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Biodiversity of Heat Resistance Soil Microfungi in Agricultural Areas of Eskisehir Province

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Abstract: Heat-resistant microfungi can survive 30 minutes of heat at 75°C and can continue to develop and deteriorate products during storage in the room conditions. The most important role in this heat resistance is based on the ability to form sexual reproduction structures called ascospores, and ascospores heat resistance depends on species, strain, pH, heating medium and other growth. *Byssoschlamys fulva* (current name; *Paecilomyces fulvus*) is the first heat-resistant microfungus recorded, and in addition to *B. nivea* (current name; *Byssoschlamys lagunculariae*), *Neosartorya fischeri* (current name; *Aspergillus fischeri*), *Talaromyces macrosporus*, *T. bacillisporus* and *Eupenicillium brefeldianum* (current; *Penicillium dodgei*) are the most common heat resistant microfungi. We investigated biodiversity of heat resistant microfungi in agricultural soils of Eskisehir Province in our study. For this purpose, four different soil samples were collected from fallow lands in east, west, north and south locations of Eskisehir Province at September 2017. Isolation process was performed by using heat treatment of soil samples and the soil dilution method. After purification step, isolates were diagnosed by using conventional methods and multi locus gene sequencing. We determined total of 3.22x10³ cfu/g colonies appertain to heat resistant microfungi and 49 isolates belong to *Aspergillus*, *Byssoschlamys*, *Penicillium* and *Talaromyces* genera. As a result, we determined that the agricultural soils have high heat resistance microfungi biodiversity that commonly known as mycotoxigenic, pathogenic and saprophytic.

Key words: Heat resistant microfungi, agricultural soils, Eskisehir, multi locus gene sequencing



Eskişehir İli Tarım Topraklarındaki Isıya Dirençli Toprak Mikrofunguslarının Biyoçeşitliliği

Öz: Isıya dayanıklı mikro mantarlar 75°C'de 30 dakika ısıya dayanabilir ve oda koşullarında depolama sırasında ürünlerde gelişmeye ve bunlarda bozulmaya devam edebilir. Bu ısı direncindeki en önemli rol, askospor adı verilen eşeyli üreme yapıları oluşturma yeteneğine dayanmaktadır ve askosporların ısı direnci; türlere, strainlere, pH, ısıtma ortamı ve diğer büyüme koşullarına bağlıdır. *Byssochlamys fulva* (geçerli isim; *Paecilomyces fulvus*), ilk kaydedilen ısıya dayanıklı mikrofungusdur ve buna ilave olarak *B. nivea* (geçerli isim; *B. lagunculariae*), *Neosartorya fischeri* (geçerli isim; *Aspergillus fischeri*), *Talaromyces macrosporus*, *T. bacillisporus* ve *Eupenicillium brefeldianum* (geçerli isim; *P. dodgei*) en yaygın ısıya dayanıklı mikrofunguslardır. Çalışmamızda, Eskişehir ilinin tarım topraklarındaki ısıya dayanıklı mikrofungusların biyoçeşitliliği araştırıldı. Bu amaçla, Eylül 2017'de Eskişehir ilinin doğu, batı, kuzey ve güney bölgelerindeki nadas alanlarından dört farklı toprak örneği toplanmıştır. Toprak örneklerinden ısıtma işlemi ve toprak seyreltme yöntemi kullanılarak izolasyon işlemi yapılmıştır. Saflaştırma aşamasından sonra, geleneksel yöntemler ve çoklu lokus gen dizilimi kullanılarak izolatlar teşhis edilmiştir. *Aspergillus*, *Byssochlamys*, *Penicillium* ve *Talaromyces* cinslerine ait 49 izolat ve ısıya dirençli mikrofunguslara ait toplam 3.22×10^3 cfu/g koloni tespit edilmiştir. Sonuç olarak, tarımsal toprakların, mikotoksijenik, patojenik ve saprofitik olarak bilinen yüksek ısıya dirençli mikofungal biyolojik çeşitliliğe sahip olduğunu belirlenmiştir.

Anahtar kelimeler: Isıya dirençli mikrofunguslar, tarım toprakları, Eskişehir, çoklu gen sekansı

Introduction

The heat resistant microfungi can be continue to their life after exposed of temperature at above or 75°C for 30 or more minutes thanks to their ascospores, chlamydospore, thick walled hyphae or sclerotia (Valik and Piecková, 2001; Houbraken and Samson, 2006; Amaeze et. al., 2010). *Aspergillus*, *Byssochlamys*, *Penicillium* and *Talaromyces* are the most common types of heat resistant microfungi (Mouchacca, 2007; Kikoku et al., 2008; Yaguchi et al., 2012). In addition, these genera are well known as their high distribution and cause of effect health on human, animal and plants such as via pathogenic activities and mycotoxin production (Asan, 2004; Demirel, 2016). The members of heat resistant microfungi are widely distribute on soil, even survive on low water activity conditions and cause to spoilage of foods (Valik and Pieckova, 2001; Yaguchi et al., 2012).

By the time, researches have focussed on heat resistant microfungi for exhibit of these sources, importance, effects on food processing workflow. For these reason, the main idea of this study are (i) isolation of heat resistant microfungi from agricultural soils in Eskişehir province, (ii) identification of isolated heat resistant microfungi by using traditional and molecular techniques, (iii) determination of heat resistant microfungi

biodiversity and distribution from agricultural soils in Eskişehir province.

Material and methods

Site description, soil sampling and characterization

The research areas are four agricultural fallow lands in four different geographical regions of Eskişehir province. The GPS and altitudes were recorded by using Garmin Fenix 3 (Garmin, Switzerland) (Table 1). Soil samples (total of 4) were collected with a sterilized trowel from 5 different profiles in 0–10 cm depth within a distance of 50 m away from each other according to Brown's technique (1958) in September 2017. Samples were transported in sterile polyethylene bags and stored at +4°C until analysed.

Some of the physical and chemical properties of soil samples such as texture, moisture, pH, organic matter, phosphor, azote, potassium, salinity, and hardness was measured in the laboratory of the Ministry of Environment, Forest, Soil and Ecology Research Institute, Eskişehir (Turkey). Percentage moisture of soil samples calculated by using formulation; % = (c–g)/g, where “c” is the weight of wet soil, and “g” is the weight of soil dried at 105 °C for 24 h.



Table.1. Location records of sampling areas

City	Sample No	GPS records (degree and decimal minutes) (DMM)		Altitude (m)
		North	East	
Eskisehir	1 (West)	39 46.8429	30 24.9482	767
	2 (North)	39 50.2406	30 30.6429	762
	3 (South)	39 43.5098	30 29.6615	954
	4 (East)	39 45.8257	30 35.5961	753

Isolation of heat resistant microfungi

Heat resistant microfungi were isolated according to Houbraken ve Samson (2006) together with some volume modifications. Briefly, one hundred grams (dry weight) of each soil sample was diluted (1:1000, v/w) in sterile distilled water into a sterile Stomacher bag. After homogenization step for 2-4 min, the Stomacher bag was treated with heat for 30 min at 75°C in a water bath. After heat treatment, samples were cooled to about 55 °C. the contents of the Stomacher bag were transferred to sterile Erlenmeyer (2000 ml) with 1000 ml melted double strength Dichloran Glycerol (DG18)-Agar. After mixing well, the agar and sample mixture were distributing into twenty large plastic Petri dishes (diameter 15 cm) and incubated at 30 °C for 14 days. The petri dishes were checked for presence of colonies after 7 days and 14 days. Emerging fungal colonies were subcultivated on Malt Extract Agar (MEA) (Samson et al., 2010) and maintained with glycerol stock (Klich, 2002) at -86°C.

Morphological and multi locus gene identification

Isolates of heat resistant microfungi were initially identified to the genus level on the basis of their microscopic and colonial characteristics. For identification of *Byssoschlamys*, *Penicillium* and *Talaromyces* species, Czapek Yeast Extract Agar (CYA; incubation at 25°C and 37°C), Malt Extract Agar (MEA; at 25°C), Yeast Extract Agar (YES; at 25°C) and Creatine Sucrose Agar (CREA; at 25°C) were used. For identification of *Aspergillus* species, CYA (at 25°C and 37°C), MEA (at 25°C) and CREA (at 25°C) were used. The isolates were incubated for 7 days. At the end of incubation, the isolates were distinguished at the species level according to their morphological and microscopic characteristics (Samson et al. 2010; 2011; 2014).

All isolates were grown on Potato Dextrose Agar (PDA) at 25°C 7 days to DNA extraction. Fungal genomic DNA was extracted using "Mobio Ultraclean Microbial DNA Isolation Kit" according to the manufacturer's instructions. Obtained DNA used as template for PCR

amplification of β -Tubulin (BenA), Bt2a (5'-GGTAACCAAATCGGTGCTGCTTTC-3'), Bt2b (5'-ACCCTCAGTGTAGTGACCCTTGCC-3') (Glass and Donaldson, 1995), Calmodulin (CaM), CL1 (5'-GA(AG)T(AT)CAAGGAGGCCTTCTC-3'), CL2 (5'-TTTTGCATCATGAGTTGGAC-3') (Serra et al., 2006), ITS, V9G-F (5'-TTACGTCCCTGCCCTTTGTA-3'), LS266-R (5'-GCATTCCCAAACAACCTCGACTC-3') (Samson et al., 2010; Schoch et al., 2012) gene regions. Reactions were performed in 25 μ l volumes containing 1 μ l genomic DNA, 2.5 μ l 2.5 μ M ITS1, 2.5 μ l 2.5 μ M ITS4, 2.5 μ l 10X Taq buffer +KCl – MgCl₂ (Fermentas), 2.5 μ l 25 mM MgCl (Fermentas), 2 μ l 2.5 mM dNTPmix, 0.25 μ l 5 U/ μ l Taq DNA polymerase (Fermentas), and 11.75 μ l sterile deionized water.

PCRs were performed using a Veriti® 96-Well Thermal Cycler (Applied Biosystems®) using initial denaturation at 94°C for 5 min, followed by 35 cycles of denaturation at 94°C for 45 s, annealing at 56 °C for 30s, extension at 72 °C for 2 min and final extension at 72°C for 6 min for ITS gene region, initial denaturation at 95°C for 3 min, followed by 30 cycles of denaturation at 95°C for 1 min, annealing at 65 °C for 55 s, extension at 72°C for 1 min and final extension at 72°C for 10 min for β -Tubulin gene region and initial denaturation at 94°C for 10 min, followed by 35 cycles of denaturation at 94°C for 50 s, annealing at 55 °C for 50 s, extension at 72°C for 1 min and final extension at 72°C for 7 min for Calmodulin gene region. PCR products were confirmed by agarose gel electrophoresis (1% w/v in 1xTAE) and visualized by GelRed staining and examined via the Gel Documentation System (Uvitec M02 4611). PCR products were purified by using EXOSAP-IT (Amersham Pharmacia Biotech, Piscataway, NJ) and used for sequencing. The ITS region was sequenced using ITS1; TCCGTAGGTGAACCTGCGG (forward), ITS4; TCCTCCGCTTATTGATATGC (reverse) (White et al., 1990) and other gen regions via their primer pairs. Sequencing reactions were performed with the Applied Biosystems (3130 XL Genetic Analyser) by the RefGen Biotechnology (<http://www.refgen.com>).



Data analysis

The sequences were compared with those deposited in the NCBI GenBank Database (Altschul et al., 1990; <https://blast.ncbi.nlm.nih.gov/Blast.cgi>). The closest Blast results are reported for each taxon.

The alignments were performed using the Muscle in MEGA X software package, together with the other sequences of morphologically and phylogenetically related type cultures that were obtained from NCBI GenBank (Kumar et al., 2018). The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) (Tamura and Nei, 1993) approach, and then selecting the topology with superior log likelihood value with 1000 bootstrap replications. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. All positions with less than 50% site coverage, containing gaps, or missing data were eliminated. *Aspergillus clavatoflavus* Raper & Fennell 1965 (EF669713, EF669686, EF669700) and *Penicillium sacculum* E.Dale 1926 (KC411707, KJ834488) was used as the out group.

Fungal author names and fungal names were standardized according to the Index Fungorum website (<http://www.indexfungorum.org/names/names.asp>).

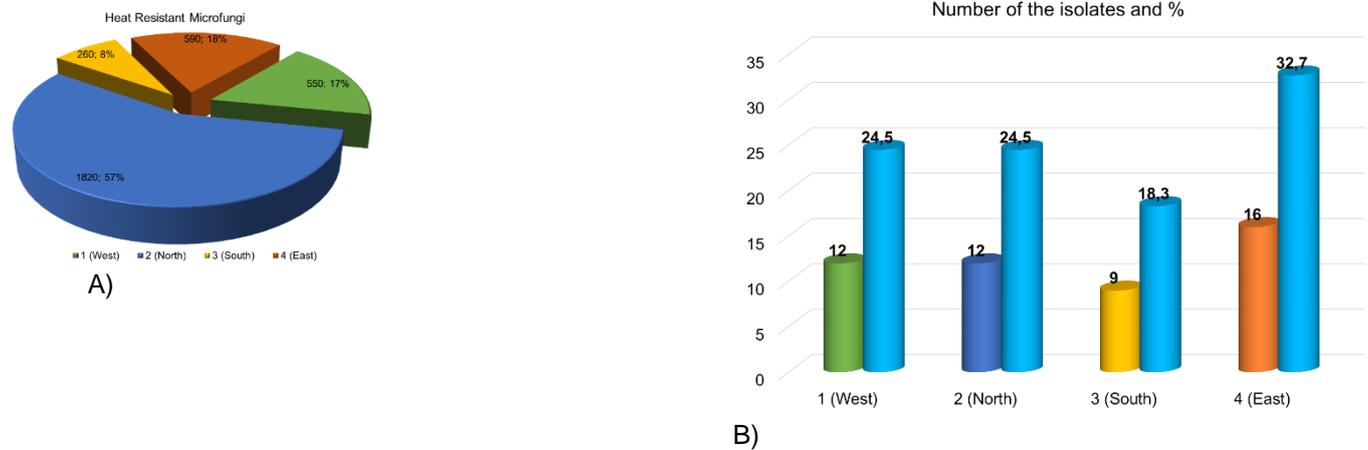
Results and discussion

The moisture values of investigated soil samples were determined between 4,65-10,67% and the lowest moisture (%) were exhibited by sample 2 (North). In addition, other soil samples showed clay soil characteristics, while the same soil sample showed clay loam soil type properties. the lowest organic carbon percentage is also determined in soil sample 2 (Table 2). The soil sample number 2, which shows low levels of moisture and organic carbon compared to other locations, was determined as the highest location in terms of colony account (cfu/g) of heat resistant microorganisms (57 %) (Figure 1A). The soil sample number 3, which has the highest values of moisture and organic matter (Table 2), showed the lowest colony account of heat resistant microorganisms (8%) (Figure 1A). Valík and Piecková (2001) showed that some of the heat resistant fungi such as *P. fulvus* Stolk & Samson 1971, *A. fischeri* Wehmer 1907 and *H. avellanea* Stolk & Samson 1971 species growth at low water activity ranging from 0,995 to 0,85. In addition, there are some records exhibited that heat resistance fungi are can be continuing to their life under the unfavourable conditions thanks to their ascospores, clamydospore, thick walled hyphae or sclerotia (Valik and Pieckova, 2001; Houbraken and Samson, 2006; Amaeze et. al., 2010). The data we have obtained with this study also supports this.

Total of 49 isolates were obtained from investigated soil samples. The highest isolates (16; 32,7%) were acquired from soil sample 4. These followed by soil samples 1 and 2 (12; 24,5%) and 3 (9; 18,3%) (Figure 1B).

Table.2. Some of the physical and chemical properties of soil samples

Soil Sample	Moister (%)	Soil Type	pH	Lime (%)	Organic Carbon (%)	Organic Matter (%)	Electric Conductivity mS/cm
1 (West)	9,11	Clay	7,89	6,70	1,12	1,94	0,21
2 (North)	4,65	Clay Loam	8,14	13,07	0,93	1,60	0,23
3 (South)	10,67	Clay	7,96	21,36	1,41	2,43	0,18
4 (East)	7,09	Clay	8,12	31,65	1,09	1,88	0,21



According to morphologic and multi-locus genes sequencing results, the isolates were found to be members of *Aspergillus* (21, 42,86%), *Byssosclamyces* (2; 4,08%), *Penicillium* (24; 48,98%) and *Talaromyces* (2; 4,08%). *Penicillium* genus was recorded as the most common genus in the agricultural soils of Eskisehir

province. Already, *Aspergillus*, *Byssosclamyces*, *Penicillium* and *Talaromyces* have the most common types of heat resistant microfungi (Mouchacca, 2007; Kikoku et al., 2008; Yaguchi et al., 2012). Five isolates of *Penicillium* genus were identified only genus level. Other 44 isolates were identified species level (Table 3).

Table 3. Biodiversity and distribution of isolates

Species Name	Number of the isolate (Locations)	Percentage	Total of the locations
<i>Aspergillus chevalieri</i> Thom & Church 1926	6 (1, 3, 4)	12,24	3
<i>A. costiformis</i> H.Z. Kong & Z.T. Qi 1995	4 (1, 2, 4)	8,16	3
<i>A. fischeri</i> Wehmer 1907	6 (2, 4)	12,24	2
<i>A. niger</i> Tiegh. 1867	2 (1, 2)	4,08	2
<i>A. ruber</i> (Jos. König, Spieck. & W. Bremer) Thom & Church 1926	3 (1, 2)	6,12	2
<i>Byssosclamyces nivea</i> Westling 1909	2 (2)	4,08	1
<i>Penicillium</i> sp.	5 (1, 2, 3, 4)	10,20	4
<i>Penicillium chrysogenum</i> Thom 1910	7 (1, 3, 4)	14,29	3
<i>P. citrinum</i> Thom 1910	1 (2)	2,04	1
<i>P. parvofructum</i> Guevara-Suarez, Cano-Canals, Cano & Stchigel 2017	1 (4)	2,04	1
<i>P. turbatum</i> Westling 1911	10 (1, 3, 4)	20,41	3
<i>Talaromyces pinophilus</i> (Hedgc.) Samson, N. Yilmaz, Frisvad & Seifert 2011	1 (2)	2,04	1
<i>Talaromyces purpureogenus</i> Samson, N. Yilmaz, Houbraken, Spierenb., Seifert, Peterson, Varga & Frisvad 2011	1 (2)	2,04	1



When we focused on biodiversity and distribution of heat resistance microfungi in soil samples, *P. turbatum* were determined as the most common (10; 20,41%) and the highest prevalence (3 locations) heat resistance fungi in agricultural soils of Eskisehir province. This is followed by *P. chrysogenum* (7; 14,29%, 3 locations), *A. chevalieri* (6; 12,24%, 3 locations), *A. fischeri* (6; 12,24%, 2

The phylogenetic relationships between the isolates belonging to *Aspergillus* and *Penicillium* members were investigated through sequencing of three loci, ITS, beta-tubulin (for *Aspergillus* and *Penicillium* members) and calmodulin (for *Aspergillus* members). The lengths of the alignments of the ITS, beta-tubulin, and calmodulin loci were 85-87 nucleotide sequences and 404 (the highest log likelihood -1849.23)-400 (the highest log likelihood -1413.64) position for ITS, 81-49 nucleotide sequences and 429 (the highest log likelihood -5239.11)-423 (the highest log likelihood -3996.52) position for beta-tubulin, 67 nucleotide sequences and 400 (the highest log likelihood -4217.95) position for calmodulin respectively as *Aspergillus* sp. and *Penicillium* sp.

Figure 2-4 shows that the members of the genera *Aspergillus* and Figure 5, 6 shows that the members of the genera *Penicillium* have almost identical topology with

locations) (Table 3). There are some records related with heat resistance fungi in some food sample in Turkey and in addition to *A. chevalieri*, *A. fumigatus*, *Paecilomyces variotii* species, some of the members of *Aspergillus* and *Penicillium* genera were identified frequently (Kocakaya Yıldız and Coksöyler, 2002; Aydın et al., 2005; Demirci and Arıcı, 2006).

respect to the ITS, beta-tubulin and calmodulin loci. A phylogenetic trees based on the three loci were constructed at higher divergence levels. For *Aspergillus* spp., 2 sections, namely *Aspergillus* and *Fumigati*, for *Penicillium* spp., 4 sections, namely *Chrysogena*, *Citrina*, *Fasciculata* and *Turbata*, could be clearly noted (Samson et al., 2014). Interestingly, some isolates belong to *Aspergillus* (26.08, 26.10, 25.56 and 26.57) and *Penicillium* (isolate codes; 26.42, 26.43, 26.46, 26.54, 26.68 and 26.73) genera exhibited different positions (marked with stars on the tree) and showed different topology from their type cultures. Because of these reasons, these isolates need to additional cooperation and description studies against to their type's cultures. Furthermore, according to Asan's checklist (2004), *A. costiformis* and *P. parvofructum* are likely to be newly recorded for Turkey.



ITS

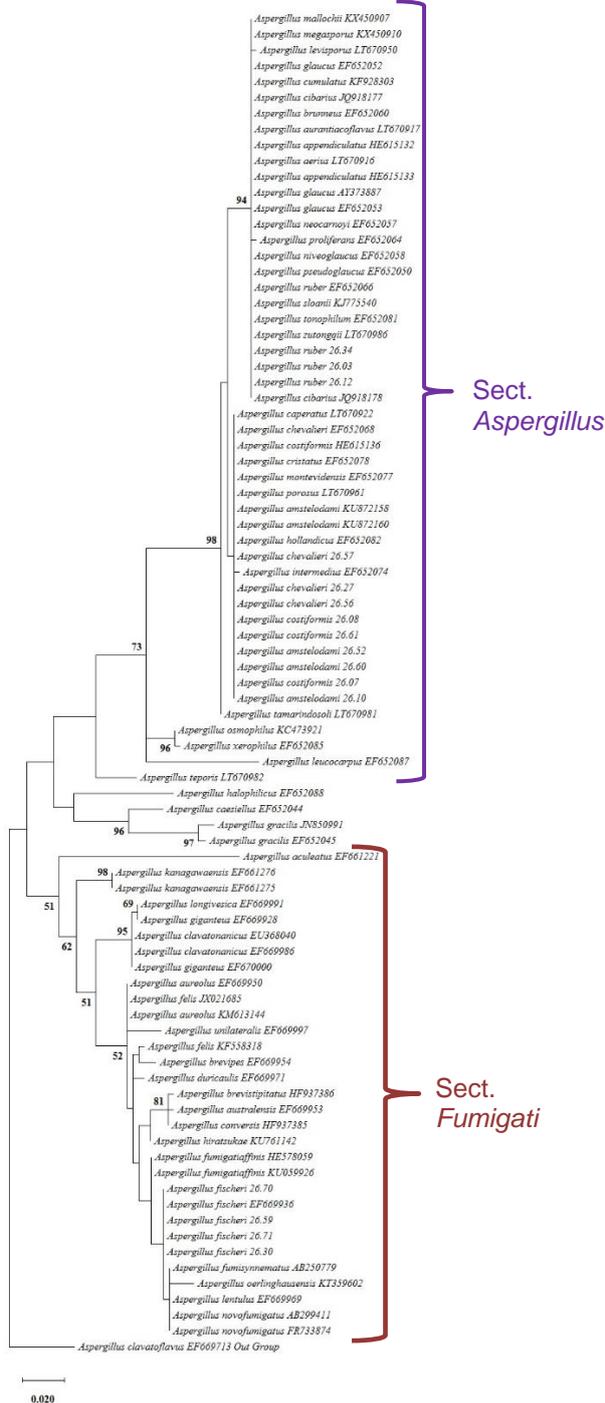


Figure 2. Best-scoring maximum likelihood tree based on ITS sequences of *Aspergillus* members showing the relationships of the newly generated sequences in this study with previously known taxa in the NCBI GenBank. The tree is rooted with *Aspergillus clavatoflavus* (EF669713) (bootstrap 1000).

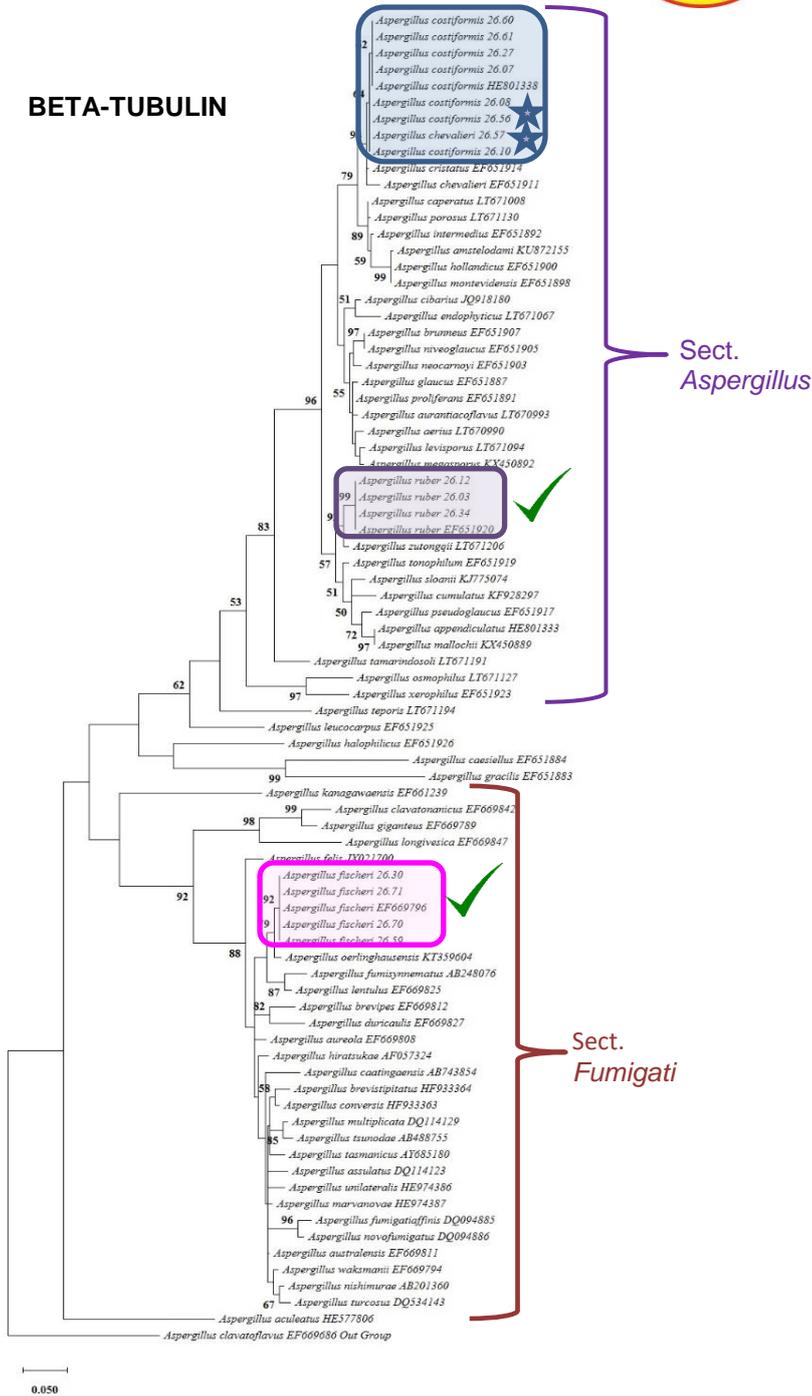


Figure 3. Best-scoring maximum likelihood tree based on beta-tubulin sequences of *Aspergillus* members showing the relationships of the newly generated sequences in this study with previously known taxa in the NCBI GenBank. The tree is rooted with *Aspergillus clavatoflavus* (EF669686) (bootstrap 1000).



CALMODULIN

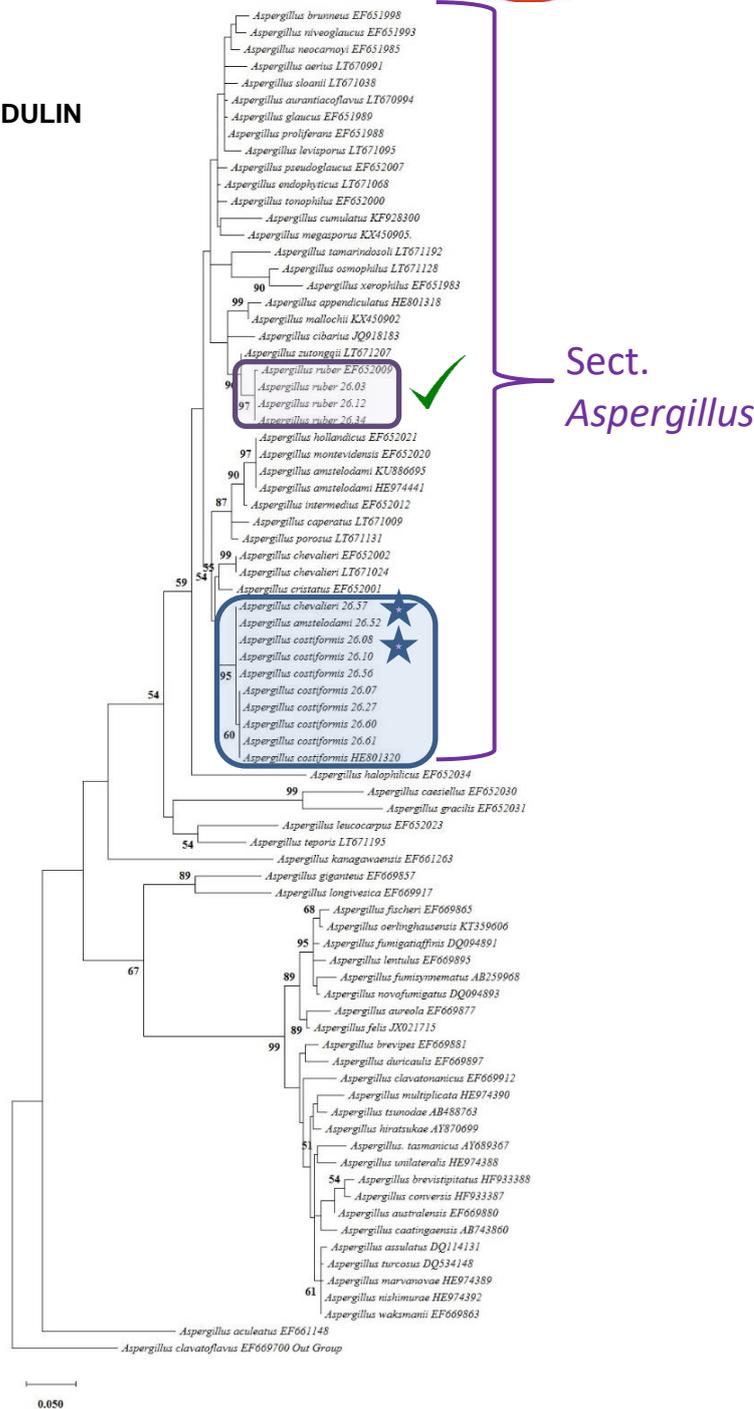


Figure 4. Best-scoring maximum likelihood tree based on calmodulin sequences of *Aspergillus* members showing the relationships of the newly generated sequences in this study with previously known taxa in the NCBI GenBank. The tree is rooted with *Aspergillus clavatoflavus* (EF669700)

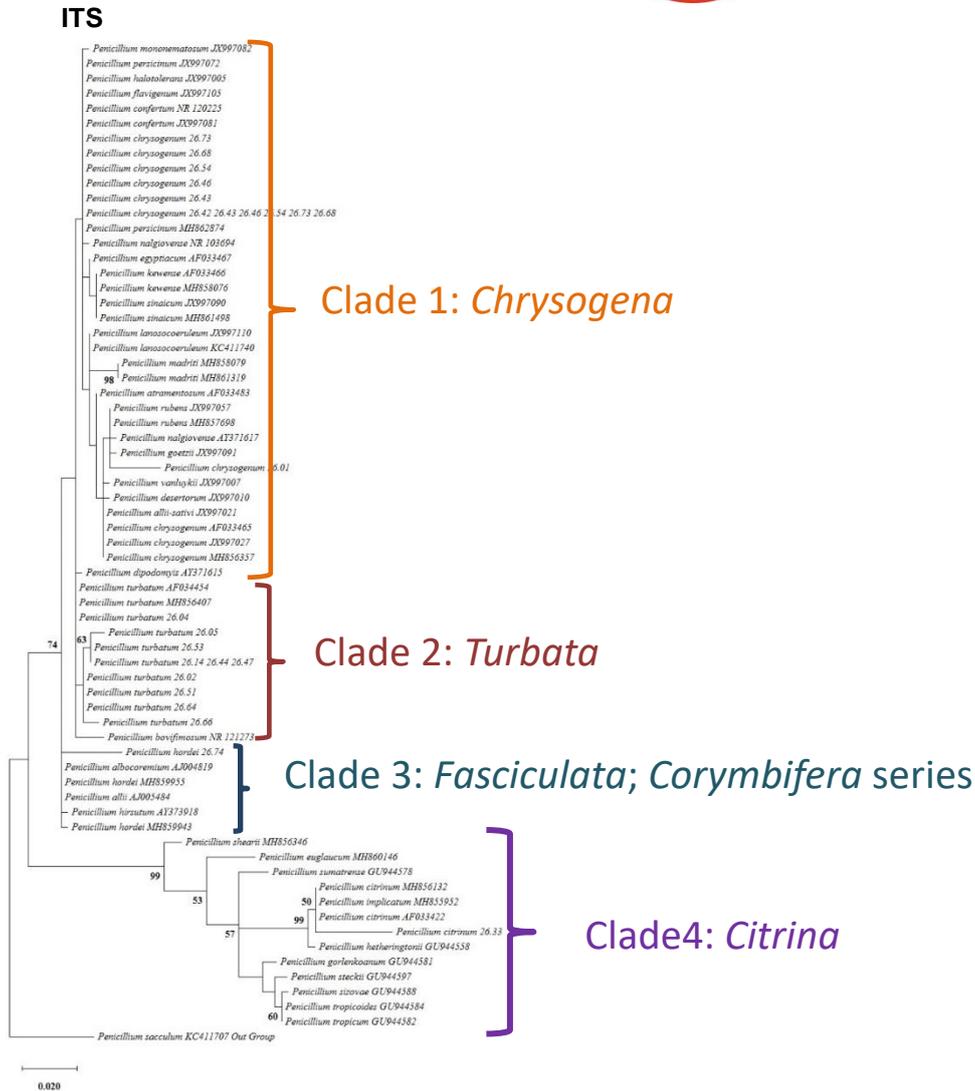


Figure 5. Best-scoring maximum likelihood tree based on ITS sequences of *Penicillium* members showing the relationships of the newly generated sequences in this study with previously known taxa in the NCBI GenBank. The tree is rooted with *Penicillium sacculum* (KC411707) (bootstrap 1000).



BETA-TUBULIN

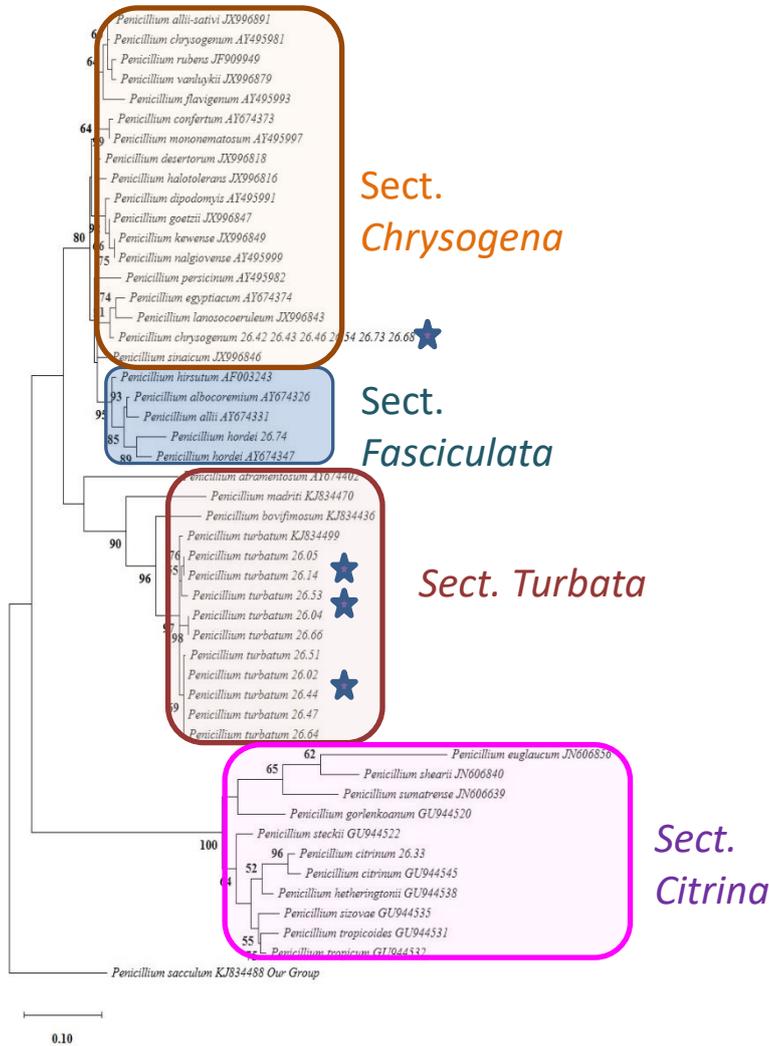


Figure 6. Best-scoring maximum likelihood tree based on beta-tubulin sequences of *Penicillium* members showing the relationships of the newly generated sequences in this study with previously known taxa in the NCBI GenBank. The tree is rooted with *Penicillium sacculum* (KJ834488) (bootstrap 1000).

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