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Preservation and molecular identification of *Aspergillus* and *Penicilium* species with ITS-PCR

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Abstract

ITS region which is common between fungal species can be clearly separated from other species by fungal identification. Molecular of identification inventory in our 300 *Aspergillus* and *Penicillium* species made and Sequence information can be used cases of 120 such sequences were compared with online gene bank with the blast of the software. As a result, *Aspergillus fumigatus* (20), *A. awamor*i (12), *A. niger* (9), *A. tubingensis* (6), *A. terreus* (9), *A. japonicas* (4), *A. oryze* (5), *A. tamari* (2), *A. versicolor* (1), *A. brassiliensis* (1), *A. flavus* (1), *Penicilium commune* (5), *P. griseofuly* (1), *P. chrysege* (6), *Penicillium restrict*cum (1) and 13 of them belong to the other fungus groups were determined. To preserve the mycelium and spores of *Aspergillus* and *Penicillium* species also been lyophilization and labeled.

Keywords: filamentous fungi, aspergillus, penicillium, dna sequencing, lyophilization

1. Introduction

Aspergillus and *Penicillium* species are abundant and common species in nature which have positive and negative influence on human activities. Some of these species are illnesses, while others are very important industrially. *Aspergillus fumigatus*, *A. flavus*, *A. terreus* and *Talaromyces* (syn. = *Penicillium*) *marneffei* are known as opportunistic pathogens for humans [1]. *Aspergillus niger* also is used for enzyme and citric acid production. *A. oryzae*, soybean, *Penicillium roqueforti*, cheese and *P. chrysogenum* are used for penicillin production [2]. Heat resistant *Byssochlamys* species may produce on pasteurized fruit juices [3]. Because of the direct effects of our lives, the correct identification of these species is very important [4]. Conventional taxonomic studies are dependent on actual phenotypic analyses [5]. Sequence analysis for taxonomic purposes in *Aspergillus* and *Penicillium* began with PCR methods at the end of 1980, followed immediately by the analysis of protein-encoding genes and the use of rRNA sequences thermal cycler units, dye-based sequence reactions, readers are already in use [6-9]. The basic principle in biodiversity and systematic studies is the protection of its vitality and preservation of the well-defined fungal cultures.

Classic parameters used in the identification of microfungi microscopic morphology, physiological tests, cultural and clinical characteristics. However, the phenotypic characters are unclear it is difficult to morphological the identification species level. Therefore, molecular approaches have been an important alternative for the identification of fungi. These methods are gene specific PCR, RFLP analysis, rRNA gene sequences, PCR fingerprinting and DNA hybridization. ITS is one of the polymorphic DNA sequences between fungal species, nowadays it is seen as a good candidate in terms of determining fungi species correctly and with this application they can be depredated to a large extend.

No matter what type of studying to be done related to microfungi, protection and sustainability of isolate is important for the continuity of study.

Generally, storage conditions of fungal species belonging to the same genus are similar [10].

Lyophilisation and liquid drying methods are difficult techniques used to preservation the fungal mycelium. These techniques are especially used for fungal spores can be stored for 20 years and more. Preservation methods for filamentous fungi, the preferred method to freeze and to store the liquid nitrogen temperature or mycelium is waiting in the freezer at -80 ° C [11]. In this study, *Aspergillus* and *Penicillium* species in our stocks were molecularly identification and stored for a long time. ITS is used for molecular identification, it is a non-coding region of Internal Transcribed Spacer-ITS and it evolves faster. This is why it is used to compare one genus species or a species of strains.

2. Materials and methods

2.1. Material

Previously isolated and recorded *Aspergillus* and *Penicillium* species in Biology Department of Adnan Menderes University stocks was used as material and identified with classical taxonomy.

2.2. DNA isolation and PCR

DNA isolations of the samples were made according to Tran-Dinh *et al*. [12]. DNA concentration and purity control of the samples were made with Nanodrop Spectrophotometer (Thermo) [13]. The isolated DNA samples were also checked by electrophoresis for 1 hour on agarose gels stained with 0.8% Safe view Classic. 5 µl of DNA samples and 3 µl of loading buffer should be mixed and loaded into agarose gel wells with 1 kilobase DNA marker (Fermentas Gene Ruler). Electrophoresis was visualized with the help of UV imaging system. Universal ITS 1 and ITS 4 primers were used for molecular identification [14]. PCR products were sent to Macrogen (Holland) company for sequencing and matched using BLASTn software [15].

3. Results and Discussion

3.1. Molecular identification of fungi

One hundred twenty samples (120) were matched with GenBank using BLASTn software (GenBank;

http://ncbi.nlm.nih.gov) and 20 of the samples were *Aspergillus fumigatus*, 12 of them were *A. awamori*, 9 of them *A. niger*, 6 of them *A. tubingensis*, 9 of them *A. terreus*, 4 of them *A. japonicus*, 5 of them *A. oryzae*, 2 of them *A. tamarii*, 5 of them *Penicilium commune*, 6 of them *P. chrysegeum*, 2 of them *Fusarium* sp., 3 of them *Mucor circinelloides*, 2 of them *Lichtheimia corymbifera*, 1 of each *Aspergillus versicolor*, *A.brassiliensis*, *A.flavus*, *Penicillium griseofulyum*, *P. restrictum*, *Fusarium proliferatum*, *F. chlamydosporum*, *Trichoderma saturnisporum*, *T. atroviride*, *T. harzinarum*, *Alternaria promicola*, *Purpurecilium lilacinum*, *Cladosporium cladosporioides*, *Eurotium cristatum* and *Cytospora* sp. were found (Table 1). Mycelia and spores of *Aspergillus* and *Penicillium* were lyophilizied and cathologed.

Table 1: Name of species and number of samples identified.

Name of the species	Number of the samples
Aspergillus fumigatus	20
Aspergillus awamori	12
Aspergillus niger	09
Aspergillus tubingensis	06
Aspergillus terreus	09
Aspergillus japonicus	04
Aspergillus oryzae	05
Aspergillus tamarii	02
Aspergillus versicolor	01
Aspergillus brassiliensis	01
Aspergillus flavus	01
Penicillium commune	05
Penicillium chrysogenum	06
Penicillium griseofulyum	01
Penicillium restrictum	01
Fusarium sp	02
Fusarium proliferatum	01
Fusarium chlamydosporum	01
Mucor circinelloides	03
Lichtheimia corymbifera	02
Trichoderma saturnisporum	01
Trichoderma atroviride	01
Trichoderma harzianum	01
Alternaria graminicola	01
Purpurecilium lilacinum	01
Cladosporium cladosporioides	01
Eurotium cristatum	01
Cytospora sp	01

3.2. Data analysis

After DNA isolation, the quantities and purity of the samples was determined using nanodrop spectrophotometer and found to be of sufficient quantity and purity for PCR. For processes of PCR, DNAs were diluted to 50 ng/µl. The PCR results were loaded on a 1.4% agarose gel using 5 µl of the sample using 2 µl loading dye and run at 90 V for 40 min. DNA marker of 100 bp (Fermentas) was used for size comparison of PCR products. Electrophoresis of PCR products was seen in Fig 1.

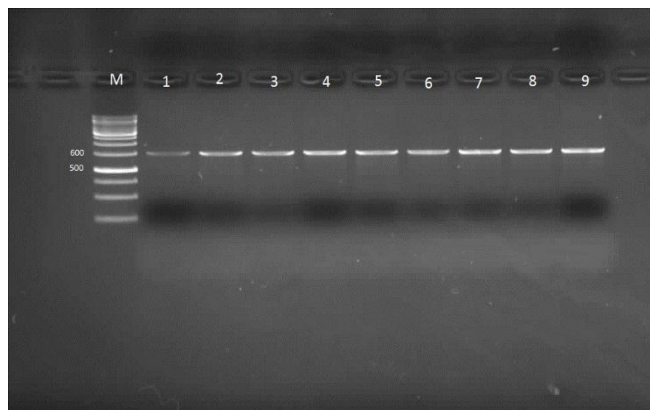

Fig 1: Electrophoresis of PCR products (M:DNA ladder 100 bp).

PCR products by passing the purification kit and agarose gel-controlled to ITS samples, developed using an enzymatic synthesis method, with a capillary system (Automatic Sequencer 3730 xl), were subjected to automated DNA sequence analysis (Macrogen Inc., Netherlands). When the sequence results were examined, it was seen that the sequence information of 60 of these 180 samples could not be compared (DNA could be broken during transport or during cleaning). Sequence analysis was performed with the MEGA6 program and the maximum parsimony tree was removed (Fig. 2). DNA sequence data was aligned with the Clustal W multiple sequence alignment programs (http://www.clustalw.genome.jp/). The sequence of ITS gene regions detected in the study was compared with the ITS gene regions (GenBank; http://ncbi.nlm.nih.gov) defined in previous studies, and the molecular recognition of the fungus species was provided.
DNA sequence analysis results were displayed using the BioEditver 7.0.9 (33) program (http://www.mbio.ncsu.edu/BioEdit/bioedit.html).

3.3. Maximum Parsimony analysis of taxa

The evolutionary history was inferred using the Maximum Parsimony method. Tree 1 out of 2 most parsimonious trees (length = 2095) is shown. The consistency index is (0.406099), the retention index is (0.900446), and the composite index is 0.373073 (0.365670) for all sites and parsimony-informative sites (in parentheses). The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches. The MP tree was obtained using the Subtree-Pruning-Regrafting (SPR) algorithm with search level 1 in which the initial trees were obtained by the random addition of sequences (10 replicates). The analysis involved 119 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions with less than 95% site coverage were eliminated. That is, fewer than 5% alignment gaps, missing data, and ambiguous bases were allowed at any position. There were a total of 388 positions in the final dataset. Evolutionary analyses were conducted in MEGA6 [16-18].

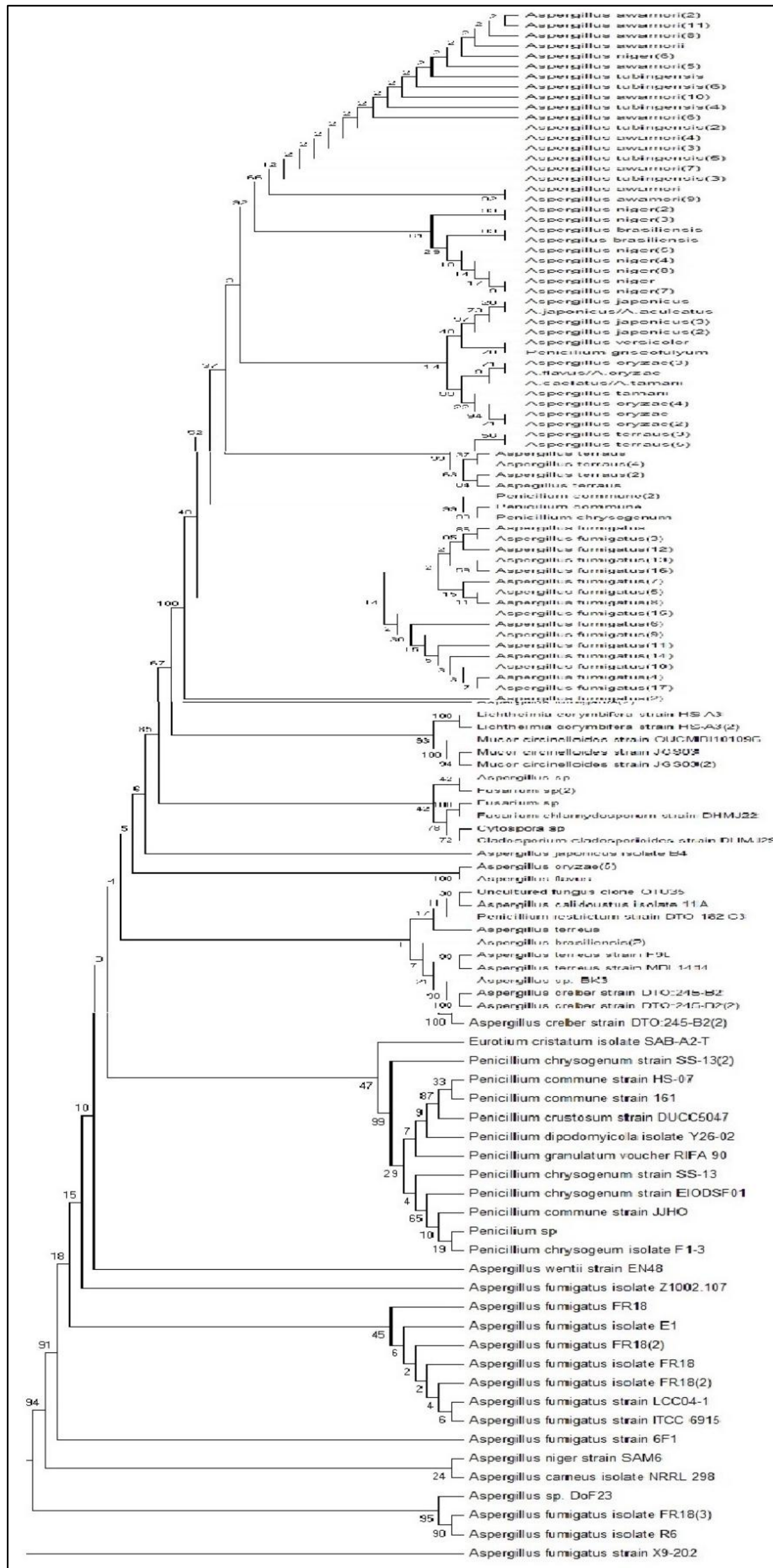

Fig 2: Maximum Parsimony analysis of taxa.

4. Conclusions

Classical parameters used in the identification of microfungi; microscopic morphology, physiological tests, culture and clinical features. However, the ambiguous nature of the phenotypic character makes morphological recognition at the species level difficult. For this reason, molecular approaches have become an important alternative in the diagnosis of microfungi. The basic principle in biodiversity and systematic studies is the preservation and preservation of well-defined fungal cultures. The *Aspergillus* and *Penicillium* species in our stocks have been molecularly diagnosed and have been stored for a long time. ITS is used for molecular diagnosis, it is a non-coding region of Internal Transcribed Spacer-ITS and it evolves faster. This is why it is used to compare one genus species or a species of strains.

Lyophilization and liquid drying methods are difficult techniques for preserving fungus scrap. These techniques are especially used for storing fungus spores for 20 years or more. The preferred method for storing fungus scrapes is to freeze and store at liquid nitrogen temperature or to keep mycelium at -80 ° C in deep freeze. One of the aims of the study is to identify the stock cultures previously isolated from various sources and diagnosed with classical taxonomy, and store them by storing them as molecular species. Stocking is the creation of the substructure of cultural collections that will be formed in our future.

One hundered twenty samples (120) were matched with GenBank using BLASTn software (GenBank; http://ncbi.nlm.nih.gov) and 20 of the samples were *Aspergillus fumigatus*, 12 of them were *A. awamori*, 9 of them *A. niger*, 6 of them *A. tubingensis*, 9 of them *A. terreus*, 4 of them *A. japonicus*, 5 of them *A. oryzae*, 2 of them *A. tamarii*, 5 of them *Penicilium commune*, 6 of them *P. chrysegeum* were found.

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6. References

1. Walsh TJ, Wissel MC, Grantham KJ, Petraitiene R, Petraitis V, Kasai M *et al*. Molecular detection and species-specific identification of medically important *Aspergillus* species by real-time PCR in experimental invasive pulmonary aspergillosis. Journal of Clinical Microbiology, 2011; 49:4150-4157.
2. Sudjaritruk T, Sirisanthana T, Sirisanthana V. Immune reconstitution inflammatory syndrome from *Penicillium marneffei* in an HIV-infected child: a case report and review of literature. Bio Med Central Infectious Diseases, 2012; 12:28.
3. Sant'Ana AS, Simas RC, Almeida CAA, Cabral EC, Rauber RH, Mallmann CA *et al*. Influence of package, type of apple juice and temperature on the production of patulin by *Byssochlamys nivea* and *Byssochlamys fulva* International Journal of Food Microbiology, 2010; 142:156-163.
4. Xu X, Yang J, An Y, Pan Y, Liu G. Over-expression of pcvA involved in vesicle vacuolar fusion affects the conidiation and penicillin production in *Penicillium chrysogenum*. Biotechnol Letters. 2012; 34:519-526.
5. Raper KB, Fennell DI. The genus *Aspergillus*. Williams and Wilkins, Baltimore, 1965.
6. Glass NL, Donaldson G. Development of primer sets designed for use with the PCR to amplify conserved genes from filamentous ascomycetes. Applied Environmental Microbiology. 1995; 61:1323-1330.
7. Edman JC, Kovacs JA, Masur H, Santi DV, Elwood HJ, Sogin ML. Ribosomal RNA sequence shows *Pneumocystis carinii* to be a member of the fungi. Nature, 1986; 334:519-522.
8. Dupont J, Dutertre M, Lafay JF, Roquebert MF, Brygoo Y. A molecular assessment of the position of stilbothamnium in the genus *Aspergillus*. In: Samson RA, Pitt JI (eds) Modern concepts in *Penicillium* and *Aspergillus* classification. Plenum, New York, 1990, 335-342.
9. Logrieco A, Peterson SW, Wicklow DT. Ribosomal RNA comparisons among taxa of the terverticillate penicillia. In: Samson RA, Pitt JI (eds) Modern concepts in *Penicillium* and *Aspergillus* classification. Plenum, New York, 1990, 343-356.
10. Onions AHS. Preservation of fungi. In the Filamentous fungi 4: Fungal echnology, 1983, 375-390.
11. Oei P. Conservation of strains and culture collections. In Mush Cultivation. Bachuys. 1996.
12. Tran-Dinh N, Pitt JI, Carter DA. Molecular genotype analysis of natural toxigenic and nontoxigenic isolate of *Aspergillus flavus* and *A. parasiticus*. Mycological Research. 1999; 103:1485-1490.
13. Turner PC, Mclennan AG, Bates AD, White MRH. Moleküler Biyoloji Önemli Notlar. Nobel Yayın, 2004; 613:346.
14. Sanger F, Coulson AR. A rapid method for determining sequences in DNA by primed synthesis with DNA polymerase. Journal of Molecular Biology. 1975; 94:441-448.
15. Hall TA. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucleic Acids Symposium Series. 1999; 41:95-98.
16. Felsenstein J. Confidence limits on phylogenies: An approach using the bootstrap. Evolution, 1985; 39:783-791.
17. Nei M, Kumar S. Molecular Evolution and Phylogenetics. Oxford University Press, New York, 2000.
18. Tamura K, Stecher G, Peterson D, Filipski A, Kumar S. MEGA6: Molecular Evolutionary Genetics Analysis version 6.0. Molecular Biology and Evolution, 2013; 30:2725-2729.