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The use of compensatory base change analysis of ITS2 as a tool in the phylogeny of *Mucorales*, illustrated by the *Mucor circinelloides* complex

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Abstract Compensatory base changes (CBCs) in helix II of rDNA ITS2, suggested as a molecular classifier for fungi, were analyzed in *Mucor circinelloides* and its varieties. Only a few CBCs were found in the complex. Three out of the four accepted formae (f. *circinelloides*, f. *lusitanicus*, f. *janssenii*) did not exhibit CBCs. One CBC was found between strains that form zygospores; consequently, CBC is not always concordant with mating experiments. Strains with two CBC are unable to breed. It is suggested that some strains of the *M. circinelloides* complex are at the beginning of speciation.

Keywords Mucor · Microevolution · Compensatory base changes

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Introduction

Mucor circinelloides is a commonly occurring soil fungus, but is also known as a causal agent of infection in immunocompromised patients (Brown 2005; Ribes et al. 2000). As a high mortality rate is typical for acute mucormycosis (Roden et al. 2005), rapid and appropriate identification of clinical strains is needed. The species was described by van Tieghem in 1875. In 1976, Schipper classified *Mucor circinelloides, M. janssenii* Lendn., *M. griseocyanus* Hagem and *M. lusitanicus* Bruderl. as a single, variable species after comparative morphological studies and interfertility tests. In a recent study, Walther et al. (2013) showed that *Mucor circinelloides* in this sense is not a clearly delimited species. In phylogenetic trees based on the large subunit (LSU) and the internal transcribed spacer (ITS) region, the four currently accepted formae of *M. circinelloides* are intermingled with morphologically intermediate strains of *M. circinelloides* and different species such as *Mucor bainieri, M. ramosissimus* as well as the sporangiola-forming *M. ctenidius* (syn. *Backusella ctenidia*) and *Ellisomyces anomalus*. Consequently, our study on *M. circinelloides* includes the complete relationship defined as the *Mucor circinelloides* complex by Walther et al. (2013).

Species recognition in *Mucorales* by Genealogical Concordance Phylogenetic Species Recognition (GCPSR) (Taylor et al. 2000) is currently hampered by the lack of appropriate single-copy markers. The biological species recognition concept (Mayr 1982) is difficult to apply in *Mucorales* because germination of zygospores has been observed in only a few species due to the lengthy period of dormancy and the particular conditions needed for every species (Gryganskyi et al. 2010). Therefore, the production of zygospores has been used instead as a single criterion (e.g. Schipper 1976) to give at least an indication of conspecifity. However, azygospores (parthenogenic zygospores) produced by a single gametangium are known in

representatives of this group (strain CBS 479.70 of forma *circinelloides* and in the only strain cultivated of *Mucor bainieri*, CBS 293.63) (Schipper 1976). Although they can be recognized by morphology, the presence of azygosporic strains in the *M. circinelloides* complex makes the search for alternative methods of species delimitation imperative.

Recently, compensatory base changes (CBCs) in the ITS2 region have been suggested as molecular classifiers for plants, animals and fungi, indicating with 93.11 % reliability whether or not two organisms belong to distinct species (Müller et al. 2007). There are several ascomycetous genera for which the ITS region is not variable enough to discriminate species (Schoch et al. 2012) and, consequently, CBCs of the ITS2 region cannot be applied in these genera. However, the ITS region in *Mucorales* is highly variable (e.g. Alastruey-Izquierdo et al. 2010), making the application of CBC seem promising. CBCs are mutations that involve both members of a pair in the double helix of an RNA transcript secondary structure with pairing being maintained (Coleman and Vacquier 2002; Gutell 1994; Ruhl et al. 2010). Coleman (2003, 2007) showed that a CBC in helix II or helix III of the ITS2 secondary structure between two organisms correlated with sexual incompatibility. Coleman (2009) proved that organisms that differ by even one CBC in the 5'-side 30 nucleotide positions of helix III of ITS2 (conserved region) are unable to cross.

The aim of the present study was to shed light on the correct taxonomic rank of the formae of *Mucor circinelloides* and related taxa, and to assess the value of CBC analysis in species delimitation in the *Mucor circinelloides* complex. The results of CBC analysis were compared with species delimitations based on molecular data (Walther et al. 2013) and on mating experiments (Schipper 1976).

Materials and methods

Eighty-nine ITS sequences of species belonging to the *Mucor circinelloides* complex were acquired from GenBank (supplementary materials S1). Eighty-five of these sequences were generated for the study of Walther et al. (2013) and originate from strains from the CBS-KNAW Fungal Biodiversity Centre (CBS; Utrecht, The Netherlands), the Instituto de Salud Carlos III National Centre of Microbiology (CNM-CM; Madrid, Spain), or the Belgian Co-ordinated Collections of Micro-organisms (IHEM; Brussels, Belgium). The assignment of strains to species or formae follows Walther et al. (2013). ITS2 of all sequences were annotated by identifying a 25-nucleotide interaction of the 5' 5.8S rDNA subunit end with 25 nucleotides of the 28S rDNA subunit 3' end (Keller et al. 2009) using the "annotate feature" (default settings) on the ITS2 website (http://its2.bioapps.biozentrum.uni-wuerzburg.de). ITS2 rDNA secondary structure of *Mucor circinelloides* f. *circinelloides* CBS 195.68 (ex-neotype strain) was folded using RNAstructure using default settings (Mathews et al. 2004), and compared with other structures for this species accessible via the ITS2 Database (Schultz et al. 2006; Selig et al. 2008; Wolf et al. 2005). Homology modeling using *M. circinelloides* f. *circinelloides* CBS 195.68 as a template was performed on all other ITS2 sequences. The ITS2 sequences with homologous structures were multi-aligned using ClustalW (Thompson et al. 1994) as implemented in 4SALE version 1.6 (Seibel et al. 2006, 2008). The CBCAnalyzer (Wolf et al. 2005) as implemented in 4SALE (Seibel et al. 2006) was used to count CBCs.

Results

No CBCs were found among 83 strains belonging to the *Mucor circinelloides* complex (Table 1). Two CBCs were detected (dark gray in Table 1; Fig. 1) between the four strains of *M. circinelloides* f. *griseocyanus* and the two strains (CBS 846.73 and CBS 526.68) that morphologically resembled forma *janssenii* but appeared as a sister group to forma *griseocyanus* in the ITS tree of Walther et al. (2013) (Table 1; Fig. 1). Although there were no CBCs between the ex-type strain of *M. circinelloides* f. *griseocyanus* (CBS 116.08) and other representatives of *M. circinelloides*, there was one CBC between this strain and the other strains assigned to this forma (pale gray in Table 1). Interestingly, this strain was also the only representative of *M. circinelloides* f. *griseocyanus* that formed zygospores with *M. circinelloides* f. *circinelloides* representatives in mating experiments (Schipper 1976).

Comparison of CBC analysis with Schipper's (1976) mating experiments (Table 2) revealed four cases of zygospore formation between strains differing by a CBC (highlighted in gray in Table 2). Thus, CBC analysis is not concordant with mating experiments at this point.

Discussion

There is only one forma (*M. circinelloides* f. *griseocyanus*) and two other strains (CBS 526.68, CBS 846.73) that have one CBC to all other representatives included in this study. Applying the criteria outlined by Müller et al. (2007), they belong to separate species with 93.11 % credibility. However, this statement should be confirmed by multilocus data. The presence of two CBCs between *M. circinelloides* f. *griseocyanus* and the strains CBS 526.68 and CBS 846.73 leads us to assume that these two groups already cannot interbreed (confirmed by Schipper's 1976 experiments) and that they could be treated as separate species, while their relation to other members of the *M. circinelloides* complex is less clear (one CBC but

Table 1 Compensatory base changes (CBCs) matrix; in gray - values different than 0

	Taxon name	Number of strains	1	2	3	4	5	6	7	8	9	10	11	12
1	*Mucor circinelloides* f. *circinelloides*	43	0	0	0	0	0	0	0	0	0	0	1	1
2	*Mucor circinelloides* (forma unassigned)	6	0	0	0	0	0	0	0	0	0	0	1	1
3	*Mucor circinelloides* f. *lusitanicus*	12	0	0	0	0	0	0	0	0	0	0	1	1
4	*Mucor circinelloides* f. *griseocyanus* strain CBS 116.08	1	0	0	0	0	0	0	0	0	0	0	1	1
5	*Mucor circinelloides* f. *janssenii*	10	0	0	0	0	0	0	0	0	0	0	1	1
6	*Mucor circinelloides* f. *janssenii*[a]	4	0	0	0	0	0	0	0	0	0	0	1	1
7	*Ellisomyces anomalus*	2	0	0	0	0	0	0	0	0	0	0	1	1
8	*Mucor bainieri*	1	0	0	0	0	0	0	0	0	0	0	1	1
9	*Mucor ctenidium*	3	0	0	0	0	0	0	0	0	0	0	1	1
10	*Mucor ramosissimus*	1	0	0	0	0	0	0	0	0	0	0	1	1
11	*Mucor circinelloides* (forma unassigned) strains CBS526.68 and CBS846.73	2	1	1	1	1	1	1	1	1	1	1	0	2
12	*Mucor circinelloides* f. *griseocyanus*	4	1	1	2	1	1	1	1	1	1	1	2	0

[a] Treated as *M. velutinosus* in Álvarez et al. 2011

interbreeding occurred in Schipper's 1976 experiments, see Table 2). Our observations may suggest that some strains in the *M. circinelloides* complex are at the beginning of speciation, and thus the decision whether or not to treat them as separate species is subjective.

The lack of CBC between ex-type cultures of *M. circinelloides* f. *griseocyanus* (CBS 116.08) and all other *M. circinelloides* representatives could mean that it is an intermediate stage between *M. circinelloides* f. *circinelloides* and *M. circinelloides* f. *griseocyanus*. It also means presumably that morphological changes precede CBC appearance. In general, forma is the lowest taxonomic rank but its concept is rather unclear. In the *M. circinelloides* complex there are several strains that were not assigned to any formae because they represented intermediate morphological characters. That fact was one of the reasons why Schipper (1976) classified *M. circinelloides* f. *circinelloides, M. circinelloides* f. *lusitanicus, M. circinelloides* f. *janssenii* and *M. circinelloides* f. *griseocyanus* in a single, variable species. However, over time, interbreeding barriers may appear between two different morphological formae. *Mucor circinelloides* f. *griseocyanus* differs from others members of this group by the presence of only one layer of large sporangiophores (Milko 1974), which may constitute the beginning of interbreeding barrier. Thus, based on CBC analysis and morphological observations, we hypothesize that *M. circinelloides* f. *griseocyanus* is a formae at the beginning of speciation, but this should be verified by detailed mating experiments.

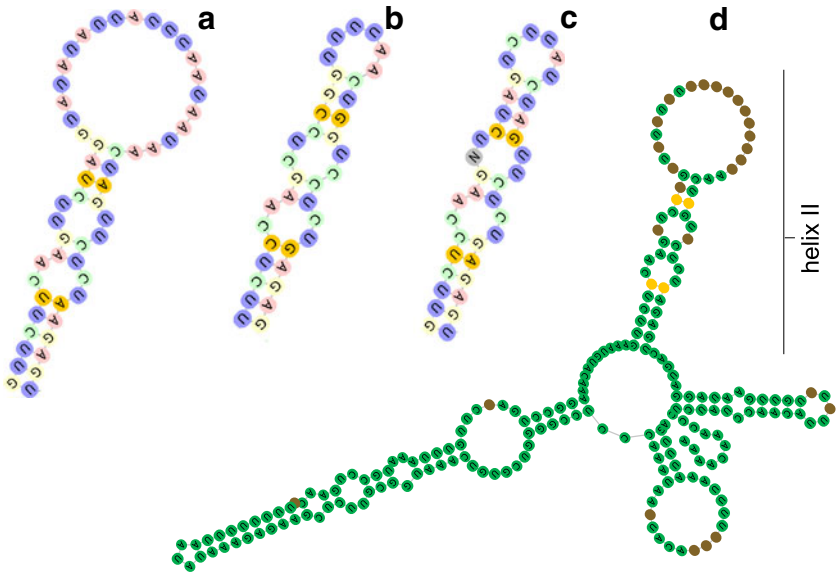

Fig. 1 **a**–**d**. Secondary structure and specific position of compensatory base changes (CBCs). **a** ITS2 secondary structure and sequence of strains CBS 526.68 and CBS 846.73, CBCs are highlighted in *yellow*. **b** ITS2 secondary structure and sequence of *M. circinelloides* f. *griseocyanus,* CBCs are highlighted in *yellow*. **c** ITS2 secondary structure and sequence of *M. circinelloides*; CBCs are highlighted in *yellow*. **d** Schematic consensus structure of ITS2 in *M. circinelloides* complex; CBC positions are shown in *yellow* and variable sites in *brown*

Based on mating experiments, Li et al. (2011) suggested that f. *circinelloides*, f. *lusitanicus* and f. *griseocyanus* (referred to as subspecies; f. *janssenii* was not included in their study) might represent discrete species, as positive mating results were observed predominantly in strains of the same forma. However, there were also two pairs of strains belonging to different formae that were able to produce zygospores (Li et al. 2011).

Like other members of the *Mucorales, Mucor circinelloides* reproduces sexually by means of zygospores resulting from conjugation of gametangia (Kirk et al. 2008). Morphologically indistinguishable compatible strains (but see Satina and Blakeslee 1925, 1926, 1928 for some weak indications of physiological differences) were named plus (+) and minus (−) by Blakeslee (1904). However, these two sexes produce different

Table 2 Comparison of CBC analysis with Schipper's (1976) mating experiment; information about zygospore formation is before slash (− lack of zygospores; Z zygospores present); number of CBCs is after slash; in gray - contradictory results of CBC analysis and mating experiments. Zygospore viability and recombination analyses were not conducted by Schipper (1976)

		circinelloides (+)		*lusitanicus* (+)	*griseocyanus* (+)		*janssenii* (+)
		CBS 172.27	CBS 192.68	CBS 969.68	CBS 198.28	CBS 223.56	CBS 205.68
circinelloides (–)	CBS 108.16	-/0	Z/0	-/0	-/1	-/1	-/0
	CBS 239.35	-/0	Z/0	-/0	-/1	-/1	-/0
	CBS 394.68	Z/0	Z/0	-/0	-/1	-/1	Z/0
lusitanicus (–)	CBS 108.17	-/0	-/0	Z/0	-/1	-/1	-/0
	CBS 276.49	-/0	-/0	Z/0	-/1	-/1	-/0
griseocyanus (–)	CBS 116.08	Z/0	Z/0	-/0	Z/1	Z/1	Z/0
	CBS 366.70	-/1	-/1	-/1	Z/0	-/0	Z/1
janssenii (–)	CBS 227.29	-/0	-/0	-/0	-/1	-/1	Z/0
	CBS 232.29	-/0	Z/0	Z/0	-/1	-/1	Z/0
	CBS 204.68	-/0	Z/0	-/0	-/1	-/1	Z/0
	CBS 365.7	-/0	-/0	-/0	-/1	-/1	Z/0
forma unassigned (–)	CBS 526.68	-/1	-/1	-/1	-/2	-/2	Z/1

pheromones, e.g. trisporic acids. The chemical interaction between two strains of opposite sex stimulates the formation of zygophores and induces carotenogenesis (Sutter 1970; Sutter et al. 1973). Thus zygospore formation has long been used as an indicator of sexual compatibility (e.g. Schipper 1973, 1976) although interspecific zygospores were known to occur. Generally, the production of a 'viable' (meaning 'typical', 'well colored', 'fully developed') zygospore was found to be sufficient evidence for successful mating and conspecifity of contrasted strains (Schipper 1973, but see Nielsen 1978 for an opposite opinion). Alastruey-Izquierdo et al. (2010) showed that interspecific zygospores occur in *Lichtheimia* and that they are poorly developed compared to their intraspecific counterparts. Schipper (1976) did not describe the zygospores in detail but neither she nor Li et al. (2011) mentioned differences in the size and color of the zygospores, indicating their interspecific nature.

On the other hand, the formation of zygospores is strongly dependent on environmental conditions and on the condition of the strains themselves (e.g. Schipper 1973). Moreover, in *Mucorales*, the pheromones produced by one mating type may be chemically modified by the other (Schachtschabel et al. 2008; Schimek and Wöstemeyer 2009). There are strains that are generally stronger and more viable, and produce zygospores more abundantly than others; there are also known examples of strains that do not produce zygospores at all ('neutral strains', Blakeslee 1904). While analyzing Schipper's (1976) mating experiments, it seems that strain CBS 116.08 could be one such more viable (Blakeslee and Cartledge 1927) strain that indicates sexual activity with various formae of *Mucor circinelloides*.

Mucorales are a phylogenetically very old group and the molecular distances between species are considerable. The weighted intraspecific ITS variability for 'zygomycetes' is 3.24 %. In contrast, for Ascomycota this variability is 1.96 % (for some species even much lower, e.g. 0.2 % for *Aspergillus fumigatus*), with much lower standard deviations than for 'zygomycetes' (Nilsson et al. 2008). The intraspecific variability in *Mucorales* differs among species but can reach more than 5 % as in *Mucor circinelloides* (5.3 %) or *Lichtheimia ramosa* (7.6 %) (Walther et al. 2013). Because of their molecular diversity, sequence differences including CBCs might not be connected with breeding barriers in *Mucorales*. For the reliable use of CBCs for species delimitation in *Mucorales*, we need a combined study on CBCs, multi-locus phylogenetic analyses, and inter- and intra-specific matings with special emphasis on zygospore details (production, germination, morphology).

Although the presence of CBCs in some strains (e.g. from forma *griseocyanus*) might indicate that speciation is underway in this group, in the ITS tree of Walther et al. (2013), forma *griseocyanus* does not take an isolated position. The sequence diversity in *M. circinelloides sensu* Schipper (1976), i.e. including all formae, is much smaller (5.3 %) than in *M. hiemalis* sensu Schipper (1973) including *M. luteus* and *M. silvaticus* (14.8 %), which obtained species status recently (Budziszewska et al. 2010; Walther et al. 2013). Taking all these findings together, we rather support the maintenance of the forma status for all formae accepted in *Mucor circinelloides* because of the presence of morphologically intermediate strains, comparatively small genetic distance among the formae, the wide absence of CBCs, and the contrasts in formation of zygospores between the formae. More detailed studies on sexual reproduction in this group and comprehensive multi-locus analysis are necessary for a final decision.

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