Biochemical markers in taxonomy of the genus *Cunninghamella*

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Summary

The chemical composition of fatty acids and ubiquinones was studied in 18 strains of *Cunninghamella*, to establish quantitative and qualitative differences within the genus. Fatty acids analysis has shown the presence of four groups. Ubiquinone analysis, through high performance liquid chromatography (HPLC), demonstrated the existence of three different groups based on the ubiquinone type. The average percentage of fatty acids of the species *Cunninghamella elegans* and *Cunninghamella bertholletiae*, show variations in linolenic and stearic acids, suggesting the possibility of differentiation between the two species.

Key words Chemotaxonomy, Ubiquinone system, Fatty acids, *Cunninghamella*

Marcadores bioquímicos en la taxonomía del género *Cunninghamella*

Resumen

Se estudió la composición química de los ácidos grasos y ubiquinonas en 18 cepas de *Cunninghamella* para conocer y establecer diferencias cuantitativas y cualitativas dentro del género. El análisis de ácidos grasos mostró la presencia de cuatro grupos. El análisis de ubiquinonas, por cromatografía líquida de alta resolución (HPLC), demostró la formación de tres grupos diferentes según el tipo de ubiquinona. El porcentaje medio de ácidos grasos entre las especies *Cunninghamella elegans* y *Cunninghamella bertholletiae* mostró una variación en los ácidos linolénico y esteárico, sugiriendo la posibilidad de diferenciación entre las dos especies.

Palabras clave Quimiotaxonomía, Ubiquinonas, Ácidos grasos, *Cunninghamella*

The genus *Cunninghamella* contains species of importance in medical mycology and in biotechnological processes. Within medical mycology over seventy cases of infections caused by *Cunninghamella bertholletiae* STADEL 1911, have already been reported, including cases of sinusitis [1], endobronchial zygomycosis [2] and pulmonary infection in cancer patients and in patients with diabetes mellitus [3]. In the biotechnological field, *Cunninghamella* species such as *Cunninghamella bainieri* NAUMOV 1939 have the capacity to metabolize xenobiotics, including aromatic compounds and pharmacological drugs [4-6]. *Cunninghamella elegans* has the ability to oxidise polycyclic aromatic hydrocarbons, petroleum compounds and to degrade fluorantene, and the most common polycyclic aromatic hydrocarbon in the environment [7-9], such as the nitrated polycyclic aromatic hydrocarbons, considered as mutagenic and carcinogenic agents [10].

The taxonomy of *Cunninghamella* is based only upon morphologic and physiological aspects; however *C. elegans* and *C. bertholletiae*, show similar reproductive structures, making their identification difficult [11]. So, other parameters can serve as aids to morphological taxonomy, such as the use of biochemical markers, in the identification of microorganisms. This methodology has contributed substantially to the resolution of several problems related to aspects of classification and phylogenetics [12,13].

Because of the need for a broader knowledge of the biology of the species of the genus *Cunninghamella*, studies were made with the objective of determining the chemical composition of the ubiquinone system and fatty acids of 18 strains. In addition, the value of these parameters as additional tools for the morphologic taxonomy of the species studied, was assessed, with the purpose of establishing the inter and intraespecific relationships especially between the species of *C. elegans* and *C. bertholletiae*.

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MATERIALS AND METHODS

The eight strains of the *Cunninghamella* genus used were from the collection of the Research Center for Pathogenic Fungi and Microbial Toxicoses, Chiba University, Japan (IFM), five isolated samples from Amazônia legal (LIKA) and five strains from UFPE (URM) collection. The strains studied are listed in table 1.

Table 1. Strains used in this investigation.

Species	Strains No
Cunninghamella elegans	IFM 40505 IFM 46109 IFM 47050 LIKA 0017 LIKA 0014 LIKA 0015 LIKA 0016 IFM 47591 URM 2084 URM 3172
Cunninghamella bertholletiae	IFM 46110 IFM 46111 IFM 46114 IFM 46987 LIKA 0013
Cunninghamella blakesleeana	URM 168
Cunninghamella echinulata	URM 2136
Cunninghamella ramosa	URM 1918

Cultural conditions. The culture medium used for maintenance and for obtaining the mycelial mass was the YMB: malt extract (3 g), yeast extract (3 g), peptone, D-glucose, adding distilled water until the final volume was 1.000 ml and pH 5.8, as a solid medium 20 g of agar was added, being denominated YMA.

Extraction and preparation of ubiquinone. The ubiquinone was extracted from saponified cells with hexane, and purified by preparative thin-layer chromatography on a silica-gel plate with pure benzene as solvent [14].

Identification of ubiquinones. The purified ubiquinones were analyzed by high performance liquid chromatography (HPLC) and identified by comparison of retention times of the samples with the following standard of ubiquinones: Q6 Coenzyme, Q7 Coenzyme, Q9 Coenzyme, Q10 Coenzyme (obtained from Sigma Chemical Company - USA). The analysis was made using a Cosmosil column 5C18-P (4,6x250mm), on the following conditions: eluent: methanol-isopropanol (2:1, v/v), flow rate: 1.0 ml/min, UV detector: 270nm.

Extraction and methylation of fatty acids. The fatty acids were converted to methyl ester according to Dunlap and Perry's method (1967), described by Durham and Kloos [15].

Gas chromatography The analyses were carried out in a gas chromatograph equipped with capillary column HR-SS-10 (0.125X50M), using helium as carrier gas. The injector and detector (FID) temperature was 250 °C, oven temperature at 130 °C, starting and increasing to 170 °C in 1 °C/min, to 180 °C em 3 °C/min, kept isothermically for 10 min. The fatty acids were identified through the comparison of the retention time of the peaks of the samples with the standard. Relative amounts of methyl esters CFA were calculated through the integration of the areas of the peaks. The standard fatty acids were: myristic acid (C14:0), palmitic acid (C16:0), stearic acid (C18:0), oleic acid (C18:1), linolenic acid (C18:3) and γ-linolenic acid (C18:3) (obtained from Sigma Chemical Company - USA).

RESULTS

*Ubiquinone system analysis***.** The *Cunninghamella* genus was divided in three groups based on the ubiquinone system. The results are shown in table 2.

It was observed in this study that the isolates of *Cunninghamella* contained Q6 ubiquinone, Q9 ubiquinone and Q10 ubiquinone.

Within the group of isolates studied three groups were recognized based on the ubiquinone type. Group I was distinguished by having Q6 ubiquinone as the main compound, and group II by Q9 ubiquinone. Both of them subdivided in A, with the presence of Q10 ubiquinone and subgroup B differing by the absence of Q10 ubiquinone and group III constituted by a higher percentage of Q9 ubiquinone.

C. bertholletiae (IFM46987, IFM46110 and IFM46111) and *C. elegans* (IFM40505, IFM47050, IFM46109 and IFM47591) species were united into group IA. *Cunninghamella ramosa* (URM1918) and *C. bertholletiae* (IFM46114 and L13) were united into group IB. The *C. bertholletiae* species was characterized into this group as all the strains present the Q6 ubiquinone as the main compound. Group IIA contained only the *C. elegans* isolate URM3172. Group IIB included the *C. elegans* isolates L14, L15, L16, L17and URM2084 and *Cunninghamella blakesleeana* URM168.

The species *Cunninghamella echinulada* (URM2136) was characterized by its higher Q9 ubiquinone percentage (91,91%) amongst all of the strains studied and constituted group III.

*Fatty acids chemical composition analysis***.** Five fatty acids were found in all of the strains of the studied genus (Table 3). Oleic acid (C18:1) was the main compound, followed by palmitic (C16:0), linolenic (C18:3), γ–linolenic (C18:3), stearic (C18:0), and myristic acids (C14:0). Myristic acid (C14:0) was found in low percentages in all of isolates, except for strain URM 168 of *C. blakesleeana*.

The composition of the fatty acids shows the existence of four groups: Group I has higher concentrations of palmitic, oleic and γ–linolenic acids; group II higher concentrations of palmitic and oleic acids; group III higher concentrations of palmitic, oleic and linolenic acids and group IV only higher concentrations of oleic acid, as shown in table 3.

Three strains of the species *C. elegans* are included within group I (L15, L17 and IFM47591).

Group II was subdivided into: IIA with high levels of linolenic acid and low levels of myristic acid. The species *C. bertholletiae* (L13) and *C. elegans* (L14, L16) are included. IIB has high levels of stearic acid and low levels of linolenic acid. Three representatives of *C. elegans* are included, IIC has high levels of linolenic acid and low levels of stearic acid, and includes *C. bertholletiae* (IFM46114, IFM46111 and IFM46987) and *C. elegans* (40505), and subgroup IID has high levels of γ–linolenic and low levels of stearic acid, and includes *C. blakesleeana* (URM168) and *C. ramosa* (URM1918).

Group III consisted solely of *C. bertholletiae* (IFM46110) isolates but group IV contained *C. echinulata* (URM2136) and *C. elegans* (URM2084).

The *C. bertholletiae* (IFM46987, IFM46110 and IFM46111) isolates could not be separated from *C. elegans* (IFM40505, IFM47050, IFM46109 and IFM47591)

Table 2. Distribution of the groups formed using ubiquinone system as chemotaxonomic marker in *Cunninghamella* strains.

Group I CoQ 6 main		Group II CoQ 9 main		Group III Major percentage
A Presence / CoQ 10	B Absence / CoQ 10	A Presence / CoQ 10	B Absence / CoQ 10	CoQ 9
C. bertholletie IFM46987	*C. ramosa* URM1918	*C. elegans* URM3172	*C. elegans* L14	*C. echinulata* URM2136
C. bertholletiae IFM46110	*C. bertholletiae* IFM46114		*C. elegans* L15	
C. bertholletiae IFM46111	*C. bertholletiae* L13		*C. elegans* L16	
C. elegans IFM40505			*C. elegans* L17	
C. elegans IFM47050			*C. elegans* URM2084	
C. elegans IFM46109			*C. blakesleeana* URM168	
C. elegans IFM47591				

Table 3. Distribution of fatty acids in groups.

Group I C16:0, C18:1, C18:3	Group II C16:0 and C18:1	Group III C16:0, 18:1, C18:3	Group IV C18:1
C. elegans (L17) *C. elegans* (L15) *C. elegans* (IFM47591)	IIA C14:0(low percentage) + C18:3 (high percentage) *C. elegans* (L14) *C. bertholletiae* (L13) *C. elegans* (L16) IIB C18:0 (high percentage) + C18:3 (low percentage) *C. elegans* (URM3172) *C. elegans* (IFM47050) *C. elegans* (IFM46109) IIC C18:0 (low percentage) + C18:3 (high percentage) *C. elegans* (IFM40505) *C. bertholletiae* (IFM46114) *C. bertholletiae* (IFM46987) *C. bertholletiae* (IFM46111) IID C18:3 g (high percentage) + C18:0 (low percentage) *C. blakesleeana* (URM168) *C. ramosa* (URM1918)	*C. bertholletiae* (IFM46110)	*C. echinulata* (URM168) *C. elegans* (URM2084)

Table 4. Fatty acids chemical composition average percentage in *C. elegans* and *C. bertholletiae*.

Species	Average percentage (average ± error standard)					
	C14:0	C16:0	C18:0	C18:1	C18:3	C18:3 (γ)
C. elegans	1.37 ± 0.11	23.39 ± 0.84	11.98 ± 1.63	36.63 ± 1.44	13.17 ± 1,55	13.56 ± 1.94
C. bertholletiae	1.63 ± 0.24	24.89 ± 0.88	8.21 ± 0.74	36.47 ± 1.42	18.56 ± 1.05	10.24 ± 1.21

as both species are included in ubiquinones Group IA. The same was true for fatty acids, where *C. elegans* belongs to group IIC as well as *C. bertholletiae* (IFM46987 and IFM46111). Although *C. ramosa* (URM1918) and *C. berhtolletiae* (IFM46114 and L13) belong to the same ubiquinone group (IB) they can be separated through the fatty acids Group IID. *C. elegans* (URM3172) differed from other strains in being confined to ubiquinone Group IIA. The especies in ubiquinone group IIB (*C. blakesleeana* URM168 and *C. elegans* L14, L15, L16, L17 and URM2084), could be separated according to fatty acids group IID. The species *C. echinulata* (URM2136) was separately characterized in ubiquinone group III since it shows the highest percentage of Q9 ubiquinone, in the same way as it has been found in fatty acids of group IV. The average percentage between the isolates of *C. elegans* and *C. bertholletiae* species are shown in table 4.

Through *C. elegans* and *C. bertholletiae* fatty acids chemical composition average percentage, differences in stearic and linolenic acids values have been observed, showing the possibility of its use as an additional parameter to morphophysiologic taxonomy in the separation process between the two species; however the *C. elegans* IFM40505 and *C. bertholletiae* IFM46987 species, have shown near values in its chemical composition, suggesting a more detailed analysis of those two species through molecular biology techniques.

DISCUSSION

The ubiquinone system found in this study has already been studied in other fungi [16-19]. Billon-Grand [20,21] discussed the importance of the smaller compounds of ubiquinone on yeast taxonomy and suggested a standard on analysis conditions for an easier reproduction.

Okada *et al*. [22] also found through the smaller compounds, an additional parameter to characterize *Malbranchea* and *Coccidioides*. The smaller ubiquinone peaks within the strains studied were also found in *Cunninghamella*. In higher fungi, Basidiomycetes, Ascomycetes and Deuteromycetes, most of the fungi have shown a system consisting of Q9 or Q10 ubiquinone [18]. Q6 and Q9 ubiquinone were found as the main peaks in species of *Cunninghamella*.

Many authors describe the usage of ubiquinone systems in a generic and intrageneric level in fungi [18,23]. However, Shubert and Kreisel [24], Kuraishi *et al*. [25] and Yagushi *et al*. [26] also obtained satisfactory results at the infrageneric level. The results obtained in the present study allowed the characterization of *C. bertholletiae* species, as it shows Q6 ubiquinone as the main compound. It could not nevertheless suggest a differentiation between *C. elegans* and *C. bertholletiae*.

No fatty acid that has not already been previously found in other Zygomycetes fungi lipids was detected in this study [27-29]. The fatty acids C16:0, C18:0 and C18:1 are universally found in fungi, and were also found in *Cunninghamella*.

Shaw suggested that the γ-linolenic acid is a characteristic of the Zygomycetes. This fatty acid was also found in all of the species studied, in agreement with the other authors [30,31].

Welch [32] comments on the scant diversity of fatty acids in filamentous fungi compared to other microorganisms. In this study, the strains differed only in relative concentration of each fatty acid. Qualitative variations were found only for *C. blakesleeana* URM168. The higher percentages of fatty acids found within *Cunninghamella* strains were of oleic and palmitic acids. Fatty acid studies in *Mucor* and *Endogone pisiformis* species also showed higher percentages of oleic acid, followed by palmitic acid [33,34]. Martinez [35] reported fatty acid composition of *Ganoderma* (Basidiomycetes) species collected in different countries [35]. The results obtained in the present study with *Cunninghamella* showed similarities in fatty acids chemical compositions of strains collected in different countries, the results being comparable to those obtained by Martinez [35].

The results of the fatty acid composition in *C. elegans* were also in accordance with those of Stahl and Klug [31], who analyzed the chemical composition of the *C. elegans* and five representatives of Zygomycetes. The proportions between the fatty acids were the same as those obtained for the *C. elegans* strains included in this study.

The use of fatty acids as an additional tool in yeast and bacteria taxonomy has been described, but this approach could not be used in some cases [36,32], for example, for strains of *Microsporum* and *Trichophyton* [37]. However, in some cases, the use of both chemical parameters (ubiquinone system and fatty acid) is required for the analysis. The study of ubiquinone chemical composition and fatty acids has already been described by other authors [12]. The fatty acids chemical composition associated with the ubiquinone system and morphophysiological characteristics allow the characterization of the *C. blakesleeana*, *C. ramosa*, *C. echinulata*, *C. bertholletiae* and *C. elegans* species. The results obtained in this study confirm the need for the union of both of those chemical parameters.

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