**Preliminary studies on the evolution of carbon assimilation abilities within Mucorales**

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**Abstract**

Representatives of Mucorales belong to one of the oldest lineages of terrestrial fungi. Although carbon is of fundamental importance for fungal growth and functioning, relatively little is known about enzymatic capacities of Mucorales. The evolutionary history and the variability of the capacity to metabolize different carbon sources among representatives of the order Mucorales was studied using Phenotypic Microarray Plates. The ability of 26 strains belonging to 23 nonpathogenic species of Mucorales to use 95 different carbon sources was tested. Intraspecies variability of carbon assimilation profiles was lower than interspecies variation for some selected strains. Although similarities between the phylogenetic tree and the dendrogram created from carbon source utilization data were observed, the ability of the various strains to use the analyzed substrates did not show a clear correlation with the evolutionary history of the group. Instead, carbon assimilation profiles are probably shaped by environmental conditions.

**Research highlights:**

* Carbon assimilation profiles of Mucorales are strongly variable
* The observed pattern reflects several gains and losses of particular characters
* None of the analyzed substrates showed dependence on the Mucorales evolutionary history
* Carbon assimilation capacities are shaped by environmental conditions

**Key words:** Mucorales, carbon assimilation, evolution, species identification

1. **Introduction**

Representatives of Mucorales belong to one of the oldest lineages of terrestrial fungi. They belong to subphylum Mucoromycotina, which is divided into three lineages: Mortierellales, Endogonales and Mucorales (Hibbett et al. 2007). However, the order Mortierellales is currently placed within a new, separate subphylum: Mortierellomycotina (Hoffmann et al. 2011). All of these fungi probably appeared on Earth in the Precambrian, and they constituted a significant component of the Carboniferous terrestrial biota (Berbee & Taylor 2010; Krings et al. 2013). Although commonly known as ubiquitous saprotrophs, Mucorales representatives may also be pathogens of animals, including humans. Recently, their role in the etiology of fungal infections in humans has been considered increasingly important, particularly in warm-climate countries (Voigt et al. 2009; Muszewska et al. 2014).

For more than 150 years, Mucorales were identified using only morphological features. However, recent phylogenetic studies proved that this order is polyphyletic (Kirk et al. 2008; O'Donnell et al. 2001; Voigt & Wöstemeyer 2001). Hoffmann et al. (2013) proposed a new taxonomic structure for the families within the order Mucorales, which better reflects the phylogenetic relationships within the group. Several traditionally-used morphological characters were revealed to be a result of convergent evolution (homoplasy). Therefore, finding new, synapomorphic diagnostic features for the clades is of particular importance. In certain groups of fungi such as yeasts, carbon assimilation profiles are now a standard feature included in species description (Yurlowa & de Hoog 1997).

Carbon is of fundamental importance for fungal growth and functioning because it is a backbone component of various organic substances. However, it occurs mainly in complex forms that are not easily accessible for fungi and other microorganisms. The main sources of nutrients for fungi are plant-, animal- and fungal-derived organic matter, which are accessed by fungal saprotrophs, pathogens or symbionts. However it is the plant material, composed mainly of cellulose and other polysaccharides (such as hemicelluloses and pectin) and complex aromatic compounds such as lignin, that is most commonly used. Fungi produce a wide variety of extracellular enzymes that are responsible for the decomposition of polymers into smaller compounds that can be assimilated by the cells. Fungi vary in their abilities to use different carbon compounds. In general, these differences are due to either the permeability of the cell wall or the presence of specific enzymes. It has been shown that fungi differ strongly in their carbon nutrition capacities (Madan & Thind 1998). The ability of the fungus to use different carbon sources available in the substrate can therefore be perceived as one of the main factors shaping the potential for a given fungal taxon to occupy a particular niche, in addition to abiotic factors such as temperature, humidity and pH.

Mucorales are commonly called "sugar fungi". They grow well on media rich in simple organic compounds, but they are unable to assimilate more complex organic compounds such as cellulose and lignin, in contrast to Ascomycetes and Basidiomycetes (Hesseltine & Ellis 1973). However, some recent reports suggest that Mucorales are also able to produce enzymes that allow for the degradation of more complex polymers. These include cellobiohydrolases, endoglukanases and ß-glucosidases in *Mucor circinelloides* (Saha 2004), and tyrosinases and lignin peroxidases in *Rhizopus oryzae* (León- Santiesteban et al. 2008). Recently, the ability of *Umbelopsis isabellina* to degrade some phenolic compounds was shown (Janicki et al. 2015). Based on these findings, the traditional opinion that Mucorales are only "sugar fungi" should be revised, as their role during the decomposition of organic matter is much more important than previously thought.

Although some mucoralean fungi are commonly used in biotechnology, fermentation processes, production of steroids or biotransformation of carotenoids (Feofilova et al. 2009; Voigt et al. 2009), relatively little is known about their enzymatic capacity. The existing knowledge is restricted to only a few well-studied taxa.

To date, the most comprehensive analyses of carbon assimilation profiles within Mucorales were made by Schwartz et al. (2007) and Vastag et al. (1998). They included a total of 62 strains (57 in the first study and 5 in the second study) representing 15 species from Mucorales. The main objective of both studies was to develop species-specific carbon assimilation profiles, enabling the identification of pathogenic taxa. They tested a total of approximately 80 different carbon sources and determined the carbon assimilation profiles characteristic for most of the analyzed species (including *Lichtheimia corymbifera* and *Rhizomucor miehei*). However, both studies were focused mainly on pathogenic species. The development of similar profiles for more species, especially non-pathogenic species, is needed.

The main objective of this study is to explore diversity in carbon utilization capacities and possible correlation of the metabolic pattern with phylogeny.

1. **Materials and methods**
   1. *Isolates and culture techniques*

This study included 26 strains of Mucorales belonging to 23 species, including 16 type strains provided by the Centraalbureau voor Schimmelcultures and the Warsaw University Herbarium. The phylogenetic position of selected strains is shown in Fig. 1. The positions of samples used in earlier studies of carbon assimilation profiles in Mucorales are also indicated. Most of the selected strains were representatives of Lichtheimiaceae (containing genera: *Circinella, Thamnostylum, Fennelomyces, Zychaea* and *Lichtheimia*). Some strains from Mucoraceae were used as background for comparison purposes.Three strains of *Thamnidium elegans* and two strains of *Circinella muscae* were used in order to preliminary evaluate intraspecific variability. Identification numbers of all isolates are presented in Table 1. The identity of all strains was confirmed by sequencing of the internal transcribed spacer (ITS) region and with standard morphological identification procedures. Before starting the analyses, fungi were cultured on 4% PGA medium (Potato-Glucose Agar; Sigma-Aldrich, Switzerland) for five days at 17°C.

* 1. *Determination of carbon assimilation profiles*

Phenotypic Microarray Plates PM1 (Biolog Inc., USA) were used to test the capacity of the analyzed strains to use 95 different carbon sources, varying from simple sources such as monosaccharides to more complex polymers (Table 1). Phenotype MicroArrays use Biolog's redox assays, engaging cell respiration or fermentation as a universal reporter and provide precise quantitation of phenotypes. If the phenotype is strongly "positive" in a well, the cells are metabolically active, reducing a tetrazolium dye and forming a strong color. If their metabolic activity is slowed or stopped less color or no color is formed. Spores were suspend in FF inoculation fluid deficient in carbon sources (Biolog Inc., USA) to produce a final optical density of 0.036 A at 590 nm (equivalent of approx. 4 x 105 CFU/ml). Although the detailed Biolog PM formulation is proprietary, some details of used media composition are given by Bochner et al. (2001). Spore suspensions were then inoculated on PM1 plates and incubated in the aerobic Omnilog incubator plate reader (Biolog Inc., USA) for 72 h at 20°C. The metabolic activity was measured kinetically by determining the colorimetric reduction of a tetrazolium dye. Data were collected approximately every 10 min over a 72-h period. This was a sufficient time for color development in the positive control wells, while the negative control wells remained colorless. The analysis of each strain was repeated three times. Preliminary data analysis was done using the Biolog Kinetic and Parametric software (Biolog Inc., USA). Colorimetric values for wells containing carbon substrates were blanked against the control well.

* 1. *Comparison of carbon assimilation capacities and phylogenetic mapping*

The obtained data were converted to categorical measurements. The result was considered to be positive when (i) a difference between the metabolic activities of the first and third days of incubation was observed and (ii) when at least two replicates out of three were positive. The similarity between analyzed strains was evaluated using the Jaccard similarity coefficient (Jaccard 1912) and Bray-Curtis dissimilarity coefficient (Bray & Curtis 1957). Dendrograms were produced with an unweighted pair-group algorithm by using arithmetic average (UPGMA) linkages. All statistical analyses were performed with PAST ver. 2.17c (Paleontological Statistics Software; Hammer et al. 2001).

Reconstruction of the evolution of carbon assimilation abilities was carried out with Mesquite (Maddison & Maddison 2015). Reconstruction of ancestral states was carried out using the Maximum Parsimony and the Maximum Likelihood methods. For each trait, the phylogenetic dispersion and the frequency of estimated gene gain and loss was determined. The phylogenetic tree used in all analyses was calculated based on SSU sequences using the Maximum Likelihood method, 100 bootstrap replicates, and the GTR model of evolution as implemented in SeaView (Gouy et al. 2010).

1. **Results**
   1. *Carbon assimilation profiles*

The analyzed strains differed significantly in their ability to use different carbon sources. All three repetitions for each strain were relatively consistent. None of the analyzed strains was able to use all 95 carbon sources. Two strains, *Thamnostylum repens* and *Helicostylum cordense*, had the highest capacities to assimilate different carbon sources (approx. 80% of all substrates). On average, approximately 40 substrates were absorbed per strain, i.e. less than 50% (Fig. 1.).

The metabolic activity on five sugars (D-cellobiose, maltose, α-D-glucose, D-xylose, D-galactose) was common for all analyzed strains. None of the tested isolates was able to utilize 1,2-propanediol, α-keto-butyric acid, α-hydroxy glutaric acid-γ-lactone, α-hydroxy butyric acid, 2-deoxy adenosine, glyoxylic acid, acetoacetic acid or any of the eleven analyzed phosphates as a sole source of carbon. The 79 tested media showed different results among strains. The obtained carbon assimilation profiles showed strain specificity and are presented in Table 1.

* 1. *Intra- and interspecies variability*

For two analyzed taxa, intraspecies variability was higher than expected and depended on the species. The Jaccard similarity coefficient for *Circinella muscae* was 0.96 (the sole difference was observed in D-sorbitol assimilation), while for *Thamnidium elegans* it was much lower and varied from 0.42 to 0.72. The interspecific variability ranged from 0.2 to 0.85. The intraspecific differences were found in the species’ capacity for use of phenylethylamine, tyramine, D.L-malic acid, L-malic acid, L-lactic acid, M-tartaric acid, pyruvic acid, propionic acid, tricarballylic acid, D-galacturonic acid, D-gluconic acid, D-glucosaminic acid, D-glucuronic acid, D-saccharic acid, mucic acid, adonitol, D-mannitol, L-arabinose, L-rhamnose, α-D-lactose, lactulose, D-melibiose, mono methyl succinate, methyl pyruvate, p-hydroxy phenyl acetic acid, bromo succinic acid, D-alanine, D-aspartic acid, L-glutamic acid, L-asparagine, L-serine, glycyl-L-glutamic acid, β-methyl-D-glucoside, α-methyl-D-galactoside and Tween 20. Thus, all sugar acids included in the analysis showed intraspecific variability (Table 1.). After removing these carbon sources that vary within species as well as those that did not vary at all, the remaining 43 carbon sources had high species specificity. The intraspecific Jaccard similarity coefficient was 1 in both cases, while the interspecific coefficients varied from 0.1 to 0.94. However, more data on intraspecies variability of carbon assimilation capacities are needed before drawing any final conclusions on their possible usage for species identification purposes.

* 1. *Evolutionary distribution of carbon assimilation capacities*

Representatives of the genera *Umbelopsis, Syncephalastrum* and *Lichtheimia* had limited assimilation capacities relative to evolutionarily more advanced groups of Mucorales (average 28% versus 41%, Fig. 1.). There were also significant differences in the types of carbon source that were used by different species. These three taxa were unable to assimilate Tweens (polymer), keto or hydroxy acids. On the other hand, *Umbelopsis, Syncephalastrum* and *Lichtheimia* were able to assimilate dulcitol and nucleosides as sole sources of carbon (Table 1), while most of the other analyzed strains exhibited deficient or very low metabolic activity in their presence. A reduction of the assimilation repertoire was also observed for three other lineages: *Circinella muscae* and *C. umbellata*, *Backusella lamprospora*, *Pirella naumovii*, each with a different substrate specificity.

There were significant similarities between the phylogenetic tree based on DNA sequences and the dendrogram created from the carbon source utilization data (Fig. 3.). However, the cophenetic correlation is rather low (0.6). The main differences consisted of the positions of *Helicostylum cordense* and the *Backusella* clade. In both dendrograms the separation of Mucoraceae from Lichtheimiaceae is visible.

1. **Discussion**

We observed a similarity between our results and those of Schwartz et al. (2007). The carbon assimilation profiles of *Lichtheimia corymbifera* were 89% similar, and after removing substrates that varied at the intraspecific level in our observations the similarity was raised to 100%. Identification of mucoralean representatives based on standard microbiological procedures is time consuming and often uncertain (Schwartz et al. 2007). Recently, molecular techniques have been used for species identification of mucoralean representatives (Walther et al. 2013; Hyde et al. 2014). The usage of biochemical assimilation profiles could be another fast and efficient way to identify mucoralean species, as was proposed by Schwartz et al. (2007). The Biolog Phenotype MicroArrays PM1 (Biolog Inc., USA) is one of ready to use systems for testing the capacities to use different sources of carbon and the results may be obtained within two days. Moreover, it is providing metabolic capacities of analyzed strain what maybe even more important information from clinical point of view than species assignment. Although obtained results are promising, data from the analyses of more strains would be useful. Information about the influence of culture age, passage numbers and environmental conditions on carbon assimilation capacities is also important. Moreover, once the carbon assimilation profiles are available for more taxa, a simpler test based on a reduced panel of media can be designed. Further advancement is also possible, a culture-free identification method could be designed when metabolic pathways are mapped on sequenced genomes and merged with phenotypic assays to select the discriminative criteria and key enzymes enabling the utilization of 'reporter' carbon sources. Nowadays with 1KFG (1000 Fungal Genomes Project) and Zygo-Life projects underway this process might be expected soon.

None of the analyzed strains was able to assimilate any of the phosphates present on the PM1 plates, despite the fact that glucosamine-6-phosphate deaminase is present in all currently available full genomic sequences of mucoralean fungi (blastp search in 8 publicly available genomes from JGI; unpublished data ). Although some strains of *Rhizopus microsporus* lack the specific transporters, most mucoralean representatives possess the needed membrane transport proteins. Thus the inability of these taxa to use phosphates could be explained by a repression of genes encoding proteins involved in phosphate assimilation that is probably dependent on environmental conditions.

All analyzed fungi were able to use D-cellobiose as a sole source of carbon. This result suggests that among Mucorales it is not only *Mucor circinelloides* that is producing ß-glucosidases, but that it is a much more common physiological characteristic. The capacity to use D-cellobiose, which is an intermediate product of cellulose decomposition, suggests the role of Mucorales in the decomposition of organic plant matter. The several researchers indicate the presence of Mucoromycotina members in community of fungi participating in wood decay (Thormann 2006; Vořišková & Baldrian 2013). The recent analysis of the genome of *Rhizopus oryzae* (which is relatively closely related to *M. circinelloides*) showed a similar pattern. Battaglia et al. (2011) described the ability of *R. oryzae* to use plant and fungal polysaccharides as a carbon source, which is an adaptation for its saprotrophic function in soils. Our results suggest that this capacity is probably common among mucoralean representatives.

Three representatives of the primary mucoralean lineages - *Umbelopsis angularis, Syncephalastrum racemosum* and *Lichtheimia corymbifera* - were not able to use Tweens as a sole source of carbon. Tweens are lipid polymers that can be easily hydrolyzed into fatty acids and sorbitan. Sorbitan is a carbohydrate that cannot be metabolized by fungi. Therefore, if the strain grows on Tween, it uses fatty acids as a carbon source (Wyn & Ratledge 2000). Therefore, the inability to use fatty acids as a sole carbon source could be treated as an ancient evolutionary character. However, the capacity to use Tweens was described in *Mortierella alpina* (Wyn & Ratledge 2000), a species that probably belongs to a group that is evolutionarily older than Mucorales. The variable occurrence of this physiological character could instead be explained by an environmental adaptation found in Mucoromycotina representatives. The production of enzymes involved in lipid degradation is probably related to the capacity of an organism to degrade the epicuticular waxes and cuticle of plants (Salleh et al. 1990). On the other hand these strains grew well on dulcitol - sugar alcohol derivative of galactose, that is a common compound of gums, mucilages and pectin. It is isolated from bacteria, fungi and plants. It is possible that these fungi use such products no as saprotrophs but as commensals or symbionts. Recently, several *Burkholderia* strains were isolated from *Rhizopus* (Mucorales) in different locations all over the world (Lackner et al. 2009; Partida-Martinez & Hertweck 2005; Partida-Martinez et al. 2007). Notably, this over-mentioned mucoralean endobiotic *Burkholderia* is representative of a bacterial genus that is well-known for efficient lipid degradation (Matsumiya et al. 2007). One could easily imagine fungal-bacteria consortia, in which fungi decompose sugars and bacteria are responsible for lipid deterioration. Such a relationship should be studied in detail as it may significantly influence the capacity of an organism to occupy specific niches. However, for the moment *Rhizopus microsporus* is the only species of the Mucorales that was shown to possess endobiotic bacteria.

One of the objectives of this study was to reconstruct the evolutionary history of the ability to use different carbon sources among representatives of the order Mucorales. Although similarities between the phylogenetic tree (based on DNA sequences) and the dendrogram created from carbon source utilization data were observed, the ability of the various strains to use the analyzed substrates did not show a clear correlation with the evolutionary history of the group. The observed pattern of carbon assimilation capacities reflects several gains and losses of particular characters. Instead, carbon assimilation abilities are probably shaped by environmental conditions, rather than by the phylogenetic position of an organism. Finally, more studies on the ecological relationships among the studied organisms are needed for a better understanding of carbon assimilation capacities and their distribution among Mucorales.

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**Tables and figures descriptions:**

**Table 1.** Carbon assimilation capacities of the analyzed strains. Classification of carbon sources and isolation substrate of analyzed strains are given.

**Figure 1.** Simplified phylogenetic tree reflecting the distribution of strains used in this study (red squares) in comparison with former studies on carbon assimilation profiles within Mucorales (blue squares – Schwartz et al. 2007; green squares – Vastag et al. 1998). The topology of the phylogenetic tree is based on the methodology of Hoffmann et al. (2013).

**Figure 2.** Total number of used carbon sources divided into groups (color meanings are described in the figure). The phylogenetic relationships among the analyzed strains are shown.

**Figure 3.** Comparison of the dendrogram reflecting carbon assimilation capacities with the phylogenetic tree of the analyzed Mucorales strains. **A.** Dendrogram of analyzed strains produced by UPGMA linkage of the Bray-Curtis dissimilarity coefficient. The assimilation pattern is based on 43 selected carbon sources, excluding invariable sources and sources only variable at the intraspecific level. The scale represents dissimilarity. The cophenetic correlation is 0.6402. **B.** The Maximum Likelihood phylogenetic tree of analyzed strains based on combined sequences coding for 18S and 28S rRNA. Bootstrap values are given for branches.