308	**-1.995**	**2.324**	-0.479	0.332	0.427
ECD	**-2.959**	**-1.677**	0.871	-0.615	0.180	-0.071	0.249	-0.593	-0.022	-0.074
Compactness	-0.098	0.741	-0.589	-0.264	0.427	-0.712	0.765	0.094	0.158	0.092
Modification ratio	0.377	-0.119	0.125	0.264	-0.022	0.572	-0.502	0.166	-0.163	0.472

The magnitude of the canonical discrimination function coefficient indicates the weightage of the parameter in each factor in differentiating airspora types. Parameters with high magnitudes in an earlier factor also carry a higher weightage in differentiating the airspora. Abbreviated parameters: ECD = equivalent circular diameter, IOD = integrated optical density.

Table 3.16 (continued): Canonical discrimination coefficients for all pollen types.

	Function (Percentage of the total sample dispersion accounted for by the canonical variables in brackets)								
	11(95.2%)	12(96.1%)	13(96.7%)	14(97.3%)	15(97.8%)	16(98.2%)	17(98.6%)	18(98.8%)	19(99%)
Aspect	0.120	0.112	0.189	-0.051	0.007	0.143	0.105	-0.079	0.015
Area/Box	0.318	1.170	0.247	-0.102	0.954	-0.171	**1.173**	-0.135	0.568
Box X/Y	0.063	0.104	0.097	-0.050	-0.029	-0.071	-0.249	0.042	-0.111
Angle	-0.008	-0.001	0.012	0.013	-0.005	-0.012	-0.004	-0.008	-0.024
Axis (minor)	**3.622**	**-1.439**	**-3.296**	**4.157**	**2.700**	**-4.424**	**-7.141**	**-10.083**	**-10.545**
Diameter (max)	-0.129	**1.345**	**-2.015**	-0.365	0.460	**-8.756**	**-7.018**	2.703	**3.846**
Diameter (min)	0.281	**-1.065**	0.348	-2.004	**-2.177**	**-1.496**	-0.598	6.251	**1.290**
Diameter (mean)	**-5.379**	-0.242	9.758	**1.903**	**-2.041**	**-9.925**	**6.173**	**-13.881**	**-2.249**
Radius (max)	-0.188	-2.651	0.712	**1.005**	**1.270**	**4.105**	**5.543**	0.246	**-1.128**
Radius (min)	0.205	0.368	**-1.666**	-0.813	**1.334**	0.991	**2.706**	0.901	**2.859**
Perimeter	**1.382**	**-5.866**	**-1.265**	0.728	**-3.708**	-0.210	-0.454	**5.285**	**-3.223**
Radius Ratio	0.010	0.055	0.108	-0.002	0.011	-0.034	-0.054	0.049	0.032
Roundness	0.270	0.197	0.307	-0.040	0.389	-0.074	0.241	0.027	-0.048
Density (red)	-0.529	-0.178	-0.352	-0.060	0.100	-0.035	0.118	0.022	-0.004
Density (green)	0.669	0.253	0.307	-0.003	-0.133	0.099	-0.147	-0.125	0.118
Density (blue)	-0.364	-0.075	-0.127	-0.076	0.093	-0.024	-0.028	0.041	-0.056
Size (length)	0.745	**-1.342**	0.909	-0.754	-0.885	**7.417**	-2.937	**1.409**	**-2.440**
Size (width)	**-1.691**	0.027	0.610	-0.501	**1.225**	**-1.014**	0.074	0.855	0.883
IOD	**-1.647**	**4.535**	**2.941**	0.165	0.684	-0.792	**1.197**	-0.466	0.580
Perim. (convex)	**-1.526**	**4.674**	**-2.364**	**-2.588**	0.830	1.902	**-2.675**	**-4.036**	3.006
Perim. (ratio)	0.028	-0.833	0.456	0.955	-0.010	-0.041	0.824	0.708	-0.376
Area (polygon)	**2.228**	**-3.556**	**-4.129**	-0.810	**-1.544**	0.951	-0.362	**1.126**	-0.375
Fractal Dim.	0.131	-0.737	0.621	0.762	0.048	-0.083	0.488	0.104	0.476
Box Width	**1.137**	**3.220**	0.063	0.332	**2.480**	**1.314**	**2.891**	**2.106**	**1.666**
Box Height	**1.375**	**3.717**	0.501	0.456	**2.219**	0.902	**1.604**	**2.096**	**1.041**
Feret (min)	**-1.578**	2.408	0.702	0.226	0.961	**5.375**	**1.127**	**2.563**	**4.129**
Density (min)	0.436	0.607	0.045	0.379	-0.647	-0.097	0.347	0.180	-0.169
Density (max)	0.681	-0.069	0.524	-0.085	0.147	-0.056	-0.191	-0.041	0.000
Density (std.dev.)	0.307	0.457	-0.023	0.284	-0.528	-0.074	0.280	0.058	-0.087
Dendritic length	0.005	0.122	-0.235	-0.037	0.029	-0.057	-0.103	-0.140	-0.068
Dendrites	-0.031	-0.531	0.457	0.046	0.002	0.077	0.315	-0.132	-0.213
Margination	0.338	-0.068	-0.187	0.449	-0.110	0.367	0.034	-0.239	0.287
Heterogeneity	-0.336	-0.197	-0.173	-0.075	0.142	0.076	-0.080	-0.003	0.062
Clumpiness	0.004	-0.078	0.047	-0.273	0.139	-0.059	0.081	0.165	-0.529
Form Factor	**1.092**	**-5.585**	**-1.858**	-0.179	**-2.784**	**1.585**	**-1.988**	**2.496**	-0.213
ECD	0.142	**1.474**	-0.188	-0.545	-0.606	**2.569**	**2.098**	**1.421**	**1.246**
Compactness	-0.241	0.120	0.149	-0.140	0.229	0.789	0.283	0.302	0.449
Modification ratio	-0.033	-0.168	0.915	0.281	-0.399	-0.017	**-1.265**	-0.281	-0.660

The magnitude of the canonical discrimination function coefficient indicates the weightage of the parameter in each factor in differentiating airspora types. Parameters with high magnitudes in an earlier factor also carry a higher weightage in differentiating the airspora. Abbreviated parameters: ECD = equivalent circular diameter, IOD = integrated optical density.

Table 3.16 (continued): Canonical discrimination coefficients for all pollen types.

	Function (Percentage of the total sample dispersion accounted for by the canonical variables in brackets)								
	20(99.2%)	21(99.4%)	22(99.5%)	23(99.6%)	24(99.7%)	25(99.8%)	26(99.8%)	27(99.9%)	28(99.9%)
Aspect	-0.020	0.489	0.082	0.140	-0.233	-0.063	0.221	-0.048	-0.206
Area/Box	0.883	-0.517	-0.595	-0.337	0.400	-0.010	-0.142	0.058	0.254
Box X/Y	-0.015	0.041	0.053	-0.066	0.025	0.073	-0.137	0.061	0.155
Angle	-0.007	0.039	-0.013	-0.015	-0.006	0.076	-0.005	0.041	0.119
Axis (minor)	**-6.116**	**-5.479**	**8.492**	**-1.615**	0.367	**-1.318**	**1.222**	0.255	**8.206**
Diameter (max)	**3.568**	**5.611**	**10.398**	**-6.599**	**9.457**	**-4.302**	**8.562**	**-1.770**	**7.659**
Diameter (min)	**-1.283**	**-1.011**	**-1.199**	**-13.196**	**1.724**	2.093	**-8.783**	**2.674**	**6.650**
Diameter (mean)	**1.551**	0.668	**-1.396**	**11.142**	**-4.707**	0.320	**-8.158**	**2.817**	**-1.495**
Radius (max)	**-2.821**	**-6.505**	**1.092**	**4.942**	**-1.370**	0.809	**-1.570**	0.326	0.225
Radius (min)	**2.298**	**1.439**	**1.630**	**6.160**	**-1.743**	-0.023	**3.228**	**-1.761**	**-3.209**
Perimeter	**2.781**	**2.475**	**-1.426**	**2.693**	**-5.913**	-0.665	**-2.823**	**2.092**	0.591
Radius Ratio	0.054	-0.019	0.038	-0.003	0.009	0.037	0.051	0.038	0.085
Roundness	-0.125	0.152	0.146	0.005	0.003	0.155	0.489	0.587	0.416
Density (red)	0.089	0.004	-0.038	-0.008	-0.028	-0.037	0.032	-0.012	0.016
Density (green)	-0.029	0.014	-0.019	-0.010	-0.037	0.042	-0.058	0.041	0.041
Density (blue)	-0.131	-0.086	0.014	0.016	0.040	0.021	0.003	-0.030	-0.019
Size (length)	**-3.679**	**-1.209**	**-3.117**	**1.900**	**-4.171**	**4.877**	**-1.138**	**-3.416**	0.869
Size (width)	**1.140**	0.016	**6.353**	**-3.606**	**4.909**	**-6.534**	**-4.617**	**9.290**	**-9.694**
IOD	**2.499**	0.588	0.297	-0.849	-0.021	0.940	-1.277	-0.622	0.887
Perim. (convex)	**-2.645**	**-4.425**	**-1.265**	**-5.273**	**4.564**	**4.213**	**4.356**	**2.502**	**-7.382**
Perim. (ratio)	0.337	0.903	0.345	-0.102	-0.480	-0.145	0.163	-0.012	0.246
Area (polygon)	**-1.303**	**1.768**	**1.014**	0.860	**-1.377**	0.030	0.883	-0.141	-0.619
Fractal Dim.	-0.669	0.544	0.087	0.102	0.258	0.161	-0.138	-0.139	-0.010
Box Width	**2.998**	0.074	**-3.481**	**-2.318**	-0.092	**-2.606**	**-2.561**	0.630	**-2.816**
Box Height	**2.745**	0.311	**-3.408**	**-2.923**	0.162	**-2.250**	**-3.008**	0.416	**-1.971**
Feret (min)	-0.571	**2.332**	**-13.993**	**8.072**	-0.402	**3.786**	**16.161**	**-13.393**	**5.157**
Density (min)	-0.204	-0.112	0.111	-0.039	0.069	-0.066	0.110	0.015	-0.034
Density (max)	0.035	0.100	-0.022	0.021	-0.050	-0.001	0.030	0.006	-0.041
Density (std.dev.)	-0.013	-0.162	0.093	0.000	0.060	-0.074	0.104	-0.014	-0.028
Dendritic length	-0.122	-0.150	0.383	-0.498	-0.034	0.923	0.222	-0.453	-0.772
Dendrites	0.458	-0.103	-0.164	-0.100	-0.037	-0.692	0.160	0.185	0.505
Margination	0.645	-0.086	-0.149	0.054	0.383	0.168	-0.232	0.002	0.101
Heterogeneity	-0.092	0.074	-0.043	-0.047	-0.039	0.035	-0.072	0.022	0.044
Clumpiness	0.069	0.217	-0.073	0.229	0.596	0.268	-0.158	-0.053	-0.080
Form Factor	**-2.487**	-0.606	**-2.623**	0.779	-0.151	-0.843	-0.237	**1.038**	**-3.266**
ECD	**1.696**	**4.984**	**2.862**	0.292	**-1.810**	**1.442**	-0.669	-0.962	0.289
Compactness	0.317	**1.059**	0.570	0.198	-0.343	0.267	-0.217	-0.148	0.082
Modification ratio	-0.559	**-1.407**	-0.324	0.288	0.478	-0.122	0.238	0.213	-0.306

The magnitude of the canonical discrimination function coefficient indicates the weightage of the parameter in each factor in differentiating airspora types. Parameters with high magnitudes in an earlier factor also carry a higher weightage in differentiating the airspora. Abbreviated parameters: ECD = equivalent circular diameter, IOD = integrated optical density.

Table 3.16 (continued): Canonical discrimination coefficients for all pollen types.

	Function (Percentage of the total sample dispersion accounted for by the canonical variables in brackets)									
	29(99.9%)	30(99.9%)	31(100%)	32(100%)	33(100%)	34(100%)	35(100%)	36(100%)	37(100%)	38(100%)
Aspect	0.332	-0.096	0.356	-0.692	0.774	-0.223	0.581	0.179	-0.680	0.215
Area/Box	-0.330	0.058	0.020	-0.060	0.020	0.171	-0.115	0.069	0.197	-0.255
Box X/Y	0.243	0.344	0.662	**1.130**	0.208	0.252	-0.093	0.868	0.338	0.876
Angle	0.001	-0.047	0.349	0.119	-0.191	0.595	0.486	-0.454	-0.094	-0.028
Axis (minor)	**-9.813**	0.974	**4.011**	**-5.502**	**3.512**	**2.562**	**-3.339**	**1.991**	**1.741**	**-3.660**
Diameter (max)	**6.089**	**3.619**	1.673	**-1.499**	**-2.854**	-0.383	**-3.276**	**-2.623**	-0.433	**-1.644**
Diameter (min)	**1.722**	**1.005**	0.920	**-3.039**	**1.061**	**-1.760**	0.470	-0.506	0.694	-0.144
Diameter (mean)	**3.943**	**-2.492**	**-3.466**	**2.196**	-0.523	**1.222**	**1.395**	**2.226**	-0.851	-0.168
Radius (max)	**5.556**	**2.102**	0.121	-0.389	-0.830	-0.489	**1.083**	-0.315	-0.746	0.445
Radius (min)	**-2.403**	0.313	-0.481	**1.500**	-0.175	0.944	-0.169	-0.054	0.023	0.243
Perimeter	**6.424**	**-2.774**	0.450	**-1.161**	**-2.221**	-0.421	-0.985	0.070	-0.240	0.991
Radius Ratio	0.020	-0.025	-0.025	0.019	-0.087	-0.225	0.337	-0.083	**1.008**	-0.174
Roundness	-0.345	0.125	-0.390	0.204	0.526	0.121	-0.072	-0.165	-0.078	-0.020
Density (red)	0.011	-0.004	0.016	0.003	0.011	-0.005	0.001	0.008	-0.006	0.011
Density (green)	0.003	0.016	0.009	-0.029	-0.005	0.004	-0.028	-0.018	0.010	0.003
Density (blue)	-0.002	-0.008	-0.004	0.007	-0.004	-0.007	0.004	0.004	0.005	0.010
Size (length)	**-12.213**	**-11.536**	**-2.373**	**1.508**	**3.954**	-0.134	**2.501**	**3.977**	0.239	**1.461**
Size (width)	**3.093**	**-11.018**	**-3.391**	**2.051**	-0.140	-0.689	**4.277**	**1.121**	-0.741	**2.502**
IOD	0.011	**1.683**	**-2.742**	**1.780**	0.119	0.787	**3.324**	**3.687**	**-1.428**	**-4.405**
Perim. (convex)	**-5.038**	**5.027**	**3.645**	**-1.057**	**-6.263**	**-1.909**	**1.287**	**1.906**	-0.300	-0.011
Perim. (ratio)	-0.249	-0.181	-0.022	-0.001	-0.096	0.066	-0.193	0.127	-0.049	-0.065
Area (polygon)	-0.126	-0.754	**2.000**	-0.894	0.037	**-2.141**	**-1.879**	**-4.067**	0.461	**5.971**
Fractal Dim.	-0.076	-0.126	-0.021	-0.027	0.022	0.025	-0.017	0.104	-0.095	-0.054
Box Width	**-4.002**	**3.762**	-0.320	**-1.280**	**3.898**	0.797	**-2.093**	**-5.159**	**1.413**	**-4.782**
Box Height	**-2.846**	**5.959**	-0.184	**1.771**	**3.833**	**3.936**	**-1.718**	-0.395	**3.464**	**1.553**
Feret (min)	**3.763**	**4.851**	**1.435**	**2.380**	**-2.468**	-0.617	**-2.432**	-0.619	**-1.905**	-0.930
Density (min)	0.032	-0.022	-0.018	0.057	-0.016	-0.018	-0.005	-0.041	0.006	0.027
Density (max)	-0.028	0.009	0.016	-0.027	0.004	0.001	0.012	-0.008	-0.007	0.002
Density (std.dev.)	0.009	-0.034	0.022	0.040	-0.013	-0.015	-0.011	-0.046	0.001	0.032
Dendritic length	0.417	-0.320	-0.247	0.208	0.515	0.253	-0.261	-0.364	0.162	-0.016
Dendrites	-0.360	0.343	0.053	0.084	-0.331	-0.307	0.338	0.190	-0.155	0.100
Margination	0.041	-0.016	0.041	-0.045	0.048	0.004	0.010	0.029	0.006	-0.009
Heterogeneity	0.015	0.016	-0.038	-0.016	0.012	0.006	0.006	0.038	0.005	-0.022
Clumpiness	-0.067	0.078	0.005	-0.073	-0.009	-0.009	0.014	0.020	0.014	-0.044
Form Factor	**5.547**	-0.564	-0.530	**1.257**	**-1.767**	**-1.365**	0.848	**-1.223**	-0.443	**1.673**
ECD	0.351	0.165	-0.892	0.470	0.819	-0.785	**1.118**	-0.173	**-1.322**	**1.391**
Compactness	0.146	0.004	-0.119	-0.020	0.211	-0.085	0.189	0.019	-0.300	0.209
Modification ratio	0.338	-0.133	0.243	-0.283	-0.004	-0.080	0.174	0.050	0.133	-0.085

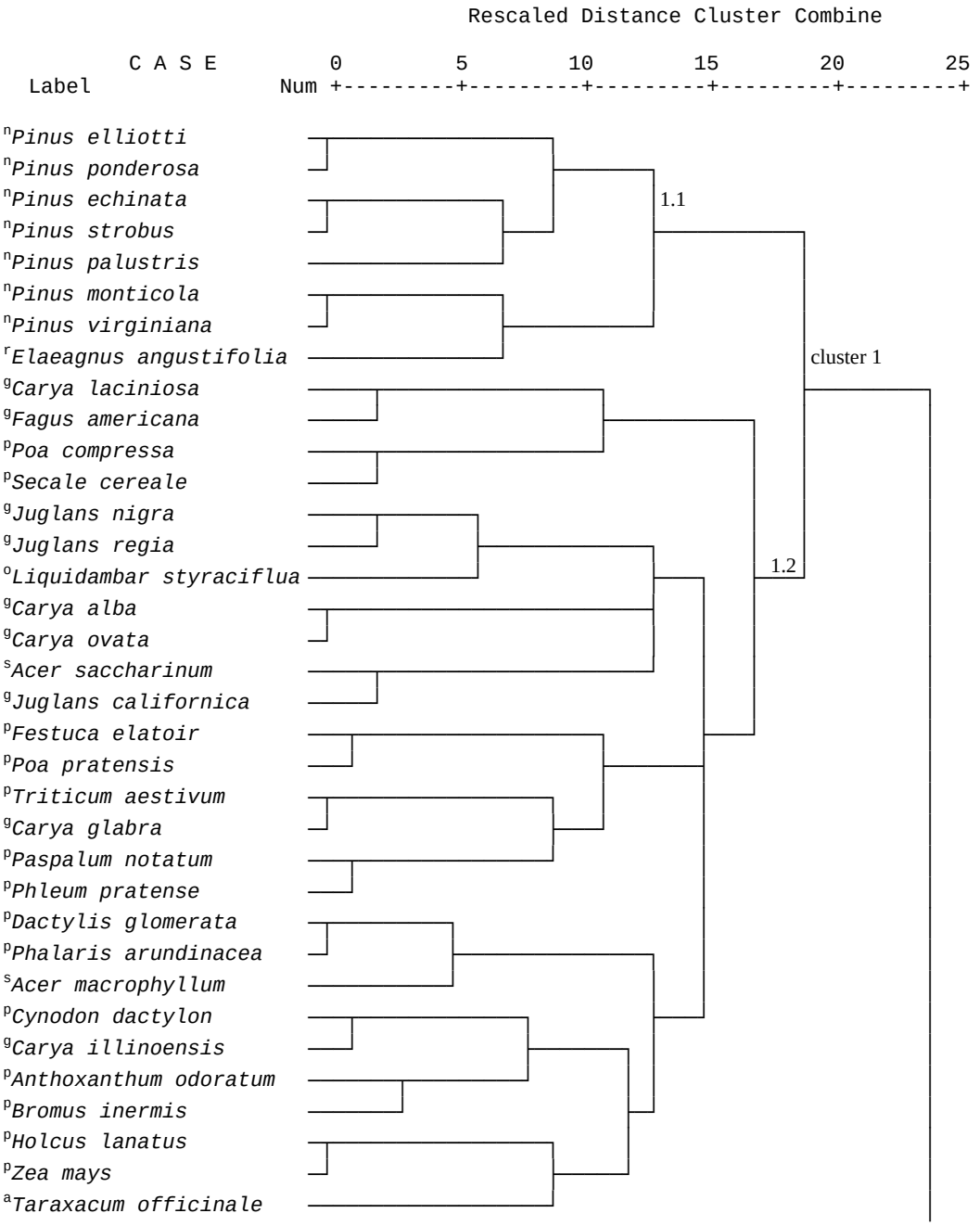
The magnitude of the canonical discrimination function coefficient indicates the weightage of the parameter in each factor in differentiating airspora types. Parameters with high magnitudes in an earlier factor also carry a higher weightage in differentiating the airspora. Abbreviated parameters: ECD = equivalent circular diameter, IOD = integrated optical density.

3.3.6 Cluster analysis

Two main clusters were obtained for the analysis of all pollen types (Figure 3.1). Cluster 1 consists of two sub-clusters with 35 pollen types while Cluster 2 can be divided into seven sub-clusters. Subcluster 1.1 consisted of mainly pollen types from the *Pinus* species (*Pinus elliotti, Pinus ponderosa, Pinus echinata, Pinus strobes, Pinus palustris, Pinus monticola* and *Pinus virginiana*) with an exception of *Elaeagnus angustifolia* from the Caryophyllales order. Fourteen out of 22 grass pollen types were grouped in Subluster 1.2.

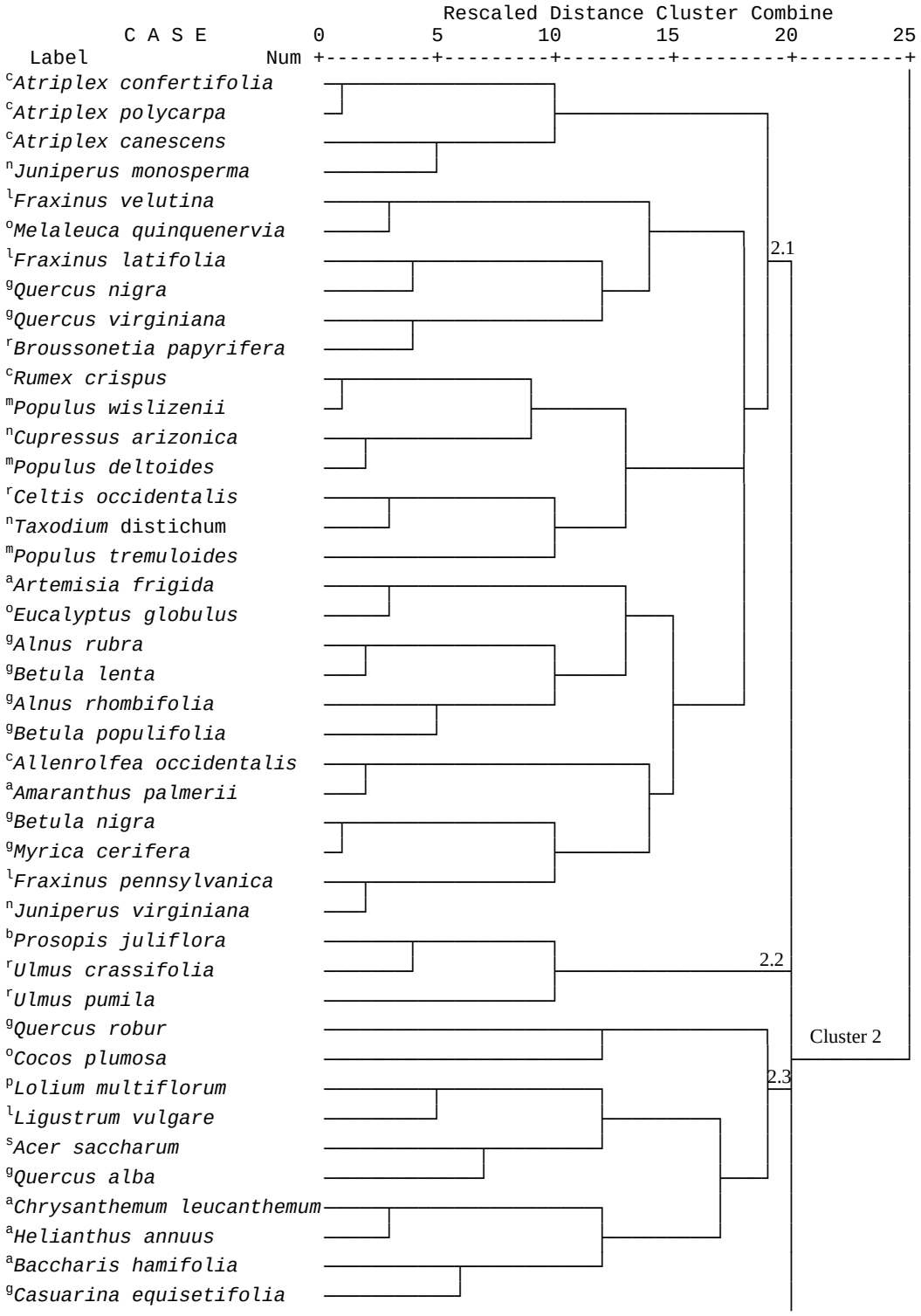
Subcluster 2.1 consisted of *Atriplex confertifolia, Atriplex polycarpa* and *Atriplex canescens, Alnus rubra, Betula lenta, Alnus rhombifolia* and *Betula populifoli.* In Subcluster 2.3 *Chrysanthemum leucanthemum, Helianthus annuus* and *Baccharis hamifolia* were closely grouped together. Subcluster 2.4 consisted of a large group of Asteraceae pollen types whihc consisted of *Baccharis sarothroides, Iva axillaris, Ambrosia acanthicarpa, Ambrosia deltoidea, Amaranthus hybridus, Hymenoclea salsola, Iva angustifolia, Ambrosia psilostachya, Ambrosia trifida, Chenopodium ambrosioides* and *Xanthium commune*. *Quercus garryana, Quercus velutina* and *Quercus kelloggii* were grouped together in Subcluster 2.6. The rest of the grass pollen types were in Subcluster 2.7. *Quercus lobata, Quercus stellata* and *Quercus dumosa*; and *Quercus agrifolia, Quercus rubra* and *Quercus macrocarpa* were clustered together in the subclusters in Subcluster 2.7. Generally, Poaceae, Asteraceae and *Quercus* pollen types were more easily clustered.

Figure 3.1: Results of the cluster analyses of all pollen types.



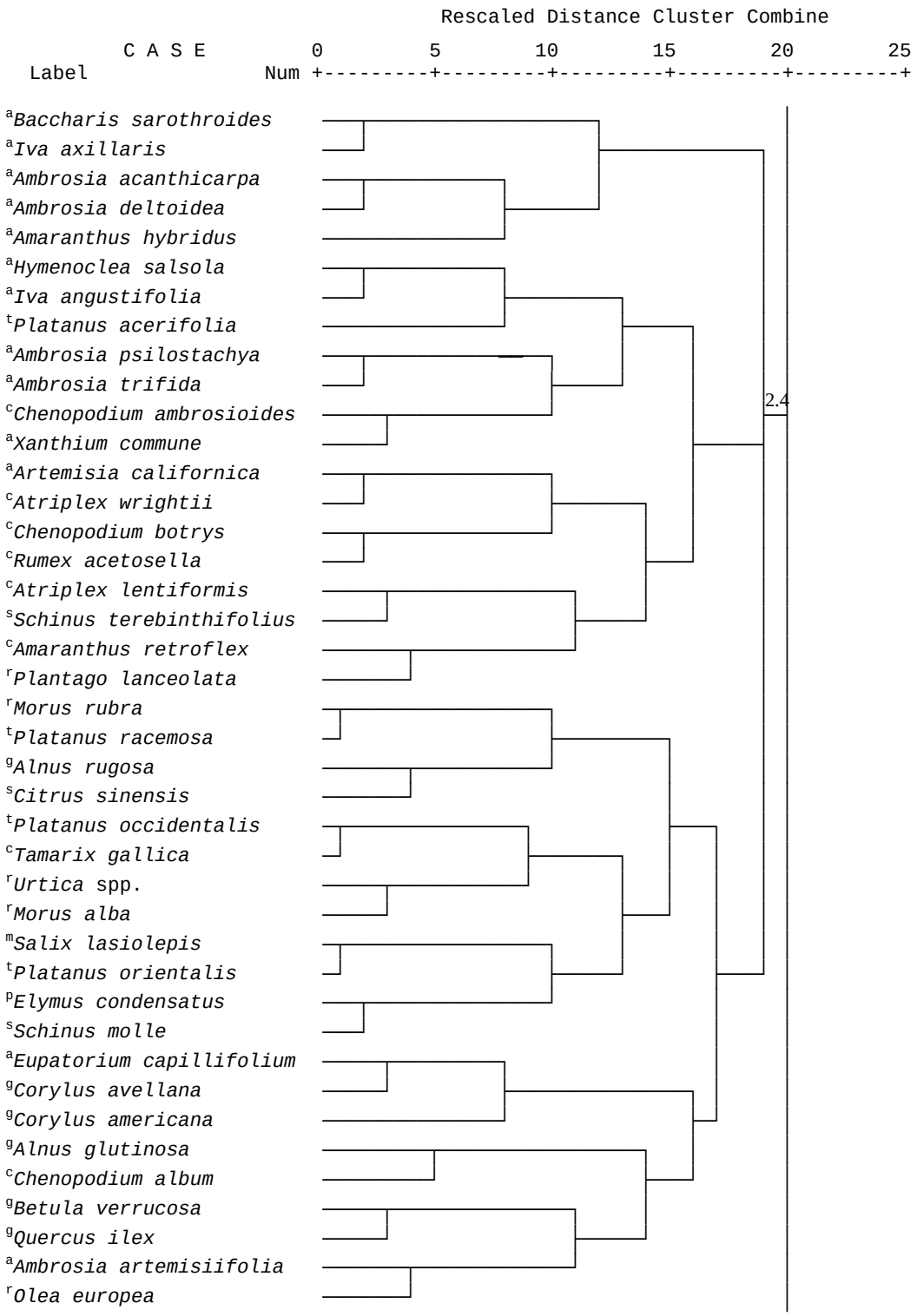
Orders of the pollen types: [a]Asterales, [p]Poales, [c]Caryophyllales, [b]Fabales, [g]Fagales, [l]Lamiales, [m]Malpighiales, [n]Pinales, [t]Proteales, [r]Rosales, [s]Sapindales and [o]others.

Figure 3.1 (continued): Results of the cluster analyses of all pollen types.



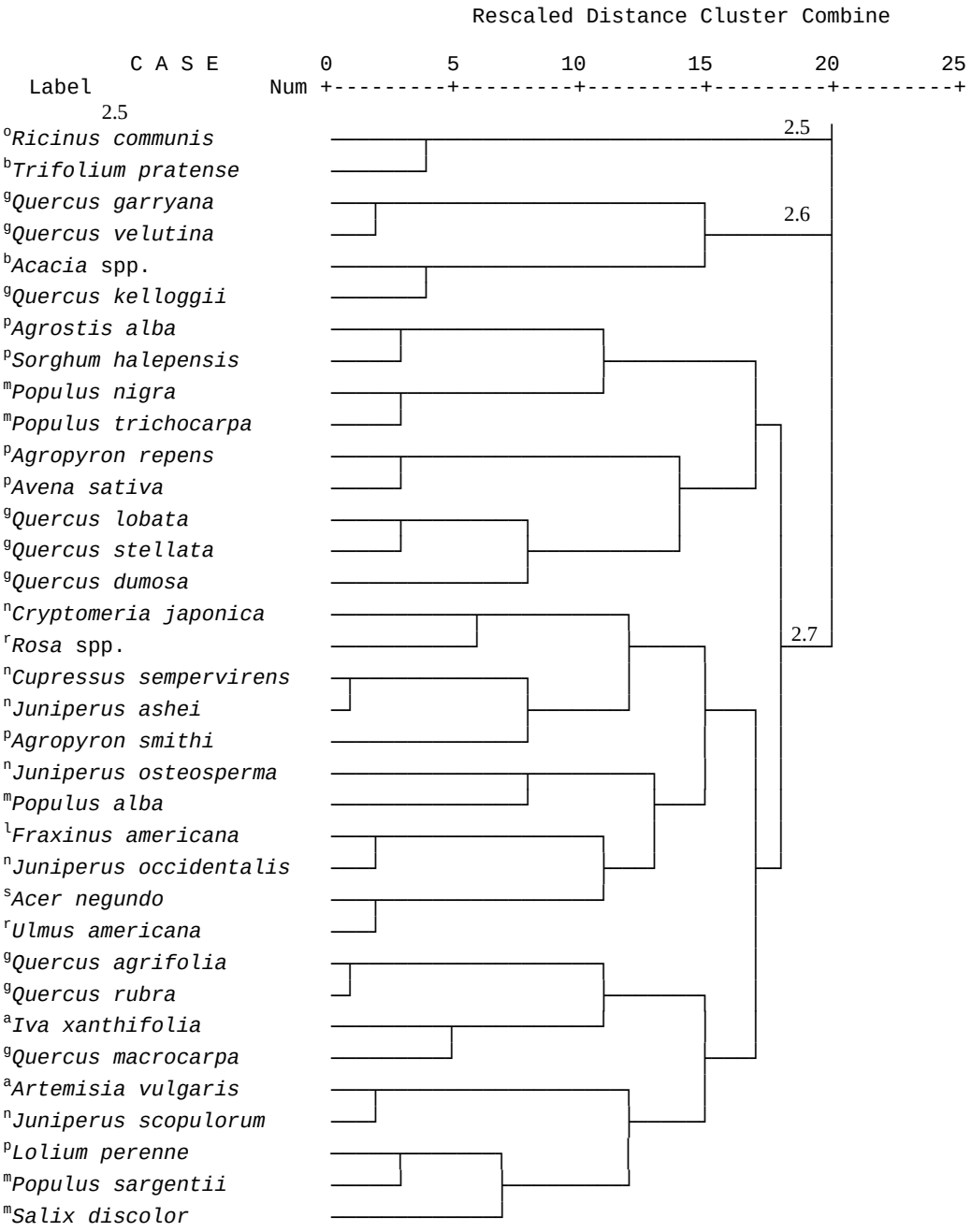
Orders of the pollen types: [a]Asterales, [p]Poales, [c]Caryophyllales, [b]Fabales, [g]Fagales, [l]Lamiales, [m]Malpighiales, [n]Pinales, [t]Proteales, [r]Rosales, [s]Sapindales and [o]others.

Figure 3.1 (continued): Results of the cluster analyses of all pollen types.



Orders of the pollen types: [a]Asterales, [p]Poales, [c]Caryophyllales, [b]Fabales, [g]Fagales, [l]Lamiales, [m]Malpighiales, [n]Pinales, [t]Proteales, [r]Rosales, [s]Sapindales and [o]others.

Figure 3.1 (continued): Results of the cluster analyses of all pollen types.



Orders of the pollen types: [a]Asterales, [p]Poales, [c]Caryophyllales, [b]Fabales, [g]Fagales, [l]Lamiales, [m]Malpighiales, [n]Pinales, [t]Proteales, [r]Rosales, [s]Sapindales and [o]others.

3.4 DISCUSSION

Local airspora types that are more morphologically dissimilar had poorer identification accuracies in comparison to airspora groups that were morphologically similar (Asteraceae, Poaceae and *Olea* look-alike pollen types). This is attributed to the lack of the differentiating parameters such as colour- (density of the RGB channels) and texture- (clumpiness, heterogeneity and margination) which were not measured during the initial work resulting in a large number of parameters required for identification. Airspora types are mostly misidentified as other airspora types with similar size and shape (Sivanesan 1984; Huang, 1981; Ellis, 1976, Erdtman, 1943). The average diameter and standard deviation of the airspora types are given in the brackets. The *Nephrolepis auriculata* spore (25.5± 2.1μm) is often misidentified as that of *Dicranopteris curranii* (28.0 ± 4.1μm) as both types are both ellipsoidal in shape or as the pollen grain of *Kyllingia polyphylla* (25.0 ± 1.6μm) which is trapezoidal to ellipsoidal. The *Casuarina equisetifolia* pollen grain (28.0 ± 2.1μm) is often misidentified as that of *Cynodon dactylon* (28.4 ± 2.2μm) as both are spheroidal. The *Elaeis guineensis* pollen grain (27.8 ± 2.0μm) is often misidentified as the spore of *Dicranopteris linearis* (28.4 ± 3.0μm) as both are triangular at the polar view. Meanwhile, the *Pithomyces maydicus* (10.4 ± 1.0μm) which is ellipsoidal is often confused with those of *Curvularia* spp. (13.2 ± 1.4μm) which are broadly fusiform to ellipsoidal. The *Dicranopteris curranii* spore is the airspora type most often confused with other local airspora. The parameters used to differentiate the local airspora are basically shape-based.

Forty parameters were required to differentiate the Poaceae (grass) pollen types owing to their high similarity to each other resulting in them being classified as “grass pollen” in airspora counts (Esch, 2004; Glassheim *et al.*, 1995; Mullins *et al.*, 1986). This can

also be seen by the mixture of parameter types which encompass size, shape, colour and texture used in differentiating a large percentage of the grass pollen studied. One hundred percent accuracy for *Zea mays* and 98.8% for *Triticum aestivum* were achieved because of the significantly larger sizes of the pollen grains of these two species. The minimum diameters for the *Zea mays* (71 ± 10.3 μm) and *Triticum aestivum* (63.3 ± 4.3 μm) pollen grains are larger then the maximum diameter of that of *Secale cereale* (59.3 ± 4.2 μm) which is the next closest in size. The average diameters of the grass pollen types in this study are between 24.7 to 54.2 μm. Misidentification as a similar subfamily or closely related subfamily would not cause much problem for studying allergies as it has been demonstrated that pollen from the same subfamily tends to cross-react (van Ree *et al*., 1992; Martin *et al*., 1985; Bernstein *et al*., 1976; Leiferman and Geich, 1976). Pollen from subfamily Pooideae has also been shown to cross-react more often with those of the Panicoideae than Chloridoideae (Esch, 2004; Weber, 2003).

Asteraceae pollen grains are spheroidal or oblately flattened, and 16.5 to 30 μm in diameter (Lewis *et al.*, 1985). The main offenders in allergy from this family are species from Artemisia, Baccharis and members of the subtribe Ambrosiinae (*Ambrosia, Hymenoclea, Iva* and *Xanthium*) (Asero 2002; Hirschwehr *et al*., 1998; Keith *et al*., 1994; Lu *et al*., 1994, Lewis *et al*., 1985). Generally, extremely low levels (<2%) of misidentification occurred between the wind- and insect-pollinated pollen types. In the insect-pollinated group, thick exines with conical, sharp-pointed spines are common features while the spines for wind-pollinated forms are greatly reduced or entirely absent (Martin *et al*., 2001; Erdtman, 1943). Even though high misidentification rates occur for pollen grains of *Hymenoclea salsola* with those of *Artemisia californica* and *Ambrosia psilostachya*, these pollen types are grouped among the major allergy of-

fenders, thus all are clinically important. Meanwhile, *Ambrosia californica* and *Iva axillaris* are in the same subtribe thus both have spinules that are reduced to small, pointed or blunt projections. *Baccharis halimifolia* and *Eupatorium capillifolium* are both grouped into the long-spined pollen grains of the Asteraceae. Similarity in surface textures coupled with overlapping sizes were the causes of misidentification in this study.

Olea look-alike pollen types were assembled based on the findings by the ASTHMA group in Europe and who are working on automating the airspora identification and subsequently, the counting process. The pollen types chosen were those that have been found to be often misidentified as *Olea*. In our study, the *Olea*-look alike pollen types is the group with the lowest percentage of misidentification. Better identification was partly due to the inclusion of pollen types from three different orders which includes Lamiales, Malpighiales and Brassicales which still have individual inherent morphological properties useful in identification. This can be seen by the pattern of misidentification which happens only between pollen types within the same family but from different genera.

Misidentification basically occurs between pollen types of the same genus due to higher shared similarities in pollen morphology and which also share similar allergenic properties. The different genera in the same family are generally observed to be differentiated mainly by size-based parameters. This is an interesting phenomenon since pollen grains in this group have overlapping size ranges mostly with diameters between 20 to 30 μm.

Pollen types that were clustered far from their closely related counterparts were those that had higher rates of misidentification. The high rates of misidentifications were

because of the availability of low numbers of parameters (38) in comparison to the very large number of taxa studied (153). Even so, the ability to identify more than 37% of the pollen types (57) with above 70% accuracy can be considered satisfactory. The number of parameters for identification or its measurement range will need to be increased to facilitate identification of a larger number of taxa. The feasibility of using pollen morphology to study taxonomic relationships is not unproven (Telleria *et al*., 2003; Lindbladh *et al*., 2002; Regalado and Sanchez, 2002; Martin *et al*. 2001; Ridder-Numan and van der Ham, 1997; Karis, 1995). However, these studies required information gathered by using tedious techniques and expensive equipment like the electron microscope.

Image analysis has been shown to be a useful tool for differentiating airspora types. The fact that the study was done using two dimensional images obtained using light microscopy is also important. The only work on airspora identification using single plane light microscopy images has been carried out on fungal spores where good differentiating abilities was also obtained (Benyon *et al*., 1999). Much of the current work in trying to automate airspora counts, especially for pollen analysis, are based on three-dimensional images obtained from the stacking of images from light microscopy (Boucher *et al*., 2002) or fluorescence imaging (Ronneberger *et al*., 2002). These methods are labour- and cost-intensive, and the large image files pose a computational problem when automation is to be carried out. Comparisons made by these workers were also between only a limited number of taxa (maximum number of taxa studied equals 26) that are taxonomically distantly related. Other studies using computer-aided recognition involved the input of quantative and qualitative data, which included the description of morphological features currently used in pollen analysis (Lebbe *et al*., 1986).

The accuracy of identification can further be improved with more surface texture parameters. The use of texture-based parameters was the basis of most of the pollen identification work regardless of whether light, fluorescence, scanning electron or transmission electron microscopy were used (Boucher *et al*., 2002; Regalado and Sanchez, 2002; Ronneberger *et al*., 2002; France *et al*., 2000, Currie *et al*., 1997; Vezey *et al*., 1993; Langford *et al*., 1990; Lebbe *et al*., 1986). The textual features used in this study are basic parameters available in most general image analysis software. Use of statistically or computationally more intensive classifiers like support vector machine (Ronneberger *et al*., 2002), active contour snake or neural network (France *et al*, 1997) and sum modified Laplacian (Boucher *et al*. 2002) have also resulted in higher accuracy in identification results.

The additional use of information such as the local airspora and surrounding vegetation composition in each area and the seasonality period will also increase the accuracy of the identification process. This is possible because airspora that does not exist in a particular location of in certain season time period can be excluded from the series of airspora that could be need to be identified. The will be inline with the final aim to use image analysis to automate the current airspora quantification process.

3.4 CONCLUSIONS

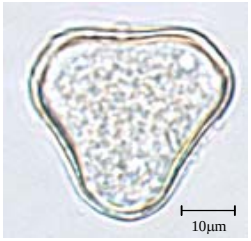
The use of image analysis coupled with light microscopy is a feasible method to use in developing an automated airspora identification and quantification system. The local airspora and pollen types which are morphologically similar can be accurately identified. Misidentifications occur mainly between pollen types that are very closely re-

lated but which also have a high tendency to share similar allergenic properties. Thus, the misidentification is not considered clinically detrimental. Shape- and size-based characters are important in the identification, but colour and textural characters are essential in the final discrimination between morphologically similar pollen types. The quantitative parameters that were measured have also been proven useful in taxonomic classification of the pollen types studied.

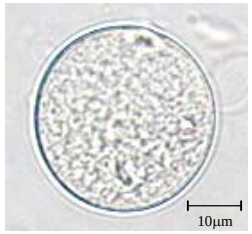
This study has been used as the basis for pursuing the development of an automated platform for identification of the local airspora. Information obtained from this study has been used as a set of guidelines for developing a fully automated system for airspora identification and quantification. Type of filters, segmentation, clump splitting solutions and useful parameters for identification were valuable information garnered from this study. Incorporation of more texture based and computational intensive classifiers has also been incorporated in this work. An identification rate of more than 99% has been demonstrated on the same set of images of local airspora used in this study. The use of more sophisticated classifiers, i.e., neural network and vector support machine have also greatly reduced the number of samples required to train the identification system. This collaborative work is currently being pursued.

Figure 3.2: Photomicrographs of the local airspora studied.

Acacia auriculiformis

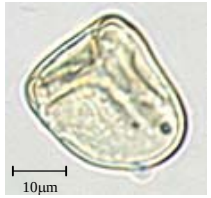


Elaeis guineensis

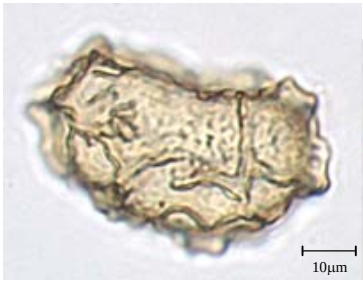


Cynodon dactylon

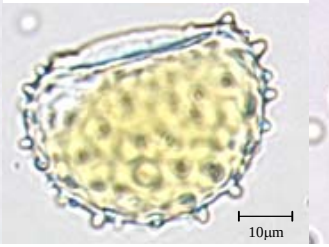
Casuarina equisetifolia



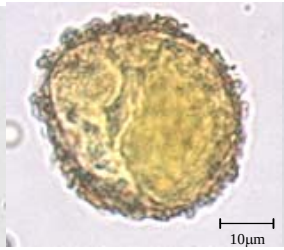
Kyllingia polyphylla



Asplenium nidus



Stenochlaena palustris



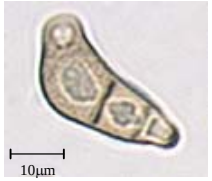
Pteridium aquilinum

Dicranopteris linearis

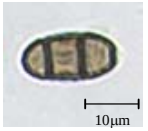
Nephrolepis auriculata

Dicranopteris linearis

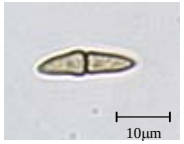
Drechslera- like spore



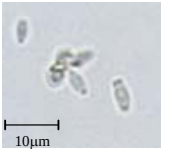
Curvularia spp.



Pithomyces maydicus



Didymosphaeria donacina



Cladosporium cladosporioides

Figure 3.3: Photomicrographs of the Poaceae pollen studied.

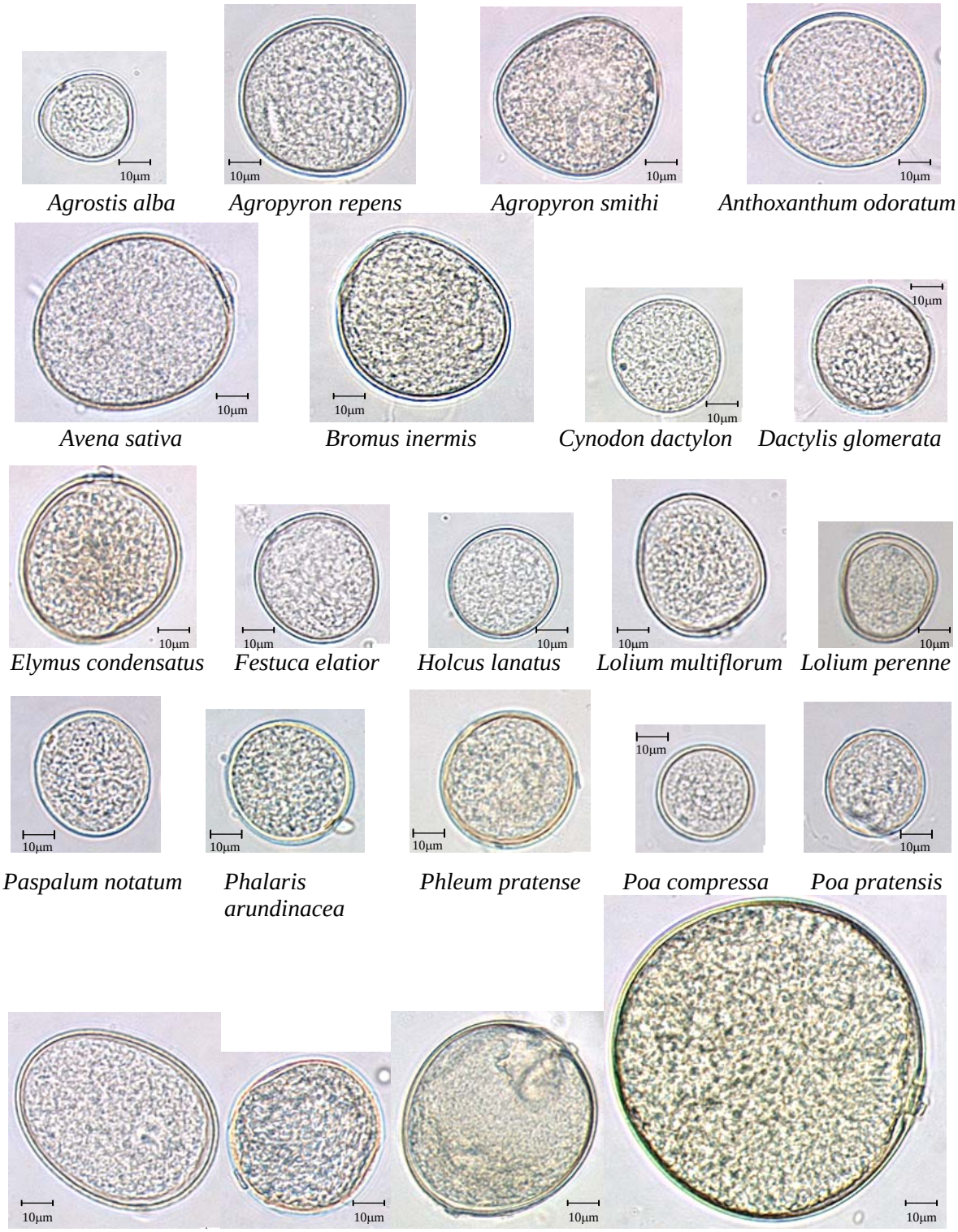
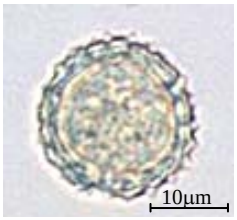
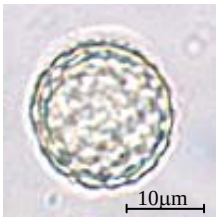


Figure 3.4: Photomicrographs of the Asteraceae pollen studied.

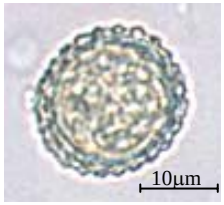


Ambrosia acanthicarpa

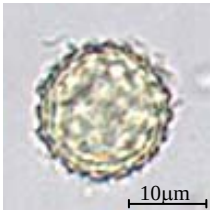


Ambrosia artemisiifolia

Ambrosia deltoidea



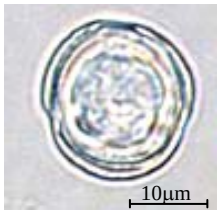
Ambrosia psilostachya



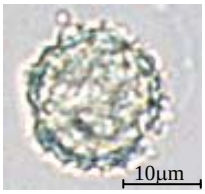
Ambrosia trifida

Artemisia californica

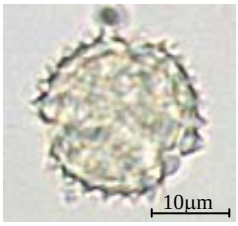
Artemisia frigida



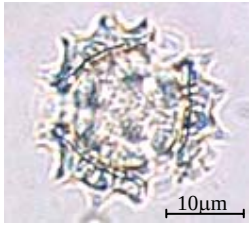
Artemisia vulgaris



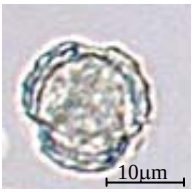
Baccharis halimifolia



Baccharis sarothroides

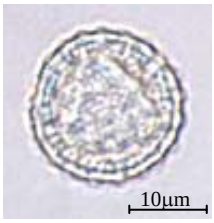


Chrysanthemum leucanthemum

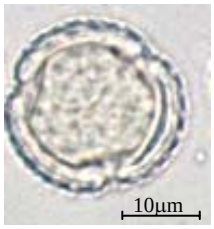


Eupatorium capillifolium

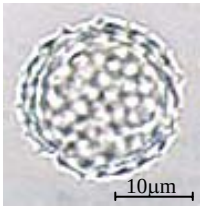
Hymenoclea salsola



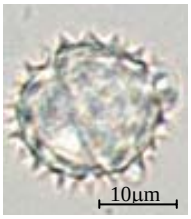
Iva angustifolia



Iva axillaris

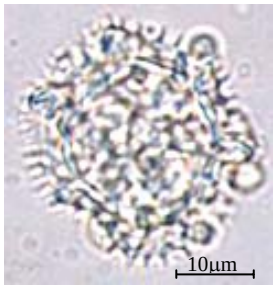


Iva xanthifolia

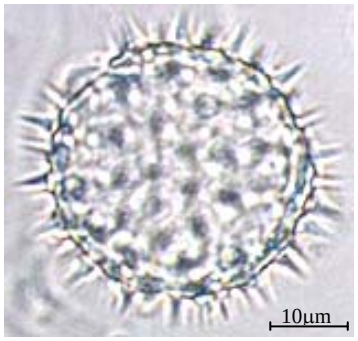


Solidago spp.

Xanthium commune



Taraxacum officinale

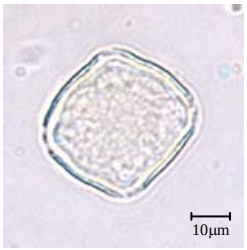


Helianthus annuus

Figure 3.5: Photomicrographs of the *Olea* look-alike pollen studied.

Brassica spp.

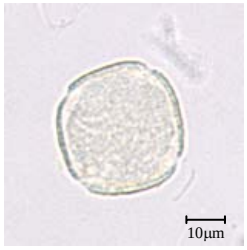
Elaeagnus angustifolia



Fraxinus americana

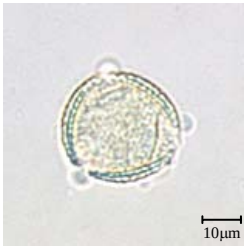
Fraxinus latifolia

Fraxinus pennsylvanica



Fraxinus velutina

Ligustrum vulgare

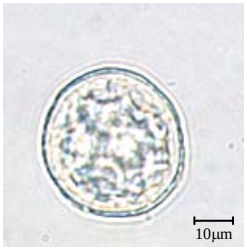


Olea europea

Populus alba

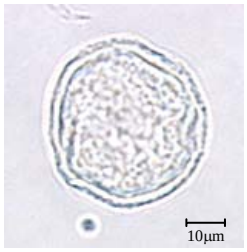
Populus deltoides

Populus nigra



Populus sargentii

Populus tremuloides



Populus trichocarpa

Populus wislizenii

Salix discolor

Salix lasiolepis

Salix nigra

CHAPTER 4: DEVELOPMENT OF A DOT IMMUNOARRAY SYSTEM FOR SIMULTANEOUS DETECTION OF A LARGE ARRAY OF ALLERGEN-SPECIFIC IGES

4.1 INTRODUCTION

4.1.1 Techniques in allergy diagnosis

Currently, allergies are mainly diagnosed based on positive skin prick tests (SPT) and the detection of specific immunoglobulin E (IgE) molecules.

In vivo tests like the commonly used SPT and intradermal test are based on the detection of histamine release in allergic individuals. For the SPT, allergens are introduced beneath the skin by pricking (Hamilton and Adkinson, 2003; Dreborg 1993, Shearer, 1989). Allergens will come into contact with tissue mast cells and bind to the adjacent specific IgEs causing cross-linking of IgEs. This in turn results in the depolarisation of mast cell membranes, entry of calcium and release of histamine with other pro-inflammatory mediators. Histamine release is responsible for the wheal and erythema produced on the skin. Reactions are then scored from 0 to 4+ based on wheal and erythema size and the presence of pseudopodia.

Currently, new technologies for diagnosing allergies are based on the *in vitro* detection of IgEs. These include radioallergosorbent (RAST) analogues and second generation methods for *in vitro* testing like those of ADVIA Centaur®, AlaSTAT®,

CARLA®, ENEA®, Hycor HY-TEC® and Pharmacia UniCAP® (Ricci *et al*., 2003; Hamilton *et al*., 1999; Plebani *et al*., 1998; Nolthe and DuBuske, 1997).

In *in vitro* immunoassays, immunosorbent matrices are utilized to bind to the allergens. Patients' sera are then added followed by incubation and washing. A conjugated anti-human IgE antibody is then added to detect the presence of IgEs, followed by further incubation and washing. Finally, an enzyme substrate is added and the levels of IgEs present are reported as corresponding values based on the levels of the enzyme response.

4.1.2 Measurement of IgE levels

The IgE molecule is normally a tissue-bound molecule and is present in nanogram levels in the serum. S.G.O. Johansson's discovery of an untypable myeloma protein termed IgND in the serum of a Swedish farmer permitted the separation and production of a large amount of proteins and rabbit anti-sera for use in immunoassays (Wide *et al*., 1967). It was later confirmed that IgND was similar to γE discovered by Ishizaka and Ishizaka (1967). Both were found to be the elusive reagin of immediate hypersensitivity reactions. This quickly resulted in the development of immunoassays for the detection of allergen specific IgEs.

Structurally, human IgEs contain two light chains (kappa, κ and lambda, λ) and two heavy epsilon (ε) chains (Roitt *et al*., 2001). The ε chains contain five structural domains (V_H, C_e1, C_e2, C_e3 and C_e4) that possess unique antigenic attributes, which confer special biological properties. The IgEs binds onto the Fcε receptors on cells, particularly the tissue mast cells and circulating basophils. Allergen cross-links IgEs

attached to basophils and/or mast cells cause degranulation and release of multiple chemical mediators which result in the manifestation of a spectrum of symptoms.

IgE in cord serum is usually below 2 IU ml^{-1} and progressively increases until 10 to 15 years of age in humans (Barbee *et al*., 1981; Wittig *et al*., 1980). After 14 years of age, total IgE levels of more than 333 kIU L^{-1} is considered abnormally elevated and strongly associated with allergic diseases when coupled with the presentation of allergic symptoms.

4.1.3 Advantages of *in vitro* techniques

Current *in vitro* techniques offer numerous advantages not available in *in vivo* ones (Dolen, 2003). Patients tested by SPT are directly exposed to the allergens thus increasing the risk of eliciting allergic reactions and the discomfort experienced during testing. Moreover, *in vivo* testing requires the patients to be closely monitored while testing and sometimes even after the test, requiring still more manpower. Fatality after undergoing skin testing for food allergens, a result of anaphylatic shock has been reported (Bernstein et al., 2004). *In vitro* tests can be requested for virtually anyone suspected to have allergies including sensitive and/or high risk individuals such as expectant mothers, the elderly and even patients with severe skin diseases even though it has been demonstrated that SPT is preferred and is a better predictor for allergic disease in children (Ricci *et al.*, 2003).

Even though the *in vivo* assay namely the skin prick test gives rapid results in 15 to 20 minutes, *in vitro* tests, which utilize solid-phase immunoassay reagents have longer shelf lives and are amenable to ongoing quality control for stability and reproducibil-

ity (Dolen, 2003). Results are also not influenced by pharmacotheraphy, thus medication need not be withdrawn prior to testing. Results are also quantitative in nature. These tests can be ordered by any clinician and expertise is only required in interpreting the results while performance of *in vivo* techniques requires training, skill and expertise which varies between individuals (Nelson, 1994).

4.1.4 Limitations of the available techniques

In vitro testing is convenient and safe for the patient. Just a single blood sample will provide sufficient samples for multiple allergy tests. However, tests are performed for one allergen at a time thus requiring a large amount of serum if large numbers of allergens are to be screened. The amount of serum required limits the number of allergens that can be tested. Additional tests will need to be requested if the panel of allergens initially tested was not chosen appropriately. All these can result in wrong diagnoses and frustration to patients and clinicians, especially when allergy to rare allergens are involved. Above all, these current methods are costly. On average, the cost of a single *in vitro* test in Singapore ranges between S$18.00 to $25.00 (charges by the National University Hospital and Allergy Laboratories Pte. Ltd.) depending on the system used and type of allergens tested.

4.1.5 Aims

Hence, we sought to develop a screening assay that could be used in research and also clinically which 1) does not expose patients to allergens, 2) enables rapid simultaneous testing of a large panel of allergen sources and 3) has a minimal cost.

4.2.1 Dotting apparatus

The dotting apparatus used in the allergen array consist of a 384-pin MULTI-BLOT™ replicator (VP386) and colony copier (VP380) from V&P Scientific (California) as well as single well plates (NUNC, USA). The replicator consists of 384 solid 1.19 mm diameter hydrophobic pins, designed to deliver 0.1 μl of liquid onto a membrane. The colony copier registers the replicator to the membrane on a single well plate for high density arrays by using four holes located on the rear of the copier frame. This results in an array of 1536 spots on a 7.5 × 11.5 cm membrane.

4.2.2 Support materials and washing buffers

Two types of membranes were tested for use as the solid phase support. The Trans-Blot nitrocellulose membrane (Bio-Rad, USA) and Hybond polyvinylidine difluoride (PVDF) membrane (Amersham Life Science, USA) were tested. Membranes were loaded with 1 μg of bovine serum albumin (BSA) per dot and air-dried. Concentrations of a commonly used detergent, Tween 20 (Duchefa, Netherlands) in washing buffers were also studied. Dotted membranes were washed with phosphate buffered saline (PBS) (37mM NaCl, 8.1mM Na_2HPO4, 2.7mM KCl, 1.4mM KH_2PO4, pH 7.4) containing 1.0%, 0.25%, 0.1%, 0.05% and 0% of Tween 20 by placing them on an orbital shaker. All membranes were stained with amido black staining solution (25% 2-propanol (v/v), 10% acetic acid (v/v), 0.5g amido black, 64.5 ml distilled water) to check for protein concentrations and blotted dry before scanning using conventional

scanners. Dot intensities on the membranes were then measured using the Olympus MicroImage™ image analysis software (Media Cybernetics, 1999) by manually setting the threshold levels of the colour intensities. The protein concentrations were expressed in units of optical density (OD), which range from 0 to 255 as defined by the software.

4.2.3 Loading efficiency of the 384-pin MULTI-BLOT™ replicator

BSA (0.2 mg ml^{-1}) in extraction buffer (137 mM NaCl, 8.1 mM Na_2HPO4, 2.7 mM KCl, 1.4 mM KH_2PO4, pH 7.4, 20% glycerol, 1 mM EDTA) was filled into a flat-bottomed 384-well plate. The dotting kit was then used to produce a membrane with 1536 dots onto the nitrocellulose membrane (Bio-Rad, USA). In total, 1 μg of BSA was loaded onto each dot. This was achieved by repeatedly dotting the same spots followed by air-drying five times after which membranes were allowed to dry overnight to ensure thorough drying. Membranes were then stained with amido black staining solution, blotted dry and scanned with conventional scanners. Subsequently, dot intensities were measured using the MicroImage™ software.

4.2.4 Allergen extracts

Skin prick extracts from ALK-Abelló S.A. (Spain) and GREER Laboratories Incorporated (USA) were purchased. However, quite a number of skin prick extracts purchased did not meet the minimum requirement of 0.2 mg ml^{-1} in total protein concentration. Thus, raw materials were purchased from Greer Laboratories Incorporated and Allergon AB (Sweden). Table 4.1 shows the allergen sources studied. All raw

materials including the local allergen sources were then homogenized using a mortal and pestle after quick freezing with liquid nitrogen and suspended in extraction buffer for either 3 or 16 hours at 4°C as indicated in Table 4.1.

The extracts were then centrifuged at 14,000 g for 10 minutes at 4°C. Supernatants were collected and the pellets discarded. Total protein concentration was then determined using the BioRad protein assay kit (Bio-Rad Laboratories, USA) based on the Bradford method (Bradford, 1976).

4.2.5 Allergen array for the detection of specific IgE

Allergen extracts prepared at 0.2 mg ml^{-1} were filled into the 384-well plates. Membranes 7.5 × 11.5 cm in size were then placed onto single-well plates. Each membrane after dotting will consist of three replicates of a full set of allergens. The actual membrane size for a set of the allergen array was approximately 2.5 × 3.8 cm. The total protein loaded for each allergen source was 1 μg, achieved by repetitively dotting five times at the same spot. All allergen sources were dotted in duplicate for each set of the array. The human serum IgE standard (75/502), purchased from the National Institute for Biological Standards and Control (NIBSC), United Kingdom was used as a positive control. BSA and extraction buffer were used as the negative protein control and buffer controls, respectively. The dotted membranes were then left to dry overnight.

After drying, membranes were blocked with 4% skimmed milk powder (Anlene™, New Zealand) (w/v) in PBS at room temperature for 1 hour. Subsequently, washing was carried out three times with PBS-T (0.05% Tween 20) (v/v) for 15, 7 and 7

Table 4.1: Allergen sources dotted onto the array.

Allergen type	Species
Pollen	*Acacia auriculiformis* (acacia)[c,3], *Agrostis alba* (bent grass)[a], *Agropyron repens* (quack grass)[a], *Alnus glutinosa* (black alder)[a], *Alopecurus prantesis* (foxtail, meadow)[a], *Ambrosia artemisiifolia* (annual ragweed)[d,3], *Amaranthus hybridus* (careless weed)[d], *Ambrosia trifida* (tall ragweed)[a], *Acer negundo* (box elder)[d], *Anthxanthum odoratum* (sweet vernal grass)[a], *Atriplex polycarpa* (allscale)[d], *Arecastrum romanzo ffianum* (queen palm)[d], *Artemisia vulgaris* (common mugwort)[a], *Avena sativa* (cultivated oats)[a], *Baccharis halimifolia* (eastern baccharis)[d], *Betula verrucosa* (white birch)[d], *Bromus mollis* (spear grass)[a], *Brassica* spp. (brassica pollen)[d], *Carpinus betulus* (hornbeam)[a], *Casuarina equisetifolia* (Australian pine)[d], *Calluna vulgaris* (heather)[f], *Chenopodium album* (lamb's quarter)[a], *Chrysanthemum leucanthemum* (ox eye daisy)[a], *Corylus avellana* (hazel)[f], *Cryptomeria japonica (*Japanese cedar)[d,3], *Cupressus arizonica* (Arizona cypress)[f], *Cupressus sempervirens* (Italian cypress)[f], *Cynodon dactylon* (Bermuda grass)[d], *Dahlia cultorum* (dahlia)[a], *Dactylis glomerata* (orchard grass)[f], *Elaeis guineensis* (oil palm)[c,3], *Eucalyptus globules* (bluegum)[d], *Fagus sylvatica* (European beech)[f], *Festuca prantesis* (meadow fescue)[f], *Fraxinus excelsior* (ash)[a], *Holcus lanatus* (velvet grass)[a], *Hordeum vulgare* (cultivated barley)[a], *Humulus lupulus* (hops)[a], *Juniperus asheisabinoides* (mountain cedar)[d], *Ligustrum vulgare* (common privet)[f], *Lolium perenne* (perennial rye grass)[f], *Medicago sativa* (alfalfa)[a], *Olea europea* (olive)[f], *Parietaria judaica* (wall pellitory)[f], *Populus deltoides* (eastern cottonwood)[d], *Phragmites communis* (reed)[f], *Philadelphus coronarius* (syringa)[a], *Phlenum pratesense*(Timothy grass)[b], *Pinus radiata* (pine)[a], *Platanus acerfolia* (plane tree)[d], *Plantago lanceolata* (English plantain)[a], *Populus nigra* (black poplar)[a], *Poa pratensis* (Kentucky bluegrass)[f], *Podocarpus polystachyus* (sea teak)[d], *Pinus strobus* (eastern white pine)[d], *Populus trichocarpa* (black cottonwood)[d], *Quercus alba* (white oak)[d], *Quercus ilex* (live oak)[f], *Quercus robur* (red oak)[a], *Robinia pseudoacacia* (false acacia)[a], *Rumex acetosella* (sorrell)[a], *Salsola kali* (Saltwalt or Russian thistle)[a], *Sambucus nigra* (European elder)[a], *Salix viminalis* (willow)[a], *Secale cereale* (cultivated rye)[a], *Schinus molle* (pepper tree)[d], *Sorghum halepense* (Johnson grass)[d], *Solidago virgaurea* (golden rod)[a], *Syringa vulgaris* (lilac)[a], *Tamarix gallica* (salt cedar)[d], *Taraxacum officinale* (dandelion)[a], *Tilia cordata* (linden)[a], *Triticum aestivum/sativum* (cultivated wheat)[a], *Ulmus americana* (American elm)[d], *Ulmus minor* (English elm)[a], *Urtica dioica* (nettle)[f], *Zea mays* (corn)[d]
Fungi	*Alternaria alternata*[d], *Aspergillus flavus* [d], *Aspergillus fumigatus* [a], *Aspergillus niger* [c], *Aspergillus terreus* [d], *Botrytis cinerea* [f], *Candida albicans* [d], *Cladosporium cladosporioides* [c], *Cladosporium fulvum* [a], *Cladosporium herbarum* [c], *Corenyspora cassicola* [c], *Curvularia brachyspora*[c,3], *Curvularia fallax*[c,3], *Curvularia inequalis*[c,3], *Curvularia lunata*[c,3], *Curvularia pallescences*[c,3], *Curvularia spicifera*[a], *Drechslerea/Bipolaris sorokiana* [c,3], *Fusarium moniliforme* [a], *Fusarium solani* [d], *Malazessia furfur* [c], *Mucor mucedo* [a], *Penicillium brevicompactum* [f], *Penicillium chrysogenum* [d], *Penicillium expansum* [a], *Penicillium notatum* [c], *Penicillium roqueforti* [a], *Rhizopus nigricans* [a], *Saccharomyces cerevisae* [a], *Stemphylium botryosum* [f], *Trichoderma viride* [d], *Trichophyton mentagrophytes* [f], *Trichophyton rubrum* [d], *Ustilago tritici*[f]

Allergen sources: [a]ALK-Abelló S.A., [b]GREER Laboratories Incorporated, [c]local sources, [d]raw materials from GREER Laboratories Incorporated, [e]raw materials for Allergon AB and [f]Sigma. Allergen sources extracted at only for 3 hour is indicated as [3] while those not indicated were extracted for 16 hours.

Table 4.1 (continued): Allergen sources dotted onto the array.

Allergen type	Species
Mites	*Acarus siro*[c,3], *Austroglycyphagus geniculatus* [c,3], *Blomia tropicalis* [c,3], *Dermatophagoides farinae* [c,3], *Dermatophagoides pteronyssinus* [c,3], *Glycophagus domesticus* [c,3], *Lepidoglyphus destructor* [c,3], *Suidasia medanensis* [c,3], *Tyrophagus putrescentiae* [c,3]
Epithelial tissue/dander	budgerigar (*Melopsittacus undulatus)*[a], cat (*Felis domesticus)*[e], cow (*Bos taurus*) [a], dog (*Canis familiaris*) [a], feather mix (chicken and duck) (*Pullus gallinaceus* and *Anas platyrhynchas*)[a], goose (*Anser anser*) [a], goat (*Capra hircus*)[b], guinea pig (*Cavia porcellus*) [a], hamster (*Cricetus cricetus*) [a], horse (*Equus caballus*) [a] and rabbit (*Oryctolagus cuniculus*) [a]
Food (animal origin)	banana prawn (*Penaeus merguiensis*)[c,3], beef (*Bos taurus*)[b], casein[b], chicken (*Pullus gallinaceus*) [a], cockles (*Anadara granosa*)[c,3], egg white [a], egg yolk [a], mackerel fish (*Scomberomorus sp.*)[b], milk, cow (*Bos taurus)* [a], milk, goat (*Capra hircus*) [a], mud crab (*Scylla olivacea*)[c,3], mussels (*Perna viridis*)[c,3], ovalbumin [a], ovomucoid [a], pork (*Sus scrofa)*[a], rabbit (*Oryctolagus cuniculus*)[a], salmon fish (*Oncorhynchus sp.*)[d], sea bream fish (*Nemipterus furcosus*)[c,3], selar fish (*Atule mate*)[c,3], sheep (*Ovis aries)*[b], squid (*Photololigo duvaucelii*)[c,3], swimming crab (*Portunus pelagicus*)[c,3], tiger prawn (*Penaeus monodon*)[c,3], tuna fish (*Thunnus sp.*)[d]
Food (plant origin)	apple (*Malus domestica)*[b], banana (*Musa* hybrids)[c], broccoli (*Brassica oleracea* var. *botrytis*)[b], cabbage (*Brassica oleracea* var. *capitata*)[b], cacao (*Theobroma cacao)*[a], carrot (*Daucus carota)*[c], chard (*Beta vulgaris* var. *cicla)*[a], corn flour (*Zea mays*)[a], garlic (*Allium sativum)*[a], gliadine (*Triticum aestivum*)[a], hazelnut (*Corylus avellana)*[a], kiwi (*Actinidia chinensis*)[c,3], orange (*Citrus sinensis)*[c], peach (*Prunus persica)*[a], peanut (*Arachys hypogaea)*[a], potato (*Solanum tuberosum*)[c], rice flour (*Oryza sativa*)[a], soya bean (*Glycine max*)[c], spinach (*Spinacia oleracea*[a], strawberry (*Fragaria vesca)*[c,3], sunflower seed (*Helianthus annuus)*[a], tofu (*Glycine max*)[c], walnut (*Junglans regia)*[a], wheat flour (*Triticum aestivum*)[a]
Insects	American cockroach (*Periplaneta americana*)[b], fire ant (*Solenopsis invicta*)[b], German cockroach (*Blatella germanica*)[b], mosquito (*Culicidae* sp.)[b], oriental cockroach (*Blatta orientalis*)[a]
Venoms	honeybee (*Apis mellifera*)[b], hornet (*Dolichovespula* spp.)[b], wasp (*Polistes* spp.)[b], yellowjacket (*Vespula* spp.)[b]
Others	horseradish peroxidase [g], latex[b], bromelain[g]
Controls	NIBSC IgE standard (positive control), bovine serum albumin (protein control)[g], extraction buffer (negative control)

Allergen sources: [a]ALK-Abelló S.A., [b]GREER Laboratories Incorporated, [c]local sources, [d]raw materials from GREER Laboratories Incorporated, [e]raw materials for Allergon AB and [f]Sigma. Allergen sources extracted at only for 3 hour is indicated as [3] while those not indicated were extracted for 16 hours.

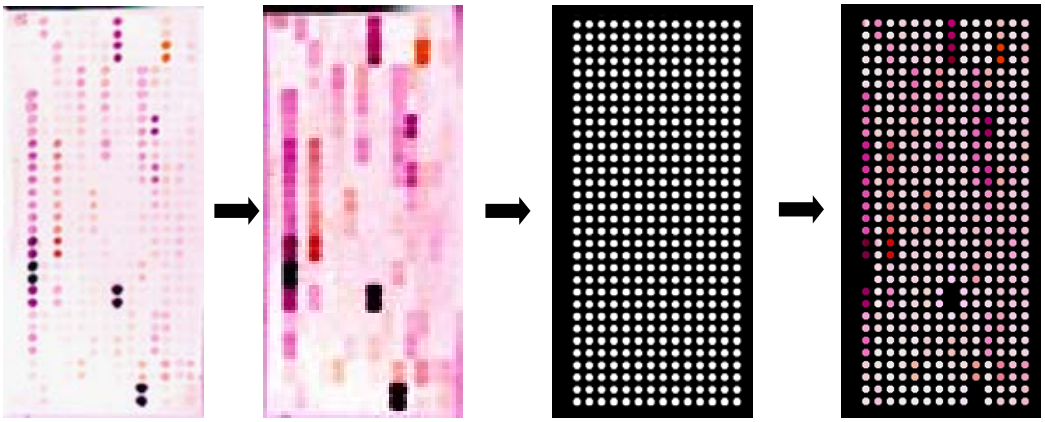
minutes, respectively. The same washing method was used throughout the assay, then the membranes were incubated with the respective patients' sera.

150 µl of serum was diluted at 1:1 (v/v) with PBS followed by overnight incubation overnight at 4°C. Membranes were then washed followed by incubation with the goat anti-human IgE ε-chain specific alkaline phosphatase conjugated antibody (Sigma, USA) at 1:800 (v/v) dilution with PBS-BSA (137 mM NaCl, 8.1 mM Na_2HPO4, 2.7 mM KCl, 1.4 mM KH_2PO4, pH 7.4, 20% glycerol, 1 mM EDTA, 1% BSA) for 2.5 hours at room temperature. Washing was then performed. The positive binding of the specific IgE to the allergen was visualised by developing with BCIP/NBT (5-bromo-4chloro-3indolyl-phosphate/nitro-blue tetrazolium) colour substrate (Promega, USA) in alkaline phosphatase buffer (100 mM Tris-HCl [pH 9.0], 150 mM NaCl, 1 mM $MgCl._2$). The membranes were then blot dried and scanned.

The reaction intensities were then measured using the Olympus MicroImage™ image analysis software. The images were processed through multiple morphological filters before the dot intensity readings were taken (Figure 4.1). First, the images were eroded using a 5 × 5 circle filter for a single pass to average out the colour of the dots. The dots on each membrane were then fitted onto a grid with 48 by 32 rings each with a diameter of 6 pixels by superimposition. Then a mask of the grid was created. The mask image was then filtered with a 5 × 5-circle single pass dilation followed by a 5 × 5-circle single pass closing to obtain solid circles. The logic operation 'AND' was performed (1^{st} operand = eroded array image and 2^{nd} operand = processed grid mask image). The final image, a superimposition of the processed grid image onto the array image, resulted in only the areas dotted with allergens is in its original colour. The rest of the image was black in colour. By using the command "automatic bright objects"

which works automatically by setting the OD range of the image using the colour histogram, the dots were segmented from the background.

Figure 4.1: Image processing sequence of the immunoarray membranes.



original membrane erode filter grid fitted mask read membrane

4.2.6 Patients and sera

In total, 508 consecutive sera from patients suspected of having allergies through clinical symptoms from 2000 to 2001, were screened. All sera were screened in duplicate. No clinical data of the patients were available for this study.

4.2.7 Allergen array validation

The Pharmacia UniCAP® system (Pharmacia Diagnostic AB, Sweden) and enzyme-linked immunosorbent assay (ELISA) were used to validate the allergen array results. Sera samples were randomly chosen for validation.

For ELISA, a total of 24 allergen sources from different groups were tested. They were pollen (*Acacia* spp., *Arecastrum romanzoffianum, Betula verrucosa, Casuarina equisetifolia, Cryptomeria japonica, Cynodon dactylon, Eucalyptus globulus, Elaeis guineensis, Olea europea, Pinus strobus, Quercus alba, Urtica dioica*), fungi (*Candida albicans, Curvularia lunata, Fusarium solani, Penicilllium notatum, Schinus mollis* and *Stemphylium botryosum*), mite (*Suidasia medanensis*), food of plant origin (soya bean [*Glycine max*], tofu [*Glycine max*]) and food of animal origin (banana prawn [*Penaeus merguiensis*], sea bream fish [*Nemipterus furcosus*] and selar fish [*Atule mate*]). A similar allergen extraction method was employed except that PBS was used instead of the extraction buffer.

Total protein (10 μg) in carbonate/bicarbonate buffer (8 mM Na_2CO_3, 17 mM $NaHCO_3$, pH 9.6) were coated overnight onto each well at 4°C. The wells were then washed three times with PBS-T. The same washing method was used through out the assay. Blocking was carried out using PBS-BSA for 30 minutes at room temperature followed by washing. 50 μl of patients' sera were then diluted 1:1 (v/v) with PBS and incubated in wells for 2.5 hours at 37°C. The wells were then washed and incubated with 2 μg ml^{-1} of a mouse anti-human IgE biotin conjugated (BD Pharmingen, USA) diluted with PBS-BSA for 2 hours at room temperature. Wells were then washed and incubated with 1:1000 (v/v) dilution of avidin-horseradish peroxidase (BD Pharmingen, USA) in PBS-BSA for 30 minutes at room temperature. This was followed by washing six times before the colour substrate 3,3´,5,5´-tetramethylbenzidine (TMB) (Sigma, USA) was added and the colour intensity read at 655 ηm.

Validation using the UniCAP® system was tested only for inhaled allergen sources. Two mite allergens (*Acarus siro* and *Dermatophagoides farinae*), 11 fungal allergen

sources (*Alternaria alternata, Aspergillus fumigatus, Botrytis cinerea, Candida albicans, Curvularia lunata, Fusarium solani, Penicillium notatum, Stemphyllum botryosum, Rhizopus nigricans, Tricoderma viride* and *Tricophyton mentagrophyt* var. *interdigitale*) and 16 pollen allergen sources (*Acacia longifolia, Arecastrum romanzoffianum, Artemisia vulgaris, Betula verrucosa, Casuarina equisetifolia, Chrysanthemum leucanthemum, Cupressus sempervirens, Cynodon dactylon, Cyryptomeria japonica, Eucalyptus* spp., *Olea europea, Pinus strobus, Schinus molle, Solidago virgaurea, Tilia cordata* and *Urtica dioica*) were tested.

4.2.8 Statistical analyses

Analyses were done using SPSS 11.5 for Windows (SPSS, USA). Effects of the types of membrane and detergent concentrations in washing buffer were analysed by ANOVA. Two standard deviations (SD) above negative reactions for each allergen source tested were used as the cut off points for positive results. All concordances were tabulated based on positive and negative reactions. Spearman's Correlation Test was used for all correlation analyses after the data was found to be not normally distribution. Examples of formulas used for concordances are as shown below.

For allergen *Acacia* spp., with 159 positives on membrane A, 150 positives on membrane B, and 135 positives on A and B there will be 349 negatives on membrane A, 334 negatives on membrane B and 334 negatives on A and B. The total number of sera tested = 508.

Inter-membrane positive concordance = number of paired positives/number of positives from the reference ×100

= 135/159 × 100

= 84.91%

Inter-membrane negative concordance = number of paired negatives/number of negatives from the reference ×100

= 334/349 × 100

= 95.70%

Total inter-membrane concordance = number of paired positives on A and B + number of paired negatives on A and B/number of membrane pairs × 100

= 135 + 334/508 × 100

= 92.32%

4.3 RESULTS

4.3.1 Support materials, washing buffer and loading efficiency

The average dot size on the membrane was 1.59 ± 0.18 mm in diameter. Protein concentrations were found to be significantly higher ($p<0.0001$) on the PVDF membrane (170.83 ± 8.93 OD) compared to that of the nitrocellulose (124.08 ± 7.92 OD). However, the background obtained using the PVDF membrane (66.42 ± 15.56 OD) was also found to be higher ($p<0.0001$) than that of the nitrocellulose (27.85 ± 9.44 OD) with no significant difference in reaction intensities when patients' sera were tested on the allergen array for these two support media. Furthermore, the use of the PVDF membrane, which requires pre-wetting with methanol before dotting, reduces its feasibility as the support medium in the allergen array owing to our repetitive dotting process.

Effects of different concentrations of Tween 20 detergent in PBS as the washing buffer are shown in Figure 4.2. Increasing concentrations of Tween 20 in the buffer showed a significant increase in the loss of proteins bound onto the membranes. The act of washing just with PBS alone causes a 4.6% loss in protein. However, we found that washing with just PBS results in a higher background compared to washing with buffers containing Tween 20%. A minimum of 0.05% Tween 20 (v/v) in the washing buffer was then used for all subsequent testing.

The dots' intensities and their ranges were found to increase with increasing amount of proteins loaded (Figure 4.3). Coefficients of variation for each pin ranged from 4.2 to 18.9% with an average 8.1% (Table 4.2).

Figure 4.2: Effects of different concentrations of Tween 20 detergent in PBS washing buffer. Means and SD (error bars) are shown.

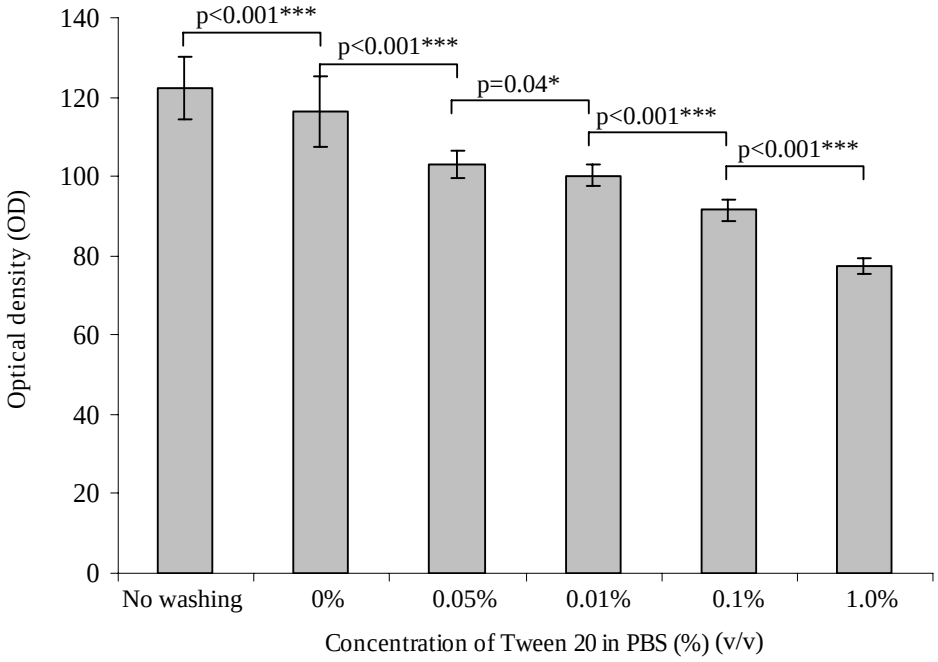


Figure 4.3: Optical density readings of the protein dots (BSA) at different concentrations. Maximum, minimum, means and SD (error bar) of dots are shown.

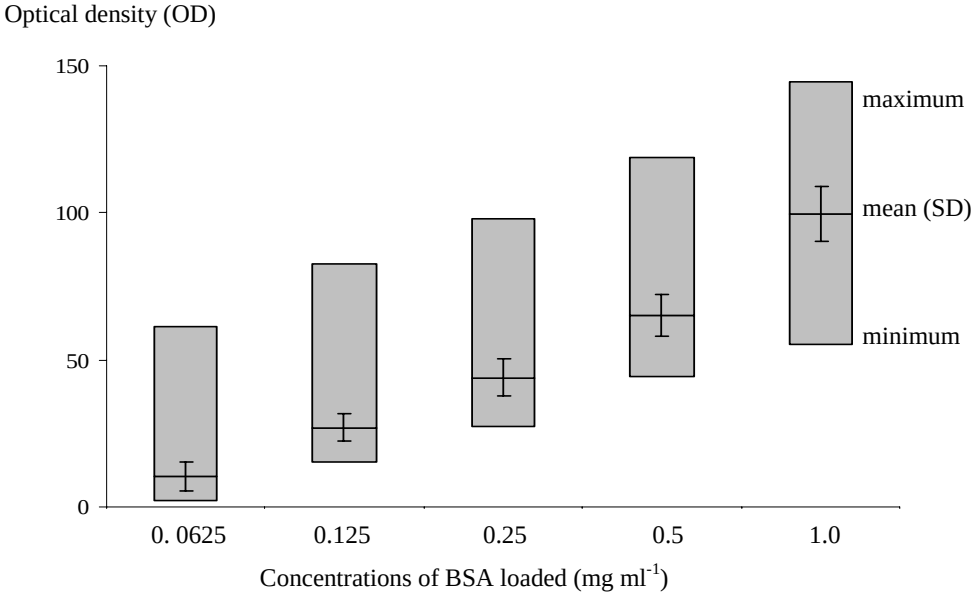


Table 4.2: Coefficients of variation of each pin on the 384-pin replicator.

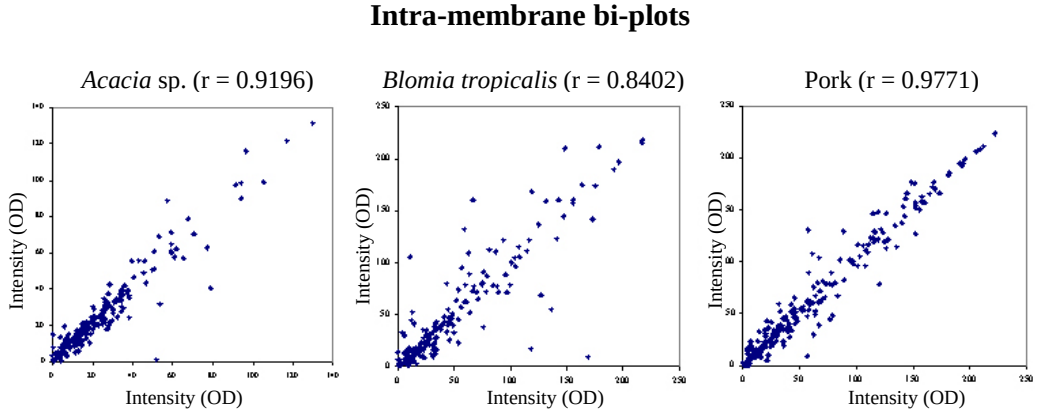
11.9	6.9	7.2	7.5	6.2	7.3	6.2	6.9	6.4	8.3	5.9	4.9	9.8	8.6	7.7	7.4	6.0	6.8	9.8	7.5	8.4	7.0	7.5	10.9
6.4	7.8	11.7	6.7	5.7	5.3	5.2	5.8	5.7	6.9	5.4	7.9	9.6	9.3	8.8	9.4	7.2	7.9	6.0	7.0	9.6	8.6	9.7	12.4
5.8	4.6	7.2	6.4	6.2	5.6	4.4	5.6	7.1	6.2	8.3	8.6	9.1	8.4	8.4	7.6	9.0	8.5	9.6	8.7	9.2	9.2	8.8	13.1
7.2	6.7	5.6	7.0	6.6	6.8	7.2	6.9	6.7	6.8	7.3	11.1	8.8	12.2	7.3	9.2	8.6	8.9	8.5	7.8	9.8	8.6	11.2	12.1
5.6	10.0	10.1	7.9	7.9	10.3	7.8	6.6	8.4	6.3	11.2	9.0	8.1	7.3	8.0	7.8	7.2	8.4	7.0	7.1	6.4	7.4	7.7	14.6
7.2	6.0	6.6	7.4	8.6	7.1	8.6	8.8	9.9	8.9	9.9	8.4	7.9	9.2	6.1	7.5	7.0	7.0	7.3	9.9	7.5	8.7	9.6	12.5
7.0	5.9	7.9	5.3	5.8	6.2	6.7	8.2	6.3	8.0	8.2	9.0	10.9	8.5	10.1	8.3	6.7	10.8	7.8	10.0	7.2	9.6	14.6	15.0
7.8	5.2	4.9	7.5	9.1	5.8	6.1	8.2	4.7	6.0	9.5	9.5	10.9	7.2	10.2	9.6	8.2	6.3	8.3	8.0	7.8	13.1	8.7	9.5
5.4	5.5	6.3	7.3	6.2	8.3	6.1	6.4	5.7	7.7	7.7	11.3	16.2	7.2	11.4	9.1	9.1	6.7	9.0	8.1	6.0	7.5	7.3	11.0
6.8	6.6	5.6	6.1	5.8	4.2	6.0	4.9	6.4	6.7	8.3	8.7	9.9	8.5	9.5	9.4	7.1	7.9	10.6	9.8	11.9	9.3	11.2	12.1
6.8	6.2	6.2	6.0	6.5	5.6	5.5	7.6	8.1	7.4	9.1	10.6	8.6	7.7	14.4	11.3	8.9	14.1	12.2	6.3	7.5	12.1	10.7	18.9
8.0	6.5	6.0	7.7	7.2	6.6	6.4	5.7	7.0	7.4	6.4	7.3	7.2	7.8	9.7	10.0	9.1	8.7	8.4	9.0	11.1	8.4	7.3	12.8
7.6	4.2	4.9	4.5	7.0	6.0	5.0	6.1	6.4	6.3	8.1	9.9	7.2	8.4	7.4	8.2	12.4	7.5	9.4	7.0	7.4	10.8	9.3	10.9
9.3	8.2	6.6	5.5	5.7	8.4	5.2	7.0	6.1	9.4	9.3	6.7	7.0	8.9	8.8	8.9	8.8	7.8	8.6	9.7	7.2	8.1	6.8	8.5
6.6	5.8	5.7	7.4	4.3	6.7	5.2	6.1	5.8	6.5	7.0	7.9	7.0	7.4	6.7	10.0	7.7	10.2	6.7	10.3	7.2	8.6	8.9	11.6
9.1	8.1	8.5	7.5	8.7	6.5	7.4	7.5	10.7	9.3	7.7	8.2	8.8	10.0	8.4	6.0	10.1	10.2	10.2	8.2	9.3	9.7	11.4	14.5

The coefficients of variation (CVs) are in percentages. The CVs were obtained from dotting 14 membranes with BSA followed by staining with amido black solution.

4.3.2 Performance of the allergen array

Intra-membrane concordances were evaluated by comparing the duplicate dots of the same allergen on a single membrane while inter-membrane concordances were evaluated using results obtained on duplicate membranes. Table 4.3 shows the inter-membrane and intra-membrane concordances for all the allergens on the array. By using results obtained from screening 483 sera, the intra-membrane concordances ranged from 83.07 to 96.65% while the inter-membrane concordances, from 61.42 to 96.46%. Inter-assay performance of the immunoarray was also studied. This was obtained by screening the same serum in duplicates at two different times. In total nine sera were used. Inter-assay correlation for the sera tested ranged from r = 0.60 to 0.86, all significant at $p<0.001$. Coefficient of variations between assays ranged from 15.24 to 63.03% with an average of 32.72%.

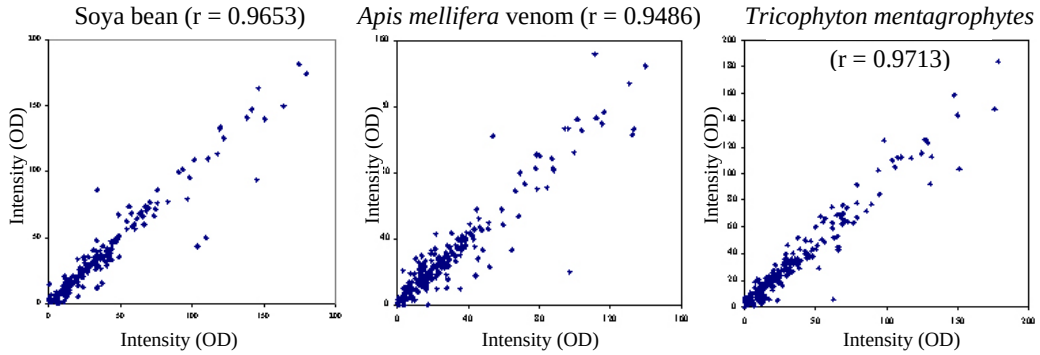
Figure 4.4: Examples of intra-membrane and inter-membrane concordance bi-plots.



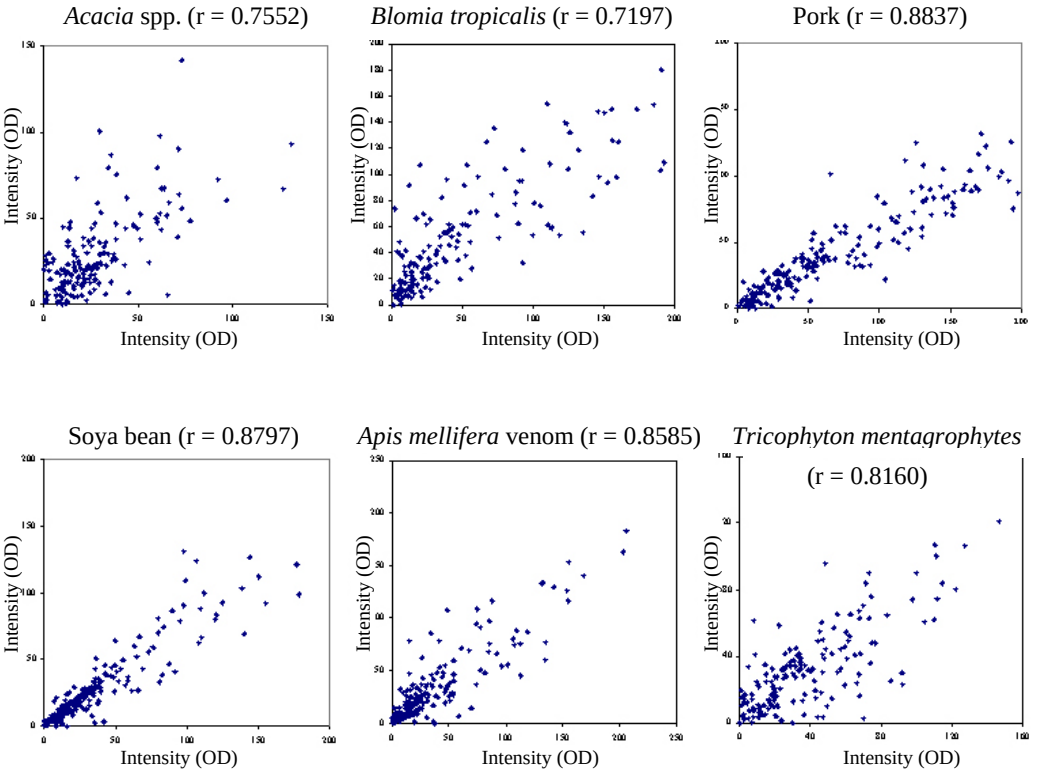
Spearman's Correlation Test analysis was used for the correlation studies. All plots shown are significantly correlated with a minimum of p = 0.05.

Figure 4.4 (continued): Examples of intra-membrane and inter-membrane concordance bi-plots.

Intra-membrane bi-plots



Inter-membrane bi-plots



Spearman's Correlation Test analysis was used for the correlation studies. All plots shown are significantly correlated with a minimum of p = 0.05.

Table 4.3: Intra-membrane and inter-membrane concordances.

Allergen	Intra-membrane Concordance (%)			Inter- membrane Concordance (%)		
	Positive (+/+)	Negative (-/-)	Total	Positive (+/+)	Negative (-/-)	Total
POLLEN						
Acacia auriculiformis	84.91	95.70	92.32	72.04	81.99	78.35
Agrostis alba	78.95	95.37	92.91	91.67	97.57	96.46
Agropyron repens	87.32	96.34	95.08	82.69	90.59	88.98
Alnus glutinosa	72.50	96.79	94.88	76.19	92.70	91.34
Alopecurus prantesis	80.23	96.68	93.90	94.55	92.96	93.31
Ambrosia artemisiifolia	84.43	97.93	94.69	85.94	96.32	93.70
Amaranthus hybridus	83.09	92.20	89.76	56.76	93.33	82.68
Ambrosia trifida	83.15	95.94	93.70	67.80	92.82	87.01
Acer negundo	88.28	94.47	92.91	59.21	96.63	85.43
Anthxanthum odoratum	74.24	92.31	89.96	77.42	82.96	82.28
Atriplex polycarpa	88.36	91.44	90.55	75.00	96.47	89.37
Arecastrum romanzaffianum	86.93	94.28	91.73	61.22	97.44	83.46
Artemisia vulgaris	76.25	96.96	93.70	95.65	80.29	83.07
Avena sativa	90.63	91.05	90.94	63.64	94.92	85.43
Baccharis halimifolia	85.14	89.72	88.39	55.56	95.12	81.10
Betula verrucosa	83.53	96.93	94.69	63.64	96.98	89.76
Bromus mollis	87.13	95.85	92.91	91.21	81.6	85.04
Brassica spp.	87.58	95.77	93.31	83.33	78.66	80.31
Carpinus betulus	80.82	97.47	95.08	75.00	87.85	85.83
Casuarina equisetifolia	88.00	92.79	91.14	80.39	88.16	85.04
Calluna vulgaris	91.89	91.94	91.93	79.69	92.11	88.98
Chenopodium album	91.61	94.79	93.90	85.06	79.04	81.10
Chrysanthemum leucanthemum	88.81	95.45	93.70	85.19	81.50	82.68
Corylus avellana	84.73	96.29	93.31	76.39	90.66	86.61
Cryptomeria japonica	85.37	96.48	94.69	97.67	54.03	61.42
Cupressus arizonica	82.86	96.12	94.29	80.56	86.70	85.83
Cupressus sempervirens	87.63	97.08	95.28	80.33	97.41	93.31
Cynodon dactylon	84.96	93.33	91.14	90.91	88.70	89.37
Dahlia cultorum	86.39	94.46	92.13	78.89	96.95	90.55
Dactylis glomerata	81.58	96.45	93.11	78.79	78.72	78.74
Elaeis guineensis	87.67	93.09	91.54	77.38	96.47	90.16
Eucalyptus globulus	82.42	95.44	93.11	77.78	95.00	91.34
Fagus sylvatica	92.00	95.15	94.69	84.91	88.56	87.80
Festuca prantesis	89.71	96.77	94.88	81.58	91.01	88.19
Fraxinus excelsior	90.97	93.13	92.52	81.48	84.97	83.86
Hordeum vulgare	96.77	94.27	94.88	75.31	92.49	87.01
Holcus lanatus	87.67	97.70	96.26	84.78	96.63	94.49
Humulus lupulus	78.30	97.51	93.50	83.33	97.87	94.09
Juniperus asheisabinoides	76.85	96.25	92.13	72.73	85.93	83.07
Ligustrum vulgare	91.34	94.23	93.50	69.86	93.37	86.61

Table 4.3 (continued): Intra-membrane and inter-membrane concordances.

Allergen	Intra-membrane Concordance (%)			Inter-membrane Concordance (%)		
	Positive (+/+)	Negative (-/-)	Total	Positive (+/+)	Negative (-/-)	Total
POLLEN (continued)						
Lolium perenne	86.73	96.96	94.69	59.32	95.90	87.40
Medicago sativa	83.33	96.34	93.11	93.94	84.57	87.01
Olea europea	88.19	91.48	90.55	79.52	96.49	90.94
Parietaria judaica	90.37	94.64	93.50	86.25	96.55	93.31
Populus deltoides	80.95	95.29	92.32	86.21	89.29	88.58
Phragmites communis	84.16	94.35	92.32	82.14	86.36	85.43
Philadelphus coronarius	87.90	94.01	92.52	75.68	93.89	88.58
Phlenum pratesense	85.53	95.60	94.09	75.56	94.74	91.34
Pinus radiata	63.89	98.85	93.90	93.55	58.30	62.60
Platanus acerfolia	89.52	98.01	96.26	81.54	95.77	92.13
Plantago lanceolata	85.15	96.56	94.29	92.31	86.77	88.19
Populus nigra	92.26	95.59	94.49	70.30	94.12	84.65
Poa pratensis	88.35	95.80	94.29	61.02	97.95	89.37
Podocarpus polystachyus	66.00	96.51	93.50	68.18	81.90	80.71
Pinus strobus	70.63	95.29	89.17	71.83	94.54	88.19
Populus trichocarpa	85.57	92.94	91.54	78.18	87.94	85.83
Quercus alba	83.33	96.15	92.52	84.71	95.27	91.73
Quercus ilex	82.30	95.95	92.91	93.65	93.72	93.70
Quercus robur	71.20	98.96	92.13	67.69	93.12	86.61
Robinia pseudoacacia	86.09	96.18	93.90	69.12	96.77	89.37
Rumex acetosella	76.12	96.15	93.50	80.43	92.31	90.16
Salsola kali	71.95	95.77	91.93	94.59	85.25	86.61
Sambucus nigra	92.52	95.26	94.69	82.35	88.17	86.61
Secale cereale	88.89	93.89	92.91	89.09	83.92	85.04
Schinus molle	85.83	92.01	90.55	93.75	81.05	84.25
Sorghum halepense	86.44	97.44	94.88	74.63	95.72	90.16
Solidago virga-aurea	86.36	94.29	92.91	96.61	80.51	84.25
Tamarix gallica	67.21	92.75	86.61	71.64	93.58	87.80
Taraxacum officinale	81.10	94.23	90.94	66.67	95.29	88.19
Tilia cordata	83.15	96.42	94.09	87.27	90.45	89.76
Triticum aestivum / sativum	90.51	96.00	94.29	35.96	93.94	73.62
Ulmus americana	88.00	94.52	92.91	82.86	92.93	90.16
Ulmus minor	83.04	94.95	92.32	87.50	89.01	88.58
Urtica dioica	86.63	90.77	89.37	84.62	87.33	86.22
Zea mays	89.86	97.03	95.08	44.00	100.00	83.46

Table 4.3 (continued): Intra-membrane and inter-membrane concordances.

Allergen	Intra-membrane Concordance (%)			Inter-membrane Concordance (%)		
	Positive (+/+)	Negative (-/-)	Total	Positive (+/+)	Negative (-/-)	Total
FUNGI						
Alternaria alternata	76.34	90.60	87.99	59.57	97.10	90.16
Aspergillus flavus	78.71	92.63	88.39	63.41	85.47	78.35
Aspergillus fumigatus	89.68	91.78	91.14	59.77	98.20	85.04
Aspergillus niger	86.49	94.48	93.90	91.30	84.42	85.04
Aspergillus terreus	88.62	92.38	91.14	76.47	94.74	87.40
Botrytis cinerea	75.64	94.19	91.34	63.64	85.24	81.50
Candida albicans	85.90	91.63	90.75	84.78	80.29	81.10
Cladosporium cladosporioides	84.97	94.37	91.54	70.59	89.94	83.46
Cladosporium fulvum	82.42	93.88	90.16	70.89	88.00	82.68
Cladosporium herbarum	80.80	92.17	89.37	62.50	96.15	86.61
Corenyspora cassicola	66.67	91.36	85.24	71.64	75.94	74.80
Curvularia brachyspora	72.58	91.15	86.61	75.38	95.24	90.16
Curvularia fallax	67.65	94.09	88.78	80.00	92.27	89.37
Curvularia inequalis	73.02	89.03	83.07	64.58	97.47	85.04
Curvularia lunata	78.99	92.29	89.17	66.15	96.30	88.58
Curvularia pallescences	75.76	95.93	93.31	84.62	93.49	92.13
Curvularia spicifera	79.52	97.88	94.88	85.11	89.37	88.58
Drechslerea/Bipolaris sorokiana	84.21	95.40	93.31	57.69	95.05	87.40
Fusarium moniliforme	86.26	90.18	88.78	79.35	94.44	88.98
Fusarium solani	87.41	94.37	92.52	87.34	89.71	88.98
Malazessia furfur	84.48	96.17	93.50	86.15	94.71	92.52
Mucor mucedo	86.00	96.81	94.69	86.67	79.90	81.10
Penicillium brevicompactum	77.65	97.64	94.29	31.37	92.61	80.31
Penicillium chrysogenum	72.62	93.16	89.76	65.79	83.33	80.71
Penicillium expansum	91.26	93.38	92.52	76.92	90.67	85.04
Penicillium notatum	87.43	93.39	91.34	68.82	94.41	85.04
Penicillium roqueforti	84.76	94.54	92.52	69.64	94.95	89.37
Rhizopus nigricans	82.56	97.16	94.69	86.96	92.31	91.34
Saccharomyces cerevisae	89.93	95.93	94.29	55.26	97.75	85.04
Stemphylium botryosum	75.32	96.75	93.50	63.89	87.61	84.25
Trichoderma viride	77.78	95.28	93.11	81.25	86.04	85.43
Trichophyton mentagrophytes	89.85	95.18	93.11	78.90	90.34	85.43
Trichophyton rubrum	85.99	89.46	88.39	84.69	92.95	89.76
MITES						
Acarus siro	91.12	92.33	91.93	69.79	96.84	86.61
Austroglycyphagus geniculatus	84.39	94.72	90.55	73.58	85.14	80.31
Blomia tropicalis	87.83	89.57	88.78	65.00	96.27	81.50
Dermatophagoides farinae	91.41	90.00	90.55	94.17	75.50	83.07
Dermatophagoides pteronyssinus	91.35	94.00	92.91	77.27	97.22	88.58
Glycophagus domesticus	74.19	92.97	88.39	65.08	93.72	86.61
Lepidoglyphus destructor	87.40	95.01	93.11	81.16	94.59	90.94
Suidasia medanensis	90.75	90.45	90.55	69.47	88.05	81.10
Tyrophagus putrescentiae	87.36	92.33	90.55	89.25	83.85	85.83

Table 4.3 (continued): Intra-membrane and inter-membrane concordances.

Allergen	Intra-membrane Concordance (%)			Inter-membrane Concordance (%)		
	Positive (+/+)	Negative (-/-)	Total	Positive (+/+)	Negative (-/-)	Total
ANIMAL-BASED FOOD						
Banana prawn	81.74	97.20	93.70	83.33	96.28	92.91
Beef	90.57	88.48	89.57	75.18	88.03	81.10
Casein	92.08	93.12	92.91	70.00	91.75	86.61
Chicken	90.41	94.46	92.72	77.78	90.41	85.04
Cockles	95.33	83.67	88.58	84.55	93.13	88.98
Egg white	72.12	94.80	90.16	54.35	93.75	86.61
Egg yolk	84.15	97.18	95.08	67.50	83.18	80.71
Mackerel fish	81.82	94.92	93.50	75.68	89.40	87.40
Milk, cow	87.15	95.14	92.32	90.82	91.03	90.94
Milk, goat	90.67	95.24	93.50	80.37	92.52	87.40
Mud crab	86.49	93.70	92.13	72.46	91.35	86.22
Mussels	91.22	91.09	91.14	76.32	95.00	86.61
Ovalbumin	94.12	96.92	96.26	74.65	97.27	90.94
Ovomucoid	77.22	97.67	94.49	79.17	82.52	81.89
Pork	95.18	91.88	93.90	83.65	97.89	88.98
Rabbit meat	95.22	92.56	94.09	76.82	93.20	83.46
Salmon fish	86.19	96.64	92.91	77.66	94.38	88.19
Sea bream fish	90.00	97.01	95.08	58.90	91.16	81.89
Selar fish	83.12	97.18	92.91	89.77	75.90	80.71
Sheep	90.61	92.40	91.54	81.48	94.96	87.80
Squid	89.10	91.19	90.55	83.54	89.71	87.80
Swimming crab	85.00	95.83	93.70	75.00	96.60	92.52
Tiger prawn	82.35	95.70	92.13	86.30	91.16	89.76
Tuna fish	87.21	96.73	93.50	75.27	95.03	87.80
PLANT-BASED FOOD						
Apple	89.04	93.56	92.91	90.00	86.27	87.01
Banana	80.72	96.71	94.09	77.55	94.63	91.34
Cabbage	92.35	95.19	94.09	73.87	97.90	87.40
Cacao	88.50	96.46	94.69	84.51	88.52	87.40
Carrot	92.47	95.42	94.88	75.00	92.78	88.58
Chard	84.94	85.13	85.04	85.25	90.91	88.19
Corn flour	95.68	91.33	92.72	73.74	95.48	87.01
Garlic	88.44	95.01	93.11	95.00	90.80	92.13
Gliadine	77.38	94.10	91.34	41.82	95.48	83.86
Hazelnut	88.79	95.41	93.90	65.28	98.35	88.98
Orange	83.52	95.92	93.70	88.68	92.54	91.73
Peach	92.57	94.72	94.09	73.49	94.74	87.80
Peanut	93.75	97.09	96.46	74.07	96.00	91.34
Potato	81.53	92.02	88.78	49.41	94.08	79.13
Rice flour	82.31	95.84	91.93	81.40	97.02	91.73

Table 4.3 (continued): Intra-membrane and inter-membrane concordances.

Allergen	Intra-membrane Concordance (%)			Inter-membrane Concordance (%)		
	Positive (+/+)	Negative (-/-)	Total	Positive (+/+)	Negative (-/-)	Total
PLANT-BASED FOOD (continued)						
Soya bean	91.87	96.66	94.69	79.13	97.12	88.98
Spinach	84.07	96.45	90.94	69.75	91.85	81.50
Strawberry	86.99	95.86	93.31	47.50	94.83	79.92
Sunflower seed	87.80	93.51	92.13	79.41	90.32	87.40
Tofu	87.95	93.27	91.54	79.00	90.91	86.22
Walnut	86.67	97.85	95.87	89.09	92.96	92.13
Wheat flour	96.06	94.75	95.08	85.54	93.57	90.94
EPITHELIAL TISSUES/DANDER						
Budgerigar	87.93	97.78	96.65	65.71	96.35	92.13
Cat	91.08	94.02	93.11	74.68	93.14	87.4
Cow	94.23	95.41	94.69	93.75	95.74	94.49
Dog	91.83	92.83	92.32	86.33	86.96	86.61
Feather mix (chicken and duck)	81.73	96.78	93.70	76.92	84.65	83.07
Goose feather	81.93	97.41	94.88	81.08	85.71	85.04
Goat	75.93	95.38	89.17	76.34	95.03	88.19
Guinea pig	71.72	97.07	92.13	73.21	90.91	87.01
Hamster	79.66	97.33	95.28	73.33	97.32	94.49
Horse	77.97	97.33	95.08	66.67	94.95	90.94
Rabbit	90.55	93.96	93.11	98.57	67.39	75.98
INSECTS						
American cockroach	87.20	94.52	92.72	92.59	92.49	92.52
Fire ant	91.61	93.97	93.31	71.43	95.29	87.4
German cockroach	87.26	93.45	91.54	67.01	88.54	80.31
Mosquito	88.51	96.67	94.29	63.41	90.12	81.50
Oriental cockroach	85.84	94.68	92.72	87.30	84.82	85.43
VENOMS						
Apis mellifera	88.08	92.06	90.55	74.31	86.90	81.50
Dolichovespula spp.	86.83	87.68	87.40	76.29	92.36	86.22
Polistes spp.	79.00	93.38	90.55	82.76	88.78	87.40
Vespula spp.	88.82	96.25	93.90	72.63	98.11	88.58
OTHERS						
Horseradish peroxidase	92.71	95.63	95.08	83.93	90.40	88.98
Latex	83.57	95.11	91.93	77.63	91.01	87.01
Bromelain	92.05	94.28	93.50	85.58	87.33	86.61

4.3.3 Allergen array validation

4.3.3.1 *Immunoarray versus ELISA*

For ELISA, a total of 24 allergen sources were tested (Table 4.4). Varying degrees of concordances were obtained. They are pollen (*Acacia* sp. [72.22%], *Arecastrum romanzoffianum* [40.88%], *Acacia* sp. [72.22%], *Betula verrucosa* [78.95%], *Casuarina equisetifolia* [60.87%], *Cryptomeria japonica* [56.52%], *Cynodon dactylon* [66.67%], *Elaeis guineensis* [73.91%], *Eucalyptus globulus* [52.63%], *Olea europea* [94.4%], *Pinus strobus* [90.0%], *Quercus alba* [91.30%], *Schinus mollis* [72.22%] and *Urtica dioica* [45.0%]), fungi (*Candida albicans* [70.59%], *Curvularia lunata* [75.0%], *Fusarium solani* [84.2%], *Penicillium notatum* [64.71%] and *Stemphylium botryosum* [66.66%]), mite (*Suidasia medanensis* [66.67%]), food (banana prawn [91.67%], sea bream fish [81.82%], selar fish [83.33%], soya bean [91.67%] and tofu [90.91%]). Spearman's Correlation Test for the different allergen sources tested ranged from r = 0.33 to r = 0.71 with the average of r = 0.52.

4.3.3.2 *Immunoarray versus the UniCAP® system*

The results for the immunoarray system versus the UniCAP® system are shown in Table 4.5. The concordances obtained ranged from no to 100% concordance. A general trend observed is that the immunoarray tended to have more positive results then the UniCAP system indicating a higher probability of presenting false positive results with the immunoarray when the UniCAP is used as the bench mark. Low false negative results were obtained using the immunoarray system. High concordance was obtained

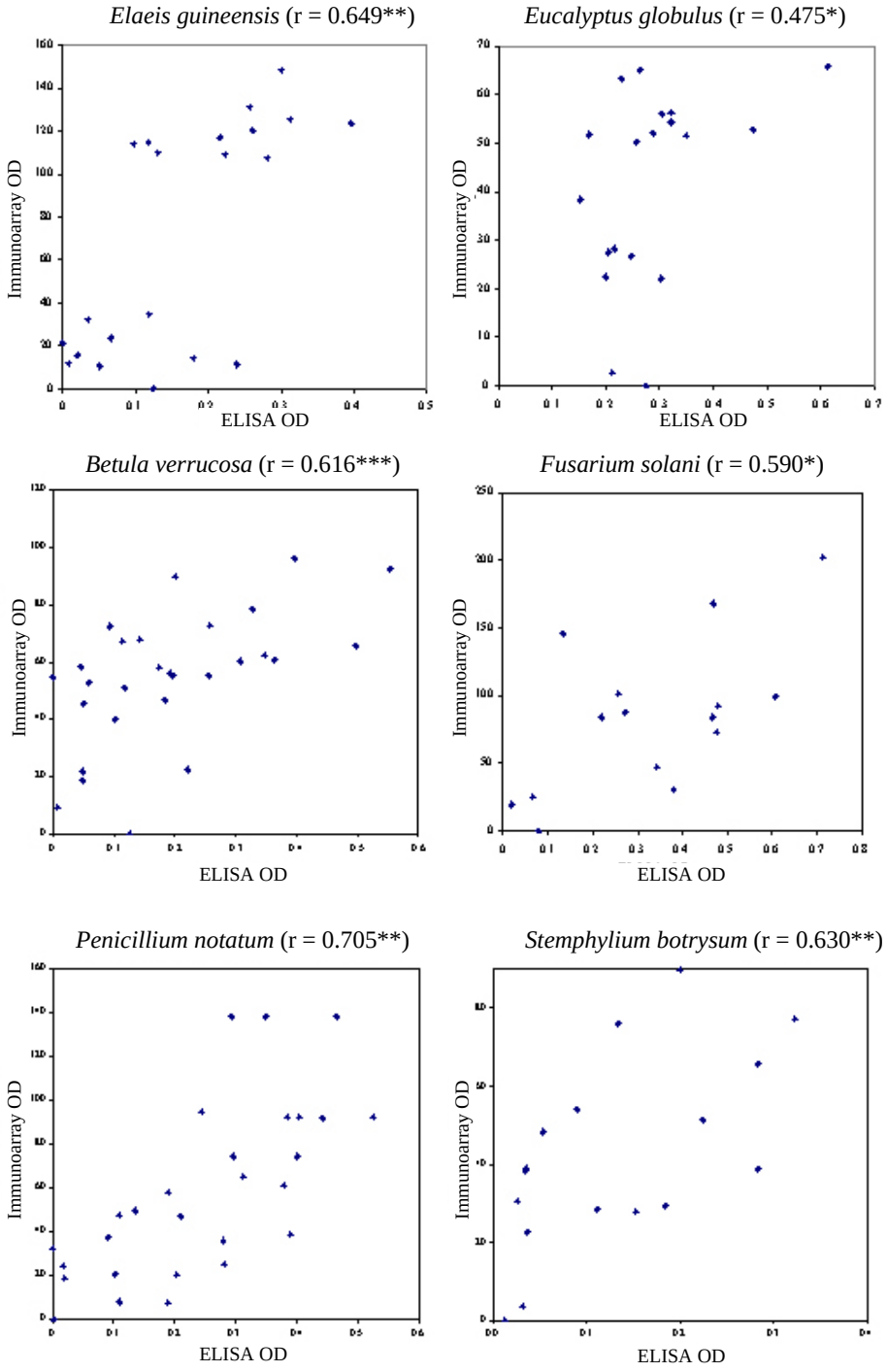
with mite, allergen sources which tended to produce very strong reaction intensities compared to those of pollen and fungus.

Table 4.4: Validation results between the immunoarray method versus the ELISA system. Allergens are arranged in descending order of concordance.

	Concordance of immunoarray versus ELISA (%)				
Allergens tested (n = 12 to 28)	+ / +	- / -	+ / -	- / +	Total
Olea europea	94.44	–	5.56	–	94.44
Banana prawn	91.67	–	8.33	–	91.67
Soya bean	91.67	–	8.33	–	91.67
Quercus alba	91.30	–	8.70	–	91.30
Tofu	90.91	–	9.09	–	90.91
Pinus strobus	90.00	0.00	0.00	10.00	90.00
Fusarium solani	84.20	0.00	0.00	15.80	84.20
Selar fish	83.33	–	16.67	–	83.33
Sea bream fish	81.82	–	18.18	–	81.82
Betula verrucosa	78.95	–	21.05	–	78.95
Curvularia lunata	75.00	–	25.00	–	75.00
Elaeis guineensis	39.13	34.78	9.09	17.00	73.91
Acacia sp.	72.22	0.00	0.00	27.78	72.22
Schinus mollis	72.22	0.00	11.11	16.67	72.22
Candida albicans	70.59	0.00	23.53	5.88	70.59
Cynodon dactylon	38.89	27.78	11.11	22.22	66.67
Suidasia medanensis	66.67	–	33.33	–	66.67
Stemphylium botryosum	55.56	11.10	27.78	5.56	66.66
Penicilllium notatum	64.71	0.00	29.40	5.89	64.71
Casuarina equisetifolia	60.87	0.00	34.79	4.34	60.87
Cryptomeria japonica	56.52	0.00	0.00	43.48	56.52
Eucalyptus globulus	10.53	42.10	4.74	42.63	52.63
Urtica dioica	55.00	40.00	5.00	0.00	45.00
Arecastrum romanzoffianum	72.72	13.60	27.28	31.84	40.88

No sera tested were indicated with a hyphen (–).

Figure 4.5: Correlation of the ELISA versus immunoarray system.



Correlation coefficient, r was analysed using Spearman's Correlation Test. p values: p = 0.001***; p = 0.01** and p = 0.05*.

Table 4.5: Validation results between the immunoarray method versus the UniCAP system. Allergens are arranged in descending order.

Allergens tested (n=16)	Concordance of immunoarray versus UniCAP (%)				
	+/+	-/-	+/-	-/+	Total
Acarus siro (d70)	35.0	35.0	25.0	5.0	70.0
Dermatophagoides pteronyssinus (d2)	43.8	37.5	6.3	12.5	81.3
Acacia spp. (t19)	0.0	37.5	56.3	6.3	37.5
Arecastrum romanzo ffianum (t72)	10.0	30.0	60.0	0.0	40.0
Artemisia vulgaris (w6)	0.0	43.8	31.3	25.0	43.8
Betula verrucosa (t3)	50.0	0.0	50.0	0.0	50.0
Casuarina equisetifolia (t73)	15.0	10.0	75.0	0.0	25.0
Chrysanthemum leucanthemum (w7)	0.0	11.1	88.9	0.0	11.1
Cryptomeria japonica (t17)	12.5	56.3	31.3	0.0	68.8
Cupressus sempervirens (t23)	20.0	20.0	60.0	0.0	40.0
Cynodon dactylon (g2)	0.0	81.3	18.8	6.3	81.3
Eucalyptus globulus (t18)	0.0	10.0	90.0	0.0	10.0
Olea europaea (t9)	40.0	20.0	60.0	0.0	60.0
Pinus strobus (t16)	0.0	75.0	25.0	0.0	75.0
Schnus molle (Rt12)	0.0	8.3	91.7	0.0	8.3
Solidago virga-aurea (w12)	100.0	0.0	0.0	0.0	100.0
Tilia cordata (t208)	12.5	12.5	75.0	0.0	25.0
Urtica dioica (w20)	25.0	6.3	68.8	0.0	31.3
Alternaria alternata (m6)	16.7	16.7	83.3	0.0	33.3
Aspergillus fumigatus (m3)	0.0	0.0	100.0	0.0	0.0
Botrytis cinerea (m7)	6.3	6.3	81.3	0.0	12.5
Candida albicans (m5)	16.7	0.0	83.3	0.0	16.7
Curvularia lunata (m16)	0.0	37.5	62.5	0.0	37.5
Fusarium solani (m9)	0.0	6.3	87.5	0.0	6.3
Penicillium notatum (m1)	6.3	12.5	87.5	0.0	18.8
Rhizopus nigricans (m11)	0.0	0.0	100.0	0.0	0.0
Stemphylium botryosum (m10)	0.0	0.0	100.0	0.0	0.0
Trichoderma viride (m15)	0.0	80.0	20.0	0.0	80.0
Trichophyton mentagrophytes (Rm211)	9.1	0.0	100.0	0.0	9.1

4.4 DISCUSSION

We have developed a dot immunoarray system for the simultaneous detection of IgE to more than 150 allergen types. The array is advantageous owing to the minute amount of sera required which is approximately 1 μl for every allergen source tested in duplicate on the same membrane. This is far less then the current conventional methods used like the ELISA including the UniCAP® system.

Good concordance results have been obtained for intra-membrane (mean = 92.50%), inter-membrane (mean = 86.55%) and inter-assay comparisons. The intra-membrane, inter-membrane and inter-assay concordances are highly dependent on the accuracy of the dotting process. A wide range of concentrations was observed when BSA was used to test the loading efficiencies of the pins. This would mostly be corrected by automating the dotting process. The current dotting process is done manually and variations between dots by the same pin on different membranes resulting in different loading concentrations were observed. The variation in speed on removing the replicator, stock well volume and dwell time of the pin on membrane are factors contributing to the differences on the volume transferred onto the membrane (datasheet from V&P Scientific, California).

The viscosity of the allergens used is another factor to be considered even though the pins are hydrophobically coated. As measured by dot intensities, different allergens were found to load in varying concentrations despite using a standardised allergen concentration of 1 μg per dot. The differences in viscosity of each allergen extracts is probably attributed to the inherent properties of the total allergen composition of the extracts. Certain foods and fungi, like ovamucoid, were found to have a high viscosity compared to the rest at the same concentration in buffers containing the same percent-

age of glycerol. The variation in the amount of allergens dotted on the membranes may contribute to the poor concordance for some of the allergen sources. When the variation of loading is large, a high standard deviation will be obtained resulting in high cut off points. This in turn will result in a higher frequency of false negative results.

Satisfactory concordance with the ELISA was also obtained. However, a wide variation of concordance was obtained when the immunoarray was compared with the UniCAP® system ranging to no concordance to 100% concordance depending on the allergen source tested. The source of allergens used could contribute to variation between the results obtained. Allergens from different sources or batches have been shown to differ in its total composition. Esch (1997) reported that the extracts prepared from *Ambrosia artemisiifolia* in 1981 contained 10 times more Amb a 1 than from pollen collected in 1987. Pure fungal cultures' composition, the strain used and culture conditions and whether the fungal mat or spent medium were collected will alter the composition of the allergens. *Alternaria alternata* has also been shown to produce significantly different quantities of the major allergen Alt a 1 only under certain condition with some strains not producing any (Esch, 1997). Vailes *et al.* (2001) compared the variability of the allergen content from allergenic products by eight manufacturers. The levels of Alt a 1 ranged from 0.01 to 6.09 $\mu g\ ml^{-1}$ while Asp f 1 ranged from 0.1 to 64 $\mu g\ ml^{-1}$. Even the composition of mite allergens in extracts is dependent on whether pure mite bodies or mites mixed with culture media were used in the preparation (Wahn *et al.*, 1988). Better concordances were obtained with the ELISA when the same sources of allergens used in the immunoarray were utilized.

The strength of the sera used to validate the reactions may also contribute to the low concordances observed between the immunoarray and the UniCAP® systems. High concordances were obtained for mite allergen sources like those for *Acarus siro* and *Dermatophagoides pteronyssinus* where a high number of positive and strong reactions were obtained (10.8% and 17.0%, respectively) compared to pollen and fungal allergen sources, which on average, have less then 1.5% of positive strong reactions. Prevalence and sera reactions of the different pollen and fungal allergen sources are also presented in Chapter 5. The performance of the array can also be further improved with the use of better defined sera. Well defined sera with their IgE levels validated with UniCAP® or allergy status confirmed with skin prick test will enable a better cut off point to be obtained. This will help to greatly reduce the number of false positive results if the UniCAP® is to be used as the gold standard.

The narrower dynamic range and different cut-off values used compared to the UniCAP® system are also factors contributing to the difference in results obtained. Differences in results obtained from different assay formats are also not foreign. Studies on the latex allergen alone have demonstrated that discordant results ranging from 9% to 25% between the UniCAP®, alaSTAT® and HY-TEC® systems (Hamilton *et al.*, 1999) and differences in sensitivity and specificities from the UniCAP® and ADVIA Centaur® systems for milk, egg, grass pollen, cat and mite allergen sources (Ricci *et al.*, 2003).

The dot immunoarray has been shown to be a useful semi-quantitative tool to screen for specific IgEs to a large amount of allergen sources simultaneously. The array can be expanded to encompass more allergens including recombinants, which have been tested on a pilot scale and in the process of being expanded. The dot immunoarray

system is also less expensive owing to the use of colorimetric precipitation detection using BCIP-NBT. A similar detection method was used by Suck *et al.* (2002) in their membrane array of natural and recombinants allergens with results corresponding to the more conventional ELISA. Fluorescence detection and usage of protein chips as the solid matrix in all the allergen microarray systems developed so far is expensive (Deinhofer *et al.*, 2004; Beyer, 2003; Fall *et al.*, 2003; Harwanegg *et al.*, 2003; Hiller *et al.*, 2002; Jahn-Schmid *et al.*, 2003; Kingsmore *et al.*, 2003; Bacarese-Hamilton *et al.*, 2002; Kim *et al.*, 2002; Wiltshire *et al.*, 2000).

4.4 CONCLUSIONS

A dot immunoarray system has been developed. This system enables the screening of more than 150 types of allergen sources in duplicate simultaneously. The amount of serum required is 150 μl per assay. This amount is less than 1 μl of serum for each allergen source tested. The assay utilised a 384-pin replicator which allows the production of a large number of arrays quickly. The results of the array were obtained by using conventional computer scanners to capture the images of the arrays followed subsequently by reading of optical density for each dot using a fixed grid. The optical density measurement was performed using a simple commercial image analysis software product. Costs of reagents were also low owing to the use of colorimetric instead of fluorescence detection. The immunoarray system also does not necessarily require a robotic system for dotting thus making it possible for use in small- to large-scale screening without the need for new expensive dotting equipment as only a scanner and simple image analysis software for quantification is sufficient.

The current estimated cost for the production of a single immunoarray is approximately S$150, thus this array can be used to screen when multiple allergies are suspected or when current tests like SPT and UniCAP failed to indicate the correct source of allergy problems. The immunoarray can also be used by food allergic people to test for the spectrum of food that they will need to avoid. Furthermore, the immunoarray can also be re-package to screen specific groups of allergen sources. This can be immediately carried out without requiring further optimisation or development work since the most of the allergen sources has been tested.

CHAPTER 5: AIRSPORA ALLERGY IN SINGAPORE

5.1 INTRODUCTION

5.1.1 Prevalence of airspora allergy

Pollen and spores have been reported to cause allergic disease exacerbations (Busse and Holgate, 1995; Wuethrich, 1989; Gergen *et al*., 1987). They afflict approximately 20% of the population in United States and other industrialized countries. More than 10% of those afflicted have significant or severe allergic diseases. The prevalence number to airspora varies depending on the population, allergens tested, techniques employed and sources of allergens.

Allergies to pollen are common. The percentage of people allergic to pollen in Greece was reported to be 40.4% (Gioulekas *et al*., 2004), 13.5% to 44.9% in Switzerland (Oertmann and Bergmann, 1997; Gassner *et al*., 1996), 29.8 to 33% in Spain (Carinanos *et al*., 2002; Armentia *et al*., 1991) and 17% in Sweden (Foucard, 1991).

Allergies caused by airborne fungal spores are equally prevalent and important (Lam *et al*., 1998; Rybnicek *et al*., 1991; Hasnain *et al*., 1985). Fungal spores are sources of allergy outdoors and indoors (Terr, 2004; Fink, 1998; Burge and Rogers, 2000). Rates for fungal allergy are 2% of the population in Sweden (Foucard, 1991) and 26.6% (Collins *et al*., 2003) or 44% in the United States (Corey *et al*., 1997).

Fern spore allergies are not well studied. The possibility of fern spores as the cause of allergy exacerbation are normally only considered after allergy to other common allergens like those from mites, pollen, fungi, epithelia/dander and foods have been ruled

out (Kofler *et al*., 2000). A prevalence of up to 50% has been reported (Bunnag *et al*., 1989). These studies were mainly done for small selected atopic populations in relation to indoor plants (Bunnag *et al*., 1989; Geller-Bernstein *et al.,* 1987) at home or as case studies (Kofler *et al*., 2000; Paulsen *et al*., 1998; Wuthrich and Johansson, 1997).

In Singapore, the prevalence of paediatric asthma rose from 3.8% in 1967 to 16.3% in 2001 (Wang *et al.*, 2004; Goh *et al.*, 1996). Prevalence of rhinitis is also high from 29.4 to 44% in children and between 13.1 to 49.8% adults (Wang *et al.,* 2004; Goh *et al.,* 1996; Ng and Tan, 1994). Skin testing on adults with rhinitis showed a maximum of 10% of the study group showing sensitisation to airspora (John *et al.,* 1996). A most recent study in by Chew *et al.* (2000) demonstrated a high frequency of positive reactions in atopics to airspora ranging from 27.7 to 33.8% for pollen, 16.5 to 30.7% to fungal spores and 20.3 to 34.2% to fern spores. Neighbouring countries like Malaysia showed rates of 23.3 to 29.5% for pollen sensitisation, 5.0 to 8.4% for fungal spores and 9.3% for fern spores in atopics (Ho *et al*., 1995; Sam *et al.,* 1998), while Indonesia showed rates of 12.1 to 22.4% for pollen, 5.0 to 13.8% for fungal spores and 11.21% for fern spores (Baratawajaja *et al*., 1999).

Plant-based foods were included in the cluster analysis as a large number of studies demonstrated food allergies resulting from exposure to pollen (mainly from tree pollen) and *vice versa* (summarized by Vieths *et al.,* 2002; Breiteneder and Ebner, 2001; Valenta and Kraft 1996). Pollen-food allergies result from the large amount of cross-reactive allergens shared by these two allergens of plant origin. Severe food allergy may result in the loss of life from anaphylaxis. A study by Magnusson *et al*. (2003) showed that the exposure to birch pollen can cause seasonal intestinal inflammation in patients with the oral allergy syndrome to birch-associated foods.

5.1.2 Aims

The development of the immunoarray system has enabled us to screen a large number of allergens simultaneously while the airspora study has provided us with information on the main airspora in Singapore. In this study we aimed to study the frequency of reactions to airspora allergens. The immunoarray system which allows simultaneous testing of a large panel of allergen sourcess provided us with the opportunity to study the possible cross-reactivity patterns among local and foreign airspora through cluster analysis. The patterns observed will serve as a reference for future cross-reactivity work.

5.2 MATERIALS AND METHODS

Sera from 1069 patients, attending and allergy clinic were screened. The dot immunoarray assay was used to screen the sera for a wide range of allergens. Two times above the standard deviation for negative sera was used as the cut off for positive reactions. Kendall's τ b correlation was used to test the correlation between the different allergen types tested. P values threshold for significant correlation were: $p<0.05$*, $p<0.01$** and $p<0.001$***. Weed and grass pollen included those of cultivated plants and flowers.

Cluster analysis was carried out to show the possible cross reactivity between the allergens namely pollen (n = 78), fern (n = 2), fungus (n = 34) and plant-based food (n = 25). Cluster analysis was performed on binarized results (positive or negative). Hierarchical cluster analysis using the Jaccard dissimilarity function was employed. The Jaccard dissimilarity index is one in which joint absences are excluded from considera-

tion. Equal weight was given to matches and non-matches. It is also known as the similarity index. Foreign allergenic pollen and fungal types were included in the screens to test for possible cross reactivity patterns. Statistical analysis was performed using the SPSS version 12 (SPSS Inc., New York) statistical software.

5.2 RESULTS

5.3.1 Detected frequencies of specific IgEs to airspora allergens

In the sera samples screened, 88.3% were found to have specific IgE to the panel of allergens tested (Figure 5.1). Food sources ranked highest while pollen (70.5%) and fungal (66.4%) allergen sources ranked second and third, respectively, in the number of positive reactions. Only 14.7% of pollen reactors were positive to one type of pollen allergen. Among those positive to only one pollen allergen, 12.6% were to *Urtica dioica*, 9.0% to *Populus trichocarpa* and 6.3% to *Populus deltoides*. The maximum number of simultaneous reactivities was to 70 pollen types. As for fungal allergens, 20.8% were positive to only one fungal allergen. Among those positive to only one fungal allergen, 14.2% were to *Penicillium roqueforti*, 10.8% to *Penicillium expansum*, 8.1% to *Aspergillus fumig*atus and 8.1% to *Penicillium chrysogenum*. The maximum number of simultaneous reaction is to 28 types of fungal allergens. Frequency of positive to the other groups of allergens like those of mites, epithelia/dander or venom that were screened can be referred to in Appendix 2.

Interestingly, the airspora types with the highest number of positive reactions to pollen were not of those found in our environment but are of foreign origin. As for fungal allergens, the highest number of reactions was not to commonly found outdoor fungi.

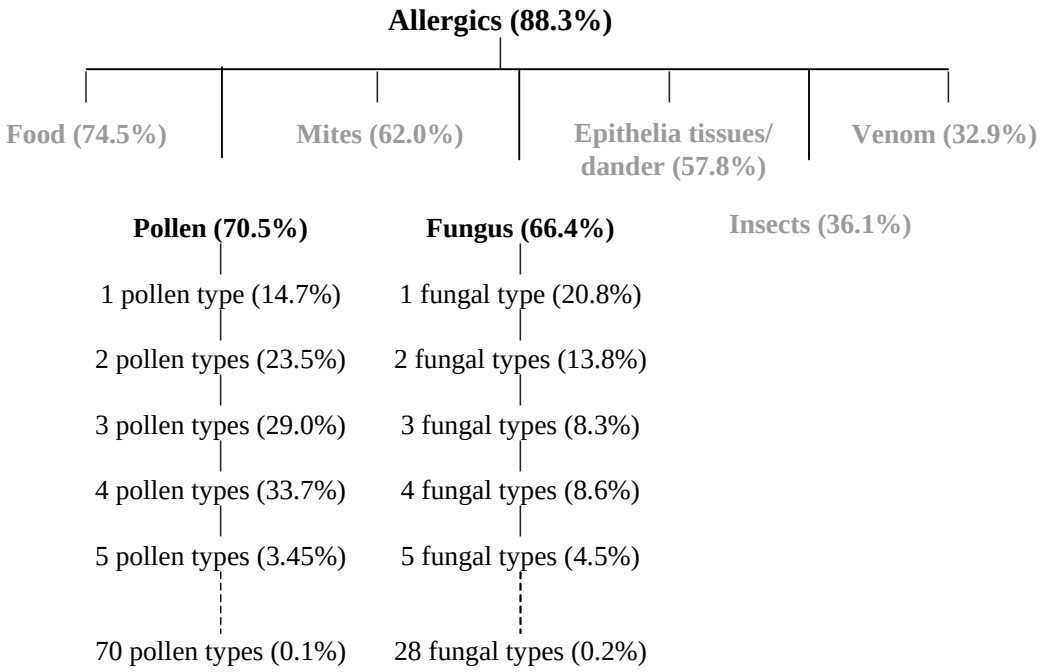
The fern allergens, *Dicranopteris linearis* and *Nephrolepis auriculata*, were excluded after the initial screening of 50 sera which did not result in any positive reactions. Reactions were highest to weed and grass pollen at 1.78 to 35.55% (Figure 5.2). Ranked second were reactions to tree pollen ranging from 0.56 to 29.93% (Figure 5.3) followed by fungal allergens at 1.59 to 27.87% (Figure 5.4). However, most of the positive reactions to weed and grass pollen (85.8%), tree pollen (86.9%) and fungi (82.1%) were low in intensities.

Eighteen pollen allergens had more than 20% positive reactions in the patients tested with *Urtica dioica* at 35.55%, followed by *Arecastrum romanzoffianum* (29.93%), *Baccharis halimifolia* (27.69%), *Philadelphus coronarius* (25.26%), *Tamarix gallica* (23.57%), *Zea mays* (23.01%), *Sorghum halepense* (22.73%), *Dahlia cultorum* (22.54%), *Populus nigra* (22.45%), *Chenopodium album* (22.26%), *Casuarina equisetifolia* (27.80%), *Ambrosia artemisiifolia* (21.42%), *Artiplex polycarpa* (21.32%), *Atriplex polycarpa* (21.33%), *Lolium perenne* (21.05%), *Chrysanthemum leucanthemum* (21.04%), *Medicago sativa* (20.65%) and *Ligustrum vulgare* (20.30%). Positive results to grasses in the sub-family Panicoideae (*Zea mays* and *Sorghum halepense*) were the highest. However, only *Populus deltoides* (1.68%), *Salix viminalis* (1.18%), *Secale cereale* (1.03%) *Syringa vulgaris* (1.07%) and *Pinus strobus* (1.03%) had of the study group strong reactions in more than 1%.

For fungal allergens, six fungal types had positive specific IgE. They were *Trichophyton mentagrophytes* (27.88%), followed by *Aspergillus terreus* (27.69%), *Penicillium*

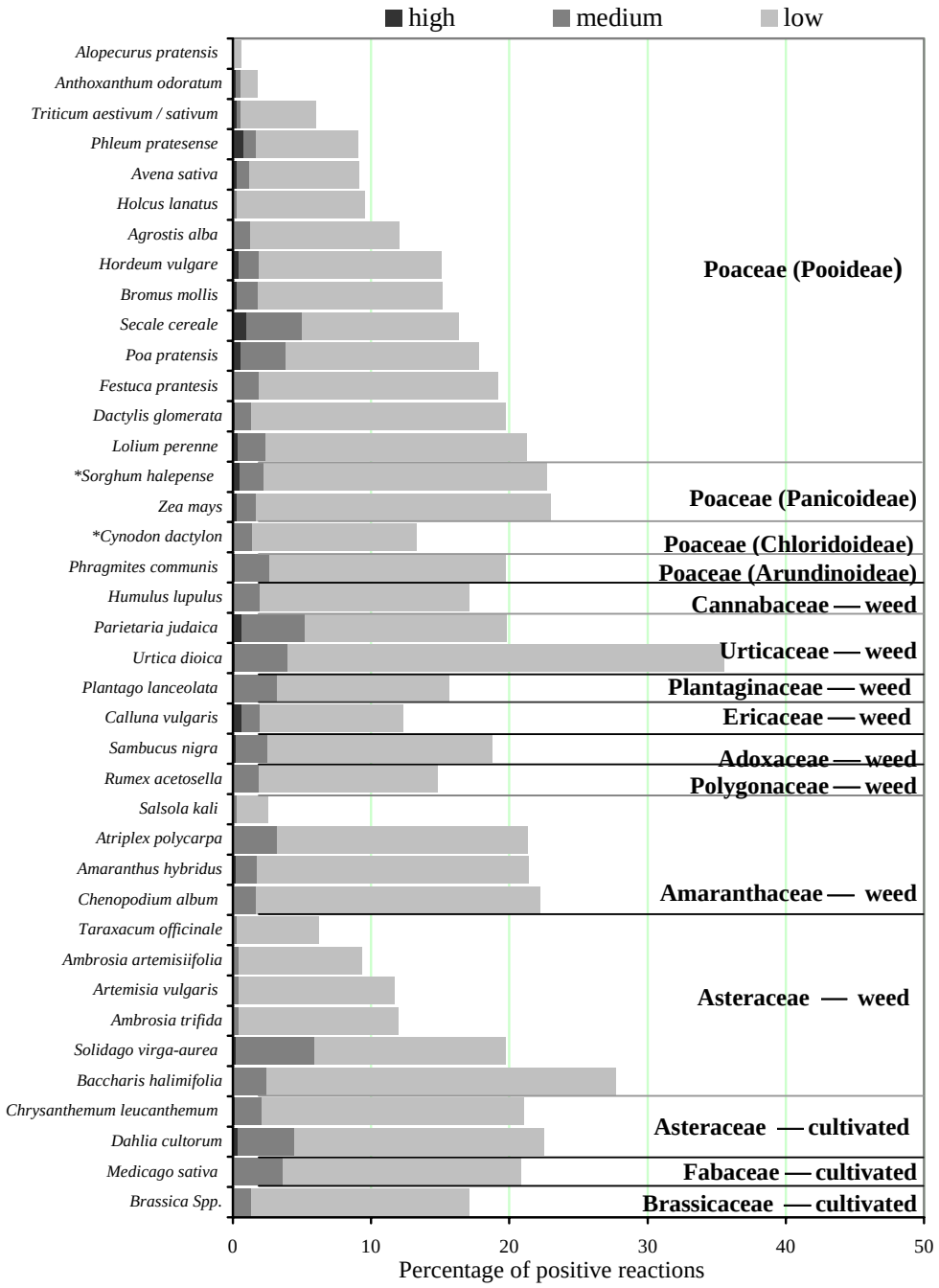
expansum (23.67%), *Cladosporium fulvum* (22.25%), *Cladosporium herbarum* (21.6%) and *Saccharomyces cerevisae* (21.33%). Only *Trichophyton rubrum* (2.53%), *Aspergillus fumigatus* (2.62%) and *Penicillium expansum* (2.34%) had of the study group strong reactions in more than 2%.

Figure 5.1: Frequency of specific IgEs detected to different types of allergens.



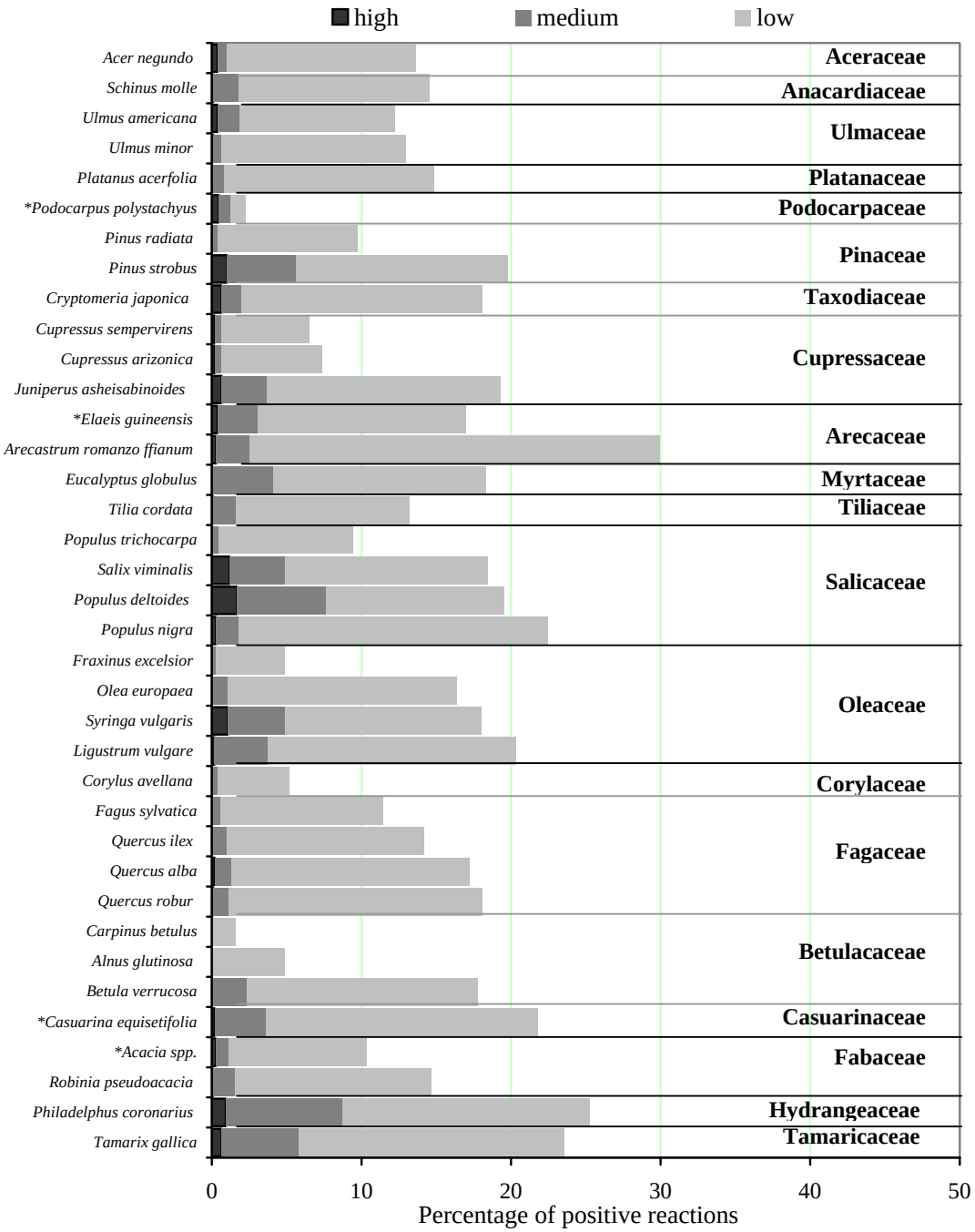
Total number of sera tested is n = 1069. Percentage of positive reactions = number of specific pollen or fungi positives / total pollen or fungal positives.

Figure 5.2: Reactions to cultivated plant, weed and grass pollen in descending order for each family or subfamily.



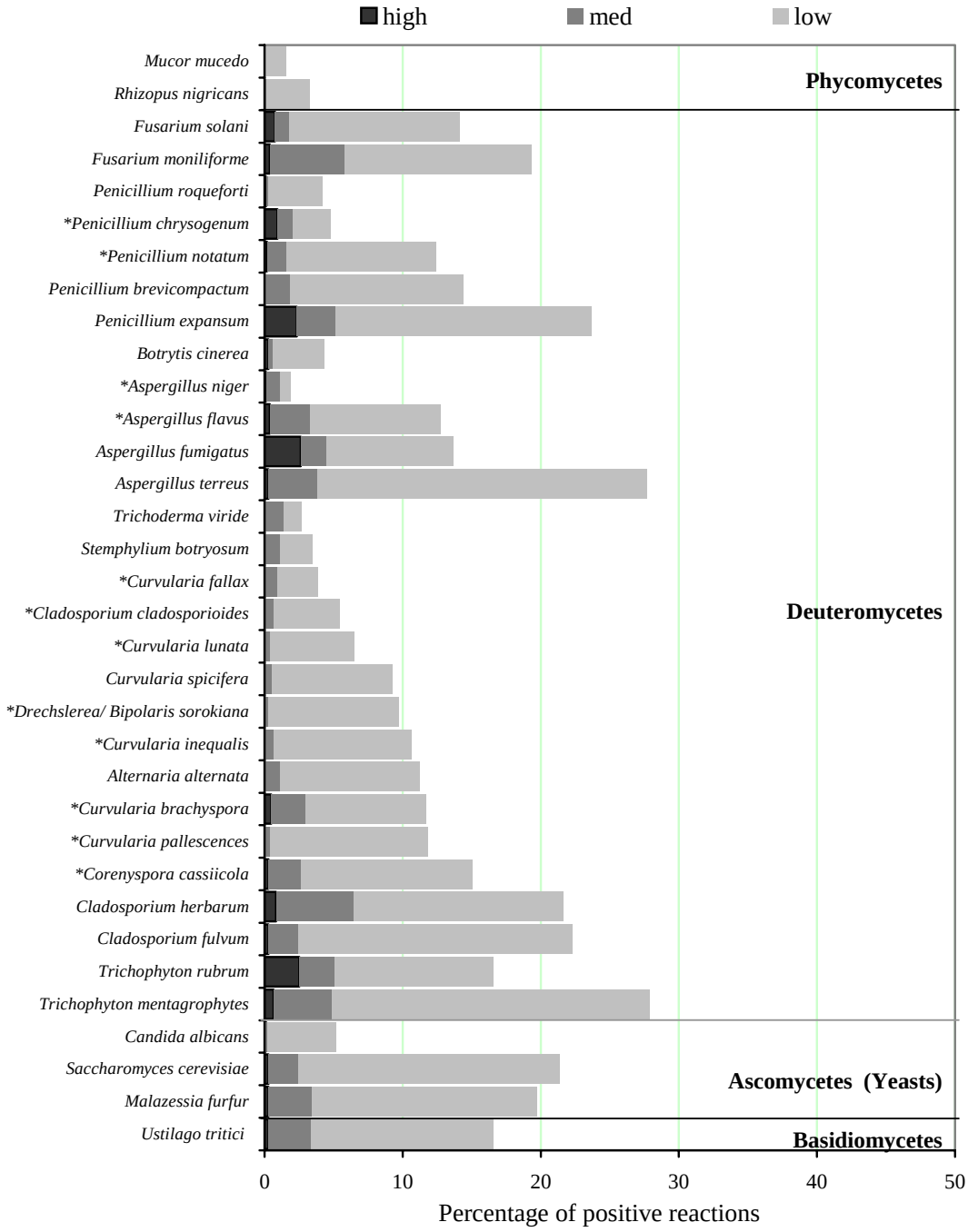
Local plant species are each marked with an asterisk (*) (Cut offs: high = 2SD + 100 OD, medium = 2SD + 50OD and low = 2SD). Allergens are divided by taxonomic order by black lines and family (sub-family) by grey lines.

Figure 5.3: Reactions to tree pollen in descending order for each family.



Local plant species are each marked with an asterisk (*) (Cut offs: high = 2SD + 100 OD, medium = 2SD + 50OD and low = 2SD). Allergens are divided by taxonomic order by black lines.

Figure 5.4: Reaction results to fungal allergens in descending order for each class.



Common local outdoor fungi are each marked with an asterisk (*) (Cut offs: high = 2SD + 100 OD, medium = 2SD + 50OD and low = 2SD). Allergens are divided by taxonomic class by black lines.

5.3.2 Cluster analyses

Cluster analysis was performed to study the pattern of reactivity among the patients in the samples that were tested. The patterns obtained may serve as a possible reference for further cross reactivity studies among the airspora allergens studied with plant-based food allergens. Since cross reactivity of fungal allergens to other allergens from pollen and plant-based food has never been reported, clustering was only carried out only among fungal allergens. A separate cluster analysis was performed for pollen with plant-based food allergens.

The pollen and plant-based food cluster analysis yielded two major clusters (Figure 5.6 sand Figure 5.7). Each cluster contains three sub-clusters. All local pollen types were in Cluster 2 except for that of *Sorghum halepense*. All Asteraceae pollen types were in Cluster 1 except for those of *Baccharis halimifolia* and *Taraxacum officinale*. *Acacia* spp. and *Elaeis guineensis* pollen were clustered together with those of the grasses *Avena sativa* and *Hordeum vulgare*.

A large number of high and significant correlations were obtained (Appendix 3). However, only correlations between the local airspora and other pollen and food types were studied owing to the confounding factors caused by the possible missing primary sensitiser counterparts (Figure 5.5). The *Casuarina equisetifolia* pollen allergen was strongly correlated with those of *Acacia* sp. (r = 0.5736***) and *Elaeis guineensis* (r = 0.5011***). The *Elaeis guineensis* pollen allergy was strongly correlated to those of *Artemisia vulgaris* (r = 0.9592***), *Acacia* sp. (r = 0.5165***) and *Ulmus americana* (r = 5242***). The *Podocarpus polystachyus* pollen allergen was strongly correlated to the allergens of *Ligustrum vulgare* pollen (r = 0.9943***), corn flour (r = 0.8047***), potato (r = 0.8336***) and *Fraxinus excelsior* pollen (r = 0.9958***).

Strong correlations were obtained for allergens that are closely related like those of *Sorghum halepense* with *Cynodon dactylon* (r = 0.7271***) and *Zea mays* pollen (r = 0.6251***). Strong correlations were also observed in allergens that were not related like the pollen of *Sorghum halepense* with those of *Betula verrucosa* (r = 0.5340***) and *Arecastrum romanzoffianum* (r = 0.5122***), walnut and *Corylus avellana* (r = 0.9391***).

Figure 5.5: Bi-plots of some local pollen with other allergens. Correlations were obtained by the Kendall τ correlation test. All correlations were significant at p value less than 0.001.

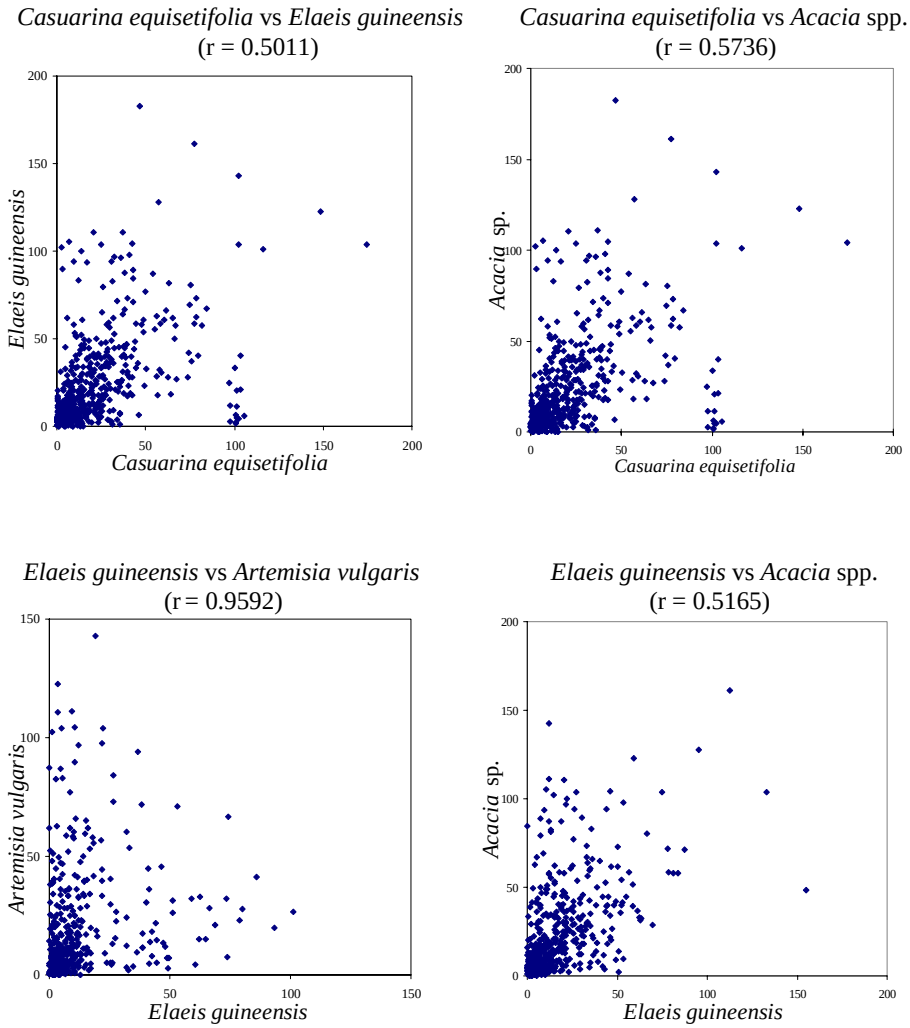


Figure 5.5 (continued): Bi-plots of some local pollen with other allergens. Correlations were obtained by the Kendall τ correlation test. All correlations were significant at p value less than 0.001.

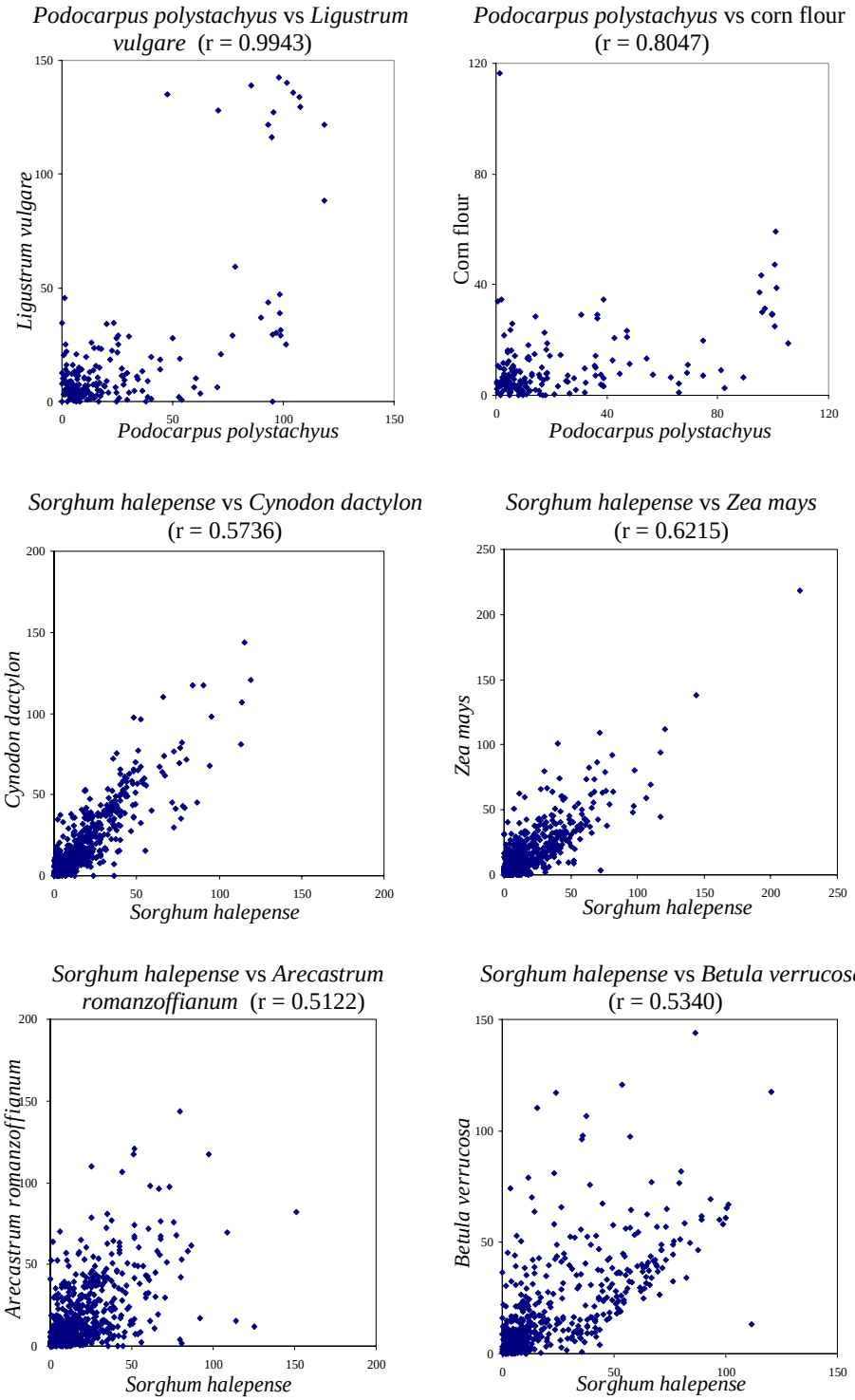
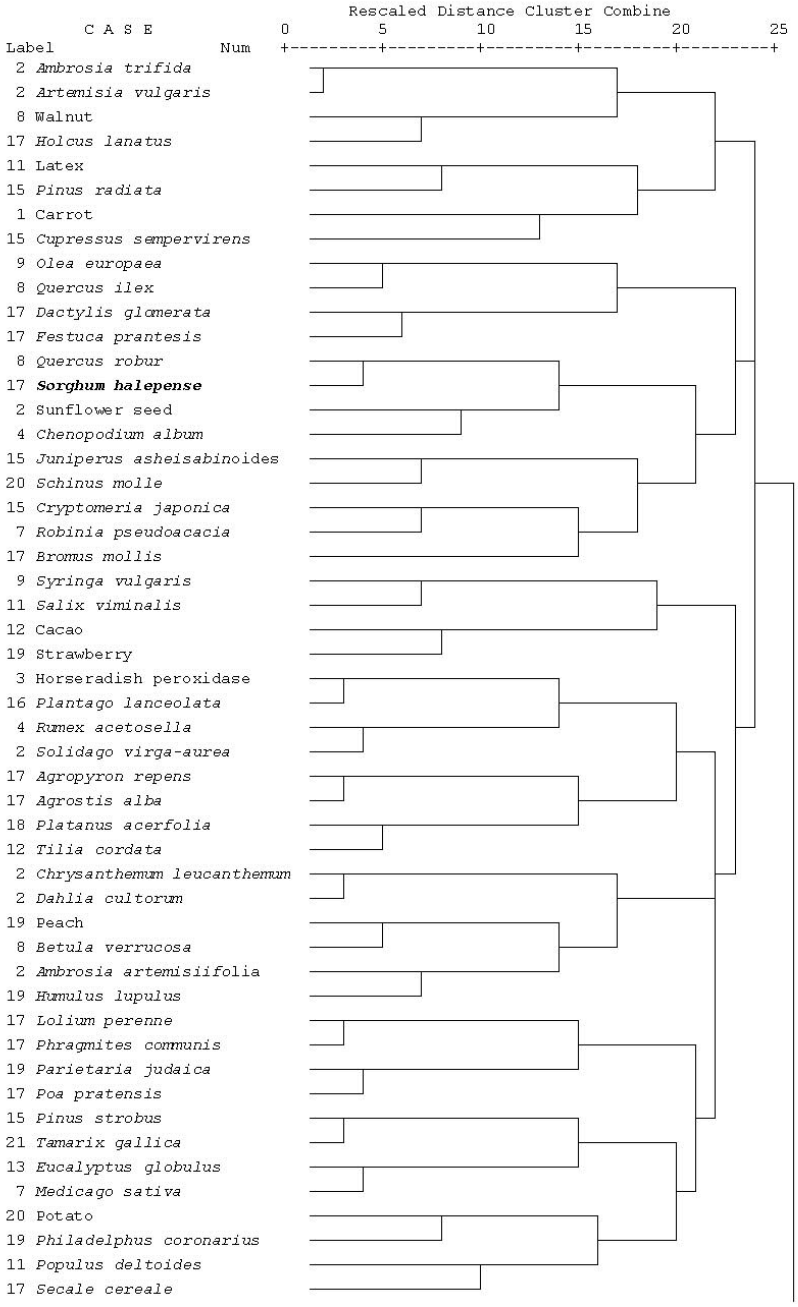
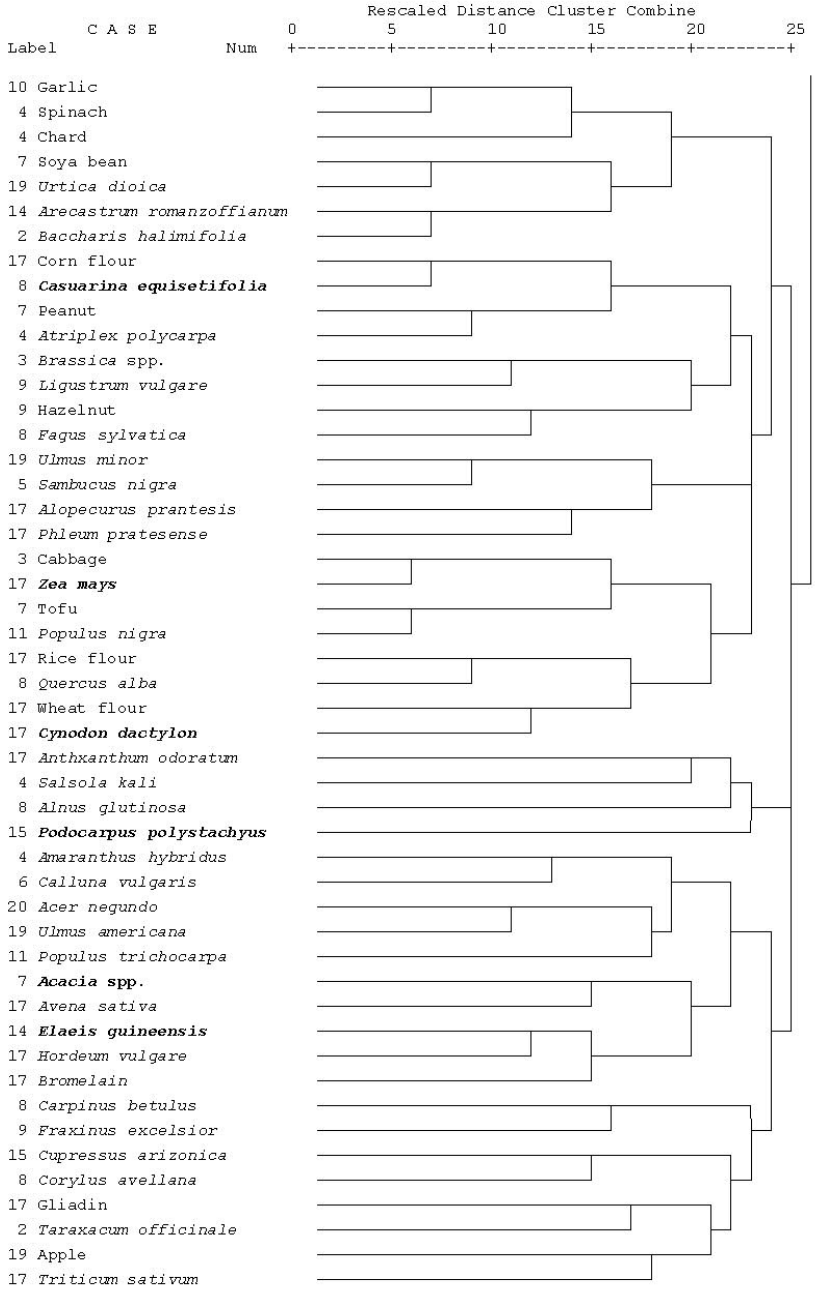


Figure 5.6: Results of cluster analysis (Cluster 1) for pollen and food-based allergens.



Pollen types are in their botanical names while food allergens are given their common names. Pollen types found locally are indicated in **bold.** The taxonomic orders are indicated by number: 1=Apiales, 2= Asterales, 3=Capparales, 4= Caryophyllales, 5= Dipsacales, 6=Ericales, 7=Fabales, 8=Fagales, 9=Lamaliales, 10=Liliales, 11= Malpighiales, 12=Malvales, 13=Myrtales, 14=Palmes, 15=Pinales, 16=Plantaginales, 17=Poales, 18=Proteales, 19=Rosales, 20=Sapindales, 21=Solanales, 22=Tamaricales, 23=Zingiberales.

Figure 5.7: Results of cluster analysis (Cluster 2) for pollen and food-based allergens.

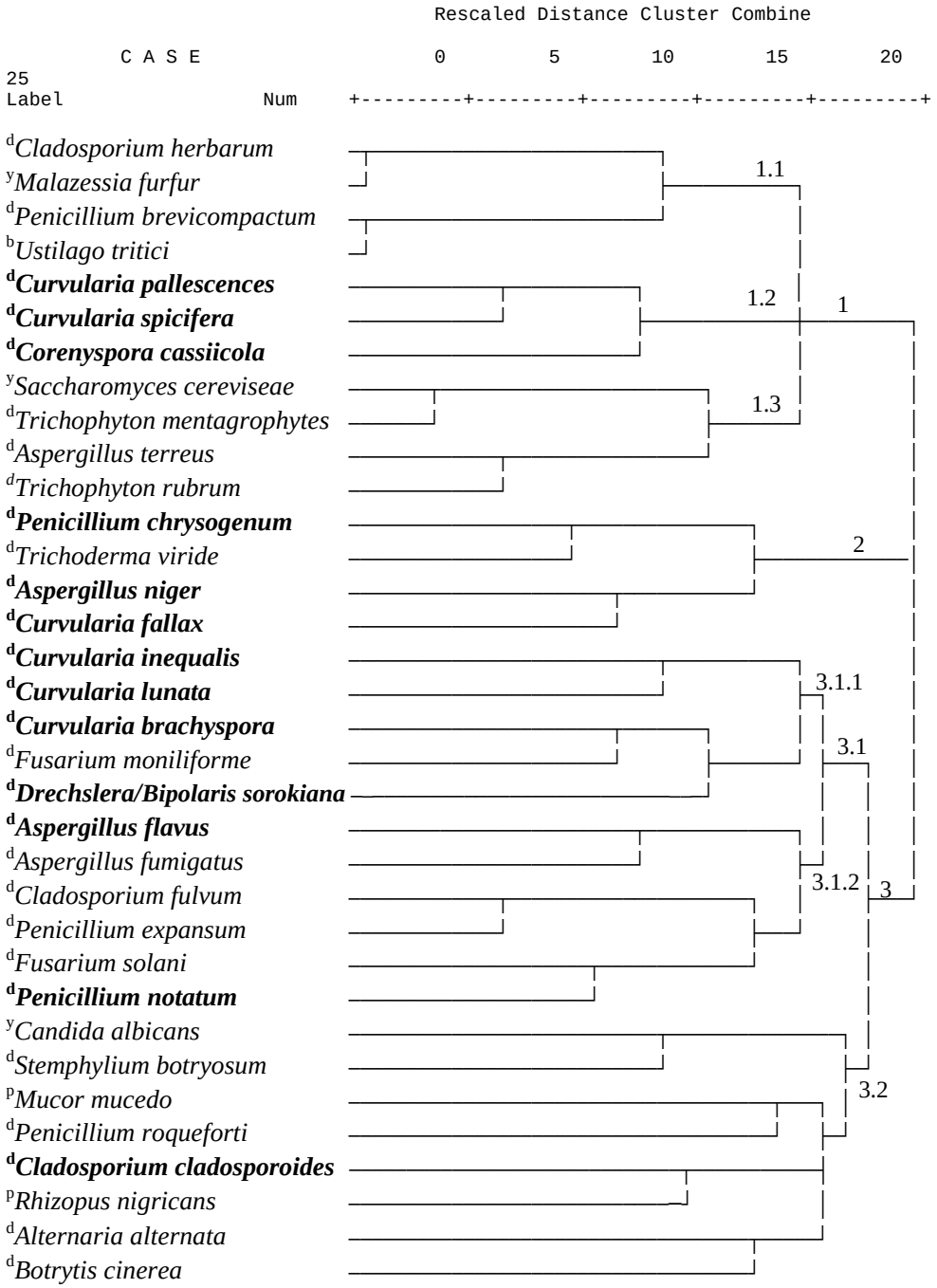


Pollen types are in their botanical names while food allergens are given their common names. Pollen types found locally are indicated in **bold.** Food allergens are in their common names. The taxonomic orders are indicated by number: 1=Apiales, 2= Asterales, 3=Capparales, 4= Caryophyllales, 5= Dipsacales, 6=Ericales, 7=Fabales, 8=Fagales, 9=Lamaliales, 10=Liliales, 11= Malpighiales, 12=Malvales, 13=Myrtales, 14=Palmes, 15=Pinales, 16=Plantaginales, 17=Poales, 18= Proteales, 19=Rosales, 20=Sapindales, 21=Solanales, 22=Tamaricales, 23=Zingiberales.

For fungal allergens, three major clusters were generated (Figure 5.8). Both *Trichophyton* species were clustered with *Aspergillus terreus* and *Saccharomyces cerevisiae* in Subcluster 1.3. *Curvularia pallescences, Curvularia spicifera* and *Corenyspora cassiicola* were clustered together in Subcluster 1.2 while the other *Curvularia* species were clustered in Cluster 3 except for *Curvularia fallax* which was together with *Aspergillus niger*.

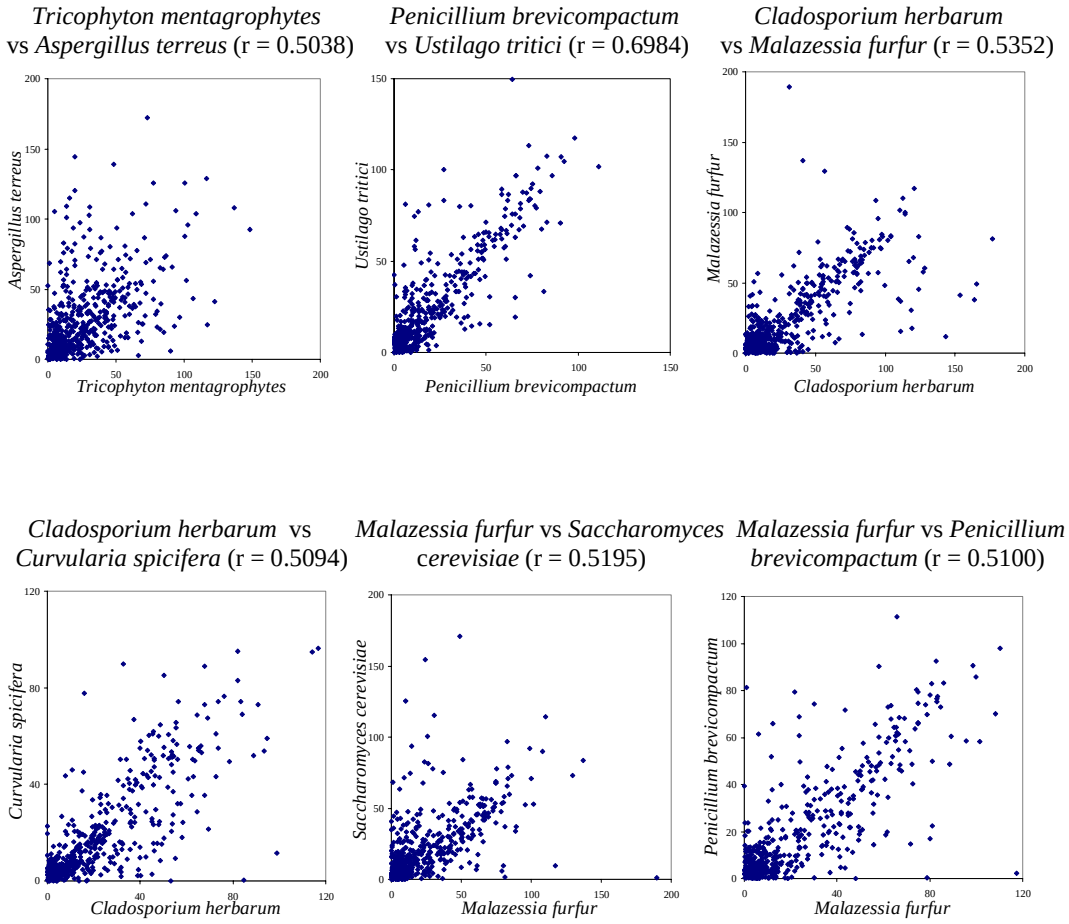
Strong correlations ($r>0.5$, $p<0.001$) were observed between *Tricophyton mentagrophytes* and *Aspergillus terreus, Penicillium brevicompactum* and *Ustilago tritici, Cladosporium herbarum* and *Malazessia furfur, Cladosporium herbarum* and *Curvularia spicifera, Malazessia furfur* and *Saccharomyces cerevisiae* and *Malazessia furfur and Penicillium brevicompactu*m (Figure 5.9). Results of the Kendall τ correlation can be referred to in Appendix 3.

Figure 5.8: Cluster analysis for fungal allergens.



Commonly found local outdoor allergens are indicated in **bold**. Classes are indicated in superscripts: [b]Basidiomycetes, [d]Deuteromycetes, [p]Phycomycetes and [y]Ascomycetes (yeasts).

Figure 5.9: Bi-plots of reaction intensities between strongly correlated fungal allergens. Correlation coefficients, r were obtained from Kendall τ correlation test with p values less than 0.001.



5.4 DISCUSSION

Only some local pollen types, i.e., *Casuarina equisetifolia, Sorghum halepense* and *Zea mays* and were found to have more than 20% positive reactions. Other pollen types were from plants that do not occur in Singapore. This may be attributed to cross reactivity properties shared with one or more local sensitizers that grows or grow in abundance here indicated by high numbers but relatively weak reactions compared to some local pollen types. Mari *et al*. (2003) demonstrated sensitivity to Bet v 1, Bet v 2 and Bet v 4 in Fagales pollen sensitised patients in a birch-free area. The two grasses *Zea mays* and *Sorghum halepense* belong to the sub-family Panicoideae, which are commonly found in Singapore followed by the Chloridoideae, which is represented only by *Cynodon dactylon*. Table 5.1 shows the corresponding common local plants that are taxonomically closely related to the foreign pollen types with high positive reactions. Some of these exotic plants have also been planted as wayside trees lining the roads or used as cut flowers or ornamental plants in local parks and gardens.

The weed, grass and cultivated plants were the majority of allergen types with more than 20% positive results. All these plant are basically plants less then 1.5 meters tall. The current spore traps are positioned on rooftops of tall building at 28 m (Clementi), 45 m (Hougang) and 61 m (Kent Ridge on a slope) above sea level. The counts for Kent Ridge were found to be constantly lower than counts for Clementi and Hougang for plants which are short in height, such as weeds and grasses in the aerobiology study. Greater pollen concentrations have been observed in traps placed at a lower height (1.5m) for herbaceous plants (Alcazar *et al*., 1998; Galan *et al*., 1995; Hart *et al*., 1994; Rantio-Lehtimaki *et al*.,

Table 5.1: List of allergens tested and possible local sensitisers.

Screened pollen type	Local closely related pollen types
Urtica dioica, Parietaria judaica	[1]*Laportea interrupta,* [1,2]*Pilea microphylla,* [1]*Pipturus argenteus,* [1]*Poikilospermum suaveolens,* [1]*Pouzolzia zeylanica*
Arecastrum romanzoffianum	[2]*Archontophoenix alexandrae,* [1,2]*Cocos nucifera,* [2]*Cyrtostachys renda,* [2]*Dypsis lutescens,* [2]*Ptychosperma macarthurii,* [2]*Roystonea oleracea,* [2]*Roystonea regia,* [2]*Veitchia merrillii,* [2]*Wodyetia bifurcata*
Baccharis halimifolia, [3]*Dahlia cultorum,* [3]*Chrysanthemum leucanthemum,* [3]*Solidago virgaaurea*	[1]*Artemisia vulgaris,* [1]*Emilia sonchifolia,* [1]*Mikania cordata,* [1]*Mikania micrantha,* [1]*Porophyllum ruderale,* [1]*Sphagneticola trilobata,* [1]*Synedrella nodiflora,* [1]*Tridax procumbens,* [1]*Vernonia cinerea,* [1]*Wallostonia biflora*
Philadelphus coronarius	[2]*Hydrangea macrophylla*
[2,4]*Zea mays, Sorghum halepense, Lolium perenne, Dactylis glomerata, Festuca prantesis, Phragmites communis*	[12]*Axonopus compressus,* [12]*Chloris barbata,* [12] *Chrysopogon aciculatus,* [12]*Dactyloctenium aegyptium,* [12]*Eleusine indica,* [12]*Eragrostis tenella,* [12]*Imperata cylindrica,* [12] *Ischaemum muticum,* [12] *Melinis repens,* [12]*Panicum maximum,* [12]*Paspalum conjugatum,* [12]*Pennisetum polystachyon,* [12]*Pennisetum purpureum,* [1]*Sporobulus indicus,* [12]*Thuarea involuta,* [12]*Urochloa mutica,* [12]*Zoysia matrella*
Populus nigra, Populus deltoides, Salix viminalis	[2]*Salix babylonica*
Chenopodium album, Atriplex polycarpa, [4]*Beta vulgaris var. cicla*	[1]*Alternanthera sessilis,* [1]*Alternanthera philoxeroides* , [2]*Amaranthus blitum,* [2]*Amaranthus lividus,* , [2]*Amaranthus spinosus,* [2]*Amaranthus tricolor,* [1,2]*Colosia argentea,* [2]*Colosia aristata,* [1]*Sphagneticola trilobata,* [4]*Spinacia oleraceae*
[1,2]*Casuarina equisetifolia*	[2]*Casuarina junghuhniana,* [2]*Casuarina nobilis,* [2]*Casuarina rumphiana,* [2]*Casuarina sumatara*
Amaranthus hybridus	[2]*Amaranthus spinosus,* [2]*Amaranthus tricolor,* [2]*Amaranthus lividus,* [2]*Amaranthus blitum,* [1,2]*Celosia argentea,* [2]*Celosia aristata*
Ligustrum vulgare	[2,3]*Jasminum multiflorum,* [2,3] *Jasminum rex,* [2,3]*Jasminum sambac*
Pinus strobus, Cryptomeria japonica	[2]*Pinus caribaea,* [2]*Pinus elliotti,* [2]*Pinus kesiya,* [2]*Pinus merkusii,* [2]*Podocarpus polystachyus,* [2]*Podocarpus rumphii,*
Juniperus asheisabinoides	[2]*Juniperus chinensis*
Sambucus nigra	[2]*Lonicera japonica*
[2]*Eucalyptus globulus*	[2]*Eucalyptus alba,* [2]*Eucalyptus camaldulensis,* [2]*Eucalyptus deglupta,* [2]*Eucalyptus ptychocarpa,* [2]*Eucalyptus robusta*
Quercus robur	[2]*Lithocarpus bennettii,* [2]*Lithocarpus cantleyanus,* [2]*Lithocarpus conocarpus,* [2]*Lithocarpus elegans,* [2]*Lithocarpus encleisacarpus,* [2]*Lithocarpus ewyckii,* [2]*Lithocarpus gracilis,* [2]*Lithocarpus hystrix,* [2]*Lithocarpus lucidus,* [2]*Lithocarpus sundaica,* [2]*Lithocarpus wallichianus,* [2]*Quercus argentata*

1 = grow wild, 2 = planted, 3 = cut flowers, 4 = food

Table compiled from Boo *et al*. (2003), Tan and Morgany (2001), Tee and Wee (2001), Turner (2000, 1995a, 1995); Keng *et al*. (1998), Tan (1997), Turner and Chin (1993), Keng (1990), Mabberley (1997) and Foo (1986), Gilliland HB (1971).

1991). Recently, Armentia *et al.* (2004) found living in towers as a risk factor of pollen allergy. This results in great concern since in Singapore more than 70% of its population live in high rise buildings on average more than 10 stories high. However this study was carried out only on subjects who lived only in building with a maximum height of eight floors.

The frequency in terms of detection of IgE levels for the local pollen types was different than previously reported. The comparison of the current results obtained and previous studied are listed in Table 5.2. All results were found to be lower than reported by Chew *et al.* (2000) except for *Casuarina equisetifolia* while higher number of positive reactions was observed between results reported by Allumoortil *et al.* (1996) and Tan and Teoh (1979). The difference between results by Chew *et al.* (2000) may be because the patients were screened whereby the statuses of allergy have been clinically confirmed (asthmatic, allergic rhinitis and/or atopic eczema) while sample used in the current screens are from patients suspected with allergies. Sources of raw materials may be another contributing factor. Differences in the composition especially in the major allergens will influence the reactivity results. Allergens from the same source but collected at different times can and do differ in their composition (Esch, 1997).

The non-positive results for the fern allergens *Dicranopteris linearis* and *Nephrolepis auriculata* are probably owed to the differences in protein extraction methods and source materials. Both fern proteins were extracted using a mixture of spores and fern fronds utilizing Trizol® (Gibco International, USA), based on the phenol-chloroform extraction method and most components were found to be highly insoluble. A similar attempt to extract allergens from ferns using the same buffer by Chew *et al.*, 2000 yielded protein levels below 0.1 mg ml^{-1}. The major allergenic protein found in *Di-*

cranopteris spp., which is a storage protein can only be found in the fern samples after a hot and prolonged dry spell (Shang HS, personal communication, 2004).

Table 5.2: Allergy tests performed in Singapore.

Patients' information	Own (2003)	Chew et al. (2000)	Allumoortil et al. (1996)	Tan and Teoh (1979)
Age of patients	Not available	mean age 13.3	mean age 26.8	10 to 73
Selection of patients	Attending allergy clinic	Asthma/Rhinitis/Atopic eczema	Rhinitis	Asthma
Gender	Not available	69(F), 107(M)	54(F), 31(M)	45(F), 93(M)
Test used	Immunoarray	FAST	Skin prick	Skin prick
Allergens studied				
Pollen				
Acacia spp.	10.4%	29.0%	11.8%	NT
Casuarina equisetifolia	21.8%	18.8%	NT	NT
Elaeis guineensis	17.0%	40.9%	NT	NT
Podocarpus polystachyus	2.3%	33.5%	NT	NT
Poaceae (grasses)	0.6% to 22.7%	NT	3.5% to 4.7%	1.1%
Salix	18.5%	NT	5.9%	NT
Plantago	15.6%	NT	NT	0.0%
Artemisia	11.7%	NT	2.4% to 3.5%	NT
Juniperus	19.3%	NT	1.2%	NT
Eucalyptus	18.3%	NT	0.0%	NT
Fungus				
Cladosporium	5.4% to 22.3%	6.8%	NT	NT
Curvularia	3.8% to 11.8%	21.0% to 22.2%	NT	NT
Drechslera-like spores	9.7% to 15.1%	14.8% to 21.6%	NT	NT
Aspergillus	1.9% to 27.7%	NT	3.5%	6.5%
Penicillium	4.2% to 23.7%	NT	4.7%	NT
Alternaria	11.2%	NT	5.9%	NT

Own = results obtained from dot immunoarray system (Chapter 4)
Chew *et al*. (2000) = results obtained from FAST.
Allumoortil *et al*. (1996) and Tan and Teoh (1979) = results obtained by the skin prick test.
NT = not tested.
Gender: F = female and M = male

Previous studies have shown that the thick cell walls of the spores that protects them from dehydration before germination make it extremely difficult to extract intact concentrated fern spore proteins (Shang, 1999). The addition of proteinase inhibitors like chymostatin (50 µg ml^{-1}), PMSF (10 µg ml^{-1}), pepstatin A (5 µg ml^{-1}) and antipin (5 µg ml^{-1}) yielded protein bands on SDS-PAGE but at very low concentrations (about 0.05 µg $µl^{-1}$). Employment of some concentrating procedures may increase concentrations but such procedures may result in protein degradation and smeary SDS-PAGE protein profiles. The use of proteinase inhibitors might also serve as an irritant thus interfering with the skin test results.

A study by Geller-Bernstein (1987) showed totally negative reactions in allergic patients, who were positive to other ferns through exposure but not to *Nephrolepis exaltata*. Much lower sensitivity rates to similar ferns were reported in Malaysia (9.3% by Ho *et al*., 1999) and Indonesia (11.21% by Baratawidjaja *et al*., 1999).

The frequency of fungal allergies among the allergy-prone individuals (66.4%) of the study group was high. This may because of the continuously high levels of outdoor fungal spores year-round (Lim *et al*., 1998) or indoors, whose development were facilitated by the hot and humid climate. The allergen of *Tricophyton mentagrophytes* had the highest sensitisation while those of *Tricophyton rubrum* had the highest number of strong reactions. These fungi have been reported to cause *tinea pedis*, a fungal infection of the interdigital toe web space as well as the skin of the feet (Devliotou-Panagliotidou *et al*., 2001; Perea *et al*., 2000; Brooks and Bender, 1996; Weitzmen and Summerbell, 1995). *Tricophyton rubrum* (58%) and *Tricophyton mentagrophytes* (10%) are common fungi isolated from patients with *tinea* in Singapore (Goh *et al*., 1994). It was also demonstrated that patients with dermatomycosis regardless of atopy status were allergic to *Tricophyton* when tested with the SPT suggesting exposure to

allergens through broken skin surfaces instead of cross-reactivities with aeroallergens (Mungan *et al*., 2001; Escalante *et al*., 2000). High numbers of reactivity to the allergen of *Tricophyton* have also been demonstrated in patients allergic to fungi (Mari *et al*., 2003; Kivity *et al*., 1992). Tri t 1 has been shown to be responsible for immediate hypersensitivity (Deuell *et al*., 1991) while Tri r 2 for delayed type hypersensitivity (Woodfolk *et al*., 1998).

Aspergillus and *Penicillium* spores account for 28% of the total local indoor fungal airspora. High reaction rates to these two fungal spores were seen in the patients' samples tested. Reactions to allergens of *Cladosporium*, a major outdoor (33.5%) and indoor (10%) fungal spore type were also relatively high. *Alternaria*, reported to be a common allergenic fungal allergen ranked 19th among 34 fungal allergens tested (Mari *et al*., 2003; Srivastava and Wadhwani, 1992). It has been reported as a common indoor and outdoor allergen (Budd, 1986). However, *Alternaria* spores occurred in less then 2% in the total indoor airspora in Singapore (Wong, 2002). *Malazessia furfur* (*Pityrosporum orbiculare*) is a common commensal from the healthy surface of healthy skin (Leeming *et al*., 1997) and *Saccharomyces cerevisiae* is commonly used in the food industry. The specific IgE to *Malazessia furfur* is commonly found in patients with the atopic eczema dermatitis syndrome (Wessels *et al*., 1991; Nordvall *et al*., 1990). Specific IgE to *Saccharomyces cerevisiae* are also commonly observed among atopic individuals (Mari *et al*., 2003).

Clustering based on close taxonomic relationships was observed for allergens of some grass types but this pattern was not observed throughout the clustering results. Most of the allergens of the common local pollen types were clustered with those of the foreign ones but not to those of food. This can be attributed to cross reactivity properties

shared by the allergens of the pollen types and/or co-sensitisation to an allergen of a related local counterpart.

A study done by Chowdhury *et al*. (1998) showed shared allergenic properties in palms (*Areca catechu, Borassus flabellifer, Cocos nucifera* and *Phoenix sylvestris*). However, most of the allergenic properties of the possible co-sensitising local counterparts were poorly or have never been studied. We will thus discuss the possible patterns and sources of cross reactivity in the reactions observed.

Reactions ranging from 0.56 to 21.33% between the allergens of members of subfamily Pooideae demonstrate the differences in reactivity of closely related pollen types. This is most probably because of the differences in allergenicity (van Ree *et al*., 1992) of major components or percentage of composition of these allergens. Studies have been carried out on the grasses especially from the Pooideae (e.g. *Agropyron, Bromus, Festuca, Lolium* and *Poa*), Chloridoideae (*Cynodon*) and Panicoideae (e.g. *Panicum, Paspalum, Sorghum*), the latter which is the major subfamily in tropical Singapore. Grasses from the same subfamily have been shown to have stronger cross-reactivities with each other (van Ree *et al*., 1992; Martin *et al*., 1985; Bernstein *et al*., 1976; Leiferman and Geich, 1976). More cross-reactivities have been shown between the Panicoideae and Pooideae than between the Panicoideae and Chloridoideae (Esch, 2004; Weber, 2003). This may account for the high number of positive reactions observed for the Pooideae allergens tested in this study although grasses from this subfamily are not found in Singapore. Reactions to Chloridoid grasses were most probably attributed to co-sensitization with local Chloridoid grasses and/or cross reactivity with Panicoid grasses. Groups 1 (95%), 2 or 3 (60%) and 5 (65 to 85%) are considered as the major grass allergens but Group 4 allergens have been reported in up to 75% of patients with grass allergies (Fahlbusch *et al*., 1998). The Phl p 4 related allergens

have been localised in apple, birch pollen, carrot root, celery root, mugwort and peanut by electron microscopy demonstrating cross reactivity to unrelated plants. This may account for the strong correlations ($r>0.8$, $p<0.001$) obtained for *Avena sativa* to walnut and seven other tree pollen types. Adverse food reactions in patients who are monosensitized to grass pollen have also been reported to be more frequent compared to mite allergic individuals. Positive correlations were obtained for allergy symptoms to grass pollen levels in the airspora (Boccafogli *et al*., 1994).

For weed pollen, cross reactivities between the weedy members of the Asteraceae family have been demonstrated between *Ambrosia, Artemisia, Helianthus* and *Solidago* (Hirschwehr *et al*., 1998; Fernandez *et al*., 1993, Sriramarao and Rao, 1993) and between the different species of the same genus, i.e., congeners (Katial *et al*., 1997).

Highly cross-reactive groups of allergens that can be found in pollen or food in unrelated families can be attributed to pan-allergens like Bet v 1 homologues (Wensing *et al*., 2002), profilins (Valenta *et al*., 1992; van Ree *et al*., 1992), calcium-binding proteins of two EF-hand motifs (Tinghino *et al*., 2002; Niederberger *et al*., 1999) and the lipid transfer proteins (LTP) (Asero *et al*., 2000) especially in the case of the oral allergy syndrome. These allergens elicit symptoms in pollen allergic individuals to a large range of allergens including food.

Bet v 1 homologous allergens have been found in trees belonging to the Fagales order (e.g., Aln g 1, Cor a 1, Car b 1, Que a 1, Cas s 1). They can also be found in food (Dau c 1). Profilins currently have been identified in plant sources such as *Artemisia*, apple, *Betula*, celery, *Helianthus, Lolium*, lychee fruit, peach, peanut, potato, tomato and wheat (Fuchs *et al*., 1997; Valenta *et al*., 1992; van Ree, 1992). LTPs have been identified in plant sources such as *Artemisia*, apricot, *Corylus*, peach and plum (Asero *et*

al., 2000). It is a major food allergen and has been shown to cause food-pollen allergies (Garcia-Selles *et al*., 2002; Pasterello *et al*., 2002; Diaz-Perales *et al*., 2000). Calcium-binding proteins of two EF-hand motifs have also been discovered as relevant cross-reactive allergens (Tinghino *et al*., 2002; Niederberger *et al*., 1999; Seibler *et al*., 1994) in alder, birch, Bermuda grass, cypress, olive and rapeseed.

Another source of cross-reactivity is the cross-reactive carbohydrate determinant (CCD) a N-linked glycan. It was discovered by Aalberse *et al*. (1981) who detected IgEs in some human sera that reacted with an antigen present in a large number of unrelated food types: buckwheat, cat dander, chicken, egg white, cow's milk, *Dermatophagoides pteronyssinus*, dog dander, grasses, honey, mussel, peanut, potato, rabbit dander, rice, soya bean, spinach, tree pollen, weed pollen, wheat and many more. It was later found that the IgEs, binding lacked specificity from inhibition studies. Follow up studies have demonstrated that CCD IgEs are largely clinically irrelevant (Ebo *et al*., 2004; Batanero *et al*., 1999; van der Veen *et al*., 1997). CCDs have also been found to cause false positive results in *in vitro* diagnostic assays (Mari *et al*., 1999). Percentages of positive reactions of each allergen, which were simultaneously positive to bromelain, are shown in Table 5.3. However, a study by Foetisch *et al*. (2003) showed that one third of patients' sera who were allergic to tomatoes have biologically relevant CCD-specific antibodies. Hence, CCD-specific IgEs should be taken into consideration for certain allergies.

To date, a large number of fungal allergens related to pathogenesis like aldehyde dehydrogenase, enolases, heat shock proteins, mangan superoxide dismutase and serine proteases have been identified (Vijay and Kurup, 2004; Horner *et al*., 1995). These allergens are mainly from highly allergenic fungal types like *Alternaria alternata, Aspergillus flavus, Aspergillus fumigatus, Candida albicans, Cladosporium herbarum,*

Malazessia furfur, Penicillium brevicompactum, Penicillium chrysogenum and *Trichophyton rubrum,* to name a few.

Fungi from closely related families have been shown to share cross-reactive properties. *Candida albicans*'s enolase has been found to cross-react with an enolase found in *Saccharomyces cerevisiae* (Ito *et al*., 1995). Patients who are SPT positive to *Curvularia lunata* allergens were also positive to those of at least five *Curvularia* species (Gupta *et al*., 2002). *Malazessia globosa* was shown to inhibit reactions to four other *Malazessia* species (Koyama *et al*., 2001). Cross-reactivities between allergens of *Aspergillus, Cladosporium* and *Fusarium* species have been demonstrated (Verma and Gangal, 1994; Vijay *et al*., 1991; Karr *et al*., 1981). Inhibition studies also demonstrated cross-reactivity between allergens of *Alternaria alternata, Curvularia, Helminthosporium, Spondylocladium* and *Stemphylium* (Hoffman *et al*., 1981).

Fungal extracts mainly contain proteins, carbohydrates as well as proteolytic and glycosidic enzymes (Vijay and Kurup, 2004). CCDs have long been considered non-allergenic but convincing evidence has shown otherwise with the discovery of IgE epitopes on fungal mannan preparations from *Malazessia furfur* (Doekes *et al*., 1993). Thus further studies on CCDs have to be carried out and may be important in certain fungal allergens. The concordance between the allergens tested and reactions to bromelain is shown in Table 5.3. Even though bromelain only represents a single type of CCD epitope, it can still be said that the large number of reactions observed are not because of unspecific CCDs alone.

A similar pattern of cross-reactivity between pollen and food allergens especially from the Rosacae (Garcia-Selles *et al*., 2002; Pasterello *et al*., 2002; Diaz-Perales *et al*., 2000), was not clearly observed. This is most probably confounded by the difference

in vegetation thus affecting the different pollen types available here. A study in Spain, demonstrated that pollen allergy is related to the area of residence (Carinanos *et al*., 2002). Similar sensitization patterns to food allergens were observed in Singapore children as in western populations (Shek and Lee, 1999). However, the major food types that cause anaphylaxis like the peanut and tree nuts were uncommon in the study. Differences in diet were hypothesized as the cause of the results obtained. With the current results, the difference in results could possibly be owed to the pollen types that the study group individuals were exposed to.

Low prevalence (less than 12%) of sensitization to plant food allergen sources like peanut, soy bean and wheat in atopic children in Singapore has repeatedly been reported (Khoo e*t al.*, 2001 and Chng et al., 1999). The frequencies and patterns of reactions seen for the food sources will need to be further evaluated by food challenge or double blind placebo control food challenge (DBPCFC) before the cause (cross-reactivity or co-sensitization) of the reactions seen can be determined.

Table 5.3: Percentages of concordance between positive results and bromelain in descending order.

Allergens	Concordance of positive reactions to bromelain (%)
Grasses	
Avena sativa	53.06
Hordeum vulgare	50.31
Triticum aestivum / Triticum sativum	48.44
Anthxanthum odoratum	47.37
Cynodon dactylon	35.92
Zea mays	33.74
Phlenum pratesense	28.87
Dactylis glomerata	25.12
Sorghum halepense	25.10
Festuca prantesis	24.88
Lolium perenne	23.35
Bromus mollis	22.22
Phragmites communis	21.33
Holcus lanatus	19.61
Poa pratensis	17.37
Alopecurus prantesis	16.67
Secale cereale	13.14
Agrostis alba	10.08
Weeds	
Taraxacum officinale	53.03
Salsola kali	51.85
Calluna vulgaris	47.73
Atriplex polycarpa	35.53
Sambucus nigra	30.85
Baccharis halimifolia	30.74
Urtica dioica	27.64
Chenopodium album	24.37
Amaranthus hybridus	21.40
Ambrosia artemisiifolia	21.00
Parietaria judaica	18.87
Humulus lupulus	17.49
Solidago virga-aurea	16.11
Ambrosia trifida	15.63
Plantago lanceolata	13.77
Artemisia vulgaris	12.80
Rumex acetosella	10.76

Table 5.3 (continued): Percentages of concordance between positive results and brom elain in descending order.

Allergens	Concordance of positive reactions to bromelain (%)
Cultivated herbaceous plants	
Brassica spp.	40.44
Dahlia cultorum	24.90
Chrysanthemum leucanthemum	20.89
Medicago sativa	18.83
Trees	
Cupressus arizonica	59.49
Ulmus americana	55.73
Carpinus betulus	52.94
Fagus sylvatica	52.46
Fraxinus excelsior	50.00
Acacia spp.	48.65
Elaeis guineensis	47.80
Corylus avellana	47.27
Acer negundo	43.15
Populus trichocarpa	41.58
Casuarina equisetifolia	40.77
Quercus alba	35.87
Ligustrum vulgare	34.56
Populus nigra	34.17
Cupressus sempervirens	32.86
Alnus glutinosa	32.69
Quercus ilex	32.24
Ulmus minor	31.65
Olea europaea	30.29
Arecastrum romanzo ffianum	26.25
Cryptomeria japonica	25.39
Quercus robur	24.87
Platanus acerfolia	22.01
Juniperus asheisabinoides	20.87
Robinia pseudoacacia	20.38
Pinus strobus	20.38
Tamarix gallica	20.24
Schinus molle	18.71
Eucalyptus globulus	17.86
Tilia cordata	17.73
Betula verrucosa	16.32
Philadelphus coronarius	15.56
Pinus radiata	15.38
Populus deltoides	13.88
Salix viminalis	12.21
Syringa vulgaris	11.90
Podocarpus sp.	4.17

Table 5.3 (continued): Percentages of concordance between positive results and bromelain in descending order.

Allergens	Concordance of positive reactions to bromelain (%)
Fungi	
Mucor mucedo	64.71
Cladosporium cladosporoides	58.62
Rhizopus nigricans	57.14
Alternaria alternata	50.83
Botrytis cinerea	50.00
Fusarium solani	47.68
Penicillium notatum	44.36
Cladosporium fulvum	42.86
Drechslerea/ Bipolaris sorokiniana	40.38
Curvularia inequalis	40.35
Candida albicans	38.18
Curvularia lunata	36.23
Penicillium expansum	35.57
Fusarium moniliforme	35.27
Aspergillus fumigatus	33.56
Trichophyton mentagrophytes	28.86
Aspergillus flavus	27.21
Penicillium roqueforti	26.67
Saccharomyces cerevisae	25.44
Ustilago tritici	25.42
Curvularia fallax	24.39
Aspergillus terreus	23.99
Curvularia spicifera	21.21
Curvularia brachyspora	20.80
Malazessia furfur	20.38
Cladosporium herbarum	19.48
Penicillium brevicompactum	18.83
Trichophyton rubrum	18.64
Corenyspora cassiicola	18.63
Stemphylium botryosum	16.22
Trichoderma viride	13.79
Penicillium chrysogenum	11.76
Curvularia pallescences	10.32
Aspergillus niger	10.00

Table 5.3 (continued): Percentages of concordance between positive results and bromelain in descending order.

Allergens	Concordance of positive reactions to bromelain (%)
Plant-based food	
Hazelnut	50.46
Wheat flour	50.00
Gliadin	48.84
Orange	46.03
Peanut	37.50
Apple	36.61
Corn flour	36.44
Rice flour	34.15
White potato	31.37
Cabbage	31.28
Soya bean	31.20
Tofu	30.39
Spinach	29.87
Sunflower seed	29.38
Garlic	26.67
Chard	24.70
Banana	21.43
Walnut	20.00
Peach	18.65
Broccoli	18.03
Cocoa	17.09
Potato	16.03
Strawberry	13.73
Carrot	11.90
Kiwi fruit	7.89

5.5 CONCLUSIONS

In this study, specific IgEs to pollen and fungal allergens were detected in the patients' sera samples. Reactions to local and also foreign airspora were observed. However, the frequency of reactions to foreign airspora allergens, e.g., *Arecastrum romanzzoffianum, Baccharis halimifolia, Philadelphus coronarius* and *Urtica dioica*, were higher than those observed for allergen from the local airspora. These patterns could be due to cross- reactivities suggesting the presence of local sensitizers that were not captured in the airspora sampling traps. Variability in the dotting process due to the inherent properties of the allergen extracts and the limitation of different assays format maybe other contributing factors to the pattern of reactions that were obtained. Under-recognition of allergic disease to pollen or fungal allergens may be possible because of this oversight. Thus, the spectrum of allergenic airspora will need to be re-considered seriously. More local plants, especially weeds, grasses and palms should be tested. The simultaneous mass screening method employed in this study also provided useful insights to possible cross-reactivity patterns that may exist. However this work will need to be further elucidated with cross inhibitions tests and *in vivo* assays. The results obtained from the immunoarray screening have provided us with a multitude of ideas for future work that can be done to better understand patterns of allergy in Singapore.

CHAPTER 6: SIGNIFICANCE, SUMMARY AND FUTURE RESEARCH WORK

These specific aims were achieved in this dissertation: 1) to evaluate the optimal method used for airspora quantification and to obtain accurate data for diurnal patterns and seasonality of airspora; 2) to evaluate image analysis as a tool for differentiating airborne spores and pollen towards the development of an automated airspora identification and quantification system; 3) to develop an array system that can simultaneously detect allergen-specific IgEs and which also only requires a small amount of sera and, 4) to study the frequency of reactions of airborne allergens, namely those of pollen, fungal and fern spores by the detection of specific IgEs and possible cross-reactivity patterns between different fungal types, between pollen types and also between pollen with plant-based foods.

The airspora study provided us with the optimal method for screening the outdoor pollen and spore samples that are collected from the Burkard seven-day volumetric spore trap. The method of screening consisting of three longitudinal traverses, 3 mm apart starting from the middle of the slide under 400× magnification, is the optimal method for airspora counts. The method consisting of 12 equally spaced vertical traverses can be employed only when diurnal patterns are of interest.

Fungal spore counts had two peaks per year with one starting in February to March and a second peak in October to November. These high peaks were also found in the seasonal patterns of two major fungal spore types: *Cladosporium* and *Didymosphaeria*. In general, two seasonal patterns were observed for pollen. The more common pattern has a peak in the later months of the year from November to March in the following

year. The second seasonal pattern for pollen has a peak in the middle of the year as observed for species of *Acacia* and *Casuarina equisetifolia*. The peak in counts for fern spores was found to be during the hottest months of the year in Singapore. High counts were obtained from May to August.

Diurnal patterns were observed in all the airspora types. High levels of ascospores were observed during the night while Deuteromycete spore counts were high during the late morning to early evening. Pollen and fern spores were high in number during the middle of the day. Correlations with meteorological parameters were also observed for daily and diurnal patterns. These correlation patterns will be used for subsequent modelling and forecasting when more daily counts are available.

During the screening process, a previously unidentified fungal spore that makes up a large proportion of the outdoor and even indoor airspora, was discovered. Conventional single spore culture methods failed to yield any results because we were unable to induce sporulation which is crucial for identification of fungi. Subsequent work by a colleague using DNA technology and available sequences in public domain databases has identified the unknown fungus as an Ascomycetes related to the Dothideomycetes and the Chaetothyriomycetes. This discovery emphasizes the need for continuous monitoring of airspora even when accurate models for forecasting are available to ensure new changes or previously missed airspora can be detected up.

Future work involving correlation with airspora load and actual allergen levels can be carried on. Contrasting results have been obtained whereby some studies showed correlation between airspora load and allergic symptoms while no correlation was observed in other studies. Correlation between actual allergen load will need to be carried out since unique patterns may be observed owing to the influence of the hot and

humid tropical climate. Collection of outdoor samples into an Eppendorf tube using a cyclone sampler from Burkard Manufacturing Limited, United Kingdom is currently carried out. Polyclonal antibodies to the major airspora allergens have been raised in rabbits for this purpose.

It has been demonstrated that image analysis, coupled with light microscopy, is a feasible and useful basis for developing an automated airspora quantification system. Local airspora and closely related pollen types, which are quite similar morphologically, can be satisfactorily differentiated. Currently, collaboration work with a graduate student in the Department of Electrical and Computer Engineering, National University of Singapore using information gathered from this work, is showing promising results. Identification accuracies of more than 99% can be achieved for the local airspora types by the extraction of more textural features and the use of intensive classifiers like support vector machines. We have since then moved on to test the system using the actual tapes from the spore traps. Improvements are actively being made on the segmentation and feature extraction modules because of the large amount of debris which are present together with the spores and pollen grains of interest. The progress made in this work has spurred us on and convinced us that a fully automated airspora identification system is possible in the future.

An immunoarray system has been developed for simultaneously screening of specific IgEs to a large panel of allergen sources ranging from those of mites, pollen, fungi, epithelia, venoms and even food types. It has been shown that the immunoarray system is a useful semiquantative tool to be used for mass screening purposes. The immunoarray system is currently being used to study the reactions to a vast number of mite recombinant proteins in the laboratory. However, variability is still involved when allergens are loaded onto the membrane. This can be greatly reduced by automa-

tion where the speed, timing and pressure applied will be more consistent compared to doing the same by hand.

The development and subsequent use of the immunoarray system to screen for the frequency of reactions to airspora allergens by detecting the presence of specific IgEs has provided us with important information. Results obtained from the screens seem to suggest an under-recognition of pollen and spore allergens locally. A large panel of foreign fungal and pollen allergen sources were included in the screens. However, some reactions to the foreign airspora allergen sources were higher than from those found locally. This has demonstrated the probable existence of other local allergenic airspora that was not captured in the sampling trap and will need to be studied. Possible cross-reactivity patterns were observed in a large number of pollen types compared to fungal spore types. However, for the pollen types the patterns were partly confounded by the absence of possible primary local sensitiser(s). Collections of possible local counterparts for further allergenicity studies are important and will shed more light on the actual co-sensitization or cross-reactivity patterns that may be observed. This will involve multiple cross inhibition studies as well as *in vivo* testing with the SPT or other *in vitro* assays such as histamine release assays, T cell proliferation assays and cytokines assays before the allergenicity of these previously unstudied allergens can be confirmed.

Finally, new and useful information has been obtained from this work. It has provided some useful solutions and answers and a multitude of future possibilities in research to better understand allergy in Singapore and the rest of the world.

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Appendix 1: List of recommended changes made to the classification of the pollen types studied by The Angiosperm Phylogeny Group (2003).

Genus	Old family names	New family names
Carya	Juglandales	Fagales
Casuarina	Casuarinales	Fagales
Citrus	Rutales	Sapindales
Fraxinus	Oleales	Lamiales
Juglans	Juglandales	Fagales
Ligustrum	Scrophuliariales	Lamiales
Myrica	Myricales	Fagales
Plantago	Plantaginales	Lamiales
Platanus	Rosales	Proteales
Populus	Salicales	Malpighiales
Ricinus	Malpighiales	Euphorbiales
Rumex	Polygonales	Caryophyllales
Salix	Salicales	Malpighiales
Tamarix	Tamaricales	Caryophyllales
Urtica	Urticales	Rosales

Appendix 2: Prevalence of other allergens screened in descending order per main grouping.

Allergens	Prevalence (%) High	Medium	Low	Total
Mites				
Blomia tropicalis	8.70	6.64	29.65	45.00
Dermatophagoides pteronyssinus	7.30	7.95	27.69	42.94
Dermatophagoides farinae	5.71	6.17	23.39	35.27
Acarus siro	2.81	4.21	19.08	26.10
Tyrophagus putrescentiae	2.71	4.86	18.24	25.82
Lepidoglyphus destructor	1.03	4.96	18.52	24.51
Austroglycyphagus geniculatus	0.47	3.74	14.97	19.18
Suidasia medanensis	1.03	2.90	12.35	16.28
Aleuroglyphus ovatus	1.35	0.85	9.98	12.18
Glycophagus domesticus	1.50	2.25	6.27	10.01
Animal-based foods				
Pig	14.41	11.23	29.00	54.63
Rabbit	11.23	11.41	26.94	49.58
Beef	9.07	10.66	26.47	46.21
Chicken	6.64	7.58	26.29	40.51
Lamb	4.99	7.96	26.85	39.79
Milk, Cow	2.62	6.74	22.92	32.27
Mussel	1.87	4.02	22.17	28.06
Tuna Fish	1.31	3.27	23.48	28.06
Cockle	3.27	4.12	19.08	26.47
Banana prawn	1.59	3.55	21.23	26.38
Tiger prawn	1.68	4.86	19.55	26.10
Milk, Goat	2.53	5.99	16.56	25.07
Mud crab	1.03	9.07	14.78	24.88
Salmon Fish	0.84	4.30	19.08	24.23
Selar fish	0.94	2.53	19.55	23.01
Ovomucoid	0.00	1.31	14.97	16.28
Ovalbumin	0.19	0.94	14.87	16.00
Squid	1.03	2.34	11.51	14.87
Sea bream fish	0.09	0.84	10.85	11.79
Milk, sheep	0.21	2.15	7.73	10.09
Swimming crab	0.84	1.50	6.83	9.17
Casein	0.28	1.31	7.02	8.61
Egg yolk	0.00	0.19	4.86	5.05
Egg white	0.09	0.37	3.37	3.84
Mackerel Fish	0.00	0.00	3.09	3.09

Two standard deviations from negative reactions were used as the cut off points. Levels of reactions: high = 2SD + 100 OD; medium = 2SD + 50OD; and low = 2SD

Appendix 2 (continued): Prevalence of other allergens screened in descending order per main grouping.

	Prevalence (%)			
Allergens	**High**	**Medium**	**Low**	**Total**
Epithelia				
Cow	13.28	13.19	26.85	53.32
Dog	5.14	8.51	23.67	37.32
Cat	1.87	4.21	19.93	26.01
Goat	1.22	3.18	17.77	22.17
Guinea pig	0.56	5.05	11.23	16.84
Rabbit	0.09	1.12	7.58	8.79
Feathers mix (duck and chicken)	0.00	0.28	8.14	8.42
Budgerigar	0.28	0.37	2.99	3.65
Horse	0.00	0.19	1.96	2.15
Hamster	0.28	0.37	1.40	2.06
Goose	0.09	0.00	0.47	0.56
Venom				
Apis mellifera	1.59	4.21	24.70	30.50
Vespula spp.	0.23	0.69	5.55	6.47
Polistes spp.	0.10	0.29	5.11	5.49
Dolichovespula spp.	0.89	1.79	2.68	5.36
Insect				
Mosquito	0.75	2.90	19.93	23.57
Fire ant	0.00	1.59	15.62	17.21
German cockroach	0.56	2.53	14.13	17.21
Oriental cockroach	0.47	1.68	14.13	16.28
American cockroach	0.19	0.75	6.74	7.67

Two standard deviations from negative reactions were used as the cut off points. Levels of reactions: high = 2SD + 100 OD; medium = 2SD + 50OD; and low = 2SD

Appendix 3: Correlations between the different pollen types and plant-based foods studied.

Allergens	Apple	Cabbage	Cacao	Carrot	Chard	Corn flour	Garlic	Gliadin	Hazelnut	Peach	Peanut	Potato	Rice flour
Apple	1												
Cabbage		1				0.56156***	0.59857***			0.52662***			0.59799***
Cacao			1										
Carrot				1						0.54181***			
Chard					1								
Corn flour		0.56156***				1			0.5337***		0.52879***		0.54115***
Garlic		0.59857***					1			0.52832***			0.55432***
Gliadin								1	0.54974***			0.5838*	
Hazelnut						0.5337***		0.54974***	1		0.53984***		
Peach		0.52662***		0.54181***			0.52832***			1			0.51698***
Peanut						0.52879***			0.53984***		1		
Potato								0.5838*				1	
Rice flour		0.59799***				0.54115***	0.55432***			0.51698***			1
Soya bean		0.56546***				0.53022***	0.55207***						0.53708***
Spinach							0.57143***						
Strawberry			0.66208***						0.8777***		0.7478**		
Sunflower seed		0.52805***					0.6258***			0.56541***			0.50981***
Tofu		0.6321***				0.51831***	0.61566***			0.52692***			0.63958***
Walnut				0.56247***			0.51656***			0.71179***			
Wheat flour		0.54581***				0.56038***	0.50529***		0.53182***				0.66162***
Banana				0.9709***									
Orange													
Brocolli						0.62564***		0.50117***	0.57601***				
Kiwi			0.62245***						0.8008*				
Acacia spp.						0.53953***			0.56352***				
Acer negundo			0.6138*			0.51078***		0.50776***	0.50853***			0.6995**	
Alnus glutinosa													0.8936***
Arecastrum romanzo ffianum		0.5154***					0.60844***						0.51146***
Betula verrucosa				0.56995***						0.65265***			
Carpinus betulus					0.9007***								
Casuarina equisetifolia		0.50401***				0.65619***					0.50365***		
Cryptomeria japonica							0.52212***			0.55994***			
Cupressus arizonica													
Cupressus sempervirens										0.53041***			
Elaeis guineeis													
Eucalyptus globulus										0.50227***			
Fagus sylvatica													
Fraxinus excelsior													
Juniperus asheisabinoides							0.52471***			0.54665***			
Olea europaea													

Appendix 3 (continued): Correlations between the different pollen types and plant-based foods studied.

Allergens	Apple	Cabbage	Cacao	Carrot	Chard	Corn flour	Garlic	Gliadin	Hazelnut	Peach	Peanut	Potato	Rice flour
Pinus radiata										0.52682***			
Pinus strobus				0.52832***						0.61879***			
Platanus acerfolia		0.51716***		0.53684***			0.54902***			0.68875***			0.55119***
Podocarpus polystachyus					0.6578*	0.8047***			0.6497*			0.8336***	
Populus deltoides	0.8725***			0.51913***						0.51108***			
Populus nigra		0.61538***				0.51583***	0.67688***			0.53222***			0.66832***
Populus trichocarpa										0.5022			
Quercus alba		0.59948***				0.51891***	0.54071***		0.50632***				0.57174***
Quercus ilex		0.53152***					0.53189***						
Quercus robur		0.57329***					0.62309***			0.6379***			0.62143***
Robinia pseudoacacia							0.50739***			0.60894***			0.53436***
Schnius molle													
Tamarix gallica				0.51573***			0.5256***			0.54155***			
Tilia cordata		0.51688***		0.5476***			0.53334***			0.67481***			
Ulmus americana													
Ulmus minor		0.53374***											0.55054***
Syringa vulgaris	0.7552**												
Agropyron repe				0.51652***						0.58999***			
Agrostis alba				0.50187***						0.59247***			
Alopecurus prantesis		0.50708***								0.51513***			0.58119***
Amaranthus hybridus									0.54271***			0.6934**	
Ambrosia artemisiifolia		0.54256***					0.59848***			0.6725***			0.53008***
Ambrosia trifida							0.52164***			0.63097***			
Anthxanthum odoratum					0.7423**		0.626*						
Artemisia vulgaris										0.66868***			
Atriplex polycarpa		0.54824***				0.64147***			0.51803***		0.52818***		
Avena sativa													
Baccharis halimifolia		0.54349***				0.51747***	0.54596***						0.52788***
Brassica Spp		0.53913***				0.50067***							0.56622***
Bromus mollis													
Calluna vulgaris			0.887***							0.5538			
Chenopodium album		0.54923***					0.60435***			0.57325***			0.59984***
Chrysanthemum leucanthemum		0.51319***		0.5207***			0.57435***			0.62472***			0.5166***
Corylus avellana					0.7392**		0.984***			0.5667		0.5263	
Cynodon dactylon		0.62548***					0.69416***			0.58221***			0.62469***
Dactylis glomerata							0.55097***			0.51877***			
Dahlia cultorum				0.52171***			0.56142***			0.5976***			
Festuca prantesis							0.5158***			0.50312***			

Appendix 3 (continued): Correlations between the different pollen types and plant-based foods studied.

Allergens	Apple	Cabbage	Cacao	Carrot	Chard	Corn flour	Garlic	Gliadin	Hazelnut	Peach	Peanut	Potato	Rice flour
Holcus lanatus		0.5331***					0.52555***			0.63328***			0.5275***
Hordeum vulgare			0.8794***			0.60065***			0.57115***				
Humulus lupulus				0.56958***						0.61966***			
Pinus radiata										0.52682***			
Pinus radiata				0.52832***						0.61879***			
Pinus strobus		0.51716***		0.53684***			0.54902***			0.68875***			0.55119***
Platanus acerfolia					0.6578*	0.8047***			0.6497*			0.8336***	
Podocarpus polystachyus	0.8725***			0.51913***						0.51108***			
Populus deltoides		0.61538***				0.51583***	0.67688***			0.53222***			0.66832***
Populus nigra										0.5022			
Populus trichocarpa		0.59948***				0.51891***	0.54071***		0.50632***				0.57174***
Quercus alba		0.53152***					0.53189***						
Quercus ilex		0.57329***					0.62309***			0.6379***			0.62143***
Quercus robur							0.50739***			0.60894***			0.53436***
Robinia pseudoacacia													
Schnius molle				0.51573***			0.5256***			0.54155***			
Tamarix gallica		0.51688***		0.5476***			0.53334***			0.67481***			
Tilia cordata													
Ulmus americana		0.53374***											0.55054***
Ulmus minor	0.7552**												
Syringa vulgaris				0.51652***						0.58999***			
Agropyron repe				0.50187***						0.59247***			
Agrostis alba		0.50708***								0.51513***			0.58119***
Alopecurus prantesis									0.54271***			0.6934**	
Amaranthus hybridus		0.54256***					0.59848***			0.6725***			0.53008***
Ambrosia artemisiifolia							0.52164***			0.63097***			
Ambrosia trifida					0.7423**		0.626*						
Anthxanthum odoratum										0.66868***			
Artemisia vulgaris		0.54824***				0.64147***			0.51803***		0.52818***		
Atriplex polycarpa													
Avena sativa		0.54349***				0.51747***	0.54596***						0.52788***
Baccharis halimifolia		0.53913***				0.50067***							0.56622***
Brassica Spp													
Bromus mollis			0.887***							0.5538			
Calluna vulgaris		0.54923***					0.60435***			0.57325***			0.59984***
Chenopodium album		0.51319***		0.5207***			0.57435***			0.62472***			0.5166***
Chrysanthemum leucanthemum					0.7392**		0.984***			0.5667		0.5263	
Corylus avellana		0.62548***					0.69416***			0.58221***			0.62469***
Cynodon dactylon							0.55097***			0.51877***			
Dactylis glomerata				0.52171***			0.56142***			0.5976***			
Dahlia cultorum							0.5158***			0.50312***			

Appendix 3 (continued): Correlations between the different pollen types and plant-based foods studied.

Allergens	Apple	Cabbage	Cacao	Carrot	Chard	Corn flour	Garlic	Gliadin	Hazelnut	Peach	Peanut	Potato	Rice flour
Holcus lanatus		0.5331***					0.52555***			0.63328***			0.5275***
Hordeum vulgare			0.8794***			0.60065***			0.57115***				
Humulus lupulus				0.56958***						0.61966***			
Ligustrum vulgare													
Lolium perenne							0.52198***			0.52294***			
Medicago sativa				0.54883***			0.54262***			0.60524***			
Parietaria judaica								0.6029*					
Philadelphus coronarius	0.9699***							0.9794***					
Phlenum pratesee			0.7321**					0.50313***	0.50895***				
Phragmites communis													
Plantago lanceolata										0.57583***			
Poa prateis													
Rumex acetosella				0.54315***						0.62907***			
Salsola kali					0.6301*								
Sambucus nigra		0.5514***				0.5485***							
Secale cereale									0.9248***				
Solidago virga aurea	0.7004**			0.53182***						0.55257***			
Sorghum halepense		0.59214***					0.6599***			0.63265***			0.55802***
Taraxacum officinale			0.8992***							0.9171***		0.5933*	
Triticum aestivum/ sativum													0.51953***
Zea mays		0.64708***					0.62988***						0.57886***
Urtica dioica		0.53079***					0.55833***						
Salix viminalis			0.53169***										
Horseradish peroxidase							0.51106***			0.61026***			
Latex													
Bromelain													

Appendix 3 (continued): Correlations between the different pollen types and plant-based foods studied.

Allergens	Soya bean	Spinach	Strawberry	Sunflower seed	Tofu	Walnut	Wheat flour	Banana	Orange	Brocolli	Kiwi	*Acacia spp.*	*Acer negundo*
Apple													
Cabbage	0.56546***			0.52805***	0.6321***		0.54581***						
Cacao			0.66208***								0.62245***		0.6138*
Carrot						0.56247***		0.9709***					0.15180***
Chard													0.27465***
Corn flour	0.53022***				0.51831***		0.56038***			0.62564***		0.53953***	0.51078***
Garlic	0.55207***	0.57143***		0.6258***	0.61566***	0.51656***	0.50529***						0.33713***
Gliadin										0.50117***			0.50776***
Hazelnut			0.8777***				0.53182***			0.57601***	0.8008*	0.56352***	0.50853***
Peach				0.56541***	0.52692***	0.71179***							
Peanut			0.7478**										
Potato													0.6995**
Rice flour	0.53708***			0.50981***	0.63958***		0.66162***						
Soya bean	1	0.51792***		0.53405***	0.62719***		0.509***			0.51757***			0.41451***
Spinach	0.51792***	1.0000			0.50934***								0.29728***
Strawberry			1							0.6178	0.59907***	0.674*	
Sunflower seed	0.53405***			1	0.59614***								0.27756***
Tofu	0.62719***	0.50934***		0.59614***	1		0.54296***						0.34595***
Walnut						1							
Wheat flour	0.509***				0.54296***		1			0.58133***		0.50459***	
Banana								1					
Orange									1	0.581***			
Brocolli	0.51757***						0.58133***		0.581***	1		0.6033***	0.56227***
Kiwi			0.59907***								1		
Acacia spp.			0.674*				0.50459***			0.6033***		1	0.54708***
Acer negundo										0.56227***		0.54708***	1
Alnus glutinosa													
Arecastrum romanzo ffianum	0.59083***	0.56192***			0.55546***		0.50283***						
Betula verrucosa						0.6467***							
Carpinus betulus		0.8487***											
Casuarina equisetifolia	0.5075***									0.60718***		0.57355***	0.50903***
Cryptomeria japonica				0.59048***	0.53529***	0.51242***							
Cupressus arizonica													
Cupressus sempervirens						0.50767***			0.5162***		0.52941***		
Elaeis guineeis												0.51648***	
Eucalyptus globulus						0.5138***							
Fagus sylvatica										0.52066***			0.5098***
Fraxinus excelsior										0.50355***			
Juniperus asheisabinoides				0.53593***		0.54383***							

Appendix 3 (continued): Correlations between the different pollen types and plant-based foods studied.

Allergens	Soya bean	Spinach	Strawberry	Sunflower seed	Tofu	Walnut	Wheat flour	Banana	Orange	Brocolli	Kiwi	*Acacia spp.*	*Acer negundo*
Olea europaea									0.53356***	0.53754***			
Pinus radiata											0.54429***		
Pinus strobus						0.57604***							
Platanus acerfolia				0.55844***	0.55307***	0.66364***							
Podocarpus polystachyus		0.7161**								0.6019			
Populus deltoides						0.54562***		0.9839***					
Populus nigra	0.61485***	0.50936***		0.61403***	0.6894***		0.57204***						
Populus trichocarpa				0.8256***									0.51363***
Quercus alba	0.51975***				0.54315***		0.5587***			0.58973***			
Quercus ilex					0.50395***				0.5091***				
Quercus robur				0.63705***	0.6314***	0.61383***	0.53271***						
Robinia pseudoacacia				0.54564***	0.53077***	0.57312***							
Schnius molle													
Tamarix gallica						0.52515***							
Tilia cordata				0.52637***	0.50424***	0.69466***							
Ulmus americana													0.61259***
Ulmus minor							0.5678***			0.59648***			
Syringa vulgaris										0.8291*			
Agropyron repe						0.60955***							
Agrostis alba						0.59841***							
Alopecurus prantesis						0.50001***	0.53556***			0.5547***			
Amaranthus hybridus										0.55941***	0.6648	0.57007***	0.57854***
Ambrosia artemisiifolia				0.5901***	0.5622***	0.59281***							
Ambrosia trifida						0.62124***							
Anthxanthum odoratum		0.6157*				0.5599			0.6271				
Artemisia vulgaris						0.67659***							
Atriplex polycarpa	0.5125***						0.53722***			0.59157***		0.53319***	0.57888***
Avena sativa						0.862***							0.51195***
Baccharis halimifolia	0.55654***				0.52982***		0.55344***			0.51732***			
Brassica Spp	0.54055***				0.54135***		0.57437***						
Bromus mollis								0.9307***					
Calluna vulgaris			0.5709										
Chenopodium album				0.59467***	0.63547***	0.53635***	0.52987***						
Chrysanthemum leucanthemum				0.56722***	0.54886***	0.59078***							
Corylus avellana		0.5478				0.9391***							

Appendix 3 (continued): Correlations between the different pollen types and plant-based foods studied.

Allergens	Soya bean	Spinach	Strawberry	Sunflower seed	Tofu	Walnut	Wheat flour	Banana	Orange	Brocolli	Kiwi	*Acacia spp.*	*Acer negundo*
Cynodon dactylon	0.53395***			0.57999***	0.61588***	0.55796***	0.56859***						
Dactylis glomerata					0.5047***	0.50905***							
Dahlia cultorum				0.51592***	0.51765***	0.60091***							
Festuca prantesis													
Holcus lanatus					0.50683***	0.64529***							
Hordeum vulgare							0.50358***			0.58589***		0.57593***	0.54404***
Humulus lupulus						0.62631***							
Ligustrum vulgare													
Lolium perenne						0.50836***							
Medicago sativa						0.6039***							
Parietaria judaica											0.54697***	0.7151**	
Philadelphus coronarius												0.7575**	0.9607***
Phlenum pratesee										0.54503***	0.6062		
Phragmites communis													
Plantago lanceolata						0.55004***							
Poa prateis													
Rumex acetosella						0.66989***							
Salsola kali					0.9893***				0.9464**				
Sambucus nigra										0.58083***			
Secale cereale													
Solidago virga aurea						0.5714***							
Sorghum halepense	0.54945***	0.51281***		0.64444***	0.60504***	0.5556***	0.50664***						
Taraxacum officinale						0.7513**							
Triticum aestivum/ sativum													
Zea mays	0.56377***	0.50024***		0.50724***	0.57884***		0.53533***			0.55465***			
Urtica dioica	0.60348***	0.51223***			0.53838***								
Salix viminalis			0.5389***					0.6446		0.9306**	0.60232***	0.553	
Horseradish peroxidase						0.6187***							
Latex									0.61182***				
Bromelain													

Appendix 3 (continued): Correlations between the different pollen types and plant-based foods studied.

Allergens	*Alnus glutinosa*	*Arecastrum romanzo ffianum*	*Betula verrucosa*	*Carpinus betulus*	*Casuarina equisetifolia*	*Cryptomeria japonica*	*Cupressus arizonica*	*Cupressus sempervirensn*	*Elaeis guineeis*	*Eucalyptus globulus*	*Fagus sylvatica*	*Fraxinus excelsior*	*Juniperus asheisabinoides*
Apple													
Cabbage		0.5154***			0.50401***								
Cacao			0.31648***				0.7424**				0.8984***		
Carrot	0.5927*		0.56995***						0.7913***				
Chard				0.9007***									
Corn flour					0.65619***								
Garlic		0.60844***				0.52212***							0.52471***
Gliadin													
Hazelnut													
Peach			0.65265***			0.55994***		0.53041***		0.50227***			0.54665***
Peanut					0.50365***								
Potato	0.8457***		0.37567***				0.5924*					0.9435***	
Rice flour	0.8936***	0.51146***											
Soya bean	0.5652	0.59083***			0.5075***								
Spinach	0.8064***	0.56192***		0.8487***									
Strawberry			0.28161***	0.7553**									
Sunflower seed	0.605*					0.59048***							0.53593***
Tofu		0.55546***				0.53529***							
Walnut			0.6467***			0.51242***		0.50767***		0.5138***			0.54383***
Wheat flour		0.50283***											
Banana													
Orange								0.5162***					
Brocolli					0.60718***						0.52066***	0.50355***	
Kiwi								0.52941***					
Acacia spp.					0.57355***				0.51648***				
Acer negundo					0.50903***						0.5098***		
Alnus glutinosa	1												
Arecastrum romanzo ffianum		1											
Betula verrucosa			1			0.50318***				0.52254***			0.54604***
Carpinus betulus				1						0.8012***			
Casuarina equisetifolia					1				0.50109***				
Cryptomeria japonica			0.50318***			1							0.54063***
Cupressus arizonica							1				0.52019***		
Cupressus sempervirens								1					
Elaeis guineeis					0.50109***				1				
Eucalyptus globulus			0.52254***							1			0.55593***
Fagus sylvatica							0.52019***				1		

Appendix 3 (continued): Correlations between the different pollen types and plant-based foods studied.

Allergens	*Alnus glutinosa*	*Arecastrum romanzo ffianum*	*Betula verrucosa*	*Carpinus betulus*	*Casuarina equisetifolia*	*Cryptomeria japonica*	*Cupressus arizonica*	*Cupressus sempervirensn*	*Elaeis guineeis*	*Eucalyptus globulus*	*Fagus sylvatica*	*Fraxinus excelsior*	*Juniperus asheisabinoides*
Fagus sylvatica							0.52019***				1		
Fraxinus excelsior												1	
Juniperus asheisabinoides			0.54604***			0.54063***				0.55593***			1
Olea europaea													
Pinus radiata								0.71789***	0.5121				
Pinus strobus			0.64731***			0.50332***				0.52942***			0.52714***
Platanus acerfolia			0.64448***			0.56897***				0.56644***			0.57755***
Podocarpus polystachyus	0.6193*				0.6082*				0.5214			0.9958***	
Populus deltoides			0.56181***				0.8472***		0.5915*	0.5036***			
Populus nigra		0.5772***				0.50594***							
Populus trichocarpa													0.9237***
Quercus alba					0.54853***								
Quercus ilex	0.5934*							0.51643***					
Quercus robur			0.57874***			0.57985***							0.51679***
Robinia pseudoacacia			0.53016***			0.53109***							0.5026***
Schnius molle			0.50842***							0.53824***			0.57125***
Tamarix gallica			0.59219***						0.6424*	0.57337***			0.52949***
Tilia cordata			0.68787***			0.55862***		0.52091***		0.57911***			0.61202***
Ulmus americana									0.52419***	0.7791***			
Ulmus minor													
Syringa vulgaris					0.8189**								
Agropyron repe			0.71607***						0.6395*	0.57834***			0.54607***
Agrostis alba	0.5831*		0.71119***				0.8872***		0.5701	0.5313***			0.50897***
Alopecurus prantesis													
Amaranthus hybridus													
Ambrosia artemisiifolia		0.50355***	0.62862***			0.58814***				0.5378***			0.54417***
Ambrosia trifida	0.944***		0.57212***										
Anthxanthum odoratum													
Artemisia vulgaris	0.6709*		0.62321***						0.9592***				
Atriplex polycarpa		0.51286***			0.5931***								
Avena sativa			0.9135***			0.9253***							0.9508***
Baccharis halimifolia	0.5257	0.63344***											
Brassica Spp	0.6024*	0.51673***											
Bromus mollis				0.9858***									
Calluna vulgaris										0.9079***			0.5037
Chenopodium album		0.50051***	0.53044***			0.56133***				0.50009***			
Chrysanthemum leucanthemum			0.58767***			0.55969***		0.53191***		0.51459***			0.54654***
Corylus avellana			0.5253							0.5027			

Appendix 3 (continued): Correlations between the different pollen types and plant-based foods studied.

Allergens	*Alnus glutinosa*	*Arecastrum romanzo ffianum*	*Betula verrucosa*	*Carpinus betulus*	*Casuarina equisetifolia*	*Cryptomeria japonica*	*Cupressus arizonica*	*Cupressus sempervirensn*	*Elaeis guineeis*	*Eucalyptus globulus*	*Fagus sylvatica*	*Fraxinus excelsior*	*Juniperus asheisabinoides*
Cynodon dactylon	0.6073*	0.53287***	0.5134***			0.50249***							
Dactylis glomerata								0.55913***					
Dahlia cultorum			0.59489***			0.52491***		0.51467***		0.53891***			0.53934***
Festuca prantesis										0.51767***			
Holcus lanatus			0.55607***					0.51341***					
Hordeum vulgare					0.52586***						0.50729***		
Humulus lupulus			0.55296***							0.51156***			0.50806***
Ligustrum vulgare	0.52				0.50319***								
Lolium perenne			0.50684***							0.54842***			0.51081***
Medicago sativa			0.64779***			0.53268***		0.50108***		0.65301***			0.56404***
Parietaria judaica													
Philadelphus coronarius				0.584*							0.9034***	0.9773***	
Phlenum pratesee													
Phragmites communis				0.7463**					0.6764**	0.56797***			
Plantago lanceolata			0.59008***			0.50322***	0.8354***		0.6955**	0.57301***			0.50159***
Poa prateis				0.6991**					0.5862*	0.56121***			
Rumex acetosella			0.62406***						0.5892*				
Salsola kali		0.7949***											
Sambucus nigra					0.54048***							0.50021***	
Secale cereale													
Solidago virga aurea			0.57992***				0.9582***		0.5201	0.50143***			
Sorghum halepense		0.51215***	0.53393***			0.58056***							0.53756***
Taraxacum officinale										0.6373*			
Triticum aestivum/ sativum													
Zea mays		0.55941***											
Urtica dioica		0.57663***											
Salix viminalis					0.8926***								
Horseradish peroxidase			0.60343***			0.50056***		0.53673***		0.56271***			0.55414***
Latex								0.61216***					
Bromelain													

Appendix 3 (continued): Correlations between the different pollen types and plant-based foods studied.

Allergens	*Olea europaea*	*Pinus radiata*	*Pinus strobus*	*Platanus acerfolia*	*Podocarpus polystachyus*	*Populus deltoides*	*Populus nigra*	*Populus trichocarpa*	*Quercus alba*	*Quercus ilex*	*Quercus robur*	*Robinia pseudoacacia*	*Schnius molle*
Apple						0.8725***							
Cabbage				0.51716***			0.61538***		0.59948***	0.53152***	0.57329***		
Cacao													
Carrot			0.52832***	0.53684***	0.7206**	0.51913***							
Chard					0.6578*								
Corn flour					0.8047***		0.51583***		0.51891***				
Garlic				0.54902***			0.67688***		0.54071***	0.53189***	0.62309***	0.50739***	
Gliadin													
Hazelnut					0.6497*				0.50632***				
Peach		0.52682***	0.61879***	0.68875***		0.51108***	0.53222***	0.5022			0.6379***	0.60894***	
Peanut													
Potato					0.8336***								
Rice flour				0.55119***			0.66832***		0.57174***		0.62143***	0.53436***	
Soya bean							0.61485***		0.51975***				
Spinach					0.7161**		0.50936***						
Strawberry													
Sunflower seed				0.55844***			0.61403***	0.8256***			0.63705***	0.54564***	
Tofu				0.55307***			0.6894***		0.54315***	0.50395***	0.6314***	0.53077***	
Walnut			0.57604***	0.66364***		0.54562***					0.61383***	0.57312***	
Wheat flour							0.57204***		0.5587***		0.53271***		
Banana						0.9839***							
Orange	0.53356***									0.5091***			
Brocolli	0.53754***				0.6019				0.58973***				
Kiwi		0.54429***											
Acacia spp.													
Acer negundo								0.51363***					
Alnus glutinosa	0.7353**				0.6193*					0.5934*			
Arecastrum romanzo ffianum							0.5772***						
Betula verrucosa			0.64731***	0.64448***		0.56181***					0.57874***	0.53016***	0.50842***
Carpinus betulus													
Casuarina equisetifolia									0.54853***				
Cryptomeria japonica			0.50332***	0.56897***			0.50594***				0.57985***	0.53109***	
Cupressus arizonica													
Cupressus sempervirens		0.71789***								0.51643***			
Elaeis guineeis													
Eucalyptus globulus			0.52942***	0.56644***		0.5036***							0.53824***

Appendix 3 (continued): Correlations between the different pollen types and plant-based foods studied.

Allergens	*Olea europaea*	*Pinus radiata*	*Pinus strobus*	*Platanus acerfolia*	*Podocarpus polystachyus*	*Populus deltoides*	*Populus nigra*	*Populus trichocarpa*	*Quercus alba*	*Quercus ilex*	*Quercus robur*	*Robinia pseudoacacia*	*Schnius molle*
Fagus sylvatica													
Fraxinus excelsior													
Juniperus asheisabinoides			0.52714***	0.57755***							0.51679***	0.5026***	0.57125***
Olea europaea	1									0.66372***			
Pinus radiata		1											
Pinus strobus			1	0.61371***		0.5558***					0.54608***		
Platanus acerfolia			0.61371***	1			0.54692***				0.64867***	0.60597***	
Podocarpus polystachyus	0.6901**				1								
Populus deltoides			0.5558***			1							
Populus nigra				0.54692***			1		0.58026***	0.50686***	0.65445***	0.51294***	
Populus trichocarpa								1			0.998***	0.5655	0.7695**
Quercus alba							0.58026***		1	0.52581***			
Quercus ilex	0.66372***						0.50686***		0.52581***	1	0.52281***		
Quercus robur			0.54608***	0.64867***			0.65445***	0.998***		0.52281***	1	0.66675***	
Robinia pseudoacacia				0.60597***			0.51294***	0.5655			0.66675***	1	
Schnius molle								0.7695**					1
Tamarix gallica			0.61998***	0.5461***		0.55903***							
Tilia cordata		0.50781***	0.62681***	0.69812***		0.55326***	0.51995***	0.6675*			0.63313***	0.62871***	0.51704***
Ulmus americana		0.9998***			0.7127**	0.7927***		0.60365***					0.9317***
Ulmus minor							0.52706***		0.56363***		0.51375***		
Syringa vulgaris													
Agropyron repe			0.60109***	0.63398***		0.52828***					0.5661***	0.56961***	0.50334***
Agrostis alba			0.61664***	0.5985***		0.54162***					0.55521***	0.55648***	
Alopecurus prantesis							0.5088***				0.53259***		
Amaranthus hybridus													
Ambrosia artemisiifolia			0.55924***	0.68615***			0.56875***	0.694**			0.6563***	0.58789***	0.50308***
Ambrosia trifida				0.52967***				0.5897*			0.58855***	0.5332***	
Anthxanthum odoratum								0.52487***					
Artemisia vulgaris			0.527***	0.5815***							0.58951***	0.56305***	
Atriplex polycarpa					0.5499								
Avena sativa		0.6289*	0.6554*	0.9481***				0.53927***					0.8396***
Baccharis halimifolia							0.54879***		0.5226***				
Brassica Spp	0.54969***						0.52277***						
Bromus mollis				0.53496***									
Calluna vulgaris			0.5971*	0.7999***									
Chenopodium album			0.51449***	0.62441***			0.63967***			0.50098***	0.73971***	0.63948***	
Chrysanthemum leucanthemum		0.52739***	0.54374***	0.62243***			0.56234***	0.8129***			0.6387***	0.62046***	

Appendix 3 (continued): Correlations between the different pollen types and plant-based foods studied.

Allergens	*Olea europaea*	*Pinus radiata*	*Pinus strobus*	*Platanus acerfolia*	*Podocarpus polystachyus*	*Populus deltoides*	*Populus nigra*	*Populus trichocarpa*	*Quercus alba*	*Quercus ilex*	*Quercus robur*	*Robinia pseudoacacia*	*Schnius molle*
Corylus avellana		0.6137*				0.5571							
Cynodon dactylon			0.50795***	0.59683***			0.67185***		0.60965***	0.54276***	0.6344***	0.5336***	
Dactylis glomerata	0.55245***			0.50709***			0.50835***	0.9556***		0.66278***	0.56535***	0.51562***	
Dahlia cultorum			0.5388***	0.60384***			0.53986***	0.5531		0.50505***	0.59059***	0.57553***	
Festuca prantesis	0.50017***			0.51738***						0.55131***	0.56475***	0.54411***	
Holcus lanatus				0.52203***			0.52409***	0.8039***		0.53532***	0.6037***	0.55609***	
Hordeum vulgare					0.7355**								
Humulus lupulus				0.56425***		0.51248***					0.52877***		
Ligustrum vulgare	0.50609***				0.9943***								
Lolium perenne		0.50363***		0.54753***						0.51818***	0.56482***	0.55346***	
Medicago sativa		0.51459***	0.59694***	0.6322***		0.54922***		0.6189*			0.55975***	0.53663***	0.53198***
Parietaria judaica					0.5099								
Philadelphus coronarius					0.5056								
Phlenum pratesee					0.7745***								
Phragmites communis				0.5126***	0.5904*								
Plantago lanceolata		0.51398***	0.60374***	0.63494***							0.56685***	0.54543***	
Poa prateis		0.51348***											
Rumex acetosella			0.56139***	0.57917***		0.57037***					0.57111***	0.55378***	
Salsola kali							0.8832***			0.7586**			
Sambucus nigra									0.53435***				
Secale cereale													
Solidago virga aurea			0.54404***	0.50735***		0.6042***							0.51249***
Sorghum halepense			0.51694***	0.64303***			0.63285***		0.52815***	0.51697***	0.69495***	0.56441***	
Taraxacum officinale		0.9538***	0.5051	0.9788***							0.9997***		0.6247*
Triticum aestivum/ sativum													
Zea mays	0.50774***						0.63004***		0.66107***	0.55364***	0.51548***		
Urtica dioica							0.52235***			0.52322***			
Salix viminalis													
Horseradish peroxidase		0.54416***	0.54181***	0.58299***							0.59886***	0.58721***	
Latex		0.56904***						0.7408**		0.52746***			
Bromelain					0.7307**	0.9485***							0.9543***

Appendix 3 (continued): Correlations between the different pollen types and plant-based foods studied.

Allergens	*Tamarix gallica*	*Tilia cordata*	*Ulmus americana*	*Ulmus minor*	*Syringa vulgaris*	*Agropyron repe*	*Agrostis alba*	*Alopecurus prantesis*	*Amaranthus hybridus*	*Ambrosia artemisiifolia*	*Ambrosia trifida*	*Anthxanthum odoratum*	*Artemisia vulgaris*
Apple					0.7552**								
Cabbage		0.51688***		0.53374***				0.50708***		0.54256***			
Cacao			0.5099										
Carrot	0.51573***	0.5476***				0.51652***	0.50187***						
Chard												0.7423**	
Corn flour													
Garlic	0.5256***	0.53334***								0.59848***	0.52164***	0.626*	
Gliadin													
Hazelnut									0.54271***				
Peach	0.54155***	0.67481***				0.58999***	0.59247***	0.51513***		0.6725***	0.63097***		0.66868***
Peanut													
Potato									0.6934**				
Rice flour				0.55054***				0.58119***		0.53008***			
Soya bean													
Spinach												0.6157*	
Strawberry													
Sunflower seed		0.52637***								0.5901***			
Tofu		0.50424***								0.5622***			
Walnut	0.52515***	0.69466***				0.60955***	0.59841***	0.50001***		0.59281***	0.62124***		0.67659***
Wheat flour				0.5678***				0.53556***					
Banana													
Orange													
Brocolli				0.59648***	0.8291*			0.5547***	0.55941***				
Kiwi													
Acacia spp.									0.57007***				
Acer negundo			0.61259***						0.57854***				
Alnus glutinosa							0.5831*				0.944***		0.6709*
Arecastrum romanzo ffianum										0.50355***			
Betula verrucosa	0.59219***	0.68787***				0.71607***	0.71119***			0.62862***	0.57212***		0.62321***
Carpinus betulus													
Casuarina equisetifolia													
Cryptomeria japonica		0.55862***								0.58814***			
Cupressus arizonica													
Cupressus sempervirens		0.52091***											
Elaeis guineeis			0.52419***										
Eucalyptus globulus	0.57337***	0.57911***				0.57834***	0.5313***			0.5378***			

Appendix 3 (continued): Correlations between the different pollen types and plant-based foods studied.

Allergens	*Tamarix gallica*	*Tilia cordata*	*Ulmus americana*	*Ulmus minor*	*Syringa vulgaris*	*Agropyron repe*	*Agrostis alba*	*Alopecurus prantesis*	*Amaranthus hybridus*	*Ambrosia artemisiifolia*	*Ambrosia trifida*	*Anthxanthum odoratum*	*Artemisia vulgaris*
Fagus sylvatica													
Fraxinus excelsior													
Juniperus asheisabinoides	0.52949***	0.61202***				0.54607***	0.50897***			0.54417***			
Olea europaea													
Pinus radiata		0.50781***	0.9998***										
Pinus strobus	0.61998***	0.62681***				0.60109***	0.61664***			0.55924***			0.527***
Platanus acerfolia	0.5461***	0.69812***				0.63398***	0.5985***			0.68615***	0.52967***		0.5815***
Podocarpus polystachyus			0.7127**										
Populus deltoides	0.55903***	0.55326***	0.7927***			0.52828***	0.54162***						
Populus nigra		0.51995***		0.52706***				0.5088***		0.56875***			
Populus trichocarpa		0.6675*	0.60365***							0.694**	0.5897*	0.52487***	
Quercus alba				0.56363***									
Quercus ilex													
Quercus robur		0.63313***		0.51375***		0.5661***	0.55521***	0.53259***		0.6563***	0.58855***		0.58951***
Robinia pseudoacacia		0.62871***				0.56961***	0.55648***			0.58789***	0.5332***		0.56305***
Schnius molle		0.51704***	0.9317***			0.50334***				0.50308***			
Tamarix gallica	1	0.58945***				0.58098***	0.56771***			0.52829***			
Tilia cordata	0.58945***	1				0.69029***	0.65848***			0.61935***	0.58632***		0.62576***
Ulmus americana			1			0.6909**	0.5696		0.50024***				
Ulmus minor				1				0.61801***					
Syringa vulgaris					1				0.7112*				
Agropyron repe	0.58098***	0.69029***	0.6909**			1	0.8065***	0.51537***		0.59255***	0.55812***		0.62295***
Agrostis alba	0.56771***	0.65848***	0.5696			0.8065***	1	0.53517***		0.57336***	0.57909***		0.63658***
Alopecurus prantesis				0.61801***		0.51537***	0.53517***	1		0.50262***	0.57105***		0.55974***
Amaranthus hybridus			0.50024***		0.7112*				1				
Ambrosia artemisiifolia	0.52829***	0.61935***				0.59255***	0.57336***	0.50262***		1	0.59744***		0.62205***
Ambrosia trifida		0.58632***				0.55812***	0.57909***	0.57105***		0.59744***	1		0.70242***
Anthxanthum odoratum		0.5504										1	
Artemisia vulgaris		0.62576***				0.62295***	0.63658***	0.55974***		0.62205***	0.70242***		1
Atriplex polycarpa									0.54179***				
Avena sativa		0.9468***	0.56368***			0.8665***	0.6933**					0.54696***	0.7696**
Baccharis halimifolia													
Brassica Spp													
Bromus mollis		0.50779***				0.53119***	0.5029***			0.53723***			
Calluna vulgaris	0.8042***	0.6063*								0.8312***			
Chenopodium album		0.58521***				0.5629***	0.5213***	0.50373***		0.62887***	0.52185***		0.53237***
Chrysanthemum leucanthemum	0.54184***	0.66695***				0.58307***	0.55848***			0.61604***	0.54312***	0.6006*	0.56581***

Appendix 3 (continued): Correlations between the different pollen types and plant-based foods studied.

Allergens	*Tamarix gallica*	*Tilia cordata*	*Ulmus americana*	*Ulmus minor*	*Syringa vulgaris*	*Agropyron repe*	*Agrostis alba*	*Alopecurus prantesis*	*Amaranthus hybridus*	*Ambrosia artemisiifolia*	*Ambrosia trifida*	*Anthxanthum odoratum*	*Artemisia vulgaris*
Corylus avellana	0.788***	0.8589***									0.6355*		
Cynodon dactylon		0.56677***		0.52365***				0.54859***		0.61333***	0.52609***		0.52206***
Dactylis glomerata		0.53116***				0.50384***				0.51447***	0.50669***	0.5986*	0.50565***
Dahlia cultorum	0.57455***	0.67966***				0.59089***	0.54517***			0.5872***	0.51995***		0.54682***
Festuca prantesis		0.52473***				0.52169***				0.51782***			0.50799***
Holcus lanatus		0.58939***		0.58235***		0.53763***	0.55207***	0.6454***		0.53364***	0.67542***	0.9179***	0.64208***
Hordeum vulgare			0.54112***						0.54021***				
Humulus lupulus		0.56577***	0.7059**			0.52614***	0.52237***			0.56012***	0.57182***		0.61908***
Ligustrum vulgare													
Lolium perenne		0.56357***				0.56828***	0.52648***			0.54183***			0.51254***
Medicago sativa	0.63859***	0.68984***				0.67689***	0.61627***			0.59631***	0.5128***		0.54596***
Parietaria judaica			0.5069						0.9866***				
Philadelphus coronarius					0.62464***								
Phlenum pratesee				0.53784***									
Phragmites communis	0.51205***	0.53568***	0.8884***			0.5435***	0.50594***						
Plantago lanceolata	0.60455***	0.63432***	0.8719***			0.65834***	0.64273***			0.54699***	0.50748***		0.54638***
Poa prateis	0.52573***	0.53285***	0.6828**			0.56695***	0.52556***						
Rumex acetosella		0.614***	0.7631**			0.59851***	0.6369***			0.52958***	0.60192***		0.64629***
Salsola kali												0.57957***	
Sambucus nigra				0.63142***									
Secale cereale													
Solidago virga aurea	0.53108***	0.57276***				0.55246***	0.58211***			0.52495***	0.53284***		0.56311***
Sorghum halepense		0.5717***				0.50121***		0.51301***		0.69005***	0.55989***		0.52468***
Taraxacum officinale	0.9203***	0.9555***								0.922***	0.9676***		
Triticum aestivum/ sativum													
Zea mays				0.50795***				0.50946***		0.52252***			
Urtica dioica												0.9555***	
Salix viminalis	0.50663***				0.56501***								
Horseradish peroxidase	0.54662***	0.68564***	0.9752***			0.63771***	0.61709***			0.56456***	0.56034***		0.60535***
Latex													
Bromelain			0.51248***		0.6754*								

Appendix 3 (continued): Correlations between the different pollen types and plant-based foods studied.

Allergens	*Atriplex polycarpa*	*Avena sativa*	*Baccharis halimifolia*	*Brassica Spp*	*Bromus mollis*	*Calluna vulgaris*	*Chenopodium album*	*Chrysanthemum leucanthemum*	*Corylus avellana*	*Cynodon dactylon*	*Dactylis glomerata*	*Dahlia cultorum*	*Festuca prantesis*
Apple													
Cabbage	0.54824***		0.54349***	0.53913***			0.54923***	0.51319***		0.62548***			
Cacao						0.887***							
Carrot								0.5207***				0.52171***	
Chard									0.7392**				
Corn flour	0.64147***		0.51747***	0.50067***									
Garlic			0.54596***				0.60435***	0.57435***	0.984***	0.69416***	0.55097***	0.56142***	0.5158***
Gliadin													
Hazelnut	0.51803***												
Peach						0.5538	0.57325***	0.62472***		0.58221***	0.51877***	0.5976***	0.50312***
Peanut	0.52818***												
Potato													
Rice flour			0.52788***	0.56622***			0.59984***	0.5166***		0.62469***			
Soya bean	0.5125***		0.55654***	0.54055***						0.53395***			
Spinach													
Strawberry						0.5709			0.9871***				
Sunflower seed							0.59467***	0.56722***		0.57999***		0.51592***	
Tofu			0.52982***	0.54135***			0.63547***	0.54886***		0.61588***	0.5047***	0.51765***	
Walnut		0.862***					0.53635***	0.59078***	0.9391***	0.55796***	0.50905***	0.60091***	
Wheat flour	0.53722***		0.55344***	0.57437***			0.52987***			0.56859***			
Banana					0.9307***								
Orange													
Brocolli	0.59157***		0.51732***										
Kiwi													
Acacia spp.	0.53319***												
Acer negundo	0.57888***	0.51195***											
Alnus glutinosa				0.6024*									
Arecastrum romanzo ffianum	0.51286***		0.63344***	0.51673***									
Betula verrucosa		0.9135***											
Carpinus betulus					0.9858***								
Casuarina equisetifolia	0.5931***												
Cryptomeria japonica							0.56133***	0.55969***			0.50249***	0.52491***	
Cupressus arizonica													
Cupressus sempervirens								0.53191***			0.55913***	0.51467***	
Elaeis guineeis													
Eucalyptus globulus							0.50009***	0.51459***				0.53891***	0.51767***

Appendix 3 (continued): Correlations between the different pollen types and plant-based foods studied.

Allergens	*Atriplex polycarpa*	*Avena sativa*	*Baccharis halimifolia*	*Brassica Spp*	*Bromus mollis*	*Calluna vulgaris*	*Chenopodium album*	*Chrysanthemum leucanthemum*	*Corylus avellana*	*Cynodon dactylon*	*Dactylis glomerata*	*Dahlia cultorum*	*Festuca prantesis*
Fagus sylvatica													
Fraxinus excelsior													
Juniperus asheisabinoides								0.54654***				0.53934***	
Olea europaea				0.54969***							0.55245***		0.50017***
Pinus radiata		0.6289*						0.52739***	0.6137*				
Pinus strobus		0.6554*				0.5971*	0.51449***	0.54374***		0.50795***		0.5388***	
Platanus acerfolia		0.9481***			0.53496***	0.7999***	0.62441***	0.62243***		0.59683***	0.50709***	0.60384***	0.51738***
Podocarpus polystachyus													
Populus deltoides									0.5571				
Populus nigra			0.54879***	0.52277***			0.63967***	0.56234***		0.67185***	0.50835***	0.53986***	
Populus trichocarpa		0.53927***						0.8129***			0.9556***	0.5531	
Quercus alba			0.5226***							0.60965***			
Quercus ilex							0.50098***			0.54276***	0.66278***	0.50505***	0.55131***
Quercus robur							0.73971***	0.6387***		0.6344***	0.56535***	0.59059***	0.56475***
Robinia pseudoacacia							0.63948***	0.62046***		0.5336***	0.51562***	0.57553***	0.54411***
Schnius molle		0.8396***											
Tamarix gallica						0.8042***		0.54184***	0.788***			0.57455***	
Tilia cordata		0.9468***			0.50779***	0.6063*	0.58521***	0.66695***	0.8589***	0.56677***	0.53116***	0.67966***	0.52473***
Ulmus americana		0.56368***											
Ulmus minor										0.52365***			
Syringa vulgaris													
Agropyron repe		0.8665***			0.53119***		0.5629***	0.58307***			0.50384***	0.59089***	0.52169***
Agrostis alba		0.6933**			0.5029***		0.5213***	0.55848***				0.54517***	
Alopecurus prantesis							0.50373***			0.54859***			
Amaranthus hybridus	0.54179***												
Ambrosia artemisiifolia					0.53723***	0.8312***	0.62887***	0.61604***		0.61333***	0.51447***	0.5872***	0.51782***
Ambrosia trifida							0.52185***	0.54312***	0.6355*	0.52609***	0.50669***	0.51995***	
Anthxanthum odoratum		0.54696***						0.6006*			0.5986*	0.5566	
Artemisia vulgaris		0.7696**					0.53237***	0.56581***		0.52206***	0.50565***	0.54682***	0.50799***
Atriplex polycarpa	1		0.61252***	0.53911***									
Avena sativa		1											0.9432***
Baccharis halimifolia	0.61252***		1	0.56606***						0.51923***			
Brassica Spp	0.53911***		0.56606***	1						0.52607***			
Bromus mollis					1		0.51961***	0.55361***		0.50372***		0.52416***	0.51469***
Calluna vulgaris						1	0.6219*	0.6765**			0.8045***	0.7018**	

Appendix 3 (continued): Correlations between the different pollen types and plant-based foods studied.

Alleregens	*Atriplex polycarpa*	*Avena sativa*	*Baccharis halimifolia*	*Brassica Spp*	*Bromus mollis*	*Calluna vulgaris*	*Chenopodium album*	*Chrysanthemum leucanthemum*	*Corylus avellana*	*Cynodon dactylon*	*Dactylis glomerata*	*Dahlia cultorum*	*Festuca prantesis*
Chenopodium album					0.51961***	0.6219*	1	0.63998***		0.58968***	0.57465***	0.58142***	0.58443***
Chrysanthemum leucanthemum					0.55361***	0.6765**	0.63998***	1	0.6934**	0.56768***	0.54002***	0.71419***	0.54061***
Corylus avellana								0.6934**	1			0.905***	0.7921***
Cynodon dactylon			0.51923***	0.52607***	0.50372***		0.58968***	0.56768***		1	0.54594***	0.5517***	
Dactylis glomerata						0.8045***	0.57465***	0.54002***		0.54594***	1	0.54184***	0.68709***
Dahlia cultorum					0.52416***	0.7018**	0.58142***	0.71419***	0.905***	0.5517***	0.54184***	1	0.50962***
Festuca prantesis		0.9432***			0.51469***		0.58443***	0.54061***	0.7921***		0.68709***	0.50962***	1
Holcus lanatus							0.53449***	0.53913***		0.5787***	0.55311***	0.52041***	0.5018***
Hordeum vulgare	0.53841***	0.5473***											
Humulus lupulus		0.5918*					0.51975***	0.5604***				0.55168***	
Ligustrum vulgare	0.50631***												
Lolium perenne					0.54024***		0.5928***	0.58651***	0.6053*		0.64317***	0.56841***	0.74305***
Medicago sativa		0.8573***			0.51421***	0.7029**	0.57433***	0.64195***	0.7087**	0.50757***	0.52017***	0.6801***	0.53851***
Parietaria judaica						0.7033**							
Philadelphus coronarius													
Phlenum pratesee													
Phragmites communis		0.5751*					0.51535***	0.53749***	0.7742***		0.55864***	0.54385***	0.61561***
Plantago lanceolata							0.57946***	0.56857***				0.5768***	
Poa prateis					0.50314***			0.50711***	0.6737*		0.5214***	0.52899***	0.58616***
Rumex acetosella							0.52559***	0.52547***				0.51219***	
Salsola kali													
Sambucus nigra	0.53499***												
Secale cereale				0.7162**									
Solidago virga aurea												0.50408***	
Sorghum halepense					0.50302***		0.63389***	0.60957***		0.70228***	0.54207***	0.55676***	0.53179***
Taraxacum officinale													
Triticum aestivum/ sativum													
Zea mays			0.55908***	0.512***			0.51313***			0.68899***	0.51605***		
Urtica dioica			0.56091***	0.52053***						0.50386***			
Salix viminalis													
Horseradish peroxidase		0.5959*			0.51311***		0.57135***	0.6323***	0.6116*		0.54794***	0.65378***	0.56447***
Latex						0.975***					0.51269***		
Bromelain													

Appendix 3 (continued): Correlations between the different pollen types and plant-based foods studied.

Allergens	*Holcus lanatus*	*Hordeum vulgare*	*Humulus lupulus*	*Ligustrum vulgare*	*Lolium perenne*	*Medicago sativa*	*Parietaria judaica*	*Philadelphus coronarius*	*Phlenum pratesee*	*Phragmites communis*	*Plantago lanceolata*	*Poa prateis*	*Rumex acetosella*
Apple								0.9699***					
Cabbage	0.5331***												
Cacao		0.8794***							0.7321**				
Carrot			0.56958***			0.54883***							0.54315***
Chard													
Corn flour		0.60065***											
Garlic	0.52555***				0.52198***	0.54262***							
Gliadin							0.6029*	0.9794***	0.50313***				
Hazelnut		0.57115***							0.50895***				
Peach	0.63328***		0.61966***		0.52294***	0.60524***					0.57583***		0.62907***
Peanut													
Potato													
Rice flour	0.5275***												
Soya bean													
Spinach													
Strawberry													
Sunflower seed													
Tofu	0.50683***												
Walnut	0.64529***		0.62631***		0.50836***	0.6039***					0.55004***		0.66989***
Wheat flour		0.50358***											
Banana													
Orange													
Brocolli		0.58589***							0.54503***				
Kiwi							0.54697***		0.6062				
Acacia spp.							0.7151**	0.7575**					
Acer negundo								0.9607***					
Alnus glutinosa													
Arecastrum romanzo ffianum													
Betula verrucosa					0.50684***	0.64779***					0.59008***		0.62406***
Carpinus betulus								0.584*		0.7463**		0.6991**	
Casuarina equisetifolia		0.52586***		0.50319***									
Cryptomeria japonica						0.53268***					0.50322***		
Cupressus arizonica													
Cupressus sempervirens	0.51341***					0.50108***							
Elaeis guineeis													
Eucalyptus globulus			0.51156***		0.54842***	0.65301***				0.56797***	0.57301***	0.56121***	

Appendix 3 (continued): Correlations between the different pollen types and plant-based foods studied.

Allergens	*Holcus lanatus*	*Hordeum vulgare*	*Humulus lupulus*	*Ligustrum vulgare*	*Lolium perenne*	*Medicago sativa*	*Parietaria judaica*	*Philadelphus coronarius*	*Phlenum pratesee*	*Phragmites communis*	*Plantago lanceolata*	*Poa prateis*	*Rumex acetosella*
Fagus sylvatica		0.50729***											
Fraxinus excelsior													
Juniperus asheisabinoides			0.50806***		0.51081***	0.56404***					0.50159***		
Olea europaea				0.50609***									
Pinus radiata					0.50363***	0.51459***					0.51398***	0.51348***	
Pinus strobus						0.59694***					0.60374***		0.56139***
Platanus acerfolia	0.52203***		0.56425***		0.54753***	0.6322***				0.5126***	0.63494***		0.57917***
Podocarpus polystachyus		0.7355**		0.9943***			0.5099	0.5056	0.7745***	0.5904*			
Populus deltoides			0.51248***			0.54922***							0.57037***
Populus nigra	0.52409***												
Populus trichocarpa	0.8039***					0.6189*							
Quercus alba													
Quercus ilex	0.53532***				0.51818***								
Quercus robur	0.6037***		0.52877***		0.56482***	0.55975***					0.56685***		0.57111***
Robinia pseudoacacia	0.55609***				0.55346***	0.53663***					0.54543***		0.55378***
Schnius molle						0.53198***							
Tamarix gallica						0.63859***				0.51205***	0.60455***	0.52573***	
Tilia cordata	0.58939***		0.56577***		0.56357***	0.68984***				0.53568***	0.63432***	0.53285***	0.614***
Ulmus americana		0.54112***	0.7059**				0.5069			0.8884***	0.8719***	0.6828**	0.7631**
Ulmus minor	0.58235***								0.53784***				
Syringa vulgaris				0.5238				0.62464***					
Agropyron repe	0.53763***		0.52614***		0.56828***	0.67689***				0.5435***	0.65834***	0.56695***	0.59851***
Agrostis alba	0.55207***		0.52237***		0.52648***	0.61627***				0.50594***	0.64273***	0.52556***	0.6369***
Alopecurus prantesis	0.6454***												
Amaranthus hybridus		0.54021***					0.9866***	0.5265					
Ambrosia artemisiifolia	0.53364***		0.56012***		0.54183***	0.59631***					0.54699***		0.52958***
Ambrosia trifida	0.67542***		0.57182***			0.5128***					0.50748***		0.60192***
Anthxanthum odoratum	0.9179***												
Artemisia vulgaris	0.64208***		0.61908***		0.51254***	0.54596***					0.54638***		0.64629***
Atriplex polycarpa		0.53841***		0.50631***									
Avena sativa		0.5473***	0.5918*			0.8573***				0.5751*			
Baccharis halimifolia													
Brassica Spp													
Bromus mollis					0.54024***	0.51421***						0.50314***	
Calluna vulgaris						0.7029**	0.7033**						

Appendix 3 (continued): Correlations between the different pollen types and plant-based foods studied.

Allergens	*Holcus lanatus*	*Hordeum vulgare*	*Humulus lupulus*	*Ligustrum vulgare*	*Lolium perenne*	*Medicago sativa*	*Parietaria judaica*	*Philadelphus coronarius*	*Phlenum pratesee*	*Phragmites communis*	*Plantago lanceolata*	*Poa prateis*	*Rumex acetosella*
Chenopodium album	0.53449***		0.51975***		0.5928***	0.57433***				0.51535***	0.57946***		0.52559***
Chrysanthemum leucanthemum	0.53913***		0.5604***		0.58651***	0.64195***				0.53749***	0.56857***	0.50711***	0.52547***
Corylus avellana					0.6053*	0.7087**				0.7742***		0.6737*	
Cynodon dactylon	0.5787***					0.50757***							
Dactylis glomerata	0.55311***				0.64317***	0.52017***				0.55864***		0.5214***	
Dahlia cultorum	0.52041***		0.55168***		0.56841***	0.6801***				0.54385***	0.5768***	0.52899***	0.51219***
Festuca prantesis	0.5018***				0.74305***	0.53851***				0.61561***		0.58616***	
Holcus lanatus	1		0.52471***			0.51462***							0.5819***
Hordeum vulgare		1					0.5979*	0.6288*					
Humulus lupulus	0.52471***		1		0.51575***	0.56053***					0.53333***		0.62382***
Ligustrum vulgare				1									
Lolium perenne			0.51575***		1	0.59393***				0.71729***	0.52663***	0.62849***	
Medicago sativa	0.51462***		0.56053***		0.59393***	1				0.59345***	0.65637***	0.61217***	0.56727***
Parietaria judaica		0.5979*					1		0.9344***			0.541***	
Philadelphus coronarius		0.6288*						1					
Phlenum pratesee							0.9344***		1				
Phragmites communis					0.71729***	0.59345***				1	0.52394***	0.66152***	
Plantago lanceolata			0.53333***		0.52663***	0.65637***				0.52394***	1	0.57151***	0.59358***
Poa prateis					0.62849***	0.61217***	0.541***			0.66152***	0.57151***	1	0.50349***
Rumex acetosella	0.5819***		0.62382***			0.56727***					0.59358***	0.50349***	1
Salsola kali													
Sambucus nigra	0.50029***								0.53754***				
Secale cereale													
Solidago virga aurea	0.50518***		0.60659***			0.56754***		0.56739***			0.54039***		0.69297***
Sorghum halepense	0.56739***		0.52661***		0.54907***	0.54959***					0.50601***		
Taraxacum officinale					0.7691**	0.8665***				0.7607**	0.7154**		
Triticum aestivum/ sativum													
Zea mays	0.51277***												
Urtica dioica													
Salix viminalis				0.9614***									
Horseradish peroxidase	0.57905***		0.53375***		0.61748***	0.64972***				0.60216***	0.60515***	0.58661***	0.58615***
Latex													
Bromelain							0.9762***						0.8137***

Appendix 3 (continued): Correlations between the different pollen types and plant-based foods studied.

Allergens	*Salsola kali*	*Sambucus nigra*	*Secale cereale*	*Solidago virga aurea*	*Sorghum halepense*	*Taraxacum officinale*	*Triticum aestivum/ sativum*	*Zea mays*	*Urtica dioica*	*Salix viminalis*	Horseradish peroxidase	Latex	Bromelain
Apple				0.7004**									
Cabbage		0.5514***			0.59214***			0.64708***	0.53079***				
Cacao						0.8992***				0.53169***			
Carrot				0.53182***									
Chard	0.6301*												
Corn flour		0.5485***											
Garlic					0.6599***			0.62988***	0.55833***		0.51106***		
Gliadin													
Hazelnut			0.9248***										
Peach				0.55257***	0.63265***	0.9171***					0.61026***		
Peanut													
Potato						0.5933*							
Rice flour					0.55802***		0.51953***	0.57886***					
Soya bean					0.54945***			0.56377***	0.60348***				
Spinach					0.51281***			0.50024***	0.51223***				
Strawberry										0.5389***			
Sunflower seed					0.64444***			0.50724***					
Tofu	0.9893***				0.60504***			0.57884***	0.53838***				
Walnut				0.5714***	0.5556***	0.7513**					0.6187***		
Wheat flour					0.50664***			0.53533***					
Banana													
Orange	0.9464**											0.61182***	
Brocolli		0.58083***						0.55465***		0.9306**			
Kiwi										0.60232***			
Acacia spp.													
Acer negundo													
Alnus glutinosa													
Arecastrum romanzo ffianum	0.7949***				0.51215***			0.55941***	0.57663***				
Betula verrucosa				0.57992***	0.53393***						0.60343***		
Carpinus betulus													
Casuarina equisetifolia		0.54048***											
Cryptomeria japonica					0.58056***						0.50056***		
Cupressus arizonica													
Cupressus sempervirens											0.53673***	0.61216***	
Elaeis guineeis													

Appendix 3 (continued): Correlations between the different pollen types and plant-based foods studied.

Allergens	*Salsola kali*	*Sambucus nigra*	*Secale cereale*	*Solidago virga aurea*	*Sorghum halepense*	*Taraxacum officinale*	*Triticum aestivum/ sativum*	*Zea mays*	*Urtica dioica*	*Salix viminalis*	Horseradish peroxidase	Latex	Bromelain
Eucalyptus globulus				0.50143***							0.56271***		
Fagus sylvatica													
Fraxinus excelsior		0.50021***											
Juniperus asheisabinoides					0.53756***						0.55414***		
Olea europaea								0.50774***					
Pinus radiata						0.9538***					0.54416***	0.56904***	
Pinus strobus				0.54404***	0.51694***	0.5051					0.54181***		
Platanus acerfolia				0.50735***	0.64303***	0.9788***					0.58299***		
Podocarpus polystachyus													0.7307**
Populus deltoides				0.6042***									0.9485***
Populus nigra	0.8832***				0.63285***			0.63004***	0.52235***				
Populus trichocarpa												0.7408**	
Quercus alba		0.53435***			0.52815***			0.66107***					
Quercus ilex	0.7586**				0.51697***			0.55364***	0.52322***			0.52746***	
Quercus robur					0.69495***	0.9997***		0.51548***			0.59886***		
Robinia pseudoacacia					0.56441***						0.58721***		
Schnius molle				0.51249***		0.6247*							0.9543***
Tamarix gallica				0.53108***		0.9203***				0.50663***	0.54662***		
Tilia cordata				0.57276***	0.5717***	0.9555***					0.68564***		
Ulmus americana											0.9752***		0.51248***
Ulmus minor		0.63142***						0.50795***					
Syringa vulgaris										0.56501***			0.6754*
Agropyron repe				0.55246***	0.50121***						0.63771***		
Agrostis alba				0.58211***							0.61709***		
Alopecurus prantesis					0.51301***			0.50946***					
Amaranthus hybridus													
Ambrosia artemisiifolia				0.52495***	0.69005***	0.922***		0.52252***			0.56456***		
Ambrosia trifida				0.53284***	0.55989***	0.9676***					0.56034***		
Anthxanthum odoratum	0.57957***								0.9555***				
Artemisia vulgaris				0.56311***	0.52468***						0.60535***		
Atriplex polycarpa		0.53499***											
Avena sativa											0.5959*		
Baccharis halimifolia								0.55908***	0.56091***				
Brassica Spp			0.7162**					0.512***	0.52053***				
Bromus mollis					0.50302***						0.51311***		

Appendix 3 (continued): Correlations between the different pollen types and plant-based foods studied.

Allergens	*Salsola kali*	*Sambucus nigra*	*Secale cereale*	*Solidago virga aurea*	*Sorghum halepense*	*Taraxacum officinale*	*Triticum aestivum/ sativum*	*Zea mays*	*Urtica dioica*	*Salix viminalis*	Horseradish peroxidase	Latex	Bromelain
Calluna vulgaris												0.975***	
Chenopodium album					0.63389***			0.51313***			0.57135***		
Chrysanthemum leucanthemum					0.60957***						0.6323***		
Corylus avellana											0.6116*		
Cynodon dactylon	0.5275				0.70228***			0.68899***	0.50386***				
Dactylis glomerata					0.54207***			0.51605***			0.54794***	0.51269***	
Dahlia cultorum				0.50408***	0.55676***						0.65378***		
Festuca prantesis					0.53179***	0.5484					0.56447***		
Holcus lanatus		0.50029***		0.50518***	0.56739***			0.51277***			0.57905***		
Hordeum vulgare													
Humulus lupulus				0.60659***	0.52661***						0.53375***		
Ligustrum vulgare										0.9614***			
Lolium perenne					0.54907***	0.7691**					0.61748***		
Medicago sativa				0.56754***	0.54959***	0.8665***					0.64972***		
Parietaria judaica													0.9762***
Philadelphus coronarius				0.56739***									
Phlenum pratesee		0.53754***											
Phragmites communis						0.7607**					0.60216***		
Plantago lanceolata				0.54039***	0.50601***	0.7154**					0.60515***		
Poa prateis											0.58661***		
Rumex acetosella				0.69297***							0.58615***		0.8137***
Salsola kali	1								0.8983***				
Sambucus nigra		1						0.51991***					
Secale cereale			1				0.5763*						
Solidago virga aurea				1							0.51512***		0.6449*
Sorghum halepense					1			0.62147***	0.51061***		0.50923***		
Taraxacum officinale						1					0.6894**		
Triticum aestivum/ sativum			0.5763*				1						
Zea mays		0.51991***			0.62147***			1	0.53339***				
Urtica dioica	0.8983***				0.51061***			0.53339***	1				
Salix viminalis										1			0.825**
Horseradish peroxidase				0.51512***	0.50923***	0.6894**					1		
Latex												1	
Bromelain				0.6449*						0.825**			1

Appendix 3: Correlation between the different fungal allergens studied.

Fungal allergens	Bromelain	*Alternaria alternata*	*Aspergillus flavus*	*Aspergillus fumigatus*	*Aspergillus niger*	*Aspergillus terreus*	*Botrytis cinerea*	*Candida albicans*	*Cladosporium cladosporoides*	*Cladosporium fulvum*	*Cladosporium herbarum*	*Corenyspora cassiicola*
Bromelain	1											
Alternaria alternata	0.1578***	1										
Aspergillus flavus	0.1791***	0.1744***	1									
Aspergillus fumigatus	0.3327***	0.2799***	0.2833***	1								
Aspergillus niger	0.085***	0.1495***	0.0675***	0.1735***	1							
Aspergillus terreus	0.2559***	0.0885***	0.2477***	0.3371***	0.0649**	1						
Botrytis cinerea	0.252***	0.1862***	ns	0.1882***	0.2007***	0.0511*	1					
Candida albicans	0.2129***	0.2846***	0.1131***	0.1515***	ns	ns	0.2427***	1				
Cladosporium cladosporoides	0.427***	0.227***	0.2169***	0.3336***	0.1472***	0.2418***	0.1957***	0.2298***	1			
Cladosporium fulvum	0.4807***	0.2166***	0.2371***	0.4304***	0.0552**	0.3637***	0.244***	0.2538***	0.4***	1		
Cladosporium herbarum	0.1459***	0.0704***	0.1424***	0.2496***	ns	0.4717***	ns	0.0428*	0.2272***	0.2611***	1	
Corenyspora cassiicola	0.0662**	0.1577***	0.1726***	0.2106***	0.0582**	0.2928***	-0.0578**	ns	0.0793***	0.1606***	0.4007***	1
Curvularia brachyspora	0.0974***	0.2891***	0.2136***	0.2343***	0.0021	0.2939***	-0.059**	0.0798***	0.128***	0.1923***	0.3004***	0.3855***
Curvularia fallax	0.0434*	0.223***	0.2448***	0.219***	ns	0.3328***	ns	0.1341***	0.1256***	0.1843***	0.2971***	0.3032***
Curvularia inequalis	0.1695***	0.1865***	0.1966***	0.2522***	ns	0.3086***	ns	0.1266***	0.1757***	0.2303***	0.2686***	0.2366***
Curvularia lunata	0.1858***	0.3579***	0.2414***	0.258***	0.0968***	0.2048***	0.0659**	0.1413***	0.2797***	0.1965***	0.1991***	0.3001***
Curvularia pallescences	ns	0.1312***	0.2315***	0.2222***	-0.0781***	0.442***	-0.1048***	ns	0.0753***	0.1847***	0.4014***	0.3545***
Curvularia spicifera	0.1507***	ns	0.1032***	0.1625***	0.1304***	0.4066***	0.1373***	ns	0.19***	0.2204***	**0.5094*** **	0.2704***
Drechslerea/ Bipolaris sorokiana	0.3414***	0.2875***	0.3704***	0.3047***	0.0423*	0.2816***	0.0818***	0.1598***	0.33***	0.3148***	0.1962***	0.2332***
Fusarium moniliforme	0.2454***	0.1913***	0.2419***	0.2407***	0.0817***	0.2378***	0.0823***	0.1979***	0.2009***	0.3161***	0.1688***	0.2686***
Fusarium solani	0.4113***	0.3422***	0.2889***	0.3858***	0.1274***	0.1978***	0.2525***	0.3406***	0.3714***	0.4435***	0.1144***	0.1468***
Malazessia furfur	0.1186***	ns	0.1642***	0.1397***	ns	0.4479***	ns	ns	0.1366***	0.1772***	**0.5352*** **	0.338***
Mucor mucedo	0.29***	0.1469***	0.0479*	0.1994***	0.0479*	0.0683***	0.3213***	0.4584***	0.2663***	0.2569***	0.0749***	ns
Penicillium brevicompactum	0.1546***	ns	0.1211***	0.1933***	0.1882***	0.4302***	0.116***	-0.0444*	0.1887***	0.1937***	0.4836***	0.2916***
Penicillium chrysogenum	ns	0.2099***	0.1145***	0.048*	-0.0588**	-0.0301	ns	0.2296***	ns	0.0678***	ns	0.053**
Penicillium expansum	0.3794***	0.108***	0.106***	0.3244***	0.159***	0.3321***	0.1699***	0.118***	0.3436***	0.3929***	0.298***	0.1872***
Penicillium notatum	0.351***	0.2172***	0.1555***	0.2695***	0.066**	0.1997***	0.2647***	0.3594***	0.282***	0.3881***	0.1176***	0.018ns
Penicillium roqueforti	0.1844***	0.1485***	ns	0.1036***	-0.0707***	-0.0071	0.0602**	0.192***	0.1011***	0.166***	ns	0.0571**
Rhizopus nigricans	0.3854***	0.1402***	0.2378***	0.2609***	ns	0.1538***	0.2413***	0.2919***	0.3577***	0.314***	0.1235***	0.0613**
Saccharomyces cerevisae	0.214***	ns	0.1426***	0.2271***	ns	0.4805***	ns	ns	0.1946***	0.2792***	0.455***	0.3007***
Stemphylium botryosum	0.1695***	0.2007***	0.0479*	0.1564***	0.4254***		0.4404***	0.1465***	0.172***	0.1227***	ns	ns
Trichoderma viride	-0.1004***	0.1831***	0.1301***	0.0596**	-0.1248***	0.0231	-0.0951***	0.1375***	-0.0614**	ns	0.0448*	0.1273***
Trichophyton mentagrophytes	0.3426***	0.0605**	0.1857***	0.3225***	0.0778***	**0.5038*** **	0.1306***	0.0919***	0.3034***	0.4317***	0.4373***	0.2786***
Trichophyton rubrum	0.1179***	0.0898***	0.2011***	0.1747***	ns	0.4277***	-0.0331	ns	0.0917***	0.2441***	0.3444***	0.3895***
Ustilago tritici	0.1823***	ns	0.0972***	0.1885***	0.2294***	0.3755***	0.1106***	-0.1164***	0.2022***	0.1595***	0.3979***	0.2327***

Appendix 3 (continued): Correlation between the different fungal allergens studied.

Allergens	*Curvularia brachyspora*	*Curvularia fallax*	*Curvularia inequalis*	*Curvularia lunata*	*Curvularia pallescences*	*Curvularia spicifera*	*Drechslerea/ Bipolaris sorokiana*	*Fusarium moniliforme*	*Fusarium solani*	*Malazessia furfur*	*Mucor mucedo*
Bromelain											
Alternaria alternata											
Aspergillus flavus											
Aspergillus fumigatus											
Aspergillus niger											
Aspergillus terreus											
Botrytis cinerea											
Candida albicans											
Cladosporium cladosporoides											
Cladosporium fulvum											
Cladosporium herbarum											
Corenyspora cassiicola											
Curvularia brachyspora	1										
Curvularia fallax	0.3423***	1									
Curvularia inequalis	0.3695***	0.3402***	1								
Curvularia lunata	0.3953***	0.2995***	0.4289***	1							
Curvularia pallescences	0.3986***	0.4935***	0.2892***	0.2245***	1						
Curvularia spicifera	0.2298***	0.2291***	0.2015***	0.1265***	0.3479***	1					
Drechslerea/ Bipolaris sorokiana	0.3251***	0.2949***	0.2446***	0.3442***	0.3312***	0.2237***	1				
Fusarium moniliforme	0.2846***	0.1416***	0.2094***	0.2213***	0.153***	0.1326***	0.2692***	1			
Fusarium solani	0.1764***	0.1494***	0.1595***	0.2693***	0.0855***	0.0449*	0.3385***	0.3327***	1		
Malazessia furfur	0.2517***	0.2906***	0.2593***	0.1527***	0.4084***	0.5071***	0.2361***	0.1539***	ns	1	
Mucor mucedo	ns	0.0513*	0.1168***	0.1083***	-0.0291	ns	0.0681***	0.1796***	0.3129***	ns	1
Penicillium brevicompactum	0.2333***	0.209***	0.1995***	0.1565***	0.3202***	0.7184***	0.2263***	0.1405***	0.0636**	**0.5100*** **	ns
Penicillium chrysogenum	0.1547***	0.1882***	0.0496*	0.1432***	0.167***	-0.0565**	0.1495***	0.072***	0.1381***	-0.0433*	0.1115***
Penicillium expansum	0.1428***	0.1095***	0.2196***	0.1991***	0.1078***	0.2933***	0.2186***	0.3193***	0.2751***	0.2252***	0.1745***
Penicillium notatum	0.0821***	0.1352***	0.1439***	0.1377***	ns	0.0694***	0.1341***	0.1986***	0.414***	0.0562**	0.3687***
Penicillium roqueforti	0.061**	ns	0.062**	0.0525*	ns	-0.0513*	0.067**	0.129***	0.1834***	ns	0.2597***
Rhizopus nigricans	0.0678***	0.079***	0.1127***	0.1717***	0.0421*	0.0838***	0.2002***	0.1491***	0.3658***	0.0454*	0.3749***
Saccharomyces cerevisae	0.2717***	0.2652***	0.326***	0.166***	0.4134***	0.491***	0.2578***	0.272***	0.1328***	**0.5195*** **	0.0647**
Stemphylium botryosum	ns	-0.0854***	ns	0.0964***	-0.1482***	0.0866***	0.0762***	0.1043***	0.1991***	-0.0793***	0.1981***
Trichoderma viride	0.1942***	0.2057***	0.1039***	0.1093***	0.2555***	ns	0.1444***	0.0642**	ns	0.1096***	ns
Trichophyton mentagrophytes	0.2322***	0.2448***	0.2862***	0.1865***	0.2831***	0.4238***	0.2794***	0.3169***	0.2397***	0.3615***	0.1347***
Trichophyton rubrum	0.3278***	0.2978***	0.2363***	0.2112***	0.3947***	0.2473***	0.2198***	0.2748***	0.1668***	0.311***	ns
Ustilago tritici	0.1582***	0.1287***	0.1317***	0.123***	0.2364***	0.5912***	0.1999***	0.1596***	0.0806***	0.4214***	ns

Appendix 3 (continued): Correlation between the different fungal allergens studied.

Allergens	*Penicillium brevicompactum*	*Penicillium chrysogenum*	*Penicillium expansum*	*Penicillium notatum*	*Penicillium roqueforti*	*Rhizopus nigricans*	*Saccharomyces cerevisae*	*Stemphylium botryosum*	*Trichoderma viride*	*Trichophyton mentagrophytes*	*Trichophyton rubrum*	*Ustilago tritici*
Bromelain												
Alternaria alternata												
Aspergillus flavus												
Aspergillus fumigatus												
Aspergillus niger												
Aspergillus terreus												
Botrytis cinerea												
Candida albicans												
Cladosporium cladosporoides												
Cladosporium fulvum												
Cladosporium herbarum												
Corenyspora cassiicola												
Curvularia brachyspora												
Curvularia fallax												
Curvularia inequalis												
Curvularia lunata												
Curvularia pallescences												
Curvularia spicifera												
Drechslerea/ Bipolaris sorokiana												
Fusarium moniliforme												
Fusarium solani												
Malazessia furfur												
Mucor mucedo												
Penicillium brevicompactum	1											
Penicillium chrysogenum	-0.0955***	1										
Penicillium expansum	0.2947***	-0.0666**	1									
Penicillium notatum	0.0475*	0.1003***	0.2494***	1								
Penicillium roqueforti	-0.0558**	0.1003***	0.0991***	0.2183***	1							
Rhizopus nigricans	0.075***	0.0809***	0.2139***	0.3534***	0.2675***	1						
Saccharomyces cerevisae	0.4887***	-0.079***	0.4013***	0.0982***	ns	0.0989***	1					
Stemphylium botryosum	0.1091***	ns	0.1394***	0.1332***	ns	0.1312***	-0.0635**	1				
Trichoderma viride	ns	0.3964***	-0.1122***	-0.0061	0.1369***	ns	ns	-0.1241***	1			
Trichophyton mentagrophytes	0.3998***	-0.0785***	0.4902***	0.2434***	0.0605**	0.2277***	0.4978***	0.0408*	-0.0617**	1		
Trichophyton rubrum	0.2394***	0.1279***	0.1653***	0.1221***	0.0511*	0.0716***	0.2915***	-0.0676***	0.1161***	0.278***	1	
Ustilago tritici	**0.6984*** **	-0.1819***	0.2976***	ns	-0.0772***	0.0818***	0.4462***	0.1198***	-0.0787***	0.3691***	0.199***	1