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Project closing report

Phylogenetic and phylogenomic analysis of *Aspergillus* species

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Aspergillus is among the economically most important fungal genera. *Aspergillus* species can be both harmful and beneficial for mankind. They are responsible for a number of plant and human diseases and produce several mycotoxins. Many species cause food spoilage, but on the other hand, they are also used in the fermentation industry to produce hydrolytic enzymes, organic acids and pharmaceutically active compounds. Aspergilli are one of the more difficult groups concerning classification and identification, and several taxonomic schemes have been proposed. New molecular approaches have shown that there is a high biodiversity, but that taxa are difficult to be recognized based solely on their phenotypic characters. In our research work, the taxonomic studies of the various sections of Aspergilli were finished combining sequence-based molecular, morphological, ecological and physiological data to delimit species in the different sections. The genome wide bioinformatical investigations of the available genome sequences was ended and the loci playing an important role in the phylogenetical relationships within the *Aspergillus* genus were described in a book chapter (Mycotoxigenic Fungi. Methods in Molecular Biology, vol 1542.).

During our work, by involving relevant species of *Penicillium*, *Aspergillus* and related taxa the monophyly of *Aspergilli* was examined via both molecular biological and bioinformatical investigations. Our statistical analyses have rejected the hypothesis that the *Aspergilli* are non-monophyletic and provided robust arguments that the genus is monophyletic and clearly separated from the monophyletic genus *Penicillium*. Thus, there is no phylogenetic evidence to split *Aspergillus* into several genera and the name *Aspergillus* can be used for all the species belonging to *Aspergillus* namely the clades comprising the subgenera *Aspergillus*, *Circumdati*, *Fumigati*, *Nidulantes*, section *Cremei* and certain species, which were formerly part of the genera *Phialosimplex* and *Polypaecilum*. Moreover, section *Cremei* and the clade containing *Polypaecilum* and *Phialosimplex* are proposed as new subgenera of *Aspergillus*. The phylogenetic analysis also clearly shows that *A. clavatoflavus* and *A. zonatus* do not belong to the genus *Aspergillus*. *A. clavatoflavus* is therefore transferred to a new genus *Aspergillago* as *Aspergillago clavatoflavus* and *A. zonatus* was transferred to *Penicilliopsis* as *P. zonata* (Stud Mycol, 2016, 85:199-203.). The morphology of the taxa in the section *Cervini* is very similar and isolates assigned to these species are frequently misidentified. In our research work, a polyphasic approach was applied with macro- and micro-morphological characters, extrolite data, temperature profiles and certain sequences to examine the relationships within this section. Based on our results, section *Cervini* is comprising ten species including six newly described species*: A. acidohumus, A. christenseniae, A. novoguineensis*, *A. subnutans*, *A. transcarpathicus* and *A. wisconsinensis* (Stud Mycol, 2016, 85:65-89.). The resolution of *Aspergillus* section *Nidulantes* using multi-gene phylogenic approaches was also investigated and the gathered data were supplemented with morphological characters, extrolites and

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physiological characters. It was confirmed that many species of section *Nidulantes* produce the carcinogenic sterigmatocystin and the most important mycotoxins in this section are aflatoxins, emestrin, fumitremorgins, asteltoxins and paxillin, while other secondary metabolites are useful drugs or drug lead candidates such as echinocandins, mulundocandins, calbistrins, varitriols, variecolins and terrain. Aflatoxin B1 is produced only by four species: *A. astellatus*, *A. miraensis*, *A. olivicola*, and *A. venezuelensis*. Altogether, nine sections are accepted in subgenus *Nidulantes* subdivided in seven clades and 65 species, and 10 species are described as new (Stud Mycol, 2016, 84:1-118.). Similar procedure was applied to study the taxonomy of *Aspergillus* section *Aspergillus* (formerly *Eurotium*) includes xerophilic species. Over 500 strains from various culture collections and new isolates obtained from indoor environments and a wide range of substrates all over the world were identified. Of these, 163 isolates were subjected to comprehensive molecular phylogenetic analyses. Colony characteristics were documented on more cultivation media, and growth parameters were recorded at three incubation temperatures as well as micromorphology was examined using both light- and scanning electron microscopy to characterize each species. A number of various extrolites were identified from cultures of the strains and some of them proved to be species dependent. Mycotoxins including sterigmatocystin, aflatoxins, ochratoxins, citrinin were not detected despite previous reports on their production in this section. Adopting a mentioned polyphasic approach, 31 species were recognized, including nine new species (Stud Mycol, 2017, 88:37-135). Furthermore, the phenotypic and genotypic characterization of species were also carried out belonging to section *Fumigati* present in soils from two semi-desert areas having different geological conditions. Altogether, 23 isolates belonging to this section were recovered and identified using the previously optimized polyphasic approach including phenotypic and molecular identifications. *A. fumigatus* sensu stricto and *A. fumigateaffinis* had the highest frequency, of occurrence, while isolates closely related to *A. udagawae* and *A. felis* were rarely observed on the investigated area. *A. fumigatiaffinis* and isolates closer to *A. udagawae* were isolated for the first time from Argentinian soils and our report was the first on the occurrence of species belonging to the *A. felis* clade in South America contributied to the knowledge of the ecology of section *Fumigati* (Rev Arg Microbiol, 2017, 49:247-254.).

Within the examination of environmental isolates, the distribution and species diversity of sterigmatocystin-producing *Aspergilli* from the section *Versicolores* were also investigated in the indoor air of apartment, basements and grain mill in Croatia. The isolates belonging to the *Aspergillus* genus comprised 0.7–20% of total airborne fungi detected in the apartment. Based on sequence data, seven species were identified including the mostly dominant *A. jensenii* and *A. creber*, which was followed by *A. protuberus*, *A. venenatus*, *A. tennesseensis*, *A. amoenus*, *A. griseoaurantiacus* and certain undescribed species. All the identified isolates produced sterigmatocystin. Lower species diversity was obtained in the grain mill due to overgrowth with more propulsive fungi. Relatively high sterigmatocystin levels were detected in 52% of grain mill dust samples confirmed the presence of sterigmatocystin-producers, although this feature cannot be exclusively attributed to *Aspergilli*. (Sci Tot Environ, 2016, 562:296-304.). Furthermore, one of the undescribed species was characterized and described by us as *Aspergillus pepii* using the polyphasic approach, including sequence-based methods, morphological and physiological studies. It was also

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concluded that this new species has also sterigmatocystin producing ability similarly to the other section members (Mycol Prog, 2017, 16:63-72).

The genetic variability and aflatoxin-producing abilities of *A. flavus* isolates from different habitats under different climates were also examined. Half of the isolates derived from Central Europe, a region, where the climate change may result in the emergence of aflatoxin-producing *A. flavus* strains, while the other half were from South India, where the tropical climate favours aflatoxin-producing *A. flavus* strains. *A. flavus* strains occurring in these habitats are also potential causal agents of fungal keratitis, while indoor air may be a potential source of respiratory *A. flavus* infections, therefore isolates from infected corneas and indoor air were also included in our study, aiming to reveal the possible differences in the diversity and aflatoxin production of these different *A. flavus* lineages. Altogether, 59 *A. flavus* isolates from four different habitats were investigated. The isolates were identified and confirmed at the species level by the sequence analysis. Applying a combined analysis of UP-PCR, microsatellite, and sequence data, the four group of isolates formed separate clusters on the phylogenetic tree. Regarding the distribution of mating type genes, the dominant presence of the MAT1-1 idiomorph was observed among the examined isolates, although the distribution of MAT genes was different between the lineages. HPLC analysis revealed that none of the examined isolates collected from indoor air or maize in Central Europe were able to produce aflatoxins, while about half of the isolates from India produced these mycotoxins under the test conditions (J Basic Microbiol, 2017, 57:899-909.).

Finally, our project contributed to the comprehensive classification of the *Aspergillus*, *Penicillium, Talaromyces* and related genera belonging to the Eurotiales order of *Ascomycetes*. Within these activities, the families and genera present in the Eurotiales were overviewed and an updated subgeneric, sectional and series classification were introduced for *Aspergillus* and *Penicillium* and a comprehensive list of accepted species in the Eurotiales was provided. The relationships between families and genera of the Eurotiales were re-evaluated using a nine-gene sequence dataset, where a new series classification was introduced for *Aspergillus* and *Penicillium*. Using a phylogenetic approach combined with phenotypic, physiologic and/or extrolite data, altogether 446 *Aspergillus* species were accepted, which was subdivided in six subgenera, 27 sections (five new) and 75 series (73 new, one new combination). In this report, the existence of the section *Vargarum* was confirmed, which was named in honor of the deceased János Varga, a prominent *Aspergillus* researcher, who was the inventor and initiator of this OTKA project (Stud Mycol, 2020, 95:5-169.).

The chemotaxonomical investigations of the *Aspergillus* genus was also performed and the fatty acid profiles of the *Aspergillus* species were recorded via HPLC-UV and GCMS techniques. The examined isolates are represented 10 sections and among the species, the similarities of the fatty acid contents were examined based on peak area ratios observed on the chromatograms. The ratios of fatty acid content related to the various *Aspergillus* isolates were applied as variables in a principal component analysis. This test was able to distinguish within the isolates, but not at the species level. Although, if the representative fatty acid patterns were defined to the isolates the strains can be segregated at species level (19th Danube-Kris-Mures-Tisa (DKMT) Euroregional Conference on Environment and Health: Program and abstracts. p.65, 2017). The metabolomical investigations of the *Aspergillus* genus were also involved in our work and for this purpose the

building of the local metabolite library was necessary. Thus, based on the public databases the own secondary metabolite library was created involving the name, the molecular formula and the exact mass of each entry. Furthermore, a complex procedure capable to extract secondary and primer metabolites of fungus growing in liquid media was also developed as well as an HPLC-HRMS method for the measurement of these metabolites was also optimized including the separation and mass spectrometric parameters.

Regarding the examination of mycotoxin production properties of the *Aspergillus* species, we continued our research on airborne black Aspergilli species and *Aspergillus* species from the section *Versicolores* in the occupational and living indoor environments including their diversity and distribution of their fumonisin producing (FB1 and FB2) abilities. Unlike *Aspergillus* from the section *Versicolores*, black Aspergilli comprised a significantly lower proportion of the total airborne fungi. However, concentration of black Aspergilli was 260-fold higher in occupational environment than in living environment with domination of *A. tubingensis* and *A. welwitschiae*. Furthermore, among black Aspergilli, only *A. welwitschiae* and *A. niger* produced FB2, which was also confirmed with the presence of fum1 and fum8 genes involved in FB production (Toxicol in Vitro, 2018, 53:160–171). To test the mycotoxin production of Aspergilli section *Flavi*, numerous strains were identified also from air samples and the aflatoxin producing capacities of these isolates were analyzed by HPLC-FLD and confirmed by genes involved in the aflatoxin biosynthesis. The cytotoxic, genotoxic, and immunomodulatory properties of AFB1-producing and AFB1-non-producing *A. flavus* extracts were also compared. In A549 cells, the extract of the AFB1-producing *A. flavus* significantly decreased the cell viability, while the THP-1 macrophage-like cells were more sensitive to both type of extracts, but IC50 was obtained only for the AFB1-producing strain (Mycotoxin Res, 2019, 35:217-230). For the measurements of aflatoxins, relatively high amounts of these substances are required in their pure forms as reference standards. Chromatographic techniques based on solid stationary phases are generally used to purify these molecules, however, based on our examinations the liquid–liquid chromatographic separation is more effective alternative for this purpose. To develop a liquid–liquid chromatographic method, we tested numerous biphasic solvent systems, where the toluene/acetic acid/water (30:24:50, v/v/v/%) system was found to be the most efficient for the separation of aflatoxins. Within the instrumental analyses, the four aflatoxins, namely B1 (400 mg), B2 (34 mg), G1 (817 mg) and G2 (100 mg), were successfully isolated with 96.3%–98.2% purity from the ferment broth (4.5 L) of an *A. parasiticus* strain. The identities and purities of the purified components were confirmed, and the performance parameters of each separation step and the whole procedure was determined. The resulting recovery rates and purities were found to be sufficiently high to allow for cost-effective reference standard preparations, which is essential for the precise analytical measurements and the toxicology studies (Toxins 2019, 11, 309).

In closing, I would like to say thank for the project participants especially Sándor Kocsubé for the efforts contributed to the success of this project and to remember our good friend and colleague János Varga inventor and initiator of this project passed away in 2016. We and the *Aspergillus* community owe him an enormous gratitude for his expertise and inspiration. In the mycological community János was respected for his knowledge of the phylogeny of *Aspergillus* and

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other hyphomycetous genera. The participants of this OTKA grant, but also the Department of Microbiology at the University of Szeged and many other mycologists around the world will miss him as a colleague and a friend.

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