MOLECULAR IDENTIFICATION AND GENETIC VARIABILITY OF *ASPERGILLUS* ISOLATES CAME FROM KERATITIS CASES FROM INDIA

Nikolett BARANYI[1], Aliz SAMU[1], Sándor KOCSUBÉ[1], András SZEKERES[1], László KREDICS[1], Csaba VÁGVÖLGYI[1], Palanisami MANIKANDAN[2], János VARGA[1]

[1] Department of Microbiology, Faculty of Science and Informatics, University of Szeged, Hungary
[2] Aravind Eye Hospital and Postgraduate Institute of Ophthalmology, Coimbatore, Tamilnadu, India

Introduction

Aspergillus strains are among the most common organisms causing fungal keratitis in tropical and subtropical areas. The main risk factor for the infection is trauma by vegetable matter during agricultural activities. Among *Aspergillus* species mainly *A. flavus*, *A. terreus*, *A. fumigatus* and *A. niger* have been isolated from fungal keratitis cases. *Aspergillus flavus* is also an important pathogen of various cultivated plants including maize, cotton and peanut, and cause serious yield losses throughout the world. Since aflatoxin production is favored by moisture and high temperature, *A. flavus* is able to produce aflatoxins in warmer, tropical and subtropical climates.
In this study, *Aspergillus* isolates identified by morphological means as members of *Aspergillus* section *Flavi* were examined using molecular methods.

Materials and methods

The *Aspergillus* isolates were collected in 2010-2012 from keratitis cases in Southern India were received from Aravind Exye Hospital, Coimbatore, India.examined. Altogether 52 isolates were examined. The cultures used for the molecular studies were grown on malt peptone broth using 10 % (v/v) of malt extract and 0.1 % (w/v) bacto peptone, and incubated at 25 °C for 7 d. DNA was extracted from the cells using the Masterpure™ yeast DNA purification kit (Epicentre Biotechnol.) according to the instructions of the manufacturer. For species identification, part of the calmodulin gene was amplified and sequenced (Pildain et al. 2008). The presence of mating type idiomorphs was examined using the primer pairs developed by Ramirez-Prado et al. (2008). UP-PCR analyses were carried out according to Bulat et al., (2000). The primers used were L45, AS15inv, L15/AS19, AA2M2, L21, 3-2, AS4, AS15 (Bulat et al., 2000, Lübeck et al., 1998). The amplification process consisted of a predenaturation step for 1 min at 94 °C, followed by 35 cycles (30 s at 94 °C, 45 s at 55 °C, and 1 min at 72 °C), plus a final extension of 2 min at 72 °C. The amplification products were separated by agarose gel electrophoresis. All amplifications were repeated at least two times. The faint bands which did not appear in all repeated experiments were not counted during cluster analysis. Altogether 155 fragments were noted and a binomial matrix was created so that presence and absence of DNA fragments were scored as 1 or 0, respectively. Cluster analysis was carried out by using PHYLIP version 3.67 software package (Felsenstein, 2007). A phylogenetic tree was created by using neighbor-joining method (Saitou et al., 1987) with the program NEIGHBOR from the PHYLIP program package. An *A. tamarii* isolate was used as outgroup in these analyses. The final tree was visualized by MEGA4 (Tamura et al., 2007). Index of association tests (IA) and parsimony tree length permutation tests (PTLPTs) were performed using the MULTILOCUS 1.2 software with 1000 randomizations (Agapow and Burt, 2001). For the IA tests, the observed data were used to simulate recombination by shuffling (resampling without replacement) the alleles at each locus of the observed data. For PTLPTs, the null hypothesis was recombination, and significance was determined by the fraction of tree lengths based on resampled data that are at least as long as those based on the observed data. The PAUP software package was used for calculating the tree lengths from 1000 randomizations (Swofford, 2000).
Antifungal susceptibility tests were carried out using disc diffusion and E-test methods, while aflatoxin producing abilities of the isolates were tested in YES culture media, and determined by HPLC analysis.

Results and discussion

During this study, 52 *Aspergillus* strains isolated from keratitis cases in South India were examined. Based on morphological studies, all isolates were classified to the *A. flavus* species. For the molecular identification, part of the calmodulin gene was amplified and sequenced. As a result, 46 isolates were identified as *A. flavus*, while four as *A. tamarii,* one as *A. terreus* and one was found to belong to the *A. pseudotamarii* species (Fig. 1). That was the first case that *A. pseudotamarii* was identified from a human infection (Fig. 2). Aflatoxin producing abilities of the isolates were tested in YES culture media, and determined by HPLC analysis. Aflatoxin B_1 was produced in the range of 50,2 ng/ml to 75,3 µg/ml by *A. flavus* isolates (50% of the isolates produced aflatoxins), while the concentration of aflatoxin B_2 was between 34,4 ng/ml and 352 ng/ml. We also examined the aflatoxin producing ability of *A. pseudotamarii* also in brain heart infusion agar and RPMI media, which imitate the human body fluids. *A. pseudotamarii* produced aflatoxins only in YES medium (2,6 ng/ml aflatoxin B_2, and almost 1 µg/ml aflatoxin B_1, Fig. 3).
Antifungal susceptibility tests were carried out using disc diffusion and E-test methods. The detected antifungal susceptibility values were mostly within the value ranges determined previously for *A. flavus* isolates, although the *A. pseudotamarii* isolate proved to be more susceptible to amphotericin B than either *A. flavus* or *A. tamarii* (Baranyi et al., 2013).
Regarding the genetic variability of the isolates, all *A. flavus* isolates and the *A. pseudotamarii* strain belonged to the MAT1-1 mating type (data not shown). The skewed distribution indicates a primarily clonal structure of the population. The genetic variability of the isolates was also investigated by UP-PCR analysis (Fig. 4). The population structure of the isolates was examined using index of association tests and parsimony tree length permutation tests. The association index (IA) test involves the calculation of the variance of genetic distances in a data matrix; in recombining populations the distribution of distances should be normal with low variance, while in clonal populations the variance is high (many distant and close relatives and only a few at the mean; Taylor et al., 1999, Varga and Tóth, 2003). IA is a rescaled variance, which is zero in strictly recombining populations, and 1 in strictly clonal populations. The observed IA value is also compared to IA values obtained for 1000 artificially recombined data sets. The IA value of a clonal population should be significantly higher than the distribution of IAs for the artificially recombined data sets, while the IA of a recombining population is within the range of IA values obtained with the 1000 randomized data sets.
The association index of the *A. flavus* population was 0.8065, which was significantly higher than that of the artificially recombined data sets (0.0564; Fig. 5), indicating that the population reproduces primarily clonally. The parsimony tree length permutation test (PTLPT) is derived from methods developed for detecting signals in phylogenetic analyses (Taylor et al., 1999, Varga and Tóth, 2003). Parsimony trees are built for the observed multilocus genotypes and for data sets that have been resampled to simulate recombination as described for the IA test. A clonal population will support one or a few short, well-resolved trees, whereas a recombining population will support many, longer, and poorly resolved trees. For the *A. flavus* population, the actual tree length was 216, while the average of the tree lengths of the artificially recombined datasets was 235, indicating again that the population reproduces primarily clonally (data not shown).

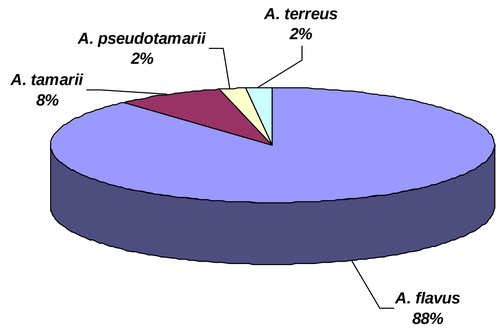

Figure 1. Species distribution of the keratitis-derived isolates based on calmodulin sequence data

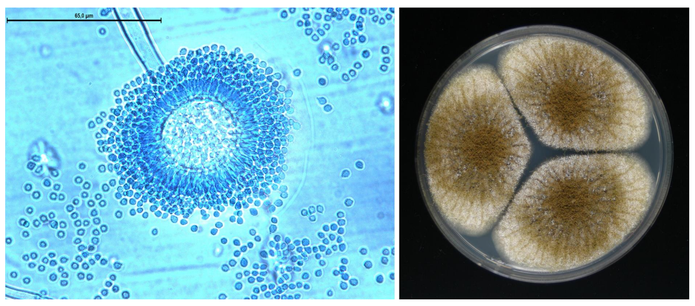

Figure 2. Conidial head and colony morphology of *A. pseudotamarii*

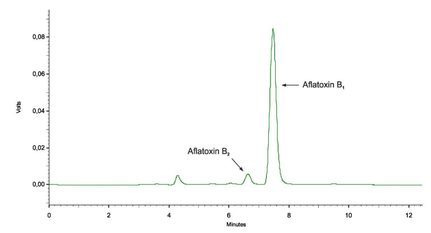

Figure 3. Aflatoxin producing abilities of *A. pseudotamarii* on YES medium

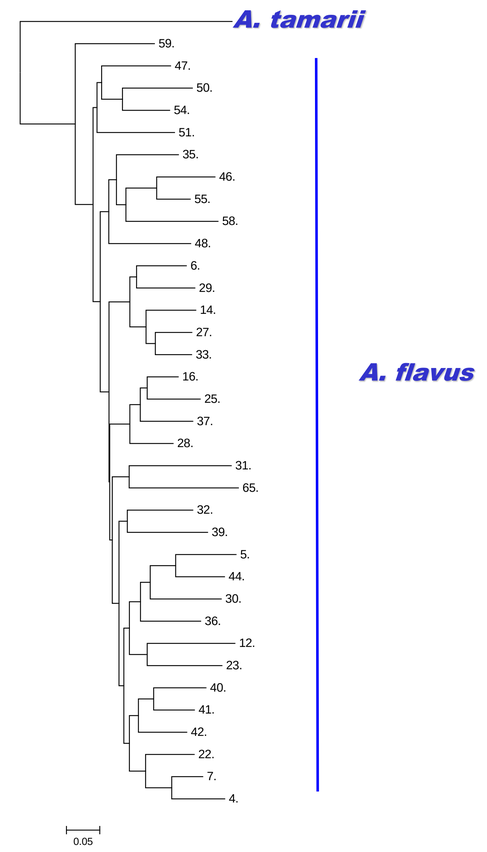

Figure 4. Neighbor joining tree of the *A. flavus* isolates based on their UP-PCR profiles

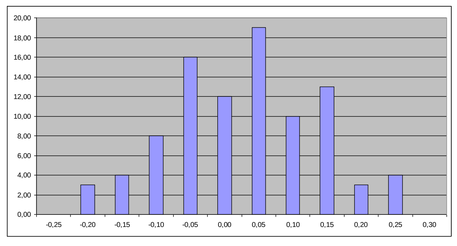

Figure 5. Distribution of IA in artificially recombined data sets of the UP-PCR data obtained for the *A. flavus* population (the actual IA of the original data set was 0.8065)

References

- Agapow, P.M. and Burt, A. 2001. Indices of multilocus linkage disequilibrium. *Mol. Ecol. Notes* 1, 101-102.
- Baranyi, N., Kocsubé, S., Szekeres, A., Raghavan, A., Narendran, V., Vágvölgyi, C., Selvam, K.P., Babu Singh, Y.R., Kredics, L., Varga, J., Manikandan, P. 2013. Keratitis caused by *Aspergillus pseudotamarii*. *Med. Mycol. Case Reports* 2, 91-94.
- Bulat, S.A., Lübeck, M., Alekhina, I.A., Jensen, D.F., Knudsen, I.M.B., Lübeck, P.S. 2000. Identification of a universally primed-PCR-derived sequence characterized amplified region marker for an antagonistic strain of *Clonostachys rosea* and development of a strain-specific PCR detection assay. *Appl. Environ. Microbiol.* 66, 4758-4763.
- Felsenstein, J. 2007. *PHYLIP (Phylogeny Inference Package).* Version 3.67 Distributed by the author. Department of Genome Sciences and Department of Biology University of Washington, Seattle.
- Lübeck, P.S., Alekhina, I.A., Lübeck, M., Bulat, S.A. 1998. UP-PCR genotyping and rDNA analysis of *Ascochyta pisi* Lib. *J. Phytopathol.* 146, 51–55.
- Pildain, M. B., Frisvad, J. C., Vaamonde, G., Cabral, D., Varga, J., Samson, R. A. (2008): Two novel aflatoxin-producing *Aspergillus* species from Argentinean peanuts. *Int. J. Syst. Evol. Microbiol.,* 58, 725-735.
- Ramirez-Prado, J.H., Moore, G.G., Horn, B.W. and Carbone, I. 2008. Characterization and population analysis of the mating-type genes in *Aspergillus flavus* and *Aspergillus parasiticus*. *Fungal Genet. Biol.* 45, 1292-1299.
- Saitou, N. and Nei, M. 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol. Biol. Evol.* 4, 406-425.
- Swofford, T. 2000. *PAUP*: Phylogenetic analysis using parsimony. version 4.0.* Sinauer Associates, Sunderland.
- Tamura, K., Dudley, J., Nei, M., Kumar, S. 2007. MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. *Mol. Biol. Evol.* 24, 1596-1599.
- Taylor, J.W., Jacobson, D.J., Fisher, M.C. 1999. The evolution of asexual fungi: reproduction, speciation and classification. *Annu. Rev. Phytopathol.* 37, 297-246.
- Varga, J. and Tóth, B. 2003. Genetic variability and reproductive mode of *Aspergillus fumigatus*: a review. *Infect. Genet. Evol.* 3, 3-17.

Acknowledgements

The authors of this study were supported by the Indian National Science Academy and the Hungarian Academy of Sciences within the frames of the INSA-HAS Indo-Hungarian bilateral exchange programme. This work was also supported by OTKA grant Nos. K84122 and K84077, and by the European Union and co-funded by the European Social Fund ("Broadening the knowledge base and supporting the long term professional sustainability of the Research University Centre of Excellence at the University of Szeged by ensuring the rising generation of excellent scientists"; TÁMOP-4.2.2/B-10/1-2010-0012).

National Development Agency
www.ujszechenyiterv.gov.hu
06 40 638 638
HUNGARY'S RENEWAL
ÚJ SZÉCHENYI TERV
The project is supported by the European Union and co-financed by the European Social Fund.