Title: *How variations in the rates of changes influence the evolution of fungi? – a comprehensive molecular evolutionary study using fungal models*
PI: Dr. Tamás Papp
Starting date: 01.01.2013
Closing date: 31.12.2016

The main goal of the project was to gain insight into the propensity and nature of rate variation and understand general patterns of species accumulation in fungi. Our efforts concentrated on the identification of nodes of the phylogenetic trees, on which the rate of molecular, phenotypic evolution or that of species accumulation changed. Further, we planned to identify the causatives of rate shifts and explosive radiations, with the aim of understanding the evolution of fungal diversity, which could provide clues about the evolutionary dynamics of species on a larger scale.

To achieve this objective, the following main tasks were included into the "Methodology" section of the research plan:
1. Data collections – including taxon sampling, establishment of a strain collection, experimental design, identification and selection of the phylogenetic marker genes and morphological and physiological characterization of the involved isolates to generate phenotypic traits for the further analyses.
2. Phylogenetic analyses – including examination of the sensitivity of taxon and gene sampling, production of large sequence datasets for Agaricales and Mucoromycotina and analyses of these datasets to infer phylogenies for the examined fungal groups.
3. Analyses of rate heterogeneity – including estimation of diversification rates and rate shifts, ancestral state reconstructions, BISSE model optimisations and other comparative analyses.

Accordingly, the following result have been achieved during the project:

1. Taxon sampling.

Considering the diversity of the studied fungal groups, Mucoromycotina and Agaricales and based on the sequence data available in GenBank and other available datasets and our previous works, the number of taxa and sequences to be involved in the analyses were determined. In case of Agaricales, about 3000 taxa representing all the 480 genera of the order were selected for taxon sampling. These 3000 taxa was used to generate a large, two-gene dataset referred to as "*diversity dataset*".

Acquisition of this huge number of samples required the involvement of collaborating partners into the study. On the other hand, one of our main goals also was to facilitate the establishment of a network of scientists working on related fields of research (i.e. fungal evolutionary studies). Therefore, a web page dedicated to the study of the Agaricales diversity was created (aDiv; https://sites.google.com/site/agaridiv2013/home) where the potential collaborators can inform about the participants, the species involved or the news and results. Results of the aDiv collaboration initiative were presented at international conferences during the project:

Szarkándi GJ, Dima B, Kocsubé S, Vágvölgyi Cs, Papp T, Nagy LG: ***The ADiv project: Analyzing rates of diversification in the Agaricales***, American Phytopathological Society and Mycological Society of America Joint Meeting, Austin, the USA, 10.08.2013-14.08.2013 Abstracts Paper 582-P., 2013

Szarkándi GJ, Dima B , Kocsubé S , Szebenyi C , Rácz N , Papp T , Vágvölgyi C , Nagy LG: ***The Agaricales Diversification (ADiv) Project (2013–2017): Results of the first year***, CBS Symposium Genera and Genomes 24-25 April 2014, Trippenhuis, Royal Netherlands Academy of Arts and Sciences, Amsterdam, the Netherlands, 2014

Numerous collaborators indicated his interest for the study and sent fungal samples, herbarium materials or sequence data, D.S. Hibbett, (USA); J. Geml, (the Nederlands), R.E. Tulloss

(USA); O. Miettinen (Finnland), W. Till (Austria), A. Shiryaev (Russia), C. Ovrebo (USA), D. Karasinski (Poland), D. Desjardin (USA), E. Davison (Australia), K. Soop (Sweden), V. Antonin (Czech Republic) and many others. These collaborations resulted in several joint publications (such as *PLOS One* 8: e56143; *Phytotaxa* 170: 13-23; *Database* 2014: Paper bau061; *Mol Biol Evol* 33: 959-970; *Mycologia* 108: 397-404; etc.).

In case of the Mucoromycotina, about 400 taxa representing the main groups of Mucorales (200 taxa) and Mortierellales (150 taxa) were selected for the analysis. International collaborators for this field of the project were also involved, such as K. Voigt (Germany), K. Kaerger (Germany), JK Misra (India), V. Garre (Spain) and others. These collaborations also manifested in joint papers, such as *Persoonia* 30: 77-93, *Mycoscience* 55: 268-274, *Virulence* 6: 395-403, etc.

The established collaborations indicate that the project generated significant international interest.

2. Creation of a strain collection containing cultures and herbarium specimens of the studied species.

Ca. 1600 dried mushroom samples obtained from international herbaria, ca. 800 other samples from field collections and ca. 250 Mucoromycotina strains obtained from strain collections and own isolations were processed. Fungal samples and isolates were deposited in the Szeged Microbiology Collection (SZMC, http://www.szmc.hu; http://www.wfcc.info/ccinfo/collection/by_id/987). The rest of the involved taxa were obtained from our own collections established prior to the present study. The aDiv web page lists the species involved in the Agaricales diversity study. These strain acquisitions and isolations contributed to the creation of the largest Mucoromycotina collection of the country (as a subcollection of the SZMC) containing approx. 450 strains (including more than 150 type strains) and representing several hundred species. Physiological, metabolic and genetic characterization of this strain collection were also carried out and used to identify life history traits and physiological characters for the further analyses.

By direct isolation of fungi or acquisition of strains and herbarium material, several specimens potentially representing new species, are obtained. For example, a new *Legeriomyces* species (*Legeriomyces hungaricus* J. K. Misra, Tamás Papp, Árpád Csernetics & Csaba Vágvölgyi, sp. nov., MycoBank no.: MB 801495) together with two new geographic records of *L. ramosus* and *Stachylina grandispora* were isolated and described. These fungi are commensalists in insect larvae and belong to the Kickxellomycotina, a basal fungal group, which belonged earlier to the former Zygomycota phylum together with Mucorales and Mortierellales. This publication is the first report on these fungi in Hungary:

Misra JK, Papp T, Csernetics Á, Vágvölgyi Cs: ***A new species of Legeriomyces and other Harpellales reported for the first time in larval insects from Hungary***, Mycoscience 55: 268-274, 2014 IF: 1.418

3. Creation of the molecular datasets (i.e. sequencing of the genes and loci selected as phylogenetic markers).

Comparative whole genome analyses were performed to identify orthologous single copy genes in published and new genome databases.

In case of the Agaricales genomes, this work was performed in cooperation with the Hibbett lab at the Clark University. This cooperation generated further international collaborative genomic studies and scientific papers:

Nagy LG, Riley R, Tritt A, Adam C, Daum C, Floudas D, Sun H, Yadav JS, Pangilinan J, Larsson KH, Matsuura K, Barry K, LaButti K, Kuo R, Ohm RA, Bhattacharya SS, Shirouzu T, Yoshinaga Y, Martin FM, Grigoriev IV, Hibbett DS: ***Comparative genomics of early-diverging mushroom-forming fungi provides insights into the origins of lignocellulose decay capabilities***, MOLECULAR BIOLOGY AND EVOLUTION 33:(4) pp. 959-970, 2016 IF: 13.649

Besides selecting sets of genes for further phylogenetic analyses, we have been involved in sequencing of whole Mucoromycotina genomes and performing RNASeq experiments in cooperation with our partners (K. Voigt, JMRC, Jena, Germany and J-L. Jany, University of Brest, France). Involvement in the Mucoromycotina genomics provided opportunity to participate in the annotation of recently sequenced genomes (such as *Lichtheimia corymbifera*, *M. circinelloides*, *M. fuscus*, *M. lanceolatus*, *M. racemosus*) and transcriptomic studies. These collaborations will be continued after the present project offering excellent opportunity to perform comparative studies. Partial results of these cooperation have been presented at international conferences and a journal paper:

Vágvölgyi C, Nagy G, Krizsán K, Jany J-L, Barbier G, Papp T: ***HMG-CoA reductase genes of a Mucor racemosus strain isolated from cheese ripening process***, In: Avalos J, Cánovas D, Corrochano LM, Ibeas JI, Limón CM (szerk.) 12th European Conference on Fungal Genetics (ECFG12). 358 p. Sevilla, Spain 23.03.2014-27.03.2014, 2014.

Csernetics Á, Tóth E, Farkas A, Nagy G, Bencsik O, Manikandan P, Vágvölgyi C, Papp T: ***Expression of a bacterial β-carotene hydroxylase in canthaxanthin producing mutant Mucor circinelloides strains***, ACTA BIOLOGICA SZEGEDIENSIS 58:(2) pp. 139-146., 2014

Nagy G, Páll O, Farkas A, Csernetics Á, Vágvölgyi Cs, Papp T: ***Function and subcellular localization of Mucor circinelloides HMG-CoA reductase 2 and 3***, In: Avalos J, Cánovas D, Corrochano LM, Ibeas JI, Limón CM (szerk.) 12th European Conference on Fungal Genetics (ECFG12). 358 p. Sevilla, Spain 2014.03.23-2014.03.27., 2014

Papp T, Nagy G, Toth D, Pall O, Vagvolgyi C: ***Functional studies on the HMG-COA reductase genes of Mucor circinelloides***, 6th Congress of European Microbiologists (FEMS 2015), Maastricht, Hollandia, 2015.06.07-2015.06.11. Paper FEMS-1009, 2015

Based on preliminary taxon sampling simulations and analysis, we focused on the nuclear ribosomal large subunit RNA gene (LSU) and the RPBII gene in case of the Agaricales and on the nuclear ribosomal LSU and SSU genes and the *tef* and RPBI sequences in case of the Mucoromycotina. All nuclear ribosomal sequences as well as RPBI, RPBII and *tef* data available in the GenBank were downloaded for the two fungal groups and analysed. These analyses were also used to select the taxa to be included in the study and adjusting the taxonomic coverage. For Mucoromycotina, genes participating in the terpene and fatty acid biosynthesis (e.g. *hmgR*, *carB*, *carRP* and genes for delta-9 and -6 desaturases), pathogenesis (e.g. *HSB*, *HOG1*, *FTR1*) and genes of certain extracellular hydrolytic enzymes (i.e. glucosidases and lipases) were also selected for sequencing and analysis.

Cultivation of the fungal strains and DNA isolation were performed from the strains and herbarium samples obtained. Primers were designed to amplified the selected markers from the fungal samples. DNA purification methods and reaction conditions for the PCR experiments were elaborated and optimized for large scale amplification. Appropriate optimization of sample preparation and PCR was crucial in case of the dried herbarium samples, especially of those stored for several years or decades. Amplification of protein coding genes, especially RPBI and RPBII, required careful primer design and PCR optimization.

DNA extractions and amplifications were performed from the selected taxa and the sequences were determined. In case of the diversity data sets, this means approx. 2500 LSU, 1000 ITS, 1000 SSU and 800 RPBI and RPBII sequences. This was enough to reach the planned coverage in the two fungal groups. Besides, the abovementioned protein coding targets for Mucoromycotina were also sequenced in selected strains. Our collaborators also sequenced a large number of markers from a wide spectrum of fungal taxa.

Results of the sequencing work concerning the Agaricales group were presented at the international conference:

Szarkándi JG, Dima B, Kocsubé S, Szebenyi C, Rácz N, Papp T, Vágvölgyi C, Nagy LG: ***The ADiv Project (Analyzing Diversification in the Agaricales, 2013-2017): Analyzing patterns of diversification in the largest group of mushroomforming basidiomycetous f***, XVth ESEB Meeting, August 10th-14th 2015 in Lausanne, Switzerland, Poster session A - POL 300, 2015

As extensive sequencing activity was carried out, sequences determined and validated (especially ITS, LSU, SSU and RPBI sequences of Mucoromycotina type and ex-type cultures) could be used as reference sequences in a broad international collaboration with the aim to develop a public database of curated and verified sequences at the NCBI covering the accepted orders of Fungi (Fungal Internal Transcribed Spacer RNA (ITS) RefSeq Targeted Loci Project: https://www.ncbi.nlm.nih.gov/bioproject/PRJNA177353/), which can serve as a reference for similarity searches:

[101], Schoch CL, Robbertse B, Kovács GM, Krizsan K, Papp T, Petkovits T, Vágvölgyi C, Federhen S: ***Finding needles in haystacks: linking scientific names, reference specimens and molecular data for Fungi,*** DATABASE: JOURNAL OF BIOLOGICAL DATABASES AND CURATION 2014: Paper bau061., 2014 IF: 3.372

4. Comparative analysis of the phenotypic traits (morphological, physiological, transcriptomic and genetic characterisation of the involved taxa).

Morphological characterization of the collection of Agaricales and Mucoromycotina samples was performed in parallel with the sequencing work. For Mucoromycotina (especially in case of the orders Mortierellales and Mucorales), scanning electronmicroscopic examinations were also performed. Thus, reference strains were assigned for several taxa and sequences deposited in the GenBank were evaluated and curated. New species and taxonomic groups also could be found and described and, in several cases, taxonomic revisions were carried out (*see below at the phylogenetic analyses*).

To obtain phenotypic (physiological and life history) traits for Mucoromycotina fungi, metabolic flexibility, metabolite and extracellular enzyme production, pathogenicity, stress resistance, carbon source assimilation, susceptibility to antifungal agents and response for different conditions (such as oxygen tension, osmotic conditions, growth temperature, etc.) affecting the fungal growth were examined and analysed. Physiological, biochemical and life history traits were used in the further phylogenetic and evolutionary analyses along with the sequence datasets to reveal localization and causatives of rate heterogeneities. In the frame of this task the following tests were carried out:

Carbon source utilization patterns of 120 Mucoromycotina strains were revealed using 70 compounds as sole carbon sources. Detailed fatty acid and lipid profiles of these isolates were also determined. Ergosterol and carotenoid content of the whole Mucoromycotina collection were also characterized. This collection was also screened for hydrolytic enzymes (i.e. glucosidases and lipases) and the presence of extrachromosomal genetic elements from viral origin. Stress response, growing abilities, susceptibility to antifungal agents and pathogenicity were tested in a set of selected taxa. Transcription and functionality of the previously selected protein coding genes and those participating in terpene and lipid metabolism and pathogenicity were also tested. These results were presented at several conferences:

Kovács SA, Juhász H, Nyilasi I, Szekeres A, Bencsik O, Papp T, Vágvölgyi Cs: ***Comparison of the fatty acid yields and profiles of different Mortierella and Umbelopsis strains***, 1. Biomedica Minikonferencia: Kutatások az SZTE Biológus Tanszékein Szeged, Hungary, 2013.12.13 Szeged: JATE Press, 2013. p. 33. (ISBN:978-963-315-157-0), 2013

Nyilasi I, Kovács AS, Juhász H, Kristó KE, Bencsik O, Szekeres A, Papp T, Vágvölgyi Cs: ***Investigation of the ω-6 and ω-3 fatty acid production of different Mortierella and related species***, ACTA MICROBIOLOGICA ET IMMUNOLOGICA HUNGARICA 60 (S): pp. 201-202, 2013

Nyilasi I, Kovács SA, Juhász H, Kristó EK, Szekeres A, Bencsik O, Vágvölgyi Cs, Papp T: ***Investigation of the polyunsaturated fatty acid production and profile of strains belonging to Mortierellales.***, 15th DKMT Euroregion Conference on Environment and Health Novi Sad, Serbia, 2013.05.16-2013.05.17. Abstracts pp. 115-116., 2013

Nyilasi I, Kovács SA, Juhász H, Kristó KE, Szekeres A, Bencsik O, Papp T, Vágvölgyi Cs: ***Different polyunsaturated fatty acid profiles in Mortierellales***, Power of Microbes in Industry and Environment 2013 Primošten, Horvátország, 2013.10.09-2013.10.12. Abstracts p. 73. (ISBN:978-953-7778-06-4), 2013

Kotogán A, Németh B, Vágvölgyi Cs, Papp T, Takó M: ***Screening for extracellular lipase enzymes with transesterification capacity in Mucoromycotina strains***, FOOD TECHNOLOGY AND BIOTECHNOLOGY 52:(1) pp. 73-82., 2014

Nyilasi I, Juhász H, Kovács AS, Bencsik O, Szekeres A, Papp T, Vágvölgyi Cs: ***Investigation of the fatty acid production and identification of the Δ9 and Δ6 desaturase genes in several Mortierella and Umbelopsis strains***, 16th Danube-Kris-Mures-Tisa (DKMT) Euroregion Conference on Environment and Health: Book of Abstracts. Konferencia helye, ideje: Arad, Románia, 2014.04.25-2014.04.26. Ara, 2014

Takó M, Bense V, Csernetics Á, Papp T, Misra JK, Vágvölgyi Cs: ***Extracellular hydrolase activities of Smittium fungi***, A Magyar Mikrobiológiai Társaság 2014. évi Nagygyűlése és EU FP7 PROMISE Regional Meeting. 82 p. Konferencia helye, ideje: Keszthely, Magyarország, 2014.10.15-2014.10.17, 2014

Kovács AS, Nyilasi I, Petkovits T, Szekeres A, Bencsik O, Papp T, Vágvölgyi Cs: ***Többszörösen telítetlen zsírsavakat termelő Mortierella fajok filogenetikai és fiziológiai vizsgálata***, In: Vágvölgyi Cs, Szekeres A (szerk.) A biológia jövője, a jövő biológusai: avagy szemelvények a magyarországi felsőoktatási intézményekben végzett tudományos munka eredm, 2014

Nagy G, Bogár É, Páll O, Voigt K, Vágvölgyi C, Papp T: ***Localization of different isoprenoid biosynthetic pathways in Mucor circinelloides***, Acta Microbiol Immunol Hung 62, 187-188, 17th International Congress of the Hungarian Society for Microbiology. Budapest, Magyarország: 2015.07.08 -2015.07.10., 2015

Nyilasi I, Dudás K, Juhász H, Kovács AS, Kecskeméti A, Bencsik O, Szekeres A, Certik M, Papp T, Vágvölgyi C: ***Expression of Δ9 and Δ6 fatty acid desaturase genes involved in polyunsaturated fatty acid biosynthesis under different culturing conditions in Mortierella and Umbelopsis***, 17th Danube-Kris-Mures-Tisa (DKMT) Euroregional Conference on Environment and Health: Program and Abstracts. 78 p. Szeged, Magyarország, 2015.06.05-2015.06.06. (ISBN:978-, 2015

Nyilasi I, Dudás K, Juhász H, Kovács SA, Kecskeméti A, Bencsik O, Szekeres A, Vágvölgyi Cs, Papp T: ***Investigation of Δ9 and Δ6 fatty acid desaturase gene expression in polyunsaturated fatty acid producing Mortierella and Umbelopsis strains***, ACTA MICROBIOL IMMUNOL HUNG 62:(S),191., 17th International Congress of the Hungarian Society for Microbiology. Budapest, Magyarország: 2015.07.08 -2015.07.10., 2015

Nyilasi I, Kristó KE, Pálffy B, Hegyi M, Chandrasekaran M, Kadaikunnan S, Alharbi NS, Papp T, Vágvölgyi Cs: ***Hygromycin B, carboxin and nourseothricin susceptibility of polyunsaturated fatty acid producing Mortierella and Umbelopsis strains***, ACTA BIOLOGICA SZEGEDIENSIS 59:(1) pp. 11-18., 2015

Kartali T, Szabó B, Nyilasi I, Vágvölgyi Cs, Papp T: ***DsRNA elements in polyunsaturated fatty acid producing Umbelopsis species***, In: Škrbić B (szerk.) 18th Danube-Kris-Mures-Tisa (DKMT) Euroregional Conference on Environment and Health: Book of abstracts. p. 116., 2016

Kartali T, Szabó B, Nyilasi I, Vágvölgyi Cs, Papp T: ***Detection of virus-like elements in different Umbelopsis species***, In: Gácser A, Pfeiffer I, Vágvölgyi Cs (szerk.) 5th CESC 2016 Central European Summer Course on Mycology, 2nd Rising Stars in Mycology Workshop. JATEPress, p. 43, 2016

Kartali T, Szabó B, Nyilasi I, Vágvölgyi Cs, Papp T: ***Screening of dsRNA elements in Mortierella species***, A Magyar Mikrobiológiai Társaság 2016. évi nagygyűlése, Keszthely, Magyarország, 2016.10.19-2016.10.21., Abstract pp.29-30, 2016

Nagy G, Hassan M, Kumar D, Grace Leo Vaz A, Bartha E, Vágvölgyi C, Voigt K, Papp T, Csernetics Á: ***Molecular background of virulence in human pathogenic Mucoralean fungi***, Annual Conference 2016 of the Association for General and Applied Microbiology (VAAM). Jena, Germany, 13.03.2016.-16.03. p. 84., 2016

Nagy G, Hassan M, Kumar D, Vaz AGL, Bartha E, Csernetics Á, Vágvölgyi C, Voigt K, Papp T: ***Characterization of virulence genes in opportunistic human pathogenic Mucoralean fungi***, 13th European Conference on Fungal Genetics (ECFG13): Abstract Book. p. 161, Paris, France, 03.04.2016.-06.04.2016., 2016

Extensive morphological characterizations, pathogenicity and antifungal susceptibility tests, metabolic and enzyme activity screenings and analyses, revealing assimilation patterns, lipid profiles and genetic variabilities allowed us to perform some detailed and comprehensive analyses to address certain particular questions concerning the biology of Mucoromycotina fungi. These studies were carried out in the frame of international collaborations and generated new scientific projects, which will be continued after finalization of the present work.

Results and methods obtained from the analyses of the terpenoid genes and the carotenoid and ergosterol composition could be used in studies where possibility and requirements to improve the fungal carotenoid biosynthesis were examined:

Csernetics Á, Tóth E, Molnár R, Szekeres A, Vágvölgyi Cs, Papp T: ***Expression of different Xanthophyllomyces dendrorhous crtS genes in the carotenoid producing Mucor circinelloides***, 15th DKMT Euroregion Conference on Environment and Health Novi Sad, Serbia, 2013.05.16-2013.05.17. Abstracts pp. 113, 2013

Nagy G, Farkas A, Csernetics Á, Bencsik O, Szekeres A, Nyilasi I, Vágvölgyi Cs, Papp T: ***Transcription analysis of the three HMG-CoA reductase genes of Mucor circinelloides***, BMC MICROBIOLOGY 14: Paper 93., 2014 IF: 2.729

Csernetics Á, Tóth E, Farkas A, Nagy G, Bencsik O, Vágvölgyi C, Papp T: ***Expression of Xanthophyllomyces dendrorhous cytochrome-P450 hydroxylase and reductase in Mucor circinelloides***, WORLD JOURNAL OF MICROBIOLOGY & BIOTECHNOLOGY 31, 321-336, 2015 IF: 1.532

Screening for extracellular enzymes also generated data, which could be exploited to find industrially exploitable hydrolytic enzymes and to test fermentation processes in environmentally friendly systems (i.e. on agricultural wastes and by-products); these results were published in journal papers:

Kotogán A, Németh B, Vágvölgyi Cs, Papp T, Takó M: ***Screening for extracellular lipase enzymes with transesterification capacity in Mucoromycotina strains***, FOOD TECHNOLOGY AND BIOTECHNOLOGY 52:(1) pp. 73-82., 2014 IF: 0.920

Takó M, Kotogán A, Krisch J, Vágvölgyi Cs, Mondal KC, Papp T: ***Enhanced production of industrial enzymes in Mucoromycotina fungi during solid-state fermentation of agricultural wastes/by-products***, ACTA BIOLOGICA HUNGARICA 66: 348-360, 2015 IF: 0.605

Takó M, Kotogán A, Papp T, Kadaikunnan S, Alharbi NS, Vágvölgyi Cs: ***Purification and properties of extracellular lipases with transesterification activity and 1,3-regioselectivity from Rhizomucor miehei and Rhizopus oryzae***, JOURNAL OF MICROBIOLOGY AND BIOTECHNOLOGY, in press, 2016 IF: 1.685

As extensive data revealed by the abovementioned physiological, biochemical and screening studies could be used in collaborative studies to improve biotechnological potential of Mucoromycotina fungi, PI and co-workers were invited to summarize the biotechnologically relevant applications of primary and secondary metabolism of these fungi. Two invited chapters were published in book series leading in fungal biology:

Papp T, Nyilasi I, Csernetics Á, Nagy G, Takó M, Vágvölgyi C: ***Improvement of Industrially Relevant Biological Activities in Mucoromycotina Fungi***, In: Schmoll, Dattenböck (Eds.) Gene Expression Systems in Fungi. Springer, pp. 97-118., 2016

Voigt K, Wolf T, Ochsenreiter K, Nagy G, Kaerger K, Shelest E, Papp T: ***Genetic and metabolic aspects of primary and secondary metabolism of the Zygomycetes***, In: Hoffmeister D (szerk.) Biochemistry and Molecular Biology. 414 p. Springer International Publishing, 2016. pp. 361-385. (The Mycota; III.) (ISBN:978-3-319-27790-5), 2016

Moreover, PI of the project was also invited to participate in the edition of a book dedicated to the practical application and management of fungal species:

Deshmukh SK, Misra JK, Tewari JP, Papp T: ***Fungi: Applications and Management Strategies***, CRC Press, Boca Raton, FL, USA, pp. 420., 2016

Data obtained from pathogenicity and antifungal tests and different physiological characterisations could be used to perform a comprehensive analysis of the human pathogenic nature of the members of the Mucoromycotina genus, *Rhizopus*. It was revealed that the clinically-relevant species of the genus are thermotolerant and monophyletic. It was supposed that, besides thermotolerance, other factors may also participate in the pathogenicity of these fungi. Nutritional and stress resistance traits did not correlate with the virulence in this study. Our results published in an international paper supported the lack of specific adaptation of *Rhizopus* fungi to warm blooded hosts:

Kaerger K, Schwartze VU, Dolatabadi S, Nyilasi I, Kovács SA, Binder U, Papp T, de Hoog S, Jacobsen ID, Voigt K: ***Adaptation to thermotolerance in Rhizopus coincides with virulence as revealed by avian and invertebrate infection models, phylogeny, physiological and metabolic flexibilty***, VIRULENCE 6:(4) pp. 395-403, 2015 IF: 5.418

Comparative data and experimental methods (antifungal testing, functional analysis of selected genes, metabolite spectra, assimilation patterns, genetic variability results, host-pathogen interactions) obtained from these studies could be used in the study of the antifungal susceptibility and pathogenicity of other filamentous fungi:

Nyilasi I, Kocsubé S, Krizsán K, Galgóczy L, Papp T, Pesti M, Nagy K, Vágvölgyi Cs: ***Susceptibility of clinically important dermatophytes against statins and different statin-antifungal combinations***, MEDICAL MYCOLOGY 52:(2) pp. 140-148, 2014 IF: 2.335.

Papp T, Nagy G, Farkas A, Csernetics Á, Vágvölgyi Cs: ***Transcription analysis of certain genes of Scedosporium (Pseudoallescheria) isolates***, e Hoog S, Meyer W, Lackner M, Tintelnot K (szerk.) Diversity and Barcoding of Medical Fungi: Novel Achievements and Masterclass: A meeting of the ISHAM Working Groups on, 2014

Krizsán K, Tóth E, Nagy LG, Galgóczy L, Manikandan P, Chandrasekaran M, Kadaikunnan S, Alharbi NS, Vágvölgyi C, Papp T: ***Molecular identification and antifungal susceptibility of Curvularia australiensis, C. hawaiiensis and C. spicifera isolated from human eye infections***, MYCOSES (2015) 58, 603-609, 2015 IF: 2.332

Tóth E, Boros É, Hoffmann A, Nagy I, Chandrasekaran M, Kadaikunnan S, Alharbi NS, Vágvölgyi C, Papp T: ***Interaction of Curvularia strains with human THP1 cells***, Acta Microbiol Immunol Hung 62:(S)231-232. 17th International Congress of the Hungarian Society for Microbiology. Budapest, Magyarország: 2015.07.08 -2015.07.10., 2015

Tóth EJ, Boros É, Hoffmann A, Vágvölgyi C, Nagy I, Papp T: ***Activation of cytokine, chemokine and chemotactic genes in THP1 cells in response to conidia and hyphae of Curvularia strains***, 13th European Conference on Fungal Genetics (ECFG13): Abstract Book. p. 178, Paris, France, 03.04.2016.-06.04.2016., 2016

Tóth EJ, Boros É, Hoffmann A, Vágvölgyi Cs, Nagy I, Papp T: ***Activation of monocytes and their production of cell signaling molecules induced by Curvulaia strains***, In: Škrbić B (szerk.) 18th Danube-Kris-Mures-Tisa (DKMT) Euroregional Conference on Environment and Health: Book of abstracts. p. 36., 2016

5. Phylogenetic analyses

After examination of the completeness, quality and combinability of the sequence data, single locus and multiple alignments were created with various methods and phylogenies were inferred and further analysed. These analyses were used to infer phylogenetic trees and data to investigate diversification and other evolutionary problems. Resulting phylogenies could be

used to address various phylogenetic and taxonomical questions regarding different sub-groups of Agaricales and Mucoromycotina with the following results:

At first, a broad spectrum phylogenetic analysis of the Agaricales including 1721 taxa was performed from the LSU sequences by Maximum Likelihood method to obtain a basic view on the phylogenetic relations in the order. Along the inferred tree, various lesser-characterized genera (with few described species, limited geographic distribution or limited accessibility by molecular methods) could be placed and several potentially new lineages could be estimated:
Szarkándi JG, Dima B, Papp T, Vágvölgyi Cs, Nagy GL: ***Phylogenetic placement of lesser-known genera in the Agaricales: the potential of elusive genera to identify distinct lineages***, In: One Fungus= Which Gene(s)? Conference Amsterdam, Hollandia, 2013.04.10-2013.04.11. Abstracts., 2013

This analysis indicated some groups, which needed more detailed studies to resolve their phylogenetic position and taxonomic questions.

Multilocus (ITS, LSU and *tef*) sequence data of the Agaricales family Bolbitiaceae was used to develop and test a method, which apply iteratively refined guide trees to improve the alignment and the phylogenetic inference. The inferred trees supported the monophyly of the core Bolbitiaceae, with the exclusion of certain genera (e.g. *Panaeolus*, *Agrocybe*, and some others formerly placed in the family).
Tóth A, Hausknecht A, Krisai-Greilhuber I, Papp T, Vágvölgyi Cs, Nagy LG: ***Iteratively Refined Guide Trees Help Improving Alignment and Phylogenetic Inference in the Mushroom Family Bolbitiaceae***, PLOS ONE 8: e56143, 2013 IF: 3.534.

Based on the comparisons of ITS and *tef* sequences of *Melanoleuca* species and morphological characterization of collected fungal specimens, a new *Melanoleuca* species and its variety, *M. juliannae* from Hungary and *M. juliannae* var. *decolorans* from Czech Republic and Italy, could be described:
Antonín V, Rimóczi I, Szarkándi JG, Dima B, Nagy LG, Papp T, Ďuriška O, Tomšovský M: ***Melanoleuca juliannae (Basidiomycota, Tricholomataceae), a new species from subgen. Urticocystis***, PHYTOTAXA 170: pp. 13-23., 2014 IF: 1.318

Based on the analysis of the Agaricales large dataset and fungal specimens from New Zealand, a group of species forming a strongly supported sister clade to *Hebeloma*, *Naucoria* and *Hymenogaster* could be discerned within the family Hymenogastraceae. Therefore, a new genus named as *Psathyloma* was described. Moreover, two new species in the genus, *P. leucocarpum* and *P. catervatim*, were also described. These species had already been known before awaiting a formal description and assigning the appropriate taxonomic position. Geographical distribution of *Psathyloma* spp. and their ectomycorrhizal relations were also surveyed. These results were published in the journal Mcyologia:
Soop K, Dima B, Szarkándi JG, Cooper J, Papp T, Vágvölgyi C, Nagy LG: ***Psathyloma, a new genus related to Hebeloma described from New Zealand.***, MYCOLOGIA 108(2):397-404., 2016 IF: 2.638

Phyolgenetic analysis of Agaricales also allowed us to reconstruct the phylogeny of the genus *Parasola* in the family Psathyrellaceae. This genus represents an enigmatic lineage of veil-less, coprinoid fungi with frequently changing taxonomic status. Phylogeny of *Parasola* (Fig. 1) was reconstructed using the ITS and LSU loci and several undescribed species were identified, of which three, *Parasola crataegi*, *P. ochracea* and *P. plicatilis-similis*, were formally described based on molecular and morphological data. Morphological descriptions for the new species and an identification key to accepted *Parasola* species were also made. Current understanding of the phylogeny of *Parasola* and its implications on character evolution were also revised and discussed. A manuscript containing these results were submitted to the journal Mycologia at the end of the last year:
Szarkándi J, Schmidt-Stohn G, Dima B, Shah H, Kocsube S, Papp T, Vagvolgyi C, Nagy L: ***The genus Parasola: phylogeny of the genus and the description of three new species***, Mycologia (submitted, under review), 2017

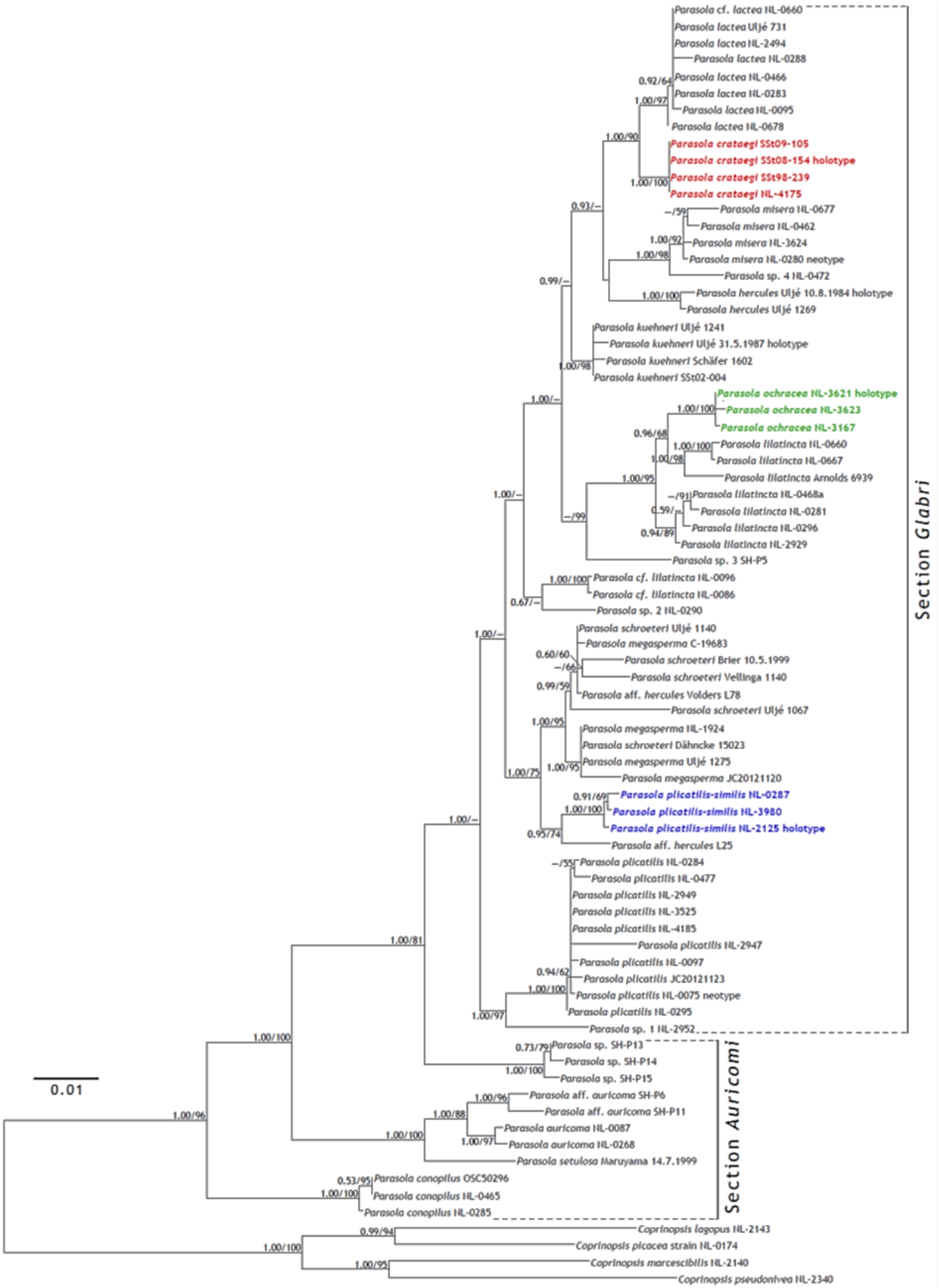

Fig. 1. 50% majority rule phylogram constructed from post-burn-in trees of the Bayesian analyses of LSU+ITS data. Numbers on the branches represent Bayesian posterior probabilities followed by ML bootstrap values. Sequences of the newly described species marked in boldface. Bar indicates 0.01 expected change per site per branch.

Finally, the biggest and most inclusive multilocus dataset of the Agaricomycetes to date was assembled to the rate heterogeneity tests and evolutionary modelling. This final dataset comprised 5326 species with three loci (5034 LSU; 1295 RPBII and 751 *tef* sequences), including 1386 newly sequenced taxa. The final superalignment contained 5737 characters. Then, maximum likelihood phylogenies were inferred for the 5541-taxon dataset. To support the inference of deep divergences in the tree a phylogenomic backbone tree were prepared using the genome data of 103 Agaricomycetes species (572 loci and 141.824 amino acid sites). This phylogenomic tree was used as a backbone constraint for the large 5326-taxon phylogeny (Fig. 2). Currently, this is the most comprehensive phylogeny of Agaricomycetes fungi. These datasets and tree were used for molecular evolutionary studies.

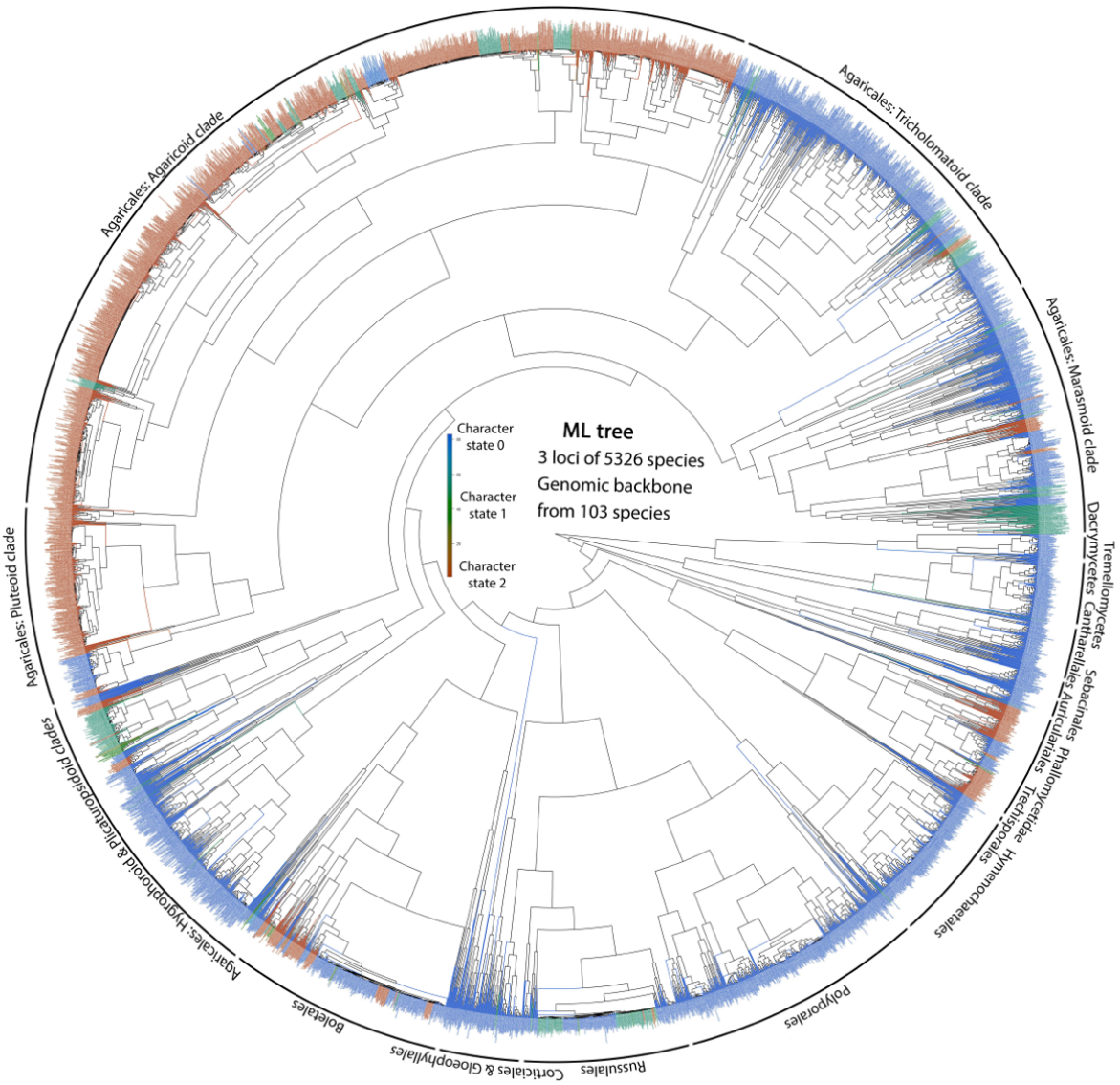

Fig. 2. Maximum likelihood tree of Agaricomycetes inferred from three loci (LSU, RPBII and *tef*) and 5326 taxa.

In case of Mucoromycotina a comprehensive phylogeny, which includes the main groups (i.e. Mortierellales, Endogonales, Umbelopsidaceae and Mucorales) in appropriate coverage and taxon sampling, was also inferred from multiple ITS, SSU and *tef* data for further analysis. This phylogeny supports the monophyly of Mucoromycotina with the maintenance of the unity of the subphylum in contrast with some recent studies, which emphasis the need of the introduction of Mortierellomycotina or Mortierellomycota for Mortierellalean fungi. At the

same time, it suggests the need to elevate Umbelopsidaceae from Mucorales to the rank of a separate order (Umbelopsidales). Umbelopsis species originally were placed in Mortierellales but later they were transferred to Mucorales (Meyer and Gams 2003. Mycol Res 107:339-350). Later, several molecular phylogenetic studies have shown that *Umbelopsis* form a basal sister-group to the Mucorales but its position remained objective of debates. Fig. 3 presents an example for Mucoromycotina phylogenies; this phylogenetic tree was inferred from combined ITS, SSU and LSU datasets

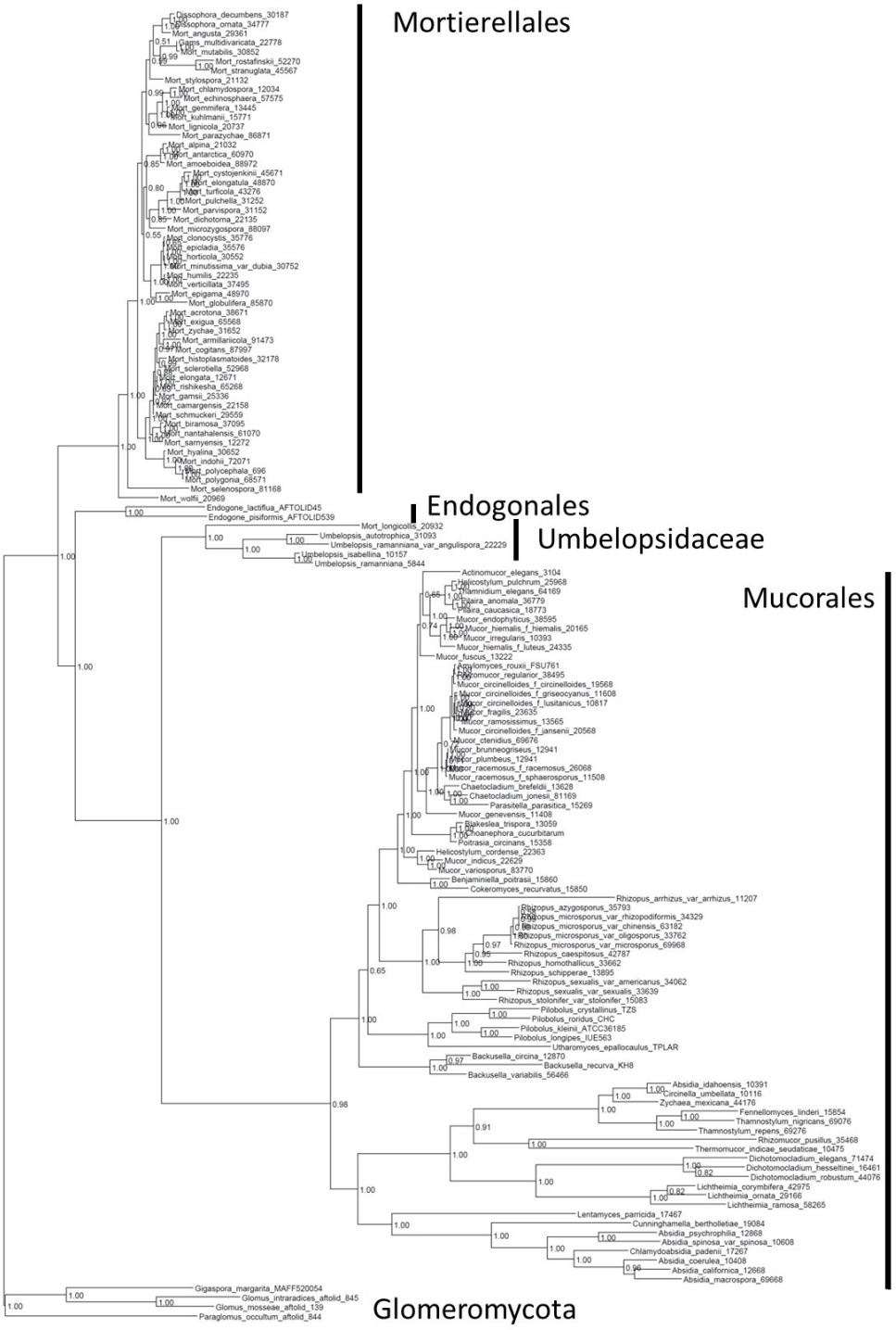

Fig. 3. 50% Majority rule phylogram constructed from post-burn-in trees of the Bayesian analyses of ITS+SSU+LSU data. Numbers on the branches represent Bayesian posterior probabilities.

Mucoromycotina sequences determined in this project was used to infer a comprehensive phylogeny of Mortierellales revealing the complete family and genus structure of the order. This study performed by an international collaboration included a large dataset of LSU and ITS sequences for more than 400 specimens containing 63 type/reference strains. As a result, traditional morphology-based classification schemes were rejected and a complete taxonomic revision of Mortierellaceae was proposed based on this phylogeny:

Wagner L, Stielow B, Hoffmann K, Petkovits T, Papp T, Vágvölgyi Cs, de Hoog GS, Verkley G, Voigt K: ***A comprehensive molecular phylogeny of the Mortierellales (Mortierellomycotina) based on nuclear ribosomal DNA***, Persoonia 30: 77-93, 2013

6. Analysis of rate heterogeneity (character evolution and diversification).

In case of Agaricomycetes (i.e. fruiting body forming fungi) evolution, fruiting body development is one of the most extensively studied questions. During early stages of morphogenesis, terrestrial species are exposed to environmental conditions, which makes early morphogenesis one of the most critical periods in the life of an organism. Enclosed development of embryos has evolved in higher plants and animals to overcome this issue. Therefore, we tested whether enclosed fruiting body development confer evolutionary benefit and if there is a trend towards enclosed primordium development in the evolution of Agaricomycetes. The character state assignment was based on whether the developing spore bearing structure (hymenophore) is open to the environment Fig 4. For these tests the dataset mentioned above (Fig. 2) was used. (To explore the robustness of the results of character state coding regime described above – three states dataset – we also worked out and tested alternative character state codings – four and five states datasets.)

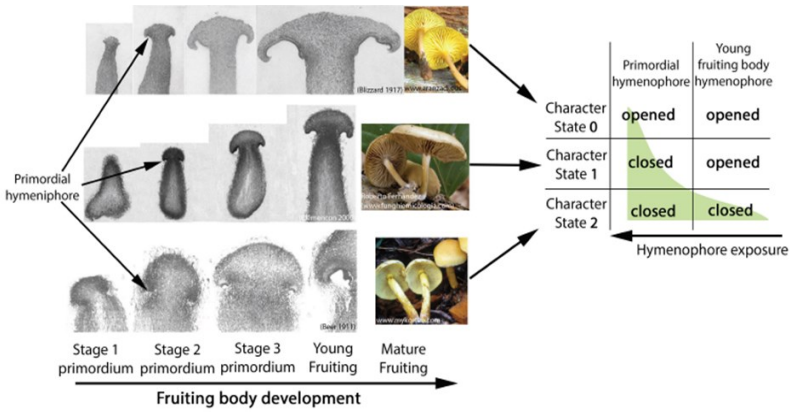

Fig. 4 Developmental types distinguished in the analysis. During open development (state 0), the hymenophore is exposed to the environment from the earliest primordial stages while during semi-enclosed development (state 1), the hymenophore is closed in the earliest primordial stages but opens later. In case of closed development (state 2), the hymenophore is closed at least until to the young fruiting body stage.

Fig. 5 shows the model of transitions between the developmental types for the three states dataset. In the model, six rate parameters (q_{ij}) were used that determine transition probabilities between character states.

For the three character states mapped on the phylogeny see Fig. 2. Analyses of developmental types provided evidence for evolution towards enclosed development in the Agaricomycetes. Both ML and Bayesian analyses suggest that the rate of the evolution of enclosed development (state 2) is on average 11.7 times higher than in the reverse direction, suggesting that enclosed developmental types tend not to revert to ancestral more opened developmental types. It was also found that losses of semi-enclosed development are surprisingly more frequent than its gains. Altogether, our results provide strong evidence for a trend towards enclosed development in the Agaricomycetes, which potentially reflects the evolutionary advantage conferred by enclosed development via protection from environmental factors and predation.

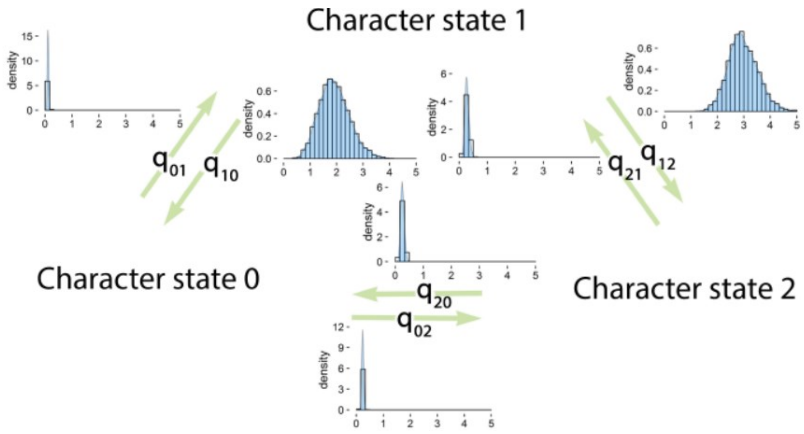

Fig. 5 The model of transitions between developmental types. The histograms show the frequency distribution of rate parameters obtained from MCMC analyses. Higher rates indicate higher probability transition between the respective states.

Diversification rate analyses were also performed. To ensure the assumption of random sampling of MuSSE, we specified the sampling fraction of each genera in accordance with the species fractions presented in Species Fungorum database (http://www.speciesfungorum.org/). For this we screened all orders of Agarycomycetes, Dacrymycetes and Tremellomycetes in Species Fungorum and gathered all species by a custom java program, which used HtmlUnit web testing framework (www.htmlunit.sourceforge.net) to filter out 'where a taxonomic opinion has been expressed'. We treated this database as species of fully resolved tree and we determined the species ratio of each genera. Then we adjusted the species number of ML tree to these hypothetical ratios by exact binomial test ($p<0.05$) using a custom R script where species iteratively deleted and added until it reached the maximum number of genus whose ratio is significantly similar to the hypothetical ratio. Diversification was modelled by the BiSSE (binary state speciation and extinction) ans MuSSE (a multistate extension of BiSSE) methods, as well as BAMM analysis (Bayesian analysis of macroevolutionary mixtures). Presently, a manuscript is under contribution from these results. Earlier, results were presented at different conferences:

Szarkándi GJ, Dima B, Kocsubé S, Papp T, Vágvölgyi Cs, Nagy LG: ***Tempo and mode of evolution among mushrooms: analysis of diversification rates in the Agaricales***, ACTA MICROBIOLOGICA ET IMMUNOLOGICA HUNGARICA 60 (S): pp. 239, 2013

Varga T, Krizsán K, Szarkándi JG, Dima B, Kiss B, Vágvölgyi C, Papp T, Nagy GL: ***Does enclosed development of young fruiting bodies confer an evolutionary advantage in the Agaricomycetes?***, 13th European Conference on Fungal Genetics (ECFG13): Abstract Book. p. 472, Paris, France, 03.04.2016.-06.04.2016., 2016, 2016

In case of Mucoromycotina, diversification rates were examined along the abovementioned large, combined datasets. These analyses suggested that the diversity Mortierellalean fungi is a result of a rapid radiation when new species had arisen during a relatively short time, especially in comparison to the Mucoralean species. Several morphological characters (such as the columella, zygospore formation, complexity of the sporangia, etc.) and physiological and genetic and life history traits (such as growth temperature, carbon source assimilation, carotenoid and fatty acid producing abilities, virulence, presence of certain extrachromosomal elements and mycoviruses, etc.) were mapped to these phylogenies and changes in the character states were analysed. Presently a manuscript is under contribution from these results.

In the frame of this study, applicability of the carbon assimilation patterns for evolutionary analysis was tested by mapping the carbon utilization data onto the multigene phylogenies inferred from a five-genes (ITS+LSU+SSU+*tef*+RPBI) dataset and a more complete three-genes (ITS+SSU+LSU) dataset. Frequency and changes of the utilization taking into account the chemical structure of the different compounds were examined in a phylogenetic context.To do this, a stochastic character mapping method was tested and optimized to our data of carbon assimilation profiles and the abovementioned trees and sequence alignments. It was found that

members of the closely related Mucorales show significant differences in their carbon source utilization patterns. Results suggested that several utilization capabilities appeared independently in many cases during the evolution of these fungi. Results were presented at various conferences:

Petkovits T, Nyilasi I, Kovács SA, Vágvölgyi Cs, Papp T: ***Stochastic character mapping of carbon source utilization data on a multigene phylogenetic tree of the Mortierellales***, ACTA MICROBIOLOGICA ET IMMUNOLOGICA HUNGARICA 60 (S): pp. 215-216., 2013

Petkovits T, Nyilasi I, Kovács SA, Vágvölgyi Cs, Papp T: ***Carbon source utilization patterns in the order Mortierellales used as characters for stochastic mapping.***, Emerging Zygomycetes, a new problem in the clinical lab: A workshop of the ECMM/ISHAM Working Group on Zygomycetes Utrecht, The Netherlands. Utrecht, Hollandia, 2013.04.0, 2013

Papp T, Petkovits T, Nyilasi I, Kovács AS, Vágvölgyi Cs: ***Analysis of carbon source utilization data combined with a multigene phylogeny of Mortierellales***, In: Avalos J, Cánovas D, Corrochano LM, Ibeas JI, Limón CM (szerk.) 12th European Conference on Fungal Genetics (ECFG12). 358 p. Sevilla, Spain 2014.03.23-2014.03.27., 2014

Other achievements

Connected to the genetic analyses of the Mucoromycotina fungi and molecular phylogenetic study of filamentous fungi, two successful PhD defence were performed in 2013 (K. Krizsán: *Characterisation of opportunistic pathogenic isolates of Cochliobolus*. Supervisor: T. Papp; University of Szeged) and 2014 (G. Nagy: *Functional analysis of HMG-CoA reductases in Mucor circinelloides*. Supervisor: T. Papp; University of Szeged) by two young researchers participating in the project.

As several students worked on the project under supervision of the researchers participated, 5 successfully defended MSc and 6 BSc diploma work were performed during the implementation time of the project.

The successful progression of the project contributed to two successful proposals for the "Lendület" program of the Hungarian Academy of Sciences. Results could be used to plan the projects. Thus, L. Nagy (senior researcher of the project) and T. Papp (PI of the project) successfully applied for the "Lendület" grant during the implementation period. Currently, L. Nagy is the PI of the "*Fungal Genomics and Evolution*" Research Group at the Biological Research Centre of the Hungarian Academy of Science (established in 2014) and T. Papp is the PI of "*Fungal Pathogenicity Mechanisms*" Research Group at the University of Szeged (established in 2016).

Results and methods of the project could be used in the educational activity of the University of Szeged and were built in the following courses: Experimental Mycology in the 2nd semester of the academic years 2014-15 and 2015-16; Phylogenetics in the 2nd semester of the year 2013-14. In connection with the fungal evolution, a course entitled as "Systematics and Evolution of Fungi" was held for MSc and PhD students in the 1st semester of the year 2015-16.