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Identification and phylogeny of spore-cyst fungi (*Ascosphaera* spp.) using ribosomal DNA sequences

D. L. ANDERSON[1], A. J. GIBBS[2] AND N. L. GIBSON[1]

[1] *CSIRO Division of Entomology, P.O. Box 1700, Canberra, ACT 2601, Australia*
[2] *Research School of Biological Science, Australian National University, Canberra ACT 2601, Australia*

The internal transcribed spacers, ITS1 and ITS2, and 5.8S region of ribosomal DNA (rDNA) from 20 species of *Ascosphaera* were amplified by PCR, and their sequences determined and compared. No variation was detected in the sequences from 23 widely distributed isolates of *A. apis*, in sequences from 11 widely distributed *A. atra* isolates, in four *A. aggregata* isolates, or in sequences from two isolates each of *A. acerosa*, *A. duoformis*, *A. flava*, *A. larvis*, *A. pollenicola* and *A. proliperda*. However, the ribosomal sequences from each of these nine species, and from another 11 species of which only a single isolate was examined, differed from one another by 0·18–30·9%. Thus these sequences provide a rapid method for identifying species, and searches using them showed that the sequence of *A. apis* rDNA recorded in the international databases is, in fact, that of *A. atra*.

The rDNA sequences also provided data for assessing the relationships of these fungi. Of the rDNA sequences in current international databases, that of *Eremascus albus* was very close to, but distinct from, those of the *Ascosphaera* species. Comparisons of the *Ascosphaera* sequences showed that most formed consistent clusters irrespective of the method of comparison used (distance matrix and parsimony), or whether the ITS1 or ITS2 portions were used; *A. acerosa* with *A. asterophora*, *A. atra* with *A. duoformis*, *A. colubrina* closely with *A. flava*, *A. larvis*, *A. major*, *A. variegata* and more distantly with *A. apis* and *A. celerrima*, and, also, *A. aggregata* with *A. subcuticulata*, *A. proliperda* and more distantly with *A. solina*. The apparent relationships of these clusters were inconsistent, depended on the alignment of several regions of 'indels', and could not be resolved. The *A. fusiformis*, *A. naganensis* and *A. osmophila* sequences showed inconsistent relationships with the others, especially that of *A. osmophila*, which had an *A. solina*-like ITS2 region, but an atypical ITS1 sequence with a large unique repetitive insertion. The clusters based on gene sequence comparisons clearly correlated with groupings based on ascospore morphology and other characters.

Spore-cyst fungi, *Ascosphaera* spp., are only found, in nature, associated with social and solitary bees of the superfamily Apoidea. The type species, *A. apis* (Maassen ex Claussen) L. S. Olive & Spiltoir, is an economically important pathogen of the honey bee, *Apis mellifera* L. (Maassen, 1913; Gilliam, 1978), and *A. aggregata* Skou is a serious pathogen of the pollinating leafcutting bee *Megachile rotundata* Fabricius (Skou, 1975; McManus & Youssef, 1984). Other species, such as *A. larvis* Bissett, *A. osmophila* Skou & Judith King, *A. proliperda* Skou and *A. subcuticulata* D. L. Anderson & N. L. Gibson are also pathogenic to bees (Skou & King, 1984; Youssef *et al.*, 1984; Bissett, 1988; Anderson & Gibson, 1997), but several have been reported to be saprotrophs in bee brood cells (Skou, 1975, 1988*a*, *b*; Skou & Hackett, 1979; Bissett, 1988; Skou & Holm, 1989; Anderson & Gibson, 1997).

The taxonomic affinities of the Ascosphaerales have been the focus of much debate (Skou, 1972, 1982*a*, 1988 *b*; Barr, 1983). Their taxonomy has traditionally been based on morphological characters and the ontogenesis of the fruiting bodies, or spore-cysts, structures not found in other fungi. Studies by Spiltoir (1955) and Spiltoir & Olive (1955) indicated that these fungi are ascomycetes because, in *A. apis*, spore-cysts develop from single-celled archicarps ('nutriocytes') into unicellular cyst-like structures containing ascogenous hyphae, asci and eventually balls of spores, called 'sporeballs'. Gäumann (1964) proposed that the Ascosphaeraceae, containing *Ascosphaera*, be accommodated in its own order, the Ascosphaerales, a taxonomy subsequently validated by Benny & Kimbrough (1980).

The groupings proposed by Gäumann (1964) were adopted by Eriksson (1984) and Eriksson & Hawksworth (1985) and supported by studies of McManus & Youssef (1984), who showed that spore-cyst development in *A. aggregata* was similar to that in *A. apis*. However, similar studies on the homothallic *A. atra* Skou & Hackett, by Kish *et al.* (1988), found a different ontogeny. These differences emphasize the difficulties in determining the taxonomic affinities of *Ascosphaera* and indicate that further comparative studies using other characters, such as genomic DNA sequence data, might be useful. Recently Berbee & Taylor (1992) studied the sequences of the 18S ribosomal DNAs (rDNAs) of 12 ascomycetes. Comparisons of these sequences confirmed the traditional ascomycete classes, the Plectomycetes, which have closed fruiting bodies, and the Pyrenomycetes, which have flask-shaped fruiting bodies. *A. apis* was found to be in the Plectomycetes, and close to *Eremascus albus* Eidam, a sapro-

trophic yeast-like ascomycete with a penchant for condiment mustard powder (Harrold, 1950).

Identification of *Ascosphaera* species has also proved difficult. Initial identification is based on morphological, biological and cultural characters. Skou (1988*a*), however, demonstrated that, although the size of ascospores seemed to be a reliable and stable character, many of the morphological characters used to identify *Ascosphaera* species vary greatly within each species. Fortunately some isolates can be cultured, and, for those that are heterothallic, mating tests are possible, provided both mating strains of each species are available, and can mate and sporulate *in vitro* on artificial culture media (Christensen & Gilliam, 1983). Homothallic species can only be identified by morphological, biological and cultural characters, which tend to be imprecise, and it is even more difficult to identify isolates that are difficult or impossible to culture. Hence it is clear that easier, more discriminatory and faster methods for identifying species of *Ascosphaera* are needed.

In the research we report in this paper, we amplified the ITS1–5.8S-ITS2 region of the rDNA of many species and isolates of *Ascosphaera* using the polymerase chain reaction (PCR). These rDNA fragments were then sequenced as a way of identifying *Ascosphaera* species and for assessing their phylogenetic relationships.

MATERIALS AND METHODS

Fungal isolates and culturing procedures

Details of the isolates and species of *Ascosphaera* examined are given in Table 1. Most were purchased from or supplied by fungal collections as pure cultures growing on slanted solid agar media in glass test-tubes plugged with cotton wool. Mycelia from each of these cultures were used for DNA extraction (see below). Species obtained from other sources were isolated on the surfaces of solid agar media of the types listed in Table 1, each medium containing 0·045 g l^{-1} of the antibiotics streptomycin sulphate and ampicillin (Boehringer–Mannheim) to inhibit bacterial growth. Pure, single-spore cultures of each of these species were obtained by the methods described by Anderson & Gibson (1997). Mycelia from each of these single-spore cultures were used for DNA extraction (see below). The identity of cultures was confirmed in mating tests (Christensen & Gilliam, 1983) and by comparing their morphological and culturing characteristics with those of known *Ascosphaera* species obtained from and identified by the Centraalbureau voor Schimmelcultures (CBS), Baarn, The Netherlands, the Agricultural Research Service Collection of Entomopathogenic Fungi (ARSEF), Ithaca, New York, U.S.A., and the Canadian Collection of Fungus Cultures (CCFC), Ontario, Canada. Identifications were also assisted by comparing the morphologies of fungal isolates with those of *Ascosphaera* species kindly lent to us by Dr J. P. Skou, Denmark.

The 20 species listed in Table 1 were used to assess the between-species rDNA sequence variation. Twenty-three isolates of *A. apis*, eleven of *A. atra*, four of *A. aggregata*, and two each of *A. acerosa* Bissett, G. Duke & Goettel, *A. duoformis* D. L. Anderson & N. L. Gibson, *A. flava* D. L. Anderson & N. L. Gibson, *A. larvis*, *A. pollenicola* Bissett and *A. proliperda* were used to test for within-species rDNA sequence variation. The *A. apis* and *A. atra* isolates came from widely distributed locations, including New Zealand, Australia and Europe (Table 1), and this allowed us to assess the amount of geographical variation in these species.

To prepare an individual isolate for DNA extraction, four 50 ml aliquots of broth culture medium in 250 ml glass flasks were inoculated with mycelium from a pure growing culture. The liquid media used are listed in Table 1. Inoculated medium was placed in a rotating water bath at 18–33 °C for up to 21 d (but usually for 6 d) or until solid clumps of mycelium developed. Some mycelia were then removed to the surface of solid agar medium and allowed to grow and sporulate so that the culture's purity and identity could be confirmed. The remainder of the mycelia were drained of liquid culture medium by vacuum, washed with dH_2O, dried, compressed between sheets of clean tissue paper and frozen at −20° until needed for DNA extraction.

DNA extraction

For DNA extraction, 0·4–0·5 g of compressed mycelium of each species and isolate was ground in liquid nitrogen to a fine powder using a pre-cooled pestle and mortar. Then, 3 ml of freshly prepared proteinase K buffer (2·0 mg milk proteinase K, 0·1 M Tris-HCl pH 8·5, 0·05 M EDTA NaCl and 1% SDS) was added, the mixture held in a water bath at 65° for 1 h, then extracted with an equal volume of phenol saturated with 10 mM Tris-HCl, and 1 mM EDTA pH 8 (TE) at room temperature for 15 min. The phases were separated by centrifuging at 2000 ***g*** for 10 min, the supernatant moved to a clean centrifuge tube, extracted as above with an equal volume of chloroform–isoamyl alcohol (24:1), and centrifuged to separate the phases. The supernatant was once more removed to a clean centrifuge tube where 2 vol. of cold ethanol were added to precipitate the DNA. The DNA was then pelleted by centrifuging at 6000 ***g*** for 10–15 min at 4°, resuspended in 75% ethanol, pelleted again at 6000 ***g*** for 5–10 min, dried at room temperature, resuspended in 500 µl TE containing 10 µg ml^{-1} RNase A and incubated at 37° for 30 min. This solution was extracted once with an equal volume of phenol–chloroform–isoamyl alcohol (25:24:1) as described above and centrifuged to separate the phases. The supernatant was removed to a clean centrifuge tube, 50 µl of 3 M sodium acetate pH 6·0 added and the DNA precipitated with 2·5 vol. of cold ethanol. Precipitated DNA was pelleted, resuspended and dried as described above, then resuspended in 200 µl of dH_2O and frozen at −20° until needed for sequencing.

rDNA amplification and sequencing

The nuclear rDNA region containing the internal transcribed spacer regions and 5.8S rDNA (ITS1–5.8S–ITS2) was amplified by PCR (Saiki *et al.*, 1988; Saiki, 1990) using the forward primer 5′-GTTTCCGTAGGTGAACCTGC-3′ (TW81) and reverse primer 5′-ATATGCTTAAGTTCAGCGGGT-3′ (AB28) (Bowman *et al.*, 1992) as described by Curran *et al.*

Table 1. Details of the *Ascosphaera* species and isolates used in this study

Species†	Host	Locality	Source* (Accession number) [Culture medium]‡
A. acerosa	*Megachile* sp.	Australia	ARSEF (5143) [SDA]
A. acerosa	*Megachile* sp.	Australia	CBS (400.96) [SDA]
A. aggregata	*Megachile rotundata*	U.S.A.	ARSEF (689)[SDAY2]
A. aggregata	Unknown	Unknown	CCFC (DAOM 211346) [SDA]
A. aggregata (2)	*M. rotundata*	U.S.A.	R. Bitner [MA20G]
A. apis (8)	*Apis mellifera*	Germany	W. Ritter [YGPSA]
A. apis	*A. mellifera*	Australia	ARSEF (5142) [YGPSA]
A. apis (3)	*A. mellifera*	Australia	D. Anderson [YGPSA]
A. apis	*A. mellifera*	New Zealand	H. Giacon [YGPSA]
A. apis (7)	*A. mellifera*	United Kingdom	A. Matheson [YGPSA]
A. apis	*A. mellifera*	U.S.A.	AQIS [YGPAA]
A. apis	*A. mellifera*	U.S.A.	CCFC (DAOM 139491) [SDA]
A. apis	*A. mellifera*	Unknown	CBS (138.22) [MA40S]
A. asterophora	*M. rotundata*	U.S.A.	CBS (152.80) [SDA]
A. atra	*M. rotundata*	Canada	CCFC (DAOM 186442) [SDA]
A. atra	*M. rotundata*	U.S.A.	CBS (524.75) [SDA]
A. atra	*A. mellifera*	New Zealand	AQIS [SDA]
A. atra	Unknown	Netherlands	CBS (506.82) [MA2]
A. atra	Unknown	Netherlands	CBS (248.82) [MA2]
A. atra	*A. mellifera*	Australia	ARSEF (5147) [SDA]
A. atra	*A. mellifera*	Australia	CBS (401.96) [SDA]
A. atra (3)	*A. mellifera*	Australia	D. Anderson [SDA]
A. atra	*A. mellifera*	Germany	W. Ritter [SDA]
A. celerrima	*Osmia cornifrons*	Japan	CBS (390.87) [MA2Y60G]
A. colubrina	*M. rotundata*	Canada	CBS (160.87) [MA20S]
A. duoformis	*A. mellifera*	Australia	ARSEF (5141) [YGPSA]
A. duoformis	*Trigona carbonaria*	Australia	CBS (403.96) [YGPSA]
A. flava	*Megachile* sp.	Australia	ARSEF (5144) [MA20G]
A. flava	*Megachile* sp.	Australia	CBS (399.96) [MA20G]
A. fusiformis	*O. cornifrons*	Japan	CBS (373.87) [MA2Y60G]
A. larvis	*M. rotundata*	Canada	CBS (163.87) [MA20S]
A. larvis	*M. rotundata*	Canada	CCFC (DAOM 186444) [MA20S]
A. major	Hymenoptera	Denmark	ARSEF (694) [SDAY2]
A. naganensis	*O. cornifrons*	Japan	CBS (374.87) [MA2Y60G]
A. osmophila	*Chalicodoma mystaceana*	Australia	CBS (381.83) [MA20S]
A. pollenicola	*M. rotundata*	Canada	CBS (159.87) [MA20S]
A. pollenicola	*M. rotundata*	Canada	CCFC (DAOM 188842) [MA20S]
A. proliperda	*M. centuncularis*	Denmark	CBS (827.70) [MA20S]
A. proliperda	Unknown	Denmark	ARSEF (695) [SDAY2]
A. solina	(Hymenoptera: Colletidae)	Australia	ARSEF (5146) [YGPSA]
A. subcuticulata	*C. aethiops*	Australia	ARSEF (5145) [SDA]
A. variegata	*M. rotundata*	Canada	CCFC (DAOM 194577) [SDA]
A. xerophila	*O. cornifrons*	Japan	CBS (376.87) [MA2Y60G]

* *Abbreviated sources*: ARSEF, United States Department of Agriculture Entomological Fungus Collection, Ithaca, New York; CCFC, Canadian Collection of Fungus Cultures, Ontario, Canada; AQIS, Australian Quarantine Inspection Service, Canberra, Australia; CBS, Central bureau voor Schimmelcultures, Baarn, The Netherlands.

† Single isolates unless otherwise indicated in parentheses.

‡ *Abbreviated culture media*: SDA, Sabouraud dextrose agar; SDAY2, Sabouraud dextrose agar + 2% yeast; YGPSA, 10 g yeast, 10 g glucose, 13.5 g KH_2PO_4, 10 g soluble starch and 20 g agar; MA2, 2% malt agar; MA20S, malt agar + 20% saccharose; MA40S, malt agar + 40% saccharose; MA20G, malt agar + 20% glucose; MA2Y60G, malt agar + 2% yeast + 60% glucose.

(1994). For PCR reactions, 2–20 ng of purified DNA was added to 15 µl of kinase treated AB28 primer, 5·0 µl 0·02 mM TW81 primer, 5·0 µl 10 × PCR buffer (670 mM Tris–HCl pH 8·8, 166 mM ammonium sulphate, 2 mg ml^{-1} gelatin, 15 mM $MgCl_2$ and 4·5% Triton X-100), 50 µM each of dATP, dCTP, dGTP and dTTP and 1 drop of oil. DNA was amplified using an automated thermal cycler (Corbett Research, Model FTS-1) to provide 5 min initial denaturation at 94° after which 2 U of Taq polymerase was added, then 30 cycles of 1 min at 94°, 1·5 min annealing at 54–55° and 2 min extension at 72°. A final extension period of 5 min at 72° completed the amplification. PCR product (5 µl) was then added to 2 µl of loading buffer, electophoresed and visualized.

DNA was sequenced from single-stranded template prepared from double-stranded PCR amplified DNA by adding a one-tenth volume of lambda exonuclease supplement buffer (775 mM glycine, 278 mM KOH and 5·8 mM $MgCl_2$) containing 1–2 U of lambda exonuclease. This solution was incubated at 37° for 30 min, then at 70° for 10 min, extracted once with an equal volume of phenol and once again with phenol–chloroform–isoamyl as described above. The super-

Table 2. GenBank accession numbers for the ribosomal DNA sequences obtained during this study from species of *Ascosphaera*

	Strain*	GenBank accession numbers
Ascosphaera apis	ARSEF 5142	U68313
A. atra	CBS 524.75	U68314
A. major	ARSEF 694	U68315
A. duoformis	ARSEF 5141	U68316
A. osmophila	CBS 381.83	U68317
A. proliperda	CBS 827.70	U68318
A. variegata	DAOM 194577	U68319
A. colubrina	CBS 160.87	U68320
A. acerosa	ARSEF 5143	U68321
A. asterophora	CBS 152.80	U68322
A. aggregata	ARSEF 689	U68323
A. fusiformis	CBS 373.87	U68324
A. celerrima	CBS 390.87	U68325
A. xerophila	CBS 376.87	U68326
A. naganensis	CBS 374.87	U68327
A. solina	ARSEF 5146	U68328
A. pollenicola	CBS 159.87	U68329
A. larvis	CBS 163.87	U68330
A. subcuticulata	ARSEF 5145	U68331
A. flava	ARSEF 5144	U68332

* See Table 1 for further details.

natant was removed to a clean centrifuge tube and a half-volume 7·5 M ammonium acetate (pH 7·4) and 2 vol. of ethanol added. The DNA was precipitated on ice for 10 min, centrifuged, washed and dried as described above and resuspended in 15 µl of dH_2O.

DNA sequencing was done using a Macrophor Sequencing System (Pharmacia) and manual sequencing protocols outlined in the manufacturer's user manual. To obtain sequence of the entire ITS1–5.8S–ITS2 rDNA region, a 5.8s sequencing primer 5′-GACATCGATGAAGAACGCAGCG-3′ was used in conjunction with the 18s forward primer given above, while a 5.8s sequencing primer 5′-AATGTGCGTTCAAAGATTC-GAT-3′ was used in conjunction with the 28s primer, also given above. Both DNA strands obtained from each species and isolate were sequenced at least five times to confirm the accuracy of the sequence data.

Sequence comparisons

FASTA was used to search the combined non-redundant GenBank, EMBL and DDBJ database on 20 Aug. 1996 when it comprised 967 040 nucleotide sequences, using the networked facilities of the Australian National Genomic Information Service. Representative sequences of each of the *Ascosphaera* species studied, together with sequences from GenBank of *A. apis* (U18362) and *E. albus* (U18359) were aligned using the CLUSTAL V program (Higgins *et al.*, 1991). The program DISCALC (G. F. Weiller, pers. comm.) was used to calculate various distance matrices from the resulting gapped sequences, or parts of them, and these were compared by the program DIPLOMO (Weiller & Gibbs, 1995). Chosen matrices were used to calculate and draw neighbour-joining trees using the programs NJTREE and TDRAW (Saitou & Nei, 1987). The aligned sequences were also compared by parsimony methods using PAUP programs (Swofford, 1993).

RESULTS

Sequence analysis

We have sequenced the complete sequences of the ITS1–5.8S–ITS2 region of the rDNA of 20 different *Ascosphaera* species, and have deposited and registered representative sequences in the GenBank data base under the accession numbers given in Table 2. Sequences obtained from all 23 isolates of *A. apis* were identical even though they were obtained from sites as far apart as the United Kingdom and Australia. This was also true for 11 isolates of *A. atra* obtained from sites as far apart as Europe and New Zealand (N.B. all our isolates of both species were grown from single spores, while the source of those from culture collections was unknown). There were also no differences between sequences from four isolates of *A. aggregata*, and two isolates each of *A. acerosa*, *A. duoformis*, *A. flava*, *A. larvis*, *A. pollenicola* and *A. proliperda*. However, sequences from each of the 20 species differed from one another to various extents (Fig. 1); from a minimum of 0·18% (in a multiple alignment including gaps) between *A. colubrina* Bissett and *A. variegata* Bissett to a maximum of 30·9% between *A. duoformis* and *A. osmophila*. The size of the ITS1–5.8S–ITS2 rDNA region of the *Ascosphaera* type species, *A. apis*, was 530 base pairs (bp). The average size of this region from all 20 species was 534 bp, the smallest being 515 bp for *A. acerosa* and the largest 571 bp for *A. osmophila*.

The presence of consistent inter-species differences in the ITS1–5.8S–ITS2 rDNA region, and the absence of intra-species variation, despite the fact that the isolates were obtained mostly as single-spore isolates and from over a large geographical range, indicated that these sequences could be used to identify *Ascosphaera* species. This was confirmed when our sequence for *A. apis* was used to search the international sequence databases using the FASTA program. It was found that the closest sequence was the *A. apis* sequence in GenBank (Accession number: U18362), that was submitted (without associated publication) by M. L. Berbee, A. Yoshimura, J. Sugiyama & J. W. Taylor and subsequently used by Berbee *et al.* (1995) when evaluating the phylogeny of fungi in the Trichocomaceae. The sequences were not, however, identical

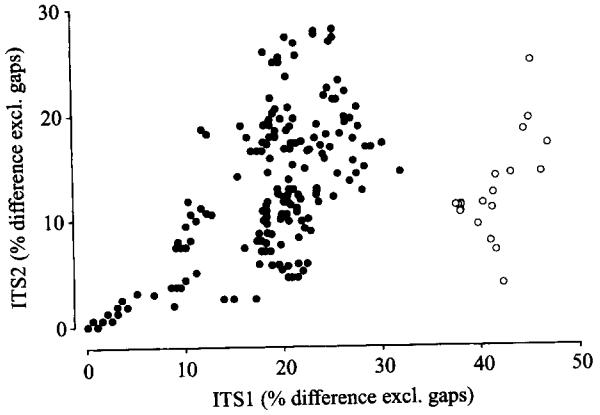

Fig. 1. Scatter plot showing the pairwise distances (% identity excluding gaps) for the ITS1 regions of 20 different species of *Ascosphaera* plotted against the ITS2 pairwise distances. The open circles show all pairwise comparisons involving *A. osmophila*.

and further comparisons showed that the database sequence was identical to our *A. atra*, not *A. apis*, sequences. A CLUSTAL V alignment of the database '*A. apis*' sequence with our *A. apis*, *A. atra* and *A. duoformis* sequences showed that, while the database sequence was identical to our *A. atra* sequences, it differed from our *A. duoformis* sequence in five positions, and from our *A. apis* sequences in 93 positions.

Phylogenetic analysis

FASTA was used to search databases with the ITS1–5.8S–ITS2 sequence of *A. apis* and found that the '*A. apis*' sequence discussed above was most similar, followed closely by that of *E. albus*. Therefore, our *Ascosphaera* sequences were aligned using CLUSTAL V with the ITS1–5.8S–ITS2 sequence of *E. albus* as the outgroup. Various pairwise and alignment parameters were tested and alignment with 647 positions, obtained using a gap penalty of 5, k-tuple of 3, 10 top diagonals and a window size of 10, seemed to be most parsimoniously aligned, and was used to provide the quantitative data given below.

The ITS1 regions of the sequences were, on average, about 1·5 times more variable than the ITS2 regions, and the intervening 5.8S regions were almost invariate; the ITS1 regions of the *Ascosphaera* sequences, excluding that of *A. osmophila*, had a mean difference of 18·4% (excluding gaps), and those of the ITS2 regions 12·7% (30·7 and 19·2% respectively when gaps were included). Although the ITS1 regions differed, on average, by 9·7% transitions and 8·7% transversions, the ITS2 regions differed by 7·6% transitions and 5·1% transversions.

Pairwise distances between the aligned sequences, or parts of them, were calculated in various ways (i.e. total nucleotide differences, transitions and transversions separately, correcting for multiple changes by 1, 2 or 4 parameter models, and correcting for nucleotide composition differences), and the results presented as neighbour-joining trees. The aligned sequences were also compared using parsimony methods to make 'branch and bound' heuristic searches for majority rule consensus trees. With three exceptions discussed below, most sequences gave the same close clusterings irrespective of the method used, or of the pairwise and alignment parameters used for CLUSTAL V, or the pairwise distance measure used for calculating the trees. One tree for the ITS2 region, excluding gapped positions, is shown in Fig. 2. The consistent clusterings were *A. acerosa* with *A. asterophora* Skou, *A. atra* with *A. duoformis*, *A. colubrina* closely with *A. flava*, *A. larvis*, *A. major* (Prökschl & Zobl) Skou, *A. variegata* and more distantly with *A. apis* and *A. celerrima* Skou, and, also, *A. aggregata* with *A. subcuticulata*, *A. proliperda* and more distantly with *A. solina* D. L. Anderson & N. L. Gibson. *A. fusiformis* Skou, *A. naganensis* Skou and *A. osmophila*, especially, did not cluster consistently.

The relationships between the clusters listed above depended most obviously on the parameters used for the alignment program, and less so on the method of free inference used, and whether the ITS1 or ITS2 regions were being compared; the alignment parameters most noticeably affected the positioning and length of runs of gaps in the alignment. This is shown clearly when the pairwise distances obtained for the ITS1 and ITS2 regions (Fig. 1) were presented as a scatter plot using the DIPLOMO program; the points for all closely related sequences, except those of *A. osmophila*, are near the diagonal line, whereas those for more distantly related pairs are spread over a wide range of ratios. Thus although most ITS1–5.8S–ITS2 rDNA sequences fall into consistent clusters, the relationships of those clusters are uncertain, perhaps because saltatory genetic changes, such as deletion or slippage (Hancock, 1995, 1996), have produced major indels resulting in the large number of gaps in some parts of the alignments, and these have obscured their relationships. This was most obvious in the sequence of *A. osmophila*. Its ITS2 region clusters closely with that of *A. solina*, but its ITS1 region seemed to be the most distinct of all the *Ascosphaera* ITS1 regions, as was shown when the ITS1 differences were plotted against the ITS2 differences (Fig. 1) using DIPLOMO, when it can be seen that all pairwise comparisons involving *A. osmophila* fall into a separate group with larger ITS1 values than expected. The alignments were, therefore, examined and much of the difference was found to result from a region of the ITS1 of *A. osmophila* with the sequence 5′..TCTCTCCTCTCCCCTCCGGGAGTAGAGG-GAGGGGGA..3′, which is repetitive and has somewhat complementary halves. When sequences of the *A. aggregata*, *A. subcuticulata*, *A. proliperda* and *A. solina* cluster, together with *A. fusiformis* and *A. osmophila*, were sequentially aligned (in the order shown in Fig. 2), removing at each step all gaps, and nucleotides with which they aligned, then both the ITS1 and ITS2 regions of the *A. osmophila* sequence clustered most closely with that of *A. solina*, and *A. fusiformis* became more closely linked with the other members of the cluster. Thus it is possible that indels, like the repetitive extra region in the ITS1 of *A. osmophila*, which was by far the longest sequence, are responsible for the 'instability' of the alignments of these sequences.

DISCUSSION

Our studies have shown that there are consistent differences between *Ascosphaera* species in the sequence of the ITS1–5.8S–ITS2 rDNA region of their genomic DNA, but we detected no differences between individuals of the same species, despite examining a significant number of samples from geographically distant sites for two species. Thus it is possible to identify to which species an *Ascosphaera* isolate belongs, even in the absence of morphological and cultural information, by determining the sequence of its ITS1–5.8S–ITS2 rDNa and comparing this with those of the published sequences. As well as being faster than existing identification methods, this approach can be used to identify species that cannot be cultivated *in vitro*, or that are homothallic and hence can only be presently identified from their morphological, biological and cultural characteristics. Nevertheless, caution should still be exercised when using rDNA sequences to identify new *Ascosphaera* species in the absence of morphological and cultural information, as some 'biological species' within the genus may yet be found to have greater genetic variability than others in their rDNA. In addition, our

	Locality	Spore length/width ratio
A. apis	Widespread	1:9
A. major	Widespread	2:6
A. variegata	Canada	3:1
A. colubrina	Canada	n.k.
A. flava	Australia	2:7
A. pollenicola	Canada	2:6
A. larvis	Canada	2:5
A. celerrima	Japan	2:8
A. fusiformis	Japan	3:9
A. proliperda	Denmark	2:2
A. aggregata	Europe/N. America	2:6
A. subcuticulata	Australia	2:3
A. solina	Australia	3:2
A. osmophila	Australia	2:3
A. atra	Widespread	2:0
A. duoformis	Australia	2:0
A. naganensis	Japan	3:1
A. xerophila	Japan	2:2
A. acerosa	Australia	4:2
A. asterophora	N. America	3:5
Eremascus albus		

Fig. 2. Neighbour-joining tree showing relationships of *Ascosphaera* species inferred from comparisons of sequences obtained from the ITS2 region, excluding gapped positions, of nuclear-encoded ribosomal DNA. n.k., not known.

sequence data, demonstrating the absence of within-species variation in the ITS1–5.8S–ITS2 rDNA region, were mostly obtained from isolates of only two species, *A. apis* and *A. atra*, which may have been through recent 'population bottlenecks' before being spread worldwide, mostly by man.

Searches of the gene sequence databases using our sequences showed that the sequence recorded in those databases as *A. apis* is significantly different from that of our *A. apis* isolates, but identical to that of our *A. atra* isolates. Hence, the database sequence is, in fact, that of *A. atra*.

Sequence comparisons showed that the various species of *Ascosphaera* we examined consistently fell into four distinct groups which we designated the '*A. apis*', '*A. aggregata*', '*A. atra*' and the '*A. acerosa*' groups (see Fig. 2). The species in these groups also share other independent characters, notably the shape and size of their ascospores, and this lends support to the sequence groupings. For example, the eight fungi in the '*A. apis* group' all have moderately large, blackish spore-cysts, with ellipsoidal to subcylindrical ascospores that are small or of average size, whereas those of the other species differ in one or more of these characters (Bissett, 1988; Anderson & Gibson, 1997). Indeed ascospores of *A. flava*, *A. larvis* and *A. pollenicola* are so similar that they cannot be distinguished (Bissett, 1988; Anderson & Gibson, 1997). Five of the six species in the '*A. aggregata* group' are pathogens, and the ascospores of *A. aggregata* and *A. subcuticulata* are not only almost indistinguishable, but, unlike all the others, are produced in spore-cysts in a single layer beneath the epicuticle of the larvae they infect, even though these are larvae of different bee species (Anderson & Gibson, 1997). Again the spores of *A. atra* and *A. duoformis*, in the '*A. atra* group', have a distinctive shape, although those of *A. duoformis* are smaller than those of *A. atra*. Both these fungi are also saprotrophs of pollen, *A. atra* on pollen collected by the leafcutting bee, *M. rotundata*, and *A. duoformis* that of the smaller social bee, *Trigona carbonaria* Smith (Skou & Hackett, 1979; Anderson & Gibson, 1997). Yet again, the two species of the '*A. acerosa* group', *A. acerosa* and *A. asterophora*, have long thin ascospores (Skou, 1982*b*; Bisset *et al.*, 1996; Anderson & Gibson, 1997). There seems to be no obvious correlation, however, between host taxonomy and the fungal rDNA taxonomy, although it is noteworthy that both *A. solina* and *A. osmophila*, which have closely related ITS2 regions, are Australian.

Even though the saprotrophic/parasitic life-style shows some clustering in the ITS1–5.8S–ITS2 taxonomy of these spore-cyst fungi, it is likely that this group of fungi readily changes life style, as the database searches showed them to be closely related not only to *E. albus*, a saprotroph, but also to *Ajellomyces capsulatus* (Kwon-Chung), McGinnis & Katz, the cause of human histoplasmosis.

Although comparisons of the rRNA sequences of these fungi clearly showed that most fell consistently into the same clusters irrespective of the method used, they did not resolve the relationships of the clusters further, and also indicated that more detailed analysis of any particular data set using any single method combined with confidence tests, such as bootstrapping, jacknifing and T-PTP, would not resolve the higher level relationships, although in bootstrapping tests with various parsimony analyses the *A. acerosa* with *A. asterophora* cluster was supported 98–100%, *A. atra* with *A. duoformis* always 100%, *A. aggregata* with *A. subcuticulata*, *A. proliperda* and *A. solina* 32–69%, and *A. apis* with *A. colubrina*, *A. flava*, *A. larvis*, *A. major*, *A. variegata* and *A. celerrima* 44–61%. It seems that saltatory genetic changes have erased clear evidence of more distant relationships from the ITS1–5.8S–ITS2 region of the genome.

Our results confirm those of Berbee & Taylor (1992), who reported that *Ascosphaera* and *Eremascus* are closely related genera. Indeed we have shown them to be very closely related.

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REFERENCES

Anderson, D. L. & Gibson, N. L. (1997). New species and isolates of spore-cyst fungi (Plectomycetes: Ascosphaerales) from Australia. *Australian Systematic Botany*. In Press.

Barr, M. E. (1983). The ascomycete connection. *Mycologia* **75**, 1–13.

Benny, G. L. & Kimbrough, J. W. (1980). A synopsis of the orders and families of Plectomycetes with keys to genera. *Mycotaxon* **12**, 1–91.

Berbee, M. L., & Taylor, J. W. (1992). Two Ascomycete classes based on fruiting-body characters and ribosomal DNA sequence. *Molecular Biology and Evolution* **9**, 278–284.

Berbee, M. L., Yoshimura, A., Sugiyama, J. & Taylor, J. W. (1995). Is *Penicillium* monophyletic? An evaluation of phylogeny in the family Trichocomaceae from 18s, 5.8s and ITS ribosomal DNA sequence data. *Mycologia* **87**, 210–222.

Bissett, J. (1988). Contribution towards a monograph of the genus *Ascosphaera*. *Canadian Journal of Botany* **66**, 2541–2560.

Bissett, J., Duke, G. & Goettel, M. (1996). *Ascosphaera acerosa* sp. nov. isolated from the alfalfa leafcutting bee, with a key to the species of *Ascosphaera*. *Mycologia* **88**, 797–803.

Bowman, B. H., Taylor, J. W., Brownlee, A. G., Lee, J., Shi-Dalu & White, J. S. (1992). Molecular evolution of the fungi: relationships of the basidiomycetes, ascomycetes, and achytridiomycetes. *Molecular Biology and Evolution* **9**, 285–296.

Christensen, M. & Gilliam, M. (1983). Notes on the *Ascosphaera* species inciting chalkbrood in honey bees. *Apidologie* **14**, 291–297.

Curran, J., Driver, F., Ballard, J. W. O. & Milner, R. J. (1994). Phylogeny of *Metarhizium*: analysis of ribosomal DNA sequence data. *Mycological Research* **98**, 547–552.

Eriksson, O. (1984). Outline of the Ascomycetes – 1983. *Systema Ascomycetum* **3**, 1–72.

Eriksson, O. & Hawksworth, D. L. (1985). Outline of the Ascomycetes – 1985. *Systema Ascomycetum* **4**, 1–79.

Gäumann, E. (1964). *Die Pilze. Grundzüge ihrer Entwicklungsgeschichte und Morphologie*, 2. Auflage. Birkhäuser: Basel.

Gilliam, M. (1978). Fungi. In *Honey Bee Pests, Predators, and Diseases* (ed. R. Morse), pp. 78–101. Cornell University Press: London.

Hancock, J. M. (1995). The contribution of DNA slippage to eukaryotic nuclear 18S rRNA evolution. *Journal of Molecular Evolution* **40**, 629–639.

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Hancock, J. M. (1966). Simple sequences and the expanding genome. *Bioessays* **18**, 421–425.

Harrold, C. E. (1950). Studies in the genus *Eremascus*. *Annals of Botany London (New Series)* **14**, 127–148.

Higgins, D. G., Bleasly, A. & Fuchs, R. (1991). CLUSTAL V: improved software for multiple sequence alignment. *CABIOS* **8**, 151–153.

Kish, L. P., Bowers, N. A., Benny, G. L. & Kimbrough, J. W. (1988). Cytological development of *Ascosphaera atra*. *Mycologia* **80**, 312–319.

Maassen, A. (1913). Weitere Mitteilungen über der seuchenhaften Brut-krankheiten der Bienen [Further communication on the epidemic brood disease of bees]. *Mitteilungen aus der Kaiserlichen Biologischen Anstalt für Land- und Forstwirtscshaft* **14**, 48–58.

McManus, W. R. & Youssef, N. N. (1984). Life cycle of the chalkbrood fungus, *Ascosphaera aggregata*, in the alfalfa leafcutting bee, *Megachile rotundata*, and its associated symptomatology. *Mycologia* **76**, 830–842.

Saiki, R. K. (1990). Amplification of genomic DNA. In *PCR Protocols* (ed. M. A. Innis, D. H. Gelfand, J. J. Sninsky & T. J. White), pp. 13–20. Academic Press: San Diego, CA.

Saiki, R. K., Gelfand, D. H., Stoffel, S., Scharf, S. J., Higuchi, R., Horn, G. T., Mullis, K. B. & Erlich, H. A. (1988). Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. *Science* **239**, 487–489.

Saitou, N. & Nei, M. (1987). The neighbour-joining method: a new method for reconstructing phylogenetic trees. *Molecular Biology and Evolution* **4**, 406–425.

Skou, J. P. (1972). Ascosphaerales. *Friesia* **10**, 1–24.

Skou, J. P. (1975). Two new species of *Ascosphaera* and notes on the conidial state of *Bettsia alvei*. *Friesia* **11**, 62–74.

Skou, J. P. (1982*a*). Ascosphaerales and their unique ascomata. *Mycotaxon* **15**, 487–499.

Skou, J. P. (1982*b*). Ascosphaera asterophora species nova. *Mycotaxon* **14**, 149–159.

Skou, J. P. (1988*a*). Japanese species of *Ascosphaera*. *Mycotaxon* **31**, 173–190.

Skou, J. P. (1988*b*). More details in support of the class Ascosphaeromycetes. *Mycotaxon* **31**, 191–198.

Skou, J. P. & Hackett, K. (1979). A new homothallic species of *Ascosphaera*. *Friesia* **11**, 265–277.

Skou, J. P. & Holm, N. (1989). *Ascosphaera tenax* species nova and a variant of *Ascosphaera aggregata*. *Mycotaxon* **35**, 211–218.

Skou, J. P. & King, J. (1984). *Ascosphaera osmophila* sp. nov., an Australian spore-cyst fungus. *Australian Journal of Botany* **32**, 225–231.

Spiltoir, C. F. (1955). Life cycle of *Ascosphaera apis* (*Pericystis apis*). *American Journal of Botany* **42**, 501–508.

Spiltoir, C. F. & Olive, L. S. (1955). A reclassification of the genus *Pericystis* Betts. *Mycologia* **47**, 238–244.

Swofford, D. L. (1993). *PAUP, Phylogenetic Analysis Using Parsimony, Version 3.1.1.* Computer program distributed by the Illinois Natural History Survey: Champaign, Illinois.

Weiller, G. F. & Gibbs, A. J. (1995). DIPLOMO: the tool for a new type of evolutionary analysis. *CABIOS* **11**, 535–540.

Youssef, N. N., Roush, C. F. & McManus, W. R. (1984). *In vivo* development and pathogenicity of *Ascosphaera proliperda* (Ascosphaeraceae) to the alfalfa leafcutting bee, *Megachile rotundata*. *Journal of Invertebrate Pathology* **43**, 11–20.