An evaluation of the fungal 'morphotype' concept based on ribosomal DNA sequences

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In studies of fungal endophyte communities, mycelia sterilia are commonly isolated from plant substrates and grouped into morphotypes on the basis of cultural characteristics. In *Polygonum multiflorum* one hundred and sixty-nine strains of mycelia sterilia were isolated and grouped into 27 morphotypes. Six randomly selected morphotypes, each with 2-3 representatives, were subsequently subjected to ribosomal DNA sequence analysis. Nucleotide sequence similarities of the rDNA internal transcribed spacer (ITS) regions and the 5.8S gene were compared using UPGMA cluster analysis. Comparison of nucleotide sequences revealed high levels of similarity (*ca*. 91.63-99.53%) among strains within morphotypes. ITS and 5.8S sequences of species within various genera from GenBank were obtained to estimate levels of nucleotide similarity within and between well-established genera and species. This study verifies on the basis of ribosomal DNA sequence analysis the validity of these 'morphotypes' as taxonomic groups. A dendogram, illustrating relatedness of the morphotypes and reference taxa from GenBank is also presented.

Key words: fungal endophytes, fungal morphospecies, mycelia sterilia, rDNA.

Introduction

In recent years a substantial number of studies have focused on the diversity of fungal endophytic communities in the tropics (Rodrigues, 1994; Fisher *et al*., 1995; Lodge *et al*., 1996; Brown *et al*., 1998; Taylor *et al*., 1999; Umali *et al*., 1999; Fröhlich *et al*., 2000), as well as in temperate regions (Dreyfuss and Petrini, 1984; Pereira *et al*., 1993; Fisher *et al*., 1994; Brown *et al*., 1998; Taylor *et al*., 1999). In most of these studies anamorphic fungi were commonly isolated and a large number of fungi that did not sporulate in culture were categorized as mycelia sterilia. In order to appreciate the considerable prevalence of these mycelia sterilia, a summary showing the proportions of mycelia sterilia found in previous studies of fungal endophytic communities is

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given in Table 1. For a given host, mycelia sterilia took up an average of 20% of the population of fungal endophytes. However, this can increase up to 54% of the population (Fisher *et al*., 1994). Since conventional classification of fungi relies heavily on reproductive structures, these mycelia sterilia are not usually given any taxonomic placements. In view of this, mycologists working on endophytes have adopted the concept of 'morphospecies' to provide a practical means to estimate endophytic fungal diversity to incorporate these non-sporulating fungal isolates.

Bills (1996) was the first to discuss the use the concept of grouping mycelia sterilia isolates into 'morphological species' on the basis of similarity in colony surface textures, hyphal pigments, exudates, margin shapes, growth rate and sporulating structures (if present). Since then numerous other studies followed the same concept in determining diversity of fungal endophytes in *Musa acuminata* (Brown *et al*., 1998); *Trachycarpus fortunei* (Taylor *et al*., 1999); *Licuala* sp. (Fröhlich and Hyde, 1999); *Bambusa tuldoides* (Umali *et al*., 1999); *Livistona chinensis* (Guo *et al*., 2000); *Licuala* sp. (Fröhlich *et al*., 2000). The use of this 'morphospecies' concept has been useful in estimating fungal numbers since the species is conventionally the basic unit in biodiversity studies. The question of greatest interest to mycologists is how good cultural similarities and differences (in the absence of sporulating structures) are at indicating species identity and species status. The confusion in terminology as well as definitions in relation to this concept needs to be addressed.

Linnaeus described morphological species as species that were defined on the basis of physical, measurable characteristics (Linnaeus, 1758). Others further described morphospecies as species defined on a set of morphological characteristics that vary within well-defined limits and this set of characters is sufficient to distinguish the species from all other species (Mayr, 1970). Brasier (1986), in discussing fungal speciation, also used morphospecies to define species that are morphologically identical, within which partially or totally reproductively isolated subgroups may be found. In the eighth edition of the Dictionary of the Fungi, the concept of morphospecies (morphological or phenetic species) is defined as 'the traditional approach recognising units that could be delimited on the basis of morphological characters, and ideally by discontinuities in several such', as distinct from biological, phylogenetic, ecological and polythetic species (Hawksworth *et al*., 1995). This concept has predominated in fungal taxonomy where, most crucially, reproductive structures constitute the main delimiting morphological characters.

Ever since Bill's (1996) discussion of the use of morphological species in studies of fungal endophytes, this concept has been used to classify endophytes

Table 1. Proportion of mycelia sterilia in fungal endophytes isolated from various plant hosts.

Host	Location	Proportion of mycelia sterilia (%)	Reference
Cuscuta reflexa	Chennai, Tamil Nadu, India	23	Suryanarayanan *et al.* (2000)
Hippohau rhamnoides	Gilbraltar Point Nature Reserve, Spain	18-24	Lindsey and Pugh (1976)
Licuala sp. and *Trachycarpus fortunei*	Australia and Brunei	11-16	Taylor *et al.* (1999); Fröhlich *et al.* (2000)
Livistona chinensis	Hong Kong	16	Guo *et al.* (1998, 2000)
Picea mariana	Canada	33	Johnson and Whitney (1992)
Quercus ilex	Switzerland	54	Fisher *et al.* (1994)
Red Mangroves	Oahu, Hawaii, USA	12-14	Lee and Baka (1973)
Sequoia sempervirens	Central California, USA	26	Espinosa-Gracia and Langenheim (1990)

Table 2. Isolates of mycelia sterilia from *Polygonum multiflorum* used in the study and their GenBank accession numbers for the 5.8S and ITS 1 & 2 sequences.

Isolate code	Morphotype	GenBank Accession No.
MS1 IS2	1	AF413034
MS1 IS264	1	AF413035
MS2 IS37	2	AF413036
MS2 IS81	2	AF413037
MS2 IS83	2	AF413038
MS3 IS106	3	AF413039
MS3 IS107	3	AF413040
MS3 IS109	3	AF413041
MS4 IS117	4	AF413042
MS4 IS139	4	AF413043
MS5 IS33	5	AF413044
MS5 IS113	5	AF413046
MS5 ISP5B3	5	AF413045
MS6 IS133	6	AF413049
MS6 IS2-1	6	AF413047
MS6 IS3-1	6	AF413048

in the absence of reproductive structures in the majority of cases. Although the strict, as well as the original, definition of the morphological species concept involves reproductive structures as central distinguishing characters, this has largely been ignored in research on fungal endophytes. Schulthess and Faeth (1998) used another term, morphotype, to described non-sporulating fungal endophytes in leaves of Arizona fescue (*Festuca arizonica*). Hawksworth *et al.*

(1995) defines morphotype as a 'group of morphologically differentiated individuals of a species of unknown or of no taxonomic significance'. For lack of a better term, 'morphotype' is used in preference over 'morphological species' or 'morphospecies' in our present discussion of classifying mycelia sterilia into morphological units.

A crucial problem in sorting fungal isolates into morphotypes based on cultural characteristics, in the absence of reproductive structures, is that one cannot be certain that these morphotypes truly reflect taxonomic units, regardless of the ranking (species/genus etc.). More rigorous alternative methods are now being used in the identification of endophytic fungal species, e.g. those involving DNA molecules (Guo *et al.*, 2000). Guo *et al.* (2000) took a representative sample of morphotypes isolated from *Livistona chinensis* (Chinese fan palm), and sequenced the 5.8S and the flanking internal transcribed spacer regions (ITS1 & 2) of the ribosomal RNA gene (rDNA). Based on phylogenetic analysis of these sequences they were able to conclude that most of these morphotypes were filamentous ascomycetes belonging or having close phylogenetic affinities to the genera *Diaporthe*, *Mycosphaerella* and *Xylaria*. Their method, however, did not specifically test whether the grouping of isolates into morphotypes had any particular phylogenetic significance. They have chosen only one representative from each of the 16 morphotypes identified, since their purpose was to identify as many fungal endophytes as possible in the host plant. Whether all isolates grouped into separate morphotypes constituted distinct taxonomic or phylogenetic units was not established.

The main aim of our study is to verify the concept of morphotypes based on DNA sequence data on mycelia sterilia isolated from a chosen host, *Polygonum multiflorum* (Fo-ti, He-shou-wu). Our approach is to examine whether similarities at the cultural level, in the absence of reproductive structures, reflect similarities at the genetic level, in particular in nucleotide similarities of a DNA molecule with phylogenetic significance. Comparison of nucleotide similarities of the 5.8S and ITS sequences is made at the level of intra- and inter- morphotypes. These similarity values will be compared with those of established fungal species and genera obtained from GenBank.

Materials and methods

Sampling of fungal strains

Endophytes were isolated from *Polygonum multiflorum* (Fo-ti, He-shou-wu) collected from Tai Po Kao Country Park, Hong Kong. Fungal endophytes were isolated according to Guo *et al.* (1998). Of the 514 fungal isolates

recovered, 169 isolates (32.9%) remained as mycelia sterilia in culture. These mycelia sterilia were grouped into 27 morphotypes based on cultural characteristics. All cultures were grown on Potato Dextrose Agar (PDA). Six morphotypes were randomly selected from these groups and 2-3 isolates from each morphotype were in turn selected to be included in this study. These isolates (16 in total) are listed in Table 2.

Comparison of cultural characteristics

Comparisons of the 16 isolates were made based on the morphotype concept. Three replicates of each of the isolates were inoculated at the same time and incubated for 4 weeks. The growth rate was determined by measuring the average diam. of the colony (cm) every two weeks. Other colony characteristics, including colour (above and reverse), elevation, texture, type of mycelium, margin shape, density, zonale and effects of the fungi on the medium, were examined after four weeks of incubation.

DNA extraction

A single hyphal tip was obtained from each culture under a dissecting microscope and grown separately on PDA at 25°C for 2 weeks. Actively growing mycelia were directly scraped off from culture plates and transferred into 1.5 mL centrifuge tubes. DNA extraction followed a modified protocol of Doyle and Doyle (1987). Approximately 0.05 g of mycelium was mixed with *ca*. 0.3 g of white quartz sand in warm (*ca*. 60°C) 2X CTAB buffer [2% (w/v) CTAB; 100 mM Tris-HCl; 1.4 M NaCl; 20 mM EDTA, pH 8.0]. Mycelium-sand mixture was ground with a glass pestle and incubated at 60°C for 1 hour before being subjected to multiple phenol:chloroform (1:1) extractions. DNA was precipitated from the purified aqueous extraction layer by ethanol precipitation at -20°C. The DNA pellet was washed (70% ethanol), dried (vacuum centrifuge) and resuspended in 100 μL TE buffer containing 0.8 μg/mL of RNase A. DNA samples were checked for purity and integrity by gel electrophoresis before storing at 4°C.

PCR amplification

Conserved fungal primers ITS4 and ITS5 (White *et al*., 1990) were used to amplify the 5.8S and flanking ITS regions of each of the isolates. DNA fragments were amplified in an automated thermal cycler (PTC-100, MJ Research, Inc., Watertown, MA, USA). Amplification was performed in a 50 μL reaction volume which contained PCR buffer (10 mM KCl, 10 mM $(NH_4)_2SO_4$, 20 mM Tris-HCl, pH 8.8, 0.1% Triton X-100), 1.5 mM MgCl2,

200 µM of each deoxyribonucleotide triphosphate, 15 pmols of each primer, *ca*. 100 ng template DNA, and 2.5 units of *Taq* DNA polymerase (Promega, Madison, WI, USA). The thermal cycling programme used was: 3 minutes initial denaturation at 95°C, followed by 30 cycles of 1 minute denaturation at 95°C, 50 seconds primer annealing at 54°C, 1 minute extension at 72°C, and a final 10 minutes extension at 72°C. PCR products were stored at 4°C. From each PCR reaction 5 µL of PCR products were examined by electrophoresis in a 2% (W/V) agarose gel with ethidium bromide (0.5 µg ml^{-1}) in TAE buffer (0.4 M Tris, 50 mM NaOAc, 10 mM EDTA, pH 7.8) and visualized under UV light.

DNA sequencing

PCR products were purified using minicolumns (Wizard PCR Preps DNA Purification System, Promega, Madison, WI, USA) according to the manufacturer's instructions. Purified PCR products were directly sequenced in an automated sequencer (ALFexpress, PharmaciaBiotech, Piscataway, NY, USA) following the instruction manual's protocol. Primers ITS2, ITS3, ITS4 and ITS5 (White *et al*., 1990) were used in the sequencing reactions. Both strands of the DNA molecule were sequenced. The thermal cycling programme was: 2 minutes initial denaturation at 94°C, followed by 36 cycles of 15 seconds denaturation at 94°C, 15 seconds primer annealing at 58°C (ITS4) or 60°C (ITS2, ITS3, ITS5), and 40 seconds extension at 72°C.

DNA sequence analysis

For each fungal strain, four separate sequences obtained for the respective primers were manually aligned and spliced to obtain a consensus sequence using the biosequence editor SeqPup v0.8 (Gilbert, 1998). During this process of alignment, individual bases were verified by comparison with the fluorescence signal printout for each sequence. Consensus sequences for the 16 strains were aligned using ClustalX (Thompson *et al*., 1994). Nucleotide sequences were then changed to numeric values (A = 1; C = 2; G = 3; T = 4) in MVSP 3.0 (Multi-Variate Statistical Package, 1998) for cluster analysis. Unweighted pair group method using arithmetic averages (UPGMA) were used as clustering method for the nucleotide sequence similarities of the 16 strains. With the help of Basic Local Alignment Search Tool (BLAST) of the National Centre for Biotechnology Information, the ITS1-5.8S-ITS2 sequences from several species of taxonomically established genera were obtained from GenBank. These nucleotide sequences were similarly treated and combined with the data of the 16 strains to generate a UPGMA similarity tree using MVSP 3.0.

Results and discussion

Sequence analysis of the 5.8S and ITS regions of the nuclear encoded rDNA have been widely used in determining taxonomic placement of fungi at different levels in recent years. Chillali *et al.* (1998) used 5.8S and ITS sequences to reveal the phylogenetic relationships among *Armillaria* species. Yao *et al.* (1999) were able to distinguish between genera and species of *Tyromyces* using sequences from the nuclear ITS regions. Guo *et al.* (2000) were able to identify endophytic fungal species using the 5.8S and ITS regions and showed that unidentified morphotypes from *Livistona chinensis* were mainly filamentous Ascomycota. Further classification of their morphotype representatives was successful to a certain extent based on sequence comparison with those obtained from GenBank. The current study utilised the same DNA region to examine the validity of the morphotype concept in a chosen plant host.

In vitro cultural and growth characteristics of the 16 strains are summarised in Table 3. On the basis of growth rate and colony morphology, these 16 strains were grouped into 6 morphotypes. The differences in colony morphology were evident and segregation into morphotypes was easily established visually and macroscopically.

The aligned data set of the rDNA 5.8S and ITS regions consisted of 700 sites. Pairwise comparisons expressed in percentage similarities of the 16 fungal strains, comprising 6 morphotypes, are shown in Table 4. Similarities within each morphotype were relatively high, within the range of 91.63% to 99.53%. Inter-morphotype was considerably less than this range. Figure 1 shows the UPGMA dendrogram generated based on similarity values of the 16 strains as well as reference strains obtained from GenBank. Isolates within each morphotypes clustered together and distinctly separated from each other. Inter-specific similarities among species of reference genera varied considerably, with species of *Xylaria* (*ca.* 84%), *Phyllosticta* (*ca.* 84%) and *Candida* (*ca.* 84.5%) being most variable. Species of *Alternaria* (*ca.* 97.6%), *Arthrobotrys* (*ca.* 95.5%) and *Colletotrichum* (*ca.* 94%) had the highest inter-specific similarities among all genera included in the study.

In terms of similarity relationships among morphotypes and reference taxa, the 6 morphotypes are generally scattered among the reference taxa (Fig. 1). MS1 is most similar to *Phyllosticta* and MS3 (*ca.*79.2%). The morphotype MS1 is, however, most probably a distinct genus based on this low similarity. MS2 has high affinity to *Alternaria*, with a similarity of *ca.* 88.2%, which is considerably higher than inter-specific similarities of the highly variable genera. However, MS2 is unlikely to be a species of *Alternaria* since the 6 species of this genus included in the study share high similarities (all within *ca.*

Table 3. Cultural characteristics of the 16 strains of mycelia sterilia from *Polygonum multiflorum* on Potato Dextrose Agar (PDA).

Morpho-type	Isolate code	Size (cm) of colony after 14 days	30 days	Shape	Colour Above	Colour Reverse	Elevation	Texture	Mycelium	Edge	Density	Zonality	Effect on medium
1	MS1 IS2	<[a]	4.5	Circular	Off white[c], green[d]	Cream[c], grayish-black[d]	Flat	Felty	Aerial	Entire	Medium	+[e]	-[e]
	MS1 IS264	<	4.2	Circular	White	Grayish	Flat	Felty	Aerial	Entire	Medium	+	-
2	MS2 IS37	<	3.3	Irregular	Cream to yellow	Yellow	Flat	Velvety	Aerial	Undulate	Spare	-	-
	MS2 IS81	<	2.9	Irregular	Cream to yellow	Yellow[c], brown[d]	Flat	Velvety	Aerial	Undulate	Spare	-	-
	MS2 IS83	<	2	Irregular	Cream to yellow	Yellow	Flat	Velvety	Immersed	Dentate	Spare	-	-
3	MS3 IS106	2.25	4.4	Circular	Greenish black	Black	Convex with papillate surface	Powdery	Immersed	Crenate	Dense	+	-
	MS3 IS107	2.7	4.8	Circular	Greenish black	Black	Convex with papillate surface	Powdery	Immersed	Crenate	Dense	+	-
	MS3 IS109	2.97	5.1	Circular	Greenish black	Black	Convex with papillate surface	Powdery	Immersed	Crenate	Dense	+	-
4	MS4 IS139	7.5	>[b]	Circular	White	White	Low convex	Cottony	Aerial	Entire	Dense	-	-
	MS4 IS117	6.8	>	Circular	White	White	Low convex	Cottony	Aerial	Entire	Dense	-	-

[a] Less than 1 cm; [b] Completely covering plate; [c] Edge of culture; [d] Centre of culture; [e] +/- present.

Table 3. (continued).

Morpho-type	Isolate code	Size (cm) of colony after		Shape	Colour		Elevation	Texture	Mycelium	Edge	Density	Zonality	Effect on medium
		14 days	30 days		Above	Reverse							
5	MS5 IS113	8.5	>	Circular	White	White	Flat	Fluffy	Aerial	Entire	Dense	-	-
	MS5 IS33	8.4	>	Circular	White	White	Flat	Fluffy	Aerial	Entire	Dense	-	-
	MS5 ISP5B3	8.3	>	Circular	White	White	Flat	Fluffy	Aerial	Entire	Dense	-	-
6	MS6 IS133	8.5	>	Circular	Off white to gray	Grayish	Flat	Hairy to fluffy	Aerial	Entire	Dense	-	-
	MS6 IS2-1	8.5	>	Circular	Off white to gray	Grayish	Flat	Hairy to fluffy	Aerial	Entire	Dense	-	-
	MS12 IS3-1	8.5	>	Circular	Off white to gray	Grayish	Flat	Hairy to fluffy	Aerial	Entire	Dense	-	-

Table 4. Similarity matrix (%) of the 5.8S and ITS 1 & 2 sequences among the 16 strains (6 morphotypes) studied.

|	MS2 IS37	MS2 IS81	MS2 IS83	MS1 IS2	MS1 IS264	MS5 IS113	MS5 IS33	MS5 ISP5B3	MS3 IS106	MS3 IS107	MS3 IS109	MS4 IS139	MS4 IS117	MS6 133	MS6 IS2-1	MS6 IS3-1
MS2 IS37	100															
MS2 IS81	91.64	100														
MS2 IS83	96.99	91.63	100													
MS1 IS2	77.53	81.81	77.81	100												
MS1 IS264	76.84	81.64	78.41	99.09	100											
MS5 IS113	76.05	80.37	77.53	82.26	82.81	100										
MS5 IS33	76.31	80.31	78	82.61	83.16	98.88	100									
MS5 ISP5B3	76.27	80.45	78.16	82.41	82.96	98.95	99.46	100								
MS3 IS106	74.04	78.86	75.25	78.32	79.02	79.97	79.84	79.99	100							
MS3 IS107	74.92	78.78	76	79.22	79.79	80.84	80.71	80.86	98.47	100						
MS3 IS109	74.42	79.24	75.63	78.7	79.4	80.37	80.24	80.38	99.29	98.92	100					
MS4 IS139	73.07	76.34	74.82	77.4	78.04	82.33	82.72	82.35	74.88	75.68	75.21	100				
MS4 IS117	75.13	78.49	77.26	80.11	80.7	85.96	85.93	86.34	78.92	79.6	79.19	94.65	100			
MS6 IS133	76.78	79.57	77.02	80.74	80.03	84.81	85.26	85.04	77.99	79.06	78.39	86.36	89.61	100		
MS6 IS2-1	77.01	79.45	76.88	80.55	79.84	84.69	84.92	84.92	78	79.08	78.4	86	89.49	99.53	100	
MS6 IS3-1	76.75	79.15	76.63	80.23	79.52	84.3	84.67	84.53	77.69	78.76	78.09	85.88	89.16	99.28	99.39	100
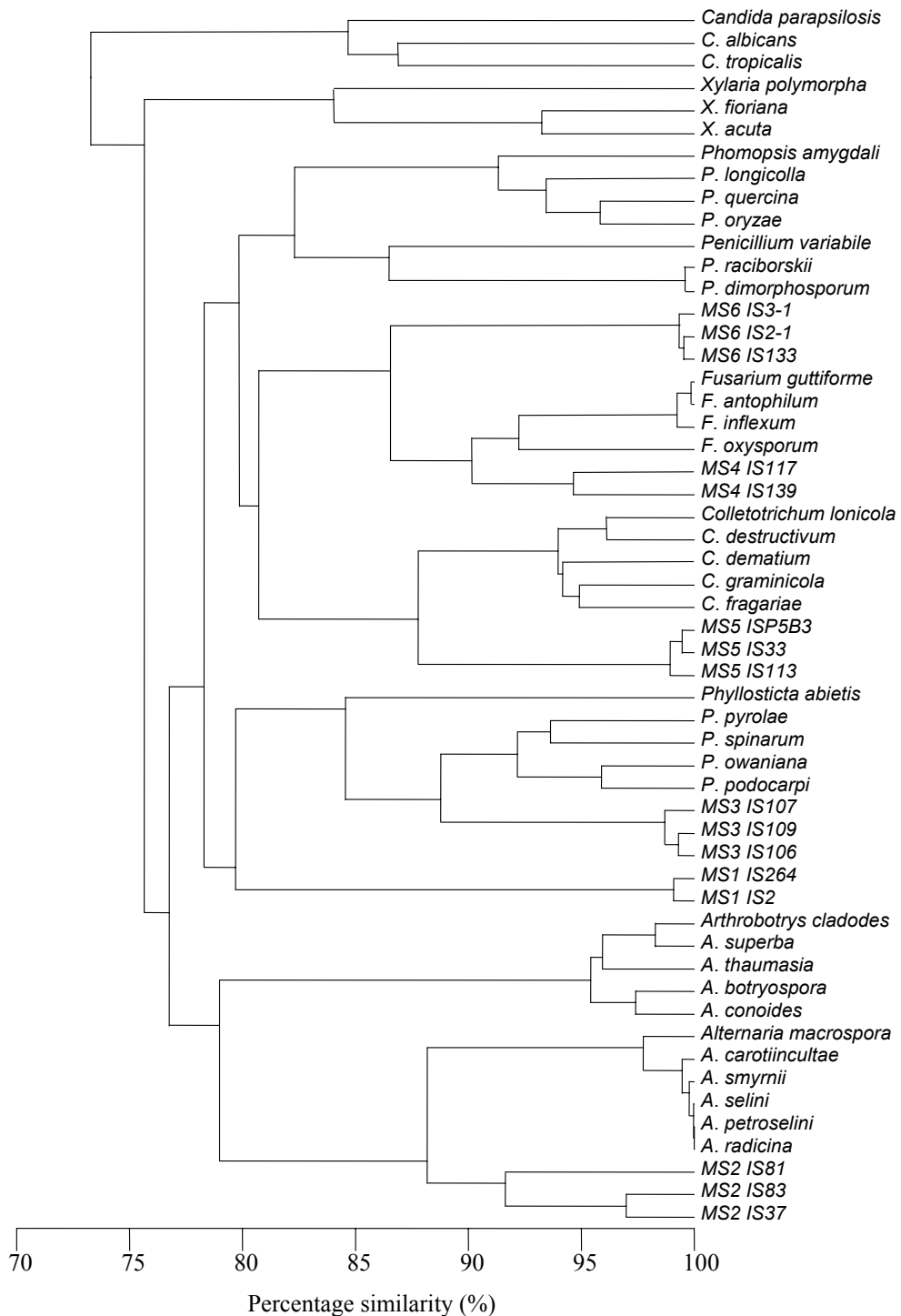
Fig. 1. Dendrogram illustrating relatedness of the 16 strains (6 morphotypes) and reference taxa based on UPGMA cluster analysis of 5.8S and ITS 1 & 2 sequence similarity.

97.6%). On the basis of sequence similarity, MS3 should belong to *Phyllosticta*. This morphotype grouped within the *Phyllosticta* cluster consisting of 5 species of this genus. MS4 is most similar to *Fusarium* with a similarity of *ca*. 90%. Similarity values within species of *Fusarium* ranged from *ca*. 92.2% to 99.8%. Although MS4 did not cluster within this genus, it is still most likely to be *Fusarium*. MS5 has close affinity to *Colletotrichum*, with a similarity of *ca*. 87.8%. Although there is a high probability that MS5 belong to the genus *Colletotrichum*, it could very well be a distinct genus. MS6, although most similar to *Fusarium* (*ca*. 86.5%), is probably a distinct genus, unlike MS4.

Although a degree of certainty exists in assigning morphotypes to genera based only on the sequence similarity comparison, cluster analysis of the 5.8S and ITS sequences of the rDNA gave results that agreed well with initial mycelia sterilia morphotype groupings. Strains within each morphotypes clearly grouped together as taxonomic units, whereas these different morphotypes being distinct taxa is unequivocal. Our data therefore supports the conventionally used morphotype concept (Guo *et al*., 1998, 2000; Taylor *et al*., 1999; Fröhlich *et al*., 2000) for this community of endophytes. In endophyte diversity studies, the assignment of different morphotypes as distinct species would appear to be justified if the results of the current study of a single plant host were a reflection of all endophytic communities. This is a crucial point of consideration as such assignment of distinct species are taken into account in estimating global fungal numbers (e.g. Hyde and Hawksworth, 1997; Fröhlich and Hyde, 1999), In fact, it appears that fungal numbers estimated by designating each morphotype into a separate species may even be an under-estimation, since our data revealed that several of the morphotypes studied were clearly distinct genera, which may comprise more than one species.

Although our morphotypes have been shown to be valid taxonomic units, the approach of not conducting phylogenetic analysis precludes the assignment of these morphotypes into definite species. One of the greatest limitations in phylogenetic analyses in this regard is the restricted number of available sequences that could be used as reference taxa, which are crucial to taxonomic placements of test sequences (Guo *et al*., 2000). Our approach was conservative although appropriate for our specific purpose.

Acknowledgements

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