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MOLECULAR TAXONOMY OF *CRYPTOCOCCUS NEOFORMANS* VARIETIES DISPLAYING PHENOTYPIC SIMILARITIES

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ABSTRACT

Cryptococcus neoformans is a pathogenic fungus and can cause life-threatening infections in humans, especially in immunocompromised patients. According to the current classification, the species consist of three varieties. The taxonomic position of these varieties is debated. We applied fluorescent Amplified Fragment Length Polymorphism (FAFLP) genotyping to analyze clinical isolates, serotypes A and D of C. neoformans. The FAFLP genotyping suggested a considerable genetic divergence between the varieties of serotypes A, D and the clinical isolates. Our FAFLP typing strategy confirms the divergence between the varieties. The FAFLP analysis might prove to be a reliable method for taxonomy, identification and typing of Cryptococcus neoformans varieties on the basis of their specific FAFLP pattern.

Introduction

Cryptococcus neoformans can cause life-threatening infections in humans, especially in immunocompromised patients. In AIDS patients the incidence rate range from 5-30%. (Mitchell and Perfect, 1998). The main sites of infection are the lungs and the central nervous system. Most skin infections are probably due to disseminated systemic infections (Schupbach et al. 1976). The *Cryptococcus neoformans* varieties are another example of clinically important yeasts with phenotypic similarities. Their taxonomic position is debated. According to the current classification, the species consists of three varieties: *C. neoformans var. neoformans* (serotype D), *C. neoformans var. grubii* (serotype A), both comprising the teleomorph *Filobasidiella neoformans var. neoformans* and *C. neoformans var. gatti* (serotype B and C) with teleomorph *Filobasidiella neoformans var. bacilispora* (2, 3). These varieties have been recognized on the basis of molecular data such as DNA fingerprinting and *URA5* sequencing (5). Boekhout et al. (2001) suggested a considerable genetic divergence between the varieties on the basis of AFLP genotyping. In a recent publication of Bicanic & Harrison (13) the two varieties have now accorded species status: *var. neoformans*, now *C. neoformans* (serotype A,D and AD; based on capsular pollysaccaride antigens) and *var. gattii*, now *C. gattii* (serotype B and C). Different cryptococcus species show differences in levels of resistance to antimycotic agents. Therefore, molecular identification methods may be more reliable than identification methods based on phenotypic characteristics (7,8).

AFLP analysis is a whole genome analysis based on restriction enzyme DNA ana-

* **Abbreviations:** AFLP – Amplified Fragment Length Polymorphism

lysis followed by specific amplification of the restriction fragments. Aim of our study is to confirm the observed genetic divergence between serotypes A and D of *C. neoformans* and to investigate the possibility to apply this AFLP strategy developed by us for typing of clinical isolates.

Materials and Methods

The investigated strains were clinical isolates of *C. neoformans* and two reference strains *C. neoformans A342 serotype A* and *C. neoformans A382 serotype D*. All strains were biochemically identified by API32.

DNA isolation

The isolation of DNA from all yeast strains was performed by modified phenol/chloroform extraction method (4). We have optimized a mini-preparation procedure for fungal DNA extraction as previously described (12).

Molecular Typing Methods

The FAFLP typing strategy is based on DNA digestion with two restriction enzymes, ligation of appropriate linkers (adaptors) to the restriction sites and PCR amplification of the polymorphic fragments with fluorescently labeled primer. Detailed scheme of adaptor and primer sequences used in the FAFLP strategy is as previously described (12).

The PCRs were performed with Ready-To-Go PCR beads (Amersham Bioscience, Piscataway NJ, USA). The PCR program was: 3 min at 94 ºC followed by 35 cycles of amplification with 45 sec at 94 ºC, 45 sec at 58 ºC, 90 sec at 72 ºC and termination 7 min at 72 ºC. These were done on GeneAmp PCR system 9700 (Applied Biosystems, USA). The PCR product was dyed with 6 µl formamide dye (98% formamide, 10 mmol EDTA pH 8.0 and 0.1% bromphenol blue) and after denaturation for 3 min at 95C, 5 µl were loaded on 6% denaturing (sequencing) polyacrilamide gel. Electrophoresis was performed on ALFexpress II automated system (Amersham Bioscience, USA) for 750 min at 55 ºC, 30 W, 60 mA, at 1500 V. Analysis of the results was performed using the software package GelCompar II (Applied Maths, Sint-Martens-Latem, Belgium). Bands were automatically identified by the software, but verified manually. Dendrogram was generated using Pearson correlation as curve-based coefficient.

Results and Discussion

The dendrogram represents clinical and reference strains analyzed. As shown, the *Cryptococcus* dendrogram **(Figure)** discriminates *C. neoformans seroptype A* from *seroptype D*. All clinical isolated are *serotype A* and form a separate grouping. From the dendrogram could be seen that the genotypes of the clinical isolates are more close to the reference *serotype A*. This is in agreement with results of Franzot, S., et al. (1998) and Boekhout T., et al. (2001) which conclude that the genotyping suggests a considerable genetic divergence between the varieties of serotypes A, D and the clinical isolates. Our FAFLP typing strategy confirms the divergence between the varieties. This divergence is more evident as demonstrated by our results. The combination of restriction enzymes *BamHI* and *PstI* with the use of appropriate adaptors and amplification primers seems to better evaluate this fare genetic relatedness. AFLP analysis in combination with fluorescently labeled primes PCR and performed on an automated sequencing machine enables standard conditions of performance and reproducibility of the results. Our previous results applying radioactively labeled primers and manual polyacrylamide 6-8% gel electrophoresis apparatus do not have this quality (data not shown). The computer assisted data elaboration is more simplified. *C. neoformans* is found worldwide in association with soil contaminated with bird (pigeon) excreta and usually causes infection in immuno-suppressed individuals. *C. gattii* is found primarily in

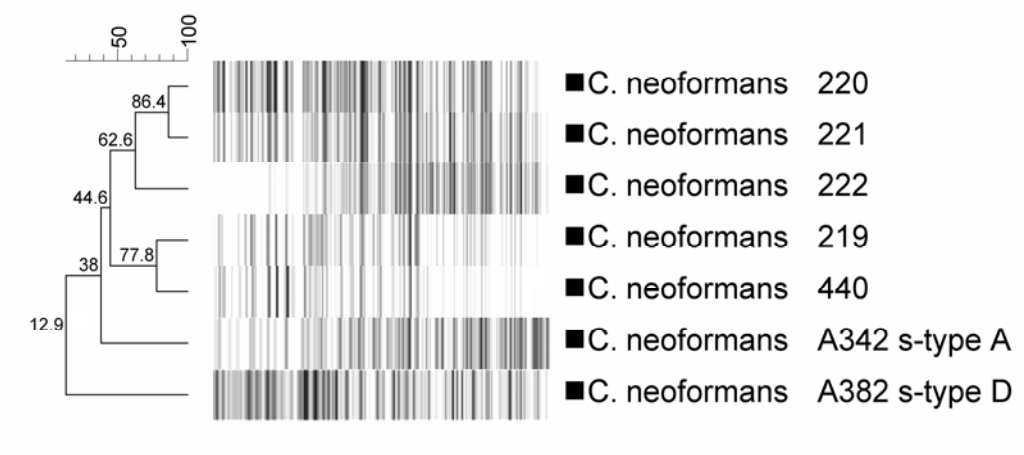

Figure. Dendrogram of the investigated *C. neoformans* serotype A and D strains.

tropical and subtropical regions and causes infection in immunocompetent individuals (13).

The FAFLP analysis might prove to be a reliable method for taxonomy, identification and typing of *Cryptococcus neoformans* varieties on the basis of their specific FAFLP pattern.

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