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# Ubiquinone system, GC contents and cellular fatty acid composition of species of the form-genus *Malbranchea* and *Coccidioides immitis* for chemotaxonomic study

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**Ubiquinone system, GC contents and cellular fatty acid (CFA) composition were analyzed as criteria for chemotaxonomy of *Malbranchea* species and *Coccidioides immitis*, which are suggested to be phylogenetically related. Based on the major ubiquinone, *Malbranchea* spp. were divided into two groups, of which one group possessed the same major ubiquinone as *C. immitis*. Similar GC contents and CFA profiles were obtained for the species of *Malbranchea* and *C. immitis*. On the basis of these criteria the relationships between the fungi are discussed.**

Key Words——*Coccidioides immitis*; fatty acid composition; GC content; *Malbranchea*; ubiquinone.

The form-genus *Malbranchea* was described by Saccardo in 1899, for a single species, *Malbranchea pulchella* Saccardo & Penig, characterized by the production of alternate arthroconidia in curved branches. On the basis of the characteristics described by Saccardo, Sigler and Carmichael (1976) proposed the addition of species that produce arthroconidia in straight branches to the genus, and amplified the genus to 15 species. However, it was emphasized that the species listed might have no phylogenetic correlation. The species of *Malbranchea* were commonly isolated from soil. Sexual states, which belong to the Onygenales (Onygenaceae and Myxotricaceae), are known in some species of *Malbranchea* (Currah, 1985).

*Coccidioides immitis* Rixford & Gilchrist is a soil-inhabiting, filamentous fungus and causative agent of coccidioidomycosis, which is the severest of all mycotic infections. The mycelia of the fungus produce asexual, airborne arthroconidia by simple fragmentation of hyphal elements (Cole and Kirkland, 1991). These arthroconidia transform into spherules packed with numerous endospores in the host tissue. The taxonomic position of *C. immitis* has been uncertain since no sexual stage has been found. Bowman and Taylor (1993) suggested that *C. immitis* has affinity with ascomycetes and is a possible member of the Onygenales, based on the analysis of the 18S ribosomal DNA (rDNA).

Some species of *Malbranchea* have been reported to resemble *C. immitis* in the general appearance of the fertile hyphae and arthroconidia, as seen in the fact that *C. immitis* was included in the genus by virtue of its having a *Malbranchea* state (Sigler and Carmichael, 1976). In spite of such close relatedness morphologically, pathogenicity like that of *C. immitis* has not been found in the species of *Malbranchea*.

Because of their morphological similarity, chemotaxonomic study of *Malbranchea* species and *C. immitis* is important. Fukushima et al. (1993) reported on the ubiquinone system of *C. immitis* and its implication for the taxonomy and identification of this fungus. In this paper, we present the results of chemotaxonomic study on the ubiquinone system, GC contents and cellular fatty acid composition (CFA) of *Malbranchea* spp., and their comparison with the results for *C. immitis*.

## Materials and Methods

**Microorganisms** The 15 species of *Malbranchea* used were as follows: *M. gypsea* Sigler & Carmichael IFM 4130, *M. sulfurea* (Miehe) Sigler & Carmichael IFM 41309, *M. aurantiaca* Sigler & Carmichael IFM 41301, *M. dendritica* Sigler & Carmichael IFM 41296, *M. chrysosporioidea* Sigler & Carmichael IFM 41294, *M. flava* Sigler & Carmichael IFM 4129, *M. graminicola* Sigler & Lacey IFM 41303, *M. albolutea* Sigler & Carmichael IFM 41545, *M. flocciformis* Sigler & Carmichael IFM 41298, *M. fulva* Sigler & Carmichael IFM 41307, *M. filamentosa* Sigler & Carmichael IFM 41300, *M. circinata* Sigler & Carmichael IFM 41295, *M. multicolor* Mannina & Mosca IFM 41297, *M. flavorosea* Sigler & Carmichael IFM 41293, *M. pulchella* Saccardo & Penzig IFM 41308. The 11 strains of *C. immitis* examined are listed in Table 2.

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**Cultivation** The species of *Malbranchea* were cultured in YM broth (0.3% yeast extract, 0.5% peptone, 0.3% malt extract, 1% glucose, pH 5.8) at 27°C on a rotary shaker and harvested at the stationary phase. Strains of *C. immitis* were grown on YM slants at 27°C for two weeks. Before analysis of ubiquinone system and CFA, fungi were sterilized with 4% formaldehyde for 48 h. Fungal cells were then collected and washed with distilled water several times. For CFA analysis, a portion of the cells was lyophilized.

**Extraction and analysis of ubiquinone** The method reported by Fukushima et al. (1993a) was adopted for this purpose.

**Extraction and analysis of cellular fatty acids** CFA extraction and methylation were carried out with the method by Durham and Kloos (1978). The methyl esters obtained were analyzed by gas-liquid chromatography using a Hewlett-Packard apparatus (model No. 5890, series II) equipped with a flame ionization detector (FID). HR-SS-10 capillary column (0.25 mm ID × 50 m L) was employed, and helium was used as the carrier gas. The temperature of the injector and FID was 250°C. The oven temperature was 130°C initially, and this was raised to 170°C at 1°C/min and to 180°C at of 3°C/min, then maintained isothermally for 10 min. A Hewlett-Packard integrator (model No. 3390) was used as recorder. Fatty acid methyl esters were identified by comparing their retention times with those of authentic compounds.

**DNA extraction** The mycelia of *Malbranchea* species were harvested at the start of the stationary phase and lyophilized after washing with distilled water. The dried material was ground thoroughly in a mortar with liquid nitrogen and transferred to an Eppendorf tube. Total DNA was isolated by the method by Raeder and Broda (1985).

**Isolation of nuclear DNA** Nuclear DNA was isolated from the total DNA by use of cesium chloride density gradients (Garber and Yoder, 1983).

**Nucleotides preparation** Nucleotides were obtained by hydrolysis of the nuclear DNA with nuclease P1 (Yamasa Shoyu Co., Chiba, Japan), by the procedure of Katayama-Fujimura et al. (1984).

**Analysis of nucleotides** HPLC was employed under the following conditions: Hewlett Packard 1050 system, equipped with YMC-Pack ODS-AQ column (150 mm L × 6.0 mm ID). Nucleotides were eluted with phosphate buffer (10 mM $H_3PO_4$, 10 mM $KH_2PO_4$, pH 3.5) at a flow rate of 1.5 ml/min, temperature 25°C, and detected by UV absorbance at 270 nm. An equimolar mixture of the four nucleotides was used as standard (Yamasa Shoyu Co., Chiba, Japan). The amount of each nucleotide in a given sample was estimated by comparison of its peak area with that of the standard.

## Results

The distribution of ubiquinones (Q) of 15 species of *Malbranchea* is summarized in Table 1, from which minor ubiquinones with relative concentrations of less than 1% are excluded. The genus was divided into two groups based on the distribution of the major ubiquinone. Eleven species had Q-10($H_2$) as the major ubiquinone, accompanied by Q-10 as the minor ubiquinone. *Malbranchea albolutea* additionally contained another minor ubiquinone, Q-9($H_2$). The second group consists of four species, *M. circinata*, *M. flavorosea*, *M. multicolor* and *M. pulchella*, which possess Q-10 as the major ubiquinone, with different profiles of minor components, as follows: Q-10($H_2$) in *M. multicolor*; Q-10($H_2$) + Q-9 in *M. circinata*; Q-9 in *M. flavorosea*; and Q-9 + Q-8 in *M. pulchella*.

The results of CFA analysis of *Malbranchea* spp. and *C. immitis* are shown in Tables 1 and 2, respectively, for

Table 1. Ubiquinone systems, GC contents and cellular fatty acid composition in *Malbranchea* species.

| Species | Ubiquinone systems (%) | | | GC (%) | Fatty acids (%) | | | |
|---|---|---|---|---|---|---|---|---|
| | Q-10 ($H_2$) | Q-10 | others | | $C_{16:0}$ | $C_{18:0}$ | $C_{18:1}$ | $C_{18:2}$ |
| *M. gypsea* | 99 | 1 | | 49.2 | 20 | 7 | 28 | 39 |
| *M. sulfurea* | 98 | 2 | | 49.7 | 23 | 9 | 14 | 50 |
| *M. aurantiaca* | 98 | 2 | | 49.4 | 12 | 4 | 33 | 48 |
| *M. dendritica* | 97 | 3 | | 48.5 | 17 | 7 | 13 | 56 |
| *M. chrysosporioidea* | 96 | 4 | | 52.3 | 13 | 7 | 15 | 64 |
| *M. flava* | 93 | 7 | | 49.1 | 15 | 8 | 12 | 60 |
| *M. graminicola* | 91 | 9 | | 46.2 | 14 | 4 | 18 | 55 |
| *M. albolutea* | 90 | 8 | 2 (Q-9($H_2$)) | 50.1 | 21 | 4 | 27 | 45 |
| *M. flocciformis* | 88 | 12 | | 50.3 | 14 | 6 | 35 | 44 |
| *M. fulva* | 88 | 12 | | 48.5 | 33 | 5 | 23 | 31 |
| *M. filamentosa* | 75 | 25 | | 50.8 | 16 | 10 | 20 | 55 |
| *M. multicolor* | 23 | 77 | | 49.4 | 16 | 10 | 14 | 56 |
| *M. circinata* | 19 | 68 | 13 (Q-9) | 52.5 | 21 | 12 | 30 | 37 |
| *M. flavorosea* | | 94 | 6 (Q-9) | 51.4 | 24 | 15 | 21 | 35 |
| *M. pulchella* | | 67 | 28 (Q-9), 5 (Q-8) | 52.3 | 20 | 5 | 37 | 35 |

Table 2. Cellular fatty acid composition and ubiquinone systems of *Coccidioides immitis*.

| Stain No. | Ubiquinone systems (%)[a] | | | Fatty acids (%) | | | |
|---|---|---|---|---|---|---|---|
| | Q-10 | Q-9 | Q-8 | $C_{16:0}$ | $C_{18:0}$ | $C_{18:1}$ | $C_{18:2}$ |
| IFM 45811 | 87 | 13 | | 18 | 9 | 32 | 34 |
| IFM 45812 | 89 | 11 | | 18 | 5 | 34 | 39 |
| IFM 45814 | 86 | 14 | | 19 | 4 | 34 | 38 |
| IFM 45815 | 91 | 9 | | 18 | 5 | 34 | 39 |
| IFM 45868 | 83 | 16 | 1 | 13 | 13 | 45 | 17 |
| IFM 45817 | 88 | 12 | | 11 | 7 | 51 | 24 |
| IFM 4945 | 90 | 5 | 5 | 8 | 9 | 53 | 27 |
| IFM 45813 | 84 | 16 | | 8 | 14 | 58 | 11 |
| IFM 45816 | 85 | 15 | | 15 | 5 | 40 | 37 |
| IFM 4935 | 90 | 5 | 5 | 17 | 12 | 26 | 41 |
| IFM 45809 | 91 | 9 | | 15 | 14 | 33 | 25 |

[a]Data of ubiquinone systems reported by Fukushima et al. (1993).

fatty acids with abundances of above 1%. Four fatty acids, palmitic acid ($C_{16:0}$), stearic acid ($C_{18:0}$), oleic acid ($C_{18:1}$) and linoleic acid ($C_{18:2}$), were commonly found in *Malbranchea* spp. and *C. immitis*. In *Malbranchea*, 13 species had $C_{18:2}$ in the highest concentration, followed by $C_{18:1}$, $C_{16:0}$ and $C_{18:0}$. Two species, *M. fulva* and *M. pulchella*, had almost equal abundances of $C_{16:0}$ and $C_{18:2}$, and $C_{18:1}$ and $C_{18:2}$, respectively, as the principal components. In the 11 strains of *C. immitis* (Table 2), the expected uniformity of CFA profile was not found. Four strains, IFM 45811, 45812, 45814 and 45815, showed almost identical CFA profiles in terms of the principal components, and IFM 45816, was also similar. Four other strains, IFM 45868, 45817, 4945 and 45813, were characterized by an abundance of more than 45.4% of the principal component, $C_{18:1}$. IFM 4935 and 45809 differed from the other strains in the high abundance (41%) of $C_{18:2}$ in the former and the overall composition in the latter.

The GC contents in the *Malbranchea* species examined ranged from 46.2% for *M. graminicola* to 52.5% mol GC for *M. circinata* (Table 1), exhibiting an intrageneric variation of 6.3%.

## Discussion

The traditional fungal taxonomy, based on morphological and some physiological characters, is not satisfactory for fungi that lack or do not produce a sexual stage (Fell and Findlay, 1987; Sugiyama et al., 1993). Chemotaxonomy has proven to be a useful tool in the identification and classification of bacteria and yeasts (Katayama-Fujimura et al., 1982; Sugiyama et al., 1993). As mentioned, *Malbranchea* spp. and *C. immitis* are morphologically related to one another; and the finding of chemotaxonomic criteria for these fungi should be contribute to the taxonomy and phylogeny of *Malbranchea* spp. as well as the convenient identification of the most hazardous fungus, *C. immitis*. The significance of the ubiquinone system in *C. immitis* was discussed in our earlier paper (Fukushima et al., 1993). Here, comparison of the ubiquinone systems in *Malbranchea* spp. (Table 1) and *C. immitis* (Table 2) revealed that the 11 species of *Malbranchea* are distinguishable from *C. immitis* by this system. Four species, *M. multicolor*, *M. circinata*, *M. pulchella* and *M. flavorosea*, had the same major ubiquinone, Q-10, as *C. immitis*, however, which means that the fungi cannot be distinguished from *C. immitis* by the major component alone.

Billon-Grand (1987, 1989) discussed the importance of minor ubiquinones in the taxonomy of the yeasts and also demonstrated the high reproducibility, qualitatively and quantitatively, of the ubiquinones in the study on standardization of growth conditions for ubiquinone analysis. As seen in Tables 1 and 2, one or more minor ubiquinones were found in all fungi tested. For the four species of *Malbranchea* mentioned above, the distribution of their minor ubiquinone molecules can serve as an additional parameter to elucidate their chemotaxonomic relation to *C. immitis*. The dihydrogenated ubiquinone molecule, Q-10($H_2$), was present as a minor ubiquinone in both *M. circinata* and *M. multicolor*, but was not found in any of the strains of *C. immitis*, in which the minor ubiquinones were found to be typical ubiquinone molecules (Table 2). Our accumulation of data on the ubiquinone systems of fungi indicates strongly that the profile of minor ubiquinones is characteristic of each fungus. Considering this possibility, the occurrence of the hydrogenated ubiquinone molecules in the above two *Malbranchea* species can serve as an additional parameter for distinguishing them from *C. immitis*. However, this parameter cannot be used to distinguish *C. immitis* from *M. pulchella* and *M. flavorosea*, which possess only typical, non-hydrogenated minor ubiquinones. Sigler and Carmichael (1976) pointed out that *C. immitis* is classified as *Malbranchea* state. Recently, Bowman and Taylor (1993) showed the phylogenetic relation of *C. immitis* with *M. dendritica*, the *Malbranchea* state of *Uncinocarpus reesii* Sigler & Orr, and *Auxarthron zuffianum* (Morini) Orr & Kuehn. The genus *Auxarthron* includes

teleomorphs of two *Malbranchea* species. As mentioned above, the ubiquinone system of *C. immitis* differs from those of the majority of *Malbranchea* spp., including *M. dendritica*. However, a more closer relation may exist with species such as *M. pulchella*, type species, that possess the same ubiquinone system.

The use of GC content in taxonomic study is mainly exclusionary: a similar GC content by itself does not indicate a close relationship, but a dissimilar GC content is a useful characteristic to exclude close relationship or conspecificity. Depending on the method used for determination, the GC values within a species do not differ by more than 1 to 3 mol %, and the differences within a genus are usually less than 10 mol % (Kurtzman, 1985; Prince et al., 1978). The HPLC method showed the advantages of good accuracy and high reproducibility for determining the GC content. Values determined by the melting temperature (Tm) method and HPLC methods were reported to differ by less than 2% in a study using basidiomycetous yeasts (Hamamoto et al., 1986).

The GC content of *C. immitis* was found to lie in the range of 49.4 to 49.6% by the Tm method (Pappagianis et al., 1985). In this study, the following means of GC contents for *Malbranchea* spp. were obtained: 50.0% for all species; 51.4% for 4 species having Q-10; 49.4% for 11 species having Q-10($H_2$). Similar GC values were found for *C. immitis* and *Malbranchea* spp., but this is not conclusive evidence of a relationship between *Malbranchea* spp. and *C. immitis*, since the GC value only allows exclusion of close conspecificity in cases of dissimilar values.

The use of CFA as a tool in the taxonomy of bacteria and yeasts has been reported, but in some cases it was found not to be useful (Boekhout and Golubev, 1993; Welch, 1991). Eijk et al. (1982) reported that fatty acid profiles of *Rhodosporidium* Banno, *Sporobolomyces* Kluyver & v. Niel and *Sporidiobolus* Nyland varied as much between strains as between species. For dermatophyte fungi, very similar CFA profiles between genera, with intraspecific variation, were demonstrated by Jones and Noble (1981). Fell and Findlay (1987) considered that lipids have considerable value as a taxonomic tool in recognition of broad phylogenetic groups.

The fatty acids $C_{16:0}$, $C_{18:0}$, $C_{18:1}$ and $C_{18:2}$ are universally present in fungi (Eijk et al., 1982), and were also found in *Malbranchea* spp. and *C. immitis* strains. The species of *Malbranchea* cannot be distinguished from each other because they have the same qualitative CFA profile and varied quantitative profiles.

CFA profiles of *C. immitis* strains were different quantitatively but the same qualitatively. The same major fatty acids for the fungus as those reported by Anderes et al. (1973) were found here. However, direct comparison of the results of different works is difficult, since CFA composition can vary with growth phase, morphological state, cultural conditions and analytical procedure (Smit et al., 1987; Gunasekaran and Hughes, 1980; Ghannoum et al., 1986). Because of the slight differences in CFA profile, this parameter cannot be used for distinguishing *C. immitis* from *Malbranchea* spp.

The three criteria of ubiquinone system, GC contents and CFA composition each had its limitations as a sole parameter for chemotaxonomical study. However, the data obtained suggest that concurrent analyses on the three criteria can serve as useful parameters to define the closest relationship between *M. circinata*, *M. flavorosea* and *M. pulchella*. These species have Q-10 as the major ubiquinone, and closely similar values of GC, CFA composition and cellulolytic activity (Sigler and Carmichael, 1976). All species belong to the *Malbranchea* group, which possess curved fertile hyphae. *Coccidioides immitis* also exhibited chemically identical characters to these species. It may be suggested that *C. immitis*, which had been classified into the group having straight fertile hyphae (Sigler and Carmichael, 1976), could be related with *M. flavorosea* and *M. pulchella*. The sexual state of *M. pulchella* is unknown, but both *M. circinata* and *M. flavorosea* have a *Myxotricum* teleomorph as sexual state. Considering biochemical, immunological and molecular data, a close phylogenetic relation between for *C. immitis* and some *Malbranchea* spp. and teleomorphic species of *Malbranchea*, in particular *Uncinocarpus reesii*, was suggested by Bowman and Taylor (1993), and Pan et al. (1994), and this supported the morphological evidence reported by Sigler and Carmichael (1976). If the species of *Malbranchea* having Q-10 had been examined, a more accentuated relationship might have been found.

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