

Differentiation of allergenic fungal spores by image analysis, with application to aerobiological counts

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Abstract

The ability of an image analysis routine to differentiate between spores of eleven allergenic fungal genera was tested using analysis based on seven basic and up to 17 more complex features, extracted from digitised images. Fungal spores of *Alternaria*, *Cladosporium*, *Fusarium*, *Aspergillus*, *Penicillium*, *Botrytis*, *Epicoccum*, *Exserohilum*, *Ustilago*, *Coprinus* and *Psilocybe* were examined in a series of experiments designed to differentiate between spores at the genus and species level. Linear and Quadratic Discriminant Analysis of feature measurements, recorded for 100 to 1600 spores per taxon, differentiated between genera and species with a high level of accuracy. Genus comparisons using only seven basic features resulted in 98% accuracy for the recognition of conidia belonging to *Cladosporium*, *Fusarium* and *Epicoccum*. Differentiation between conidia of *Aspergillus* and *Penicillium* was the least reliable, with 56% of *Aspergillus* conidia correctly identified and 41% misidentified as *Penicillium*. At the species level, conidia of *Cladosporium macrocarpum*, *Fusarium moniliforme* (microconidia), *F. oxysporum* (microconidia), *F. solani* (macroconidia), *Alternaria helianthi* and *A. brassicae* were consistently identified with 86–100% accuracy. Reduced levels of accuracy in the identification of spores by image analysis reflected similarities between species in their spore morphology. The application of image analysis to aerobiological counting methods is discussed in relation to the results obtained.

1. Introduction

Fungal spores are consistently present in the airborne environment, although levels vary according to season, climate, diurnal influences, vegetation and location, e.g., indoor versus outdoor (Ebner et al., 1992; Hasnain, 1993; Hjelmroos, 1993; Li and Kendrick, 1995a, b; Crotzer and Levetin, 1996; Garrett et al., 1997; Mitikakis et al., 1997). The most prevalent airborne fungal spores, year round in both indoor and outdoor environments, are those belonging to *Cladosporium* (Sneller and Roby, 1979; Ballero et al., 1992; Ebner et al., 1992; Li and Kendrick, 1995a, b; Garrett et al., 1997). Following *Cladosporium* in prevalence, are spores of *Alternaria*, *Aspergillus*, *Penicillium*, *Epicoccum*, *Fusarium*, *Botrytis*, *Exser-*

ohilum, *Ustilago* and *Coprinus* amongst others, in varying hierarchies (Hirsch and Sosman, 1976; Sneller and Roby, 1979; Kozak et al., 1979; Beaumont et al., 1984; Ballero et al., 1992; Ebner et al., 1992; Srivastava and Wadhwani, 1992). Of the basidiospores, *Coprinus* were the most abundant recorded in a study in Tulsa, Oklahoma, with *Ganoderma* spores also featuring prominently (Levetin, 1991).

Sensitisation to airborne fungal spores varies and is dependent upon the allergenic potency of the fungal proteins, human genetic predisposition to disease (atopy) and levels of exposure to allergens. Establishing the relationship between exposure and disease requires accurate methods for allergen identification and enumeration, and allergy assessment. Current methods assess allergic sensitisation by skin prick

testing with fungal extracts, whilst the identification and enumeration of fungal spores and pollen is conducted independently using air sampling devices. Recent research has focussed on combining the assessment of sensitisation and exposure, using nasal air samplers, immunostaining and image analysis, with the aim of improving allergy diagnosis (Tovey et al., 1998; Jones et al., 1998).

Of particular interest has been the identification and enumeration of fungal spores by image analysis. Automated recognition of fungal spores has been investigated previously for the enumeration of white-rot fungi (Jones et al., 1992). Spores held in suspension were viewed in an haemocytometer chamber under the light microscope and quantified using an automated image analysis program. By this method four microscope fields could be counted in two minutes, greatly improving the speed and accuracy of the manual operation. The identification of fungal spores to the level of genus and species on the basis of morphological features using image analysis has not been attempted previously. However, this would be an extremely useful tool in aerobiological studies that rely on manual identification and enumeration using a light microscope, as the speed and accuracy of identification could be increased and the reliance on mycological expertise would be reduced.

In this study the ability of image analysis techniques to correctly differentiate between a selection of allergenic fungi based solely on their spore morphology has been investigated. The fungi selected for study were those most commonly recorded in aeroallergen studies or recognised as highly allergenic fungi, with diverse spore morphologies, and included eight species of *Alternaria*, four species of *Cladosporium*, three species of *Fusarium*, *Penicillium chrysogenum* Thom (syn. *P. notatum* Westling), *Aspergillus fumigatus*, *Epicoccum nigrum* Link, *Botrytis cinerea* Pers. ex Nocca and Balb., *Exserohilum rostratum* (Drechsler) Leonard and Suggs (syn. *Helminthosporium halodes* Drechsler), *Ustilago nuda* (Jensen) Rostrup, *Coprinus comatus* (Mull. ex Fr.) S.F. Gray and *Psilocybe cubensis* (Earle) Singer. Spores of *Psilocybe cubensis*, have not featured in any aerobiological studies, but extracts of this basidiomycete are highly allergenic (Horner et al., 1995).

2. Materials and methods

2.1 Fungal cultures

Fungal cultures and specimens were obtained from recognised mycological herbaria in New South Wales (Table 1). Microfungi were cultured under standardised conditions; 25 °C/20 °C 12 hr alternation, with a 12 hr photoperiod and 75% relative humidity in the fungal growth chamber. Species of *Alternaria* were cultured on V8[®] juice agar, whilst *Fusarium* species were cultured on Carnation Leaf Agar (CLA) and all other microfungi were cultured on Czapek Yeast Agar (CYA). Spores were harvested from the microfungi at 7 days growth. Spores of *Coprinus comatus*, *Psilocybe cubensis* and *Ustilago nuda* were obtained directly from the herbarium specimens. Fungal spores were mounted in permanent mounting medium (50 g gelatine, 150 ml glycerine and 7 g phenol in 350 ml distilled water) on microscope slides for image analysis. *Fusarium* conidia were stained in 0.8% trypan blue, prior to mounting in permanent mountant, to facilitate imaging of the naturally hyaline conidia. *Fusarium* conidia were placed into a small drop of lactic acid on the microscope slide to aid in the uptake of the stain, followed by a small drop of 0.8% trypan blue with mixing and finally a drop of permanent mountant and a coverslip. At least one minute was allowed between operations. All other spores were mounted in their natural state.

2.2 Image analysis

Image analysis was conducted using an Olympus BH-2 light microscope, a high resolution video camera (1000×1000 square pixels at 256 grey levels) and WIT[®] (Logical Vision) image processing software. Neutral density filters were used to ensure that the light source operated at its correct colour temperature. Specimens were imaged at 50×, 100× and 200×, using the 20× or 40× objective with either a 2.5× or 5× transfer lens, depending upon the spore size. Measurements were converted to micrometres by simple conversion factors. Programs for feature extraction were developed in-house using the WIT[®] programming environment. Features extracted from fungal spores by image analysis included 7 basic features; length, width, width:length ratio, area, form factor, perimeter and roundness; and 17 more complex features; equivalent circular diameter (ECD), compactness, box area, box ratio, radius (min. and max.), modification ratio, sphericity, convex hull area,

Table 1. Fungal species, accession numbers and origin of cultures used in this study, together with the number of spores examined by image analysis.

Species	Accession no.	Origin	Spore type	# Spores imaged	
				Genera	Species
<i>Alternaria alternata</i>	FRR 2548	Sorghum, Qld	Conidium	300	100
<i>Alternaria brassicae</i>	DAR 33224	<i>Sisymbrium orientale</i> Purlewaugh, NSW	Conidium	100	100
<i>Alternaria carthami</i>	DAR 28810	Safflower, Trangie, NSW	Conidium	100	100
<i>Alternaria citri</i>	DAR 49340	<i>Citrus sinensis</i> , Gosford, NSW	Conidium	100	100
<i>Alternaria helianthi</i>	DAR 35921	Sunflower, Armidale, NSW	Conidium	100	100
<i>Alternaria infectoria</i>	FRR 5010	E. Simmons, USA	Conidium	100	100
<i>Alternaria macrospora</i>	DAR 62319	Cotton, Emerald, Qld	Conidium	100	100
<i>Alternaria solani</i>	DAR 28484	Potato, Fitzroy Falls, NSW	Conidium	100	100
<i>Aspergillus fumigatus</i>	FRR 581	City refuse, Jakarta, Indonesia	Conidium	200	0
<i>Botrytis cinerea</i>	FRR 2509	Potato haulms, Stirling, UK	Conidium	200	0
<i>Cladosporium cladosporioides</i>	FRR 3027	Dried fish, Indonesia	Conidium	400	200
<i>Cladosporium herbarum</i>	FRR 2441	Oats, Germany	Conidium	200	0
<i>Cladosporium herbarum</i>	FRR 2459	Cottage cheese, Australia	Conidium	200	0
<i>Cladosporium herbarum</i>	IRM C7	Sultana, Mildura, NSW	Conidium	200	200
<i>Cladosporium macrocarpum</i>	FRR 4198	Cheddar cheese, Australia	Conidium	200	200
<i>Cladosporium sphaerospermum</i>	FRR 4292	Margarine, Australia	Conidium	200	200
<i>Coprinus comatus</i>	DAR 26968	Parramatta, NSW	Basidiospore	200	0
<i>Epicoccum nigrum</i>	DAR 27008a	<i>Sorghum bicolor</i> , Parramatta, NSW	Conidium	200	0
<i>Exserohilum rostratum</i>	FRR 3092	Sorghum, Australia	Conidium	200	0
<i>Fusarium moniliforme</i>	F 90058	Tan Linh, Vietnam Macro	Conidium	400	200
"	"	"	Microconidium	200	200
<i>Fusarium moniliforme</i>	F 11866	Maize kernels, Australia	Microconidium	200	0
<i>Fusarium oxysporum</i>	F 7396	Carnation stem, NT Macro	Conidium	200	200
<i>Fusarium oxysporum</i>	F 8455	Soil, Darwin, NT Micro	Conidium	200	200
<i>Fusarium solani</i>	F 7315	Red spot fish, NT	Macroconidium	200	200
"	"	"	Microconidium	200	200
<i>Penicillium chrysogenum</i>	FRR 821	<i>Hyssopus</i> , Norway	Conidium	200	0
<i>Psilocybe cubensis</i>	DAR 56658	Ballina, NSW	Basidiospore	200	0
<i>Ustilago nuda</i>	DAR 55914	<i>Hordeum</i> , Sweden	Teliospore	200	0

Mycological herbaria accession designations: DAR = NSW Agriculture, Orange, NSW 2800; FRR = Division of Food Science and Technology, CSIRO, North Ryde, NSW 2113; F = Fusarium Research Laboratory, University of Sydney, NSW 2006; IRM = Institute of Respiratory Medicine, University of Sydney, NSW 2006. # Spores imaged is the number of spores for which measurements were extracted by image analysis in genera and species comparisons.

convex hull perimeter, solidity, concavity, convexity, fibre length and fibre width (min., av. and max.) (Table 2) (Russ, 1995).

2.3 Genera comparison

The comparison of genera examined spores obtained from each of the fungal isolates listed in Table 1. Thus, for *Alternaria* a total of 1000 spores were examined, *Cladosporium* 1400 spores, *Fusarium* 1600 spores, and 200 spores for each of the other genera which were represented by single species. Only the seven basic feature measurements were used in this investigation and the genera, comprising all species, were analysed using both quadratic and linear discriminant analyses.

2.4 Species comparison

Species comparisons were conducted for eight species of *Alternaria*, four species of *Cladosporium* and three species of *Fusarium*, in four separate experiments. Isolates examined and the number of spores analysed are listed in Table 1 and comprised *Alternaria alternata* (Fr.) von Keissler, *A. brassicae* (Berk.) Sacc., *A. carthami* Chowdhury, *A. citri* Ellis and Pierce emend. Bliss and Fawcett, *A. helianthi* (Hansf.) Tubaki and Nishihara, *A. infectoria* Simmons, *A. macrospora* Zimm., *A. solani* (Ellis and Martin) Sorauer, *Cladosporium cladosporioides* (Fresen.) de Vries, *C. herbarum* (Persoon: Fries) Link ex S.F. Gray, *C. macrocarpum* Preuss, *C. sphaerospermum* Penz., *Fusarium moniliforme* Sheldon, *F. oxysporum* Schlechtendahl: Fries and *F. solani* (Martius) Saccardo.

Table 2. Morphological feature measurements extracted from fungal spores by image analysis.

Feature	Formula	Units
Length (L)	Maximum projection	μm
Width (W)	Minimum orthogonal to length	μm
LW Ratio (LWR)	$\frac{\text{Width}}{\text{Length}}$	None
Area (A)	Number of Pixels	μm^2
Form Factor (FF)	$\frac{4\pi A}{P^2}$	None
Perimeter (P)	Perimeter length	μm
Roundness (R)	$\frac{4A}{\pi L^2}$	None
Equivalent circular diameter (ECD)	$\sqrt{\frac{4A}{\pi}}$	μm
Compactness (C)	$\frac{\text{ECD}}{L}$	None
Box area (BXA)	Bounding rectangle	μm^2
Box ratio (BXR)	$\frac{A}{\text{BXA}}$	None
Minimum radius (MinR)	Minimum inscribed radius at centroid	μm
Maximum radius (MaxR)	Maximum circumscribed radius at centroid	μm
Modification ratio (ModR)	$\frac{2 \cdot \text{MinR}}{L}$	None
Sphericity (SP)	$\frac{\text{MinR}}{\text{MaxR}}$	None
Convex hull area (CHA)	Area of convex hull	μm^2
Convex hull perimeter (CHP)	Perimeter length of convex hull	μm
Solidity (S)	$\frac{A}{\text{CHA}}$	None
Concavity (CCav)	$\text{CHA} - A$	μm^2
Convexity (CVex)	$\frac{\text{CHP}}{P}$	None
Fibre length (FL)	Length of medial axis	μm
Average fibre width (FW)	$\frac{\sum \text{Width at medial axis}}{\text{FL}}$	μm
Min fibre width (MinFW)	Min (width at medial axis)	μm
Max fibre width (MaxFW)	Max (width at medial axis)	μm

The first experiment examined spores of *Cladosporium*, *Fusarium* and *Alternaria* species, as a subset of the large genera study, using only the seven basic feature measurements. The number of spores analysed in this study are shown in Table 1 as the number used for genera comparisons and comprised 1000 *Alternaria*, 1400 *Cladosporium* and 1600 *Fusarium* spores. Linear and quadratic discriminant analysis was performed on the feature measurements obtained.

The second experiment examined spores of *Cladosporium*, *Fusarium* and *Alternaria*, with 20 features extracted. *Cladosporium* and *Fusarium* species were examined using 200 spores (including 200 macroconidia and 200 microconidia of *Fusarium*), whilst 100 spores were examined for each *Alternaria* species. The 20 features extracted were; length, width, width:length ratio, area, form factor, perimeter, roundness, ECD, compactness, box area, box ratio, radius (min. and max.), modification ratio, sphericity, convex hull area, convex hull perimeter, solidity, concavity and convexity. Only linear

discriminant analysis was applied to the measurements obtained.

In the third experiment only *Cladosporium* and *Fusarium* species were analysed with the same 20 features extracted from 200 spores of each species, including 200 macroconidia and 200 microconidia of *Fusarium* species. Only linear discriminant analysis was applied to the measurements obtained.

In the fourth experiment *Alternaria* species were examined in isolation, with 100 spores analysed and all 24 (7 + 17) features extracted. The additional four features used in this investigation were fibre length and fibre width (min., av. and max.). Only linear discriminant analysis was applied to the measurements obtained.

2.5 Statistical analysis

Linear and Quadratic discriminant analysis were applied to the image analysis measurements obtained in the comparison of genera, and species comparisons

Table 3. Seven basic morphological feature measurements (means) of fungal spores obtained by image analysis.

Species	Accession #	Spore type	Length μm	Width μm	W:L ratio	Area μm^2	Perimeter μm	Form factor	Roundness
<i>Alternaria alternata</i>	FRR 2548	C	28.88	10.84	0.40	244.30	67.21	0.69	0.39
<i>Alternaria brassicae</i>	DAR 33224	C	151.16	16.31	0.11	1687.41	316.26	0.22	0.10
<i>Alternaria carthami</i>	DAR 28810	C	95.13	19.24	0.22	1002.09	280.44	0.18	0.15
<i>Alternaria citri</i>	DAR 49340	C	27.02	10.32	0.42	217.52	63.01	0.72	0.42
<i>Alternaria helianthi</i>	DAR 35921	C	111.59	25.80	0.23	2238.00	239.33	0.49	0.23
<i>Alternaria infectoria</i>	FRR 5010	C	29.19	8.72	0.33	193.01	65.10	0.62	0.32
<i>Alternaria macrospora</i>	DAR 62319	C	106.62	25.40	0.27	1449.27	347.72	0.17	0.19
<i>Alternaria solani</i>	DAR 28484	C	113.60	16.81	0.15	1240.56	280.46	0.21	0.13
<i>Aspergillus fumigatus</i>	FRR 581	C	3.12	2.84	0.91	7.02	7.91	1.41	0.91
<i>Botrytis cinerea</i>	FRR 2509	C	9.25	6.89	0.75	50.01	22.50	1.24	0.75
<i>Cladosporium cladosporioides</i>	FRR 3027	C	8.07	4.63	0.64	30.61	20.45	0.90	0.63
<i>Cladosporium herbarum</i>	FRR 2441	C	10.99	5.05	0.50	44.82	23.30	1.04	0.50
<i>Cladosporium herbarum</i>	FRR 2459	C	6.45	3.63	0.60	18.64	14.29	1.14	0.59
<i>Cladosporium herbarum</i>	IRM C7	C	7.16	4.49	0.69	25.95	18.67	0.93	0.69
<i>Cladosporium macrocarpum</i>	FRR 4198	C	17.72	8.10	0.47	113.21	41.57	0.82	0.47
<i>Cladosporium sphaerospermum</i>	FRR 4292	C	5.67	3.45	0.69	15.63	14.5	20.94	0.69
<i>Coprinus comatus</i>	DAR 26968	B	15.76	10.85	0.69	134.72	37.75	1.18	0.69
<i>Epicoccum nigrum</i>	DAR 27008a	C	18.41	16.92	0.92	247.03	50.12	1.22	0.92
<i>Exserohilum rostratum</i>	FRR 3092	C	87.26	17.33	0.24	1161.23	162.18	0.62	0.23
<i>Fusarium moniliforme</i>	F 90058	M	35.84	3.60	0.10	93.20	75.92	0.20	0.09
"	"	m	7.83	2.02	0.26	12.56	17.31	0.52	0.26
<i>Fusarium moniliforme</i>	F 11866	m	7.84	1.81	0.24	11.16	14.05	0.72	0.23
<i>Fusarium oxysporum</i>	F 7396	M	33.87	4.51	0.13	102.59	75.86	0.22	0.11
<i>Fusarium oxysporum</i>	F 8455	m	4.76	1.58	0.34	5.92	10.28	0.70	0.34
<i>Fusarium solani</i>	F 7315	M	43.15	5.73	0.13	168.33	94.42	0.24	0.12
"	"	m	9.97	2.59	0.28	21.30	21.19	0.59	0.28
<i>Penicillium chrysogenum</i>	FRR 821	C	2.97	2.75	0.93	6.47	7.50	1.44	0.93
<i>Psilocybe cubensis</i>	DAR 56658	B	15.30	9.28	0.61	111.57	34.81	1.15	0.61
<i>Ustilago nuda</i>	DAR 55914	T	8.28	7.35	0.89	47.94	21.57	1.29	0.89

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in Experiment 1. Only linear discriminant analysis was applied to the results of Experiments 2, 3 and 4. Linear discriminant analysis partitions the feature space (i.e. the multi-dimensional space where each measurement feature is plotted as a separate axis or dimension) into distinct regions that are separated by flat planes or hyperplanes, whilst quadratic discriminant analysis defines the regions with curved planes or hyperplanes. Quadratic discrimination is considered more robust in the face of increasing data co-variance but requires more test data in order to define these more complex discriminant functions. In this present study sufficient data was available for both linear and quadratic analysis to be performed in experiment 1,

but only linear discriminant analysis was able to be applied in experiments 2, 3 and 4.

3. Results

3.1 Measurements by image analysis

Average measurements obtained for the seven basic morphological features are presented in Table 3. Distinct similarities were observed between the spores of the 23 species from the measurements of length, width, area and perimeter length and the derived width:length ratio, form factor and roundness para-

meters. Noticeable similarities were observed between the conidia of *A. alternata*, *A. citri* and *A. infectoria*; *C. sphaerospermum* and *C. herbarum*; *A. fumigatus* and *P. chrysogenum*; *Psilocybe cubensis* and *Coprinus comatus*; *Ustilago nuda* and *Botrytis cinerea*; *F. moniliforme* and *F. oxysporum* macroconidia and *F. moniliforme* and *F. solani* microconidia. Similarities between the spores of different genera and species can be seen in the scatter plots generated from the first two discriminant variables, that clearly show some overlap between taxa (Figures 1 and 2). Despite these similarities, statistical discrimination involving all discriminant variables (i.e. not just the first two) resulted in high levels of discrimination between the various genera and between different species.

3.2 Genera comparison

Comparisons of the morphological features of conidia, basidiospores and teliospores belonging to eleven different fungal genera using image analysis of seven basic morphological features and quadratic discriminant analysis provided a high level of separation between genera (Table 4). Conidia belonging to *Cladosporium*, *Epicoccum* and *Fusarium* (both macro and microconidia) were distinguished from each other and all other imaged spores with 98% identification accuracy (Table 4). Spores of *Psilocybe*, *Exserohilum*, *Ustilago* and *Alternaria* were also identified with 92%, 89%, 86% and 83% accuracy, respectively (Table 4). *Psilocybe* spores were mistakenly identified as *Coprinus* (4%) or *Cladosporium* (3%) spores, whilst *Ustilago* spores were mistaken to be *Botrytis* spores in 12% of cases. *Exserohilum* spores were misidentified in 11% of cases as *Alternaria* spores, whilst *Alternaria* spores were misidentified as *Exserohilum* and *Cladosporium* spores 8% of the time.

Genera that were more difficult to identify included *Botrytis*, *Penicillium*, *Coprinus* and *Aspergillus* with 69%, 67%, 63% and 56% of spores accurately identified, respectively. *Botrytis* spores were most often misidentified as *Ustilago* teliospores (27%) and infrequently as *Cladosporium* conidia (4%). *Penicillium* and *Aspergillus* conidia were confused only with each other, but *Penicillium* conidia were identified with greater accuracy than *Aspergillus* conidia (Table 4).

3.3 Species comparison

In the first experiment examining eight species of *Alternaria*, four species of *Cladosporium* and three species of *Fusarium* in a study of all genera and

Table 4. Genera comparison: results of quadratic discriminant analysis.

Genus	No. of species	Correct ID %	Confusing spores (>2%)	Confusion %
<i>Alternaria</i>	8	83	<i>Cladosporium</i>	8
			<i>Exserohilum</i>	8
<i>Aspergillus</i>	1	56	<i>Penicillium</i>	41
<i>Botrytis</i>	1	69	<i>Ustilago</i>	27
			<i>Cladosporium</i>	4
<i>Cladosporium</i>	4	98		
<i>Coprinus</i>	1	63	<i>Psilocybe</i>	32
			<i>Epicoccum</i>	4
<i>Epicoccum</i>	1	98		
<i>Exserohilum</i>	1	89	<i>Alternaria</i>	11
<i>Fusarium</i>	3	98		
<i>Penicillium</i>	1	67	<i>Aspergillus</i>	32
<i>Psilocybe</i>	1	92	<i>Coprinus</i>	4
			<i>Cladosporium</i>	3
<i>Ustilago</i>	1	86	<i>Botrytis</i>	12

species, with only seven basic feature measurements, conidia belonging to several species were identified with high levels of precision by quadratic discriminant analysis. Conidia belonging to *Cladosporium macrocarpum*, *C. sphaerospermum*, *Alternaria helianthi*, *A. brassicae* and macroconidia of *Fusarium oxysporum* and *F. solani* and microconidia of *F. moniliforme* and *F. oxysporum* were identified with greater than 90% accuracy (Table 5). *Alternaria helianthi* and *C. macrocarpum* were the most readily identifiable spores by image analysis of only seven features, with 97% and 96% of spores correctly identified, respectively. The most difficult conidia to differentiate, on the basis of only seven features, were those belonging to *C. cladosporioides*, *A. infectoria* and *A. macrospora* with 26%, 37% and 47% of spores identified correctly. All other conidia imaged were identified correctly more than 55% of the time (Table 5).

In the second experiment, which examined conidia of *Cladosporium*, *Fusarium* and *Alternaria* only, with 20 features extracted and linear discriminant analysis of feature measurements, marked differences were seen in comparison with the first experiment results (Table 5). The accuracy of identification improved for ten species, but declined for eight species (Table 5). The most notable improvements occurred for conidia belonging to *C. cladosporioides*, *A. carthami*, *A. macrospora*, *A. infectoria*, *F. moniliforme* macroconidia and *F. solani* microconidia. Although there

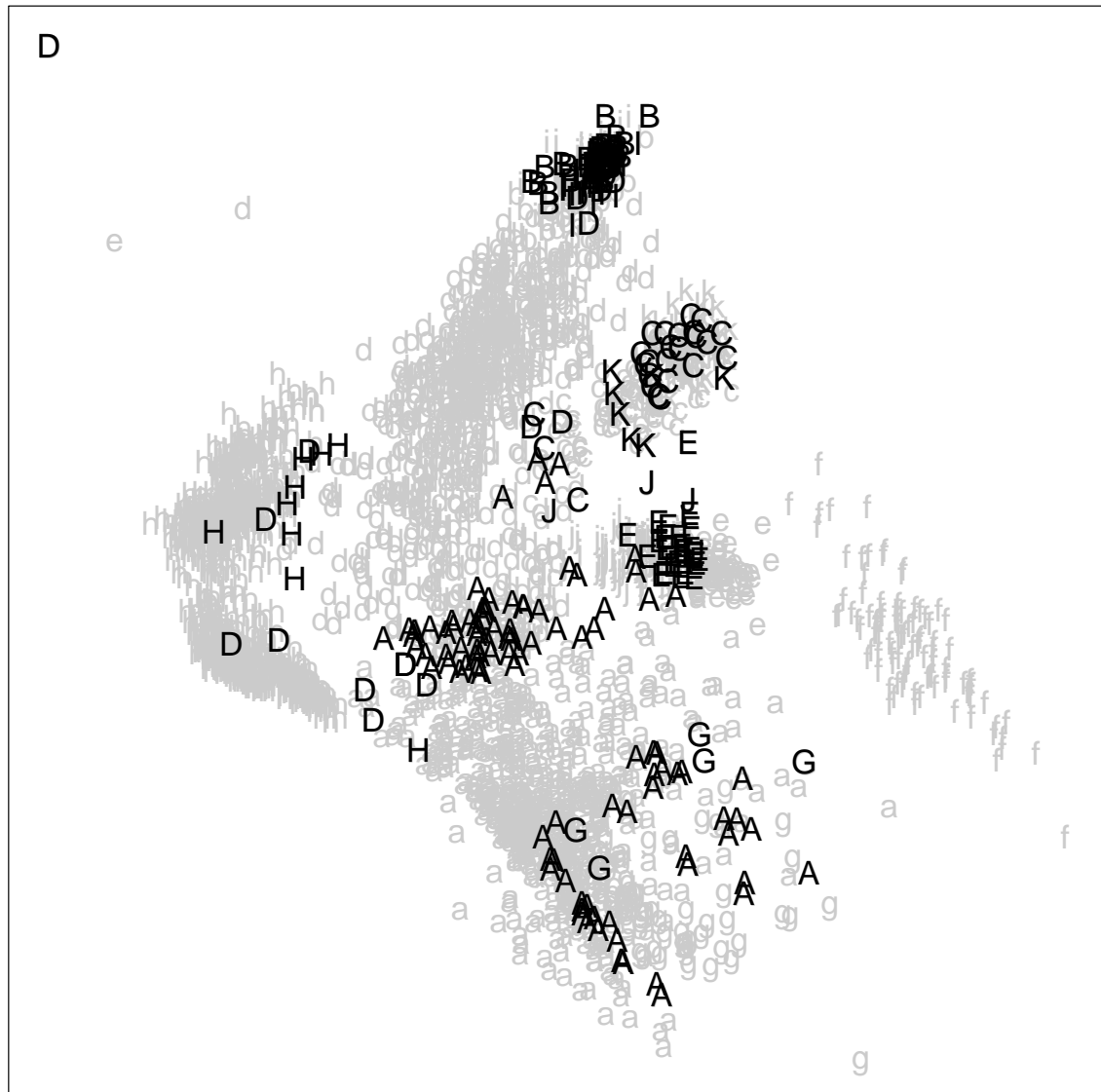


Figure 1. Scatter plot of the first and second discriminants in non-dimensional units of spores belonging to different genera based on imaged spore morphology (seven basic features) and quadratic discriminant analysis

Legend

- | | |
|-----------------------|------------------------|
| a. <i>Alternaria</i> | b. <i>Aspergillus</i> |
| c. <i>Botrytis</i> | d. <i>Cladosporium</i> |
| e. <i>Coprinus</i> | f. <i>Epicoccum</i> |
| g. <i>Exserohilum</i> | h. <i>Fusarium</i> |
| i. <i>Penicillium</i> | j. <i>Psilocybe</i> |
| k. <i>Ustilago</i> | |

Alphabetical letters (a to k) represent the actual genus to which spores belong. Lower case and lightly shaded letters represent spores that have been correctly classified by image analysis. Capital and boldface letters represent spores that have been misclassified by image analysis. The genus to which misclassified spores have been assigned by image analysis is not indicated.

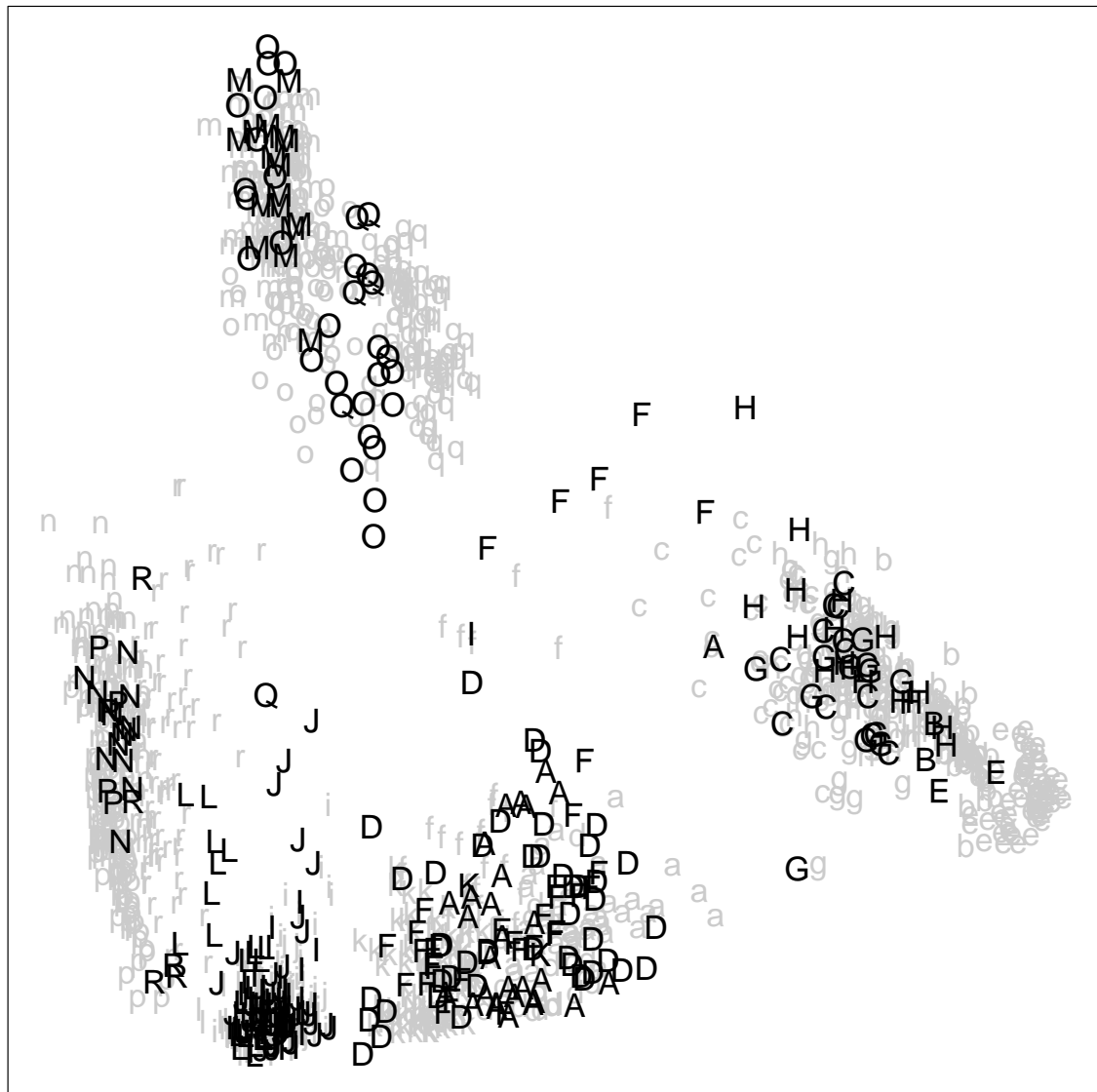


Figure 2. Scatter plot of the first and second discriminants in non-dimensional units of spores belonging to species of *Cladosporium*, *Fusarium* and *Alternaria* based on imaged spore morphology (20 features) obtained in Experiment 2 and linear discriminant analysis

Legend

- | | |
|---|---|
| a. <i>Alternaria alternata</i> | b. <i>Alternaria brassicae</i> |
| c. <i>Alternaria carthami</i> | d. <i>Alternaria citri</i> |
| e. <i>Alternaria helianthi</i> | f. <i>Alternaria infectoria</i> |
| g. <i>Alternaria macrospora</i> | h. <i>Alternaria solani</i> |
| i. <i>Cladosporium cladosporioides</i> | j. <i>Cladosporium herbarum</i> |
| k. <i>Cladosporium macrocarpum</i> | l. <i>Cladosporium sphaerospermum</i> |
| m. <i>Fusarium moniliforme</i> macroconidia | n. <i>Fusarium moniliforme</i> microconidia |
| o. <i>Fusarium oxysporum</i> macroconidia | p. <i>Fusarium oxysporum</i> microconidia |
| q. <i>Fusarium solani</i> macroconidia | r. <i>Fusarium solani</i> microconidia |

Alphabetical letters (a to r) represent the actual species to which spores belong. Lower case and lightly shaded letters represent spores that have been correctly classified by image analysis. Capital and boldface letters represent spores that have been misclassified by image analysis. The species to which misclassified spores have been assigned by image analysis is not indicated.

Table 5. Species comparisons: discrimination of *Cladosporium*, *Fusarium* and *Alternaria* conidia.

Species experiment	1	2	3	4
Scope of analysis	All genera and species	<i>Alternaria</i> , <i>Cladosporium</i> and <i>Fusarium</i>	<i>Cladosporium</i> & <i>Fusarium</i> species	<i>Alternaria</i> species only
No. features	7	20	20	24
Discriminant analysis	Quadratic	Linear	Linear	Linear
	Correct identification %			
<i>C. cladosporioides</i>	26	51	60	–
<i>C. herbarum</i>	64	43	50	–
<i>C. macrocarpum</i>	92	97	99	–
<i>C. sphaerospermum</i>	96	60	84	–
<i>F. moniliforme</i> (macroconidia)	66.5	85	83	–
<i>F. moniliforme</i> (microconidia)	94	86	93	–
<i>F. oxysporum</i> (macroconidia)	92	70	78	–
<i>F. oxysporum</i> (microconidia)	93	92	96	–
<i>F. solani</i> (macroconidia)	92	86	95	–
<i>F. solani</i> (microconidia)	68	81	94	–
<i>A. alternata</i>	78.5	53	–	43
<i>A. citri</i>	67	30	–	50
<i>A. infectoria</i>	37	63	–	80
<i>A. helianthi</i>	97	100	–	97
<i>A. brassicae</i>	90	93	–	100
<i>A. carthami</i>	53	80	–	80
<i>A. macrospora</i>	47	73	–	87
<i>A. solani</i>	73	77	–	80

Bold numbering indicates the highest level of identification accuracy achieved for that species and spore type.

was only 3% improvement in the identification of *A. helianthi* conidia, this resulted in 100% accuracy for identification of this species, the best result attained for this species in any of the experiments. Macroconidia of *F. moniliforme* were also identified with the greatest accuracy (85%) in this experiment. Conidia of *A. brassicae* were equally well identified (100%) in this experiment with 20 features and in Experiment 4 with 24 features and only *Alternaria* spores. Large declines in the accuracy of identification occurred for conidia belonging to *A. citri*, *C. sphaerospermum*, *A. alternata*, *C. herbarum* and *F. oxysporum* macroconidia, from Experiment 1 to 2.

The large declines in accuracy apparent in Experiment Two were largely offset by the removal of *Alternaria* species from the analysis in Experiment Three. The accuracy of identification improved in this analysis for most *Fusarium* and *Cladosporium* species. Improvements, from Experiment Two, were most noticeable for the identification of conidia of *C. sphaerospermum* and *C. cladosporioides* microconidia and macroconidia of *F. solani*. The

highest levels of identification accuracy, compared with all other experiments, were recorded for *C. cladosporioides*, *C. macrocarpum*, microconidia of *F. oxysporum* and both macroconidia and microconidia of *F. solani*, in this experiment with 20 features, with percent correct identifications of 60, 99, 96, 95 and 94, respectively (Table 5).

In the fourth experiment, which focussed on *Alternaria* species only, with the full set of 24 features, five of the eight species achieved their highest percentage of correctly identified conidia (Table 5). Conidia belonging to *A. brassicae* were identified with 100% accuracy, with other top scores for *A. macrospora* (87%), *A. solani*, *A. carthami* and *A. infectoria*, each with 80% of conidia correctly identified by image analysis. Accuracy of identification of conidia belonging to *A. alternata*, *A. citri* and *A. helianthi* was less than was achieved in the other experiments (Table 5).

Table 6. Misclassification rates by linear and quadratic methods of discriminant analysis.

Experiment	Linear	Quadratic
Genera comparison	15.9	10.2
Species – Experiment 1	33.8	25.9
Experiment 2	25.6	NA
Experiment 3	16.8	NA
Experiment 4	22.9	NA

3.4 Statistical analysis

Quadratic discriminant analysis provided the greatest discrimination between genera and all species in Experiment 1 of the species comparisons. The overall misclassification rates for genera and species comparisons by both linear and quadratic discriminant analysis are presented in Table 6. Only 10.2% of spores of genera were misclassified by quadratic discriminant analysis, compared with 15.9% misclassified by linear discriminant analysis. Similarly, 25.9% of spores of *Cladosporium*, *Fusarium* and *Alternaria* were misclassified by quadratic discriminant analysis, compared with 33.8% by linear discriminant analysis in Experiment 1. Misclassification rates in species comparisons were lowest in the discrimination of *Cladosporium* and *Fusarium* spores (Experiment 3) with 16.8% of conidia misclassified. The other species comparisons produced similar misclassification results, with 22.9% of *Alternaria* conidia misclassified in Experiment 4 and 25.6% of *Cladosporium*, *Fusarium* and *Alternaria* conidia misclassified in Experiment 2.

Figures 1 and 2 depict the overall discrimination between genera, and species of *Alternaria*, *Cladosporium* and *Fusarium*, respectively, based on the first two discriminant variables only, as it is not physically possible to depict the discrimination between taxa based on all variables, as a 7 or 20 dimensional space would be required for genera and species, respectively. The similarity in shape between spores of different taxa is evidenced by the proximity of their clusters in the two-dimensional space (Figures 1 and 2). In Figure 1, the cluster of letters representing *Fusarium* (h), *Epicoccum* (f) and *Cladosporium* (d) are quite distinct and further removed from the other taxa than *Penicillium* (i) and *Aspergillus* (b), the clusters of which reside in the same space at the top of the diagram. Similarly, *Ustilago* (k) and *Botrytis*

(c), *Psilocybe* (j) and *Coprinus* (e), and *Alternaria* (a) and *Exserohilum* (g) are situated close together, with some overlap between the genera. These groupings broadly illustrate the results presented in Table 4, where *Fusarium*, *Epicoccum* and *Cladosporium* spores were identified with 98% accuracy, but *Penicillium* and *Aspergillus* spores were more difficult to differentiate, as were spores belonging to *Ustilago* and *Botrytis*, *Psilocybe* and *Coprinus*, and *Alternaria* and *Exserohilum*.

In Figure 2, distinct groupings are evident, with the macroconidia of all *Fusarium* species (m, o and q) clustered together in the top left corner of the diagram. Microconidia of all *Fusarium* species (n, p and r) are clustered together in the bottom left corner of the diagram, whilst there is considerable overlap between the spore shape of three *Cladosporium* species; *C. cladosporioides* (i), *C. herbarum* (j) and *C. sphaerospermum* (l), clustered nearby (Figure 2). The fourth *Cladosporium* species; *C. macrocarpum* (k) possesses spores which display considerable similarity with those of *Alternaria alternata* (a), *A. citri* (d) and *A. infectoria* (f), all of which are short-beaked *Alternaria* species. The variability in shape or size of spores belonging to *A. infectoria* is evidenced by the spread of this taxon in the diagram. The long-beaked *Alternaria* species; *A. brassicae* (b), *A. carthami* (c), *A. macrospora* (g) and *A. solani* (h) have clustered together (right of centre), with the distinctive spores of *A. helianthi* (e) residing further to the right of this group. These groupings broadly illustrate the results presented for Experiment 2 in Table 5, where spores of *A. helianthi* were identified with 100% accuracy, but spores belonging to *A. alternata*, *A. citri*, *A. infectoria*, *C. cladosporioides*, *C. herbarum* and *C. sphaerospermum* were identified with less than 63% accuracy.

Consultation of the average measurement data (Table 3), together with experiment results (Table 4 and 5) and scatter plots (Figures 1 and 2) allow for a thorough understanding of the similarities and differences between the spores of each of the taxa examined.

4. Discussion

The measurement of only seven basic shape parameters facilitated discrimination of spores belonging to seven of the eleven investigated genera with greater than 82% accuracy. Genera that were more diffi-

cult to differentiate included *Aspergillus*, *Penicillium*, *Coprinus* and *Botrytis* with the confounding spores belonging mostly to *Penicillium*, *Aspergillus*, *Psilocybe* and *Ustilago*, respectively. Examination of the feature measurements recorded in Table 3 explains the inability of the image analysis program to differentiate well between spores of these genera, for there exists great similarity between the spore measurements for these genera. Reference to traditional taxonomic treatises confirms the similarity of the basic morphological parameters; length and width, between the genera representatives used in this study (Punithalingam and Waterston, 1970; Shepherd and Totterdell, 1988; Pitt, 1991; Klich and Pitt, 1994). However, spores belonging to *Botrytis* and *Ustilago*, and *Alternaria*, *Cladosporium* and *Exserohilum* possess distinctive characteristics which are recognised by the human eye and thus, spores belonging to these genera would never be confused with one another. In contrast, it is not surprising that conidia of *Aspergillus* and *Penicillium* were confused with one another by image analysis, as these conidia lack features distinctive to the human eye.

For several species, the highest values for correct identification were obtained with only seven feature measurements, including conidia of *C. herbarum*, *C. sphaerospermum*, *F. moniliforme* (microconidia), *F. oxysporum* (macroconidia), *A. alternata* and *A. citri*. This is despite the large similarities in morphological parameters observed between *C. sphaerospermum* and *C. herbarum* and *A. alternata* and *A. citri*. These species and *C. cladosporioides* produce variable conidial forms, in terms of both spore shape and size, which accounts for the overlap between species (Ellis, 1971). The variability within *C. herbarum* is apparent from the measurements recorded in Table 3, which demonstrate similarity between two isolates, with the third quite different. Surprisingly, extraction of further shape parameters resulted in improved identification of ten species, but reduced accuracy for eight species in Experiment 2. In the case of reduced identification accuracy, the larger number of feature measurements may have resulted in greater variability in the dataset for each species and thus less discriminatory information. In contrast, improved identification would have resulted from the measurements of the extra features verifying or enhancing those of the seven basic features, resulting in greater discriminatory power of the feature measurements.

A reduction in the number of species in comparative analyses to only species of *Cladosporium* and

Fusarium in Experiment 3, resulted in improved identification accuracy of spores belonging to all seven species compared with the results of Experiment 2. This appears to be a direct result of the reduction of the number of species in the analysis, and conidia of four species were identified with the greatest accuracy in this experiment. However, values for three species (*C. herbarum*, *C. sphaerospermum* and *F. oxysporum*) were noticeably lower than recorded with only seven basic feature measurements, so there was no added benefit from the extraction of 20 features from these species for discrimination.

The identification and discrimination of conidia belonging to *Alternaria* species was greatly improved by the extraction of 24 features from species with long-beaked spores. The four long-beaked spore types, belonging to *A. brassicae*, *A. carthami*, *A. macrospora* and *A. solani* were identified with greatest precision using all 24 feature measurements. In addition, the larger short-beaked spores of *A. infectoria* were also more easily identified by the 24 feature measurements. However, the smaller short-beaked spores of *A. alternata* and *A. citri* were identified with greatest accuracy from only seven basic feature measurements.

The accuracy of image analysis routines can be improved by refining the selection of features implemented in the analysis. The data obtained using shape parameters are now being analysed further to identify the essential or 'best' features for the overall discrimination of fungal spores and specific genera. Hagen and Hagen (1995) in their study of macroconidia of *Fusarium sambucinum sensu lato* strains by image analysis, reduced the number of features extracted from seven shape and size parameters to just three parameters; area, convexity and curvature. However, discrimination between the three, later formally recognised species, *F. venenatum* Nirenberg, *F. torulosum* (Berk. and Curt.) Nirenberg and *F. sambucinum* Fuckel *sensu stricto* (Nirenberg, 1995) was generally poor, with up to 70% of *F. torulosum* macroconidia misclassified as *F. sambucinum sensu stricto*. Oh et al. (1996), employed curvature based shape parameters (line, convex, concave and apical) to discriminate between germinated and non-germinated conidia of *Aspergillus fumigatus*, *Curvularia lunata* and *Fusarium solani* in an automated system. This study extends the work of Oh et al. (1996), by applying similar image analysis methods to the differentiation of a select group of non-viable spores for precise taxonomic identification.

The application of image analysis routines to the identification and differentiation of fungal spores in aerobiological samples would require further research using laboratory cultures to encompass a wider range of fungi and to refine the technique, as there are many complicating factors that would need to be overcome. These include the large range of fungal spores present in the environment, morphological variability due to environmental factors, the presence of inorganic particles and artefacts and the concentrations in which these spores and contaminating particles occur. Further analyses of laboratory samples are required to examine spores of Ascomycetes, Myxomycetes and Basidiomycetes and their similarities to the Imperfect Fungi investigated in this study. For instance, an image analysis routine for use in aerobiological sampling would need to be able to differentiate between macroconidia of *Fusarium* and ascospores of *Leptosphaeria*, as well as differentiating between *Pithomyces* conidia and short-beaked *Alternaria* conidia. Identification of *Sporobolomyces* and *Tilletiopsis* spores by image analysis would also aid aerobiological monitoring, as these spores are frequently and abundantly found in the air and are difficult to enumerate.

Image analysis of morphological features is clearly a powerful tool. However, it is likely that even with the extraction of a greater number of features, or with more detailed or powerful statistical analysis, these methods will never be 100 percent accurate at all times. This preliminary investigation into the ability of image analysis to differentiate between a select group of allergenic fungal spores prepared from laboratory cultures and herbarium specimens has demonstrated the potential of this technique, but further research is required before implementation in the field.

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