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Isolation and Identification of *Penicillium toxicarium*: A New Record for Turkey

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Abstract: In this study *Penicillium toxicarium* was cited for the first time as a new record of *Penicillium* in Turkey. This species was isolated from decaying wood samples in Usak province, Turkey. Morphological, colonial and molecular identification were performed. Fungal DNA Isolation Kit and universal internal transcribed spacer (ITS) primers were used for molecular identification.

Keywords: *Penicillium toxicarium* , *Penicillium* ,Turkey, Fungi, Mycobiota ,Record

Penicillium toxicarium'un İzolasyonu Ve İdentifikasyonu: Türkiye İçin Yeni Kayıt

Öz: Bu çalışmada *Penicillium toxicarium* Türkiye'de ilk kez yeni bir *Penicillium* türü olarak gösterildi. Bu tür, Türkiye'nin Uşak ilinde çürüyen ağaç örneklerinden tespit edilmiştir. Makroskobik ve mikroskobik morfolojiksine göreve moleküler tani yöntemlerine gore tanımlama yapıldı. Fungal DNA İzolasyon Kiti ve evrensel primerler kullanılarak ITS bölgsi dizi analizleri moleküler tanımlama için kullanıldı.

AnahtarKelimeler: *Penicillium toxicarium, Penicillium*, Türkiye, Mantarlar, Mikobiyota, Kayıt

Introduction

Traditionally, fungal species determination depends on media culturing and macro or microscopic appearances identification.

However, these methods have disadvantages, as the evaluation of fungal species according to the existence or not of fruiting structures, the incapacity of cultural growth of specific types, and the uneasy recognizing to the species level depending on microscopic appearances. Hence, molecular methods have been found as rapid and easy methods for identifying of fungi to species level and found as a dependable replacement to conventional techniques (Kiraz N, 2015).

Generally the recognition of fungal species molecularly depends on the amplification and sequencing of (ITS) region of the genetic material of fungi, which shows greatliability to vary or change with in the species or even in community of the similar species. ITS primers (ITS1 and ITS4) have been used widely in molecular studies of fungi identification (Irinyi et al., 2015).

Penicillium genus includes a wide range of species that look similar. Morphology, colonyforms, metabolite description and molecular informations have been used recentlyfor taxonomy of this genus.

Molecular phylogenetic examinations depending on (ITS) have been applied for distinguishing of the *Penicillium* species (Berbee et al.,1995; Peterson, 2000b).

There are over 250 species of *Penicillium* genus (Visagie et al., 2014; Abastabar et al., 2016). Many of *Penicillium* species had been isolated and identified from the different places of Turkey. A check list documented by Asan, (2000) gave about 159 species of *Penicillium* in 2000, and later data base added 66 species to the earlier list, to reach the total number of *Penicillium* species isolated in Turkey to 225 as of February 10, 2015 (Asan, 2004).

After the set wo checklist, the number of novel *Penicillium* species isolated in Turkey continued in increase (Çakır and Maden, 2015; Kolanlarli et al., 2019). Recent check list that published in 2020 have been included a huge list of certain fungi found inTurkey (Sesli et al., 2020).

Although many of published articles and variety of *Penicillium sp.* that had been isolated in Turkey, up to date we couldn't find one published article about isolation of *Penicillium toxicarium* I. Miyake ex C. Ramírez in Turkey.

So the purpose of this paper is to record for the first time the isolation and identification of *Penicillium toxicarium* in Turkey.

Materials and Methods

Materials

For fungus isolation process two media have been used which are Potato Dextrose Agar (PDA) (Merck 110130) and Rose Bengal Agar (RBA). Czapek-Dox Agar (CDA) and Malt Extract Agar (MEA) (Merck 105398) have been used for macroscopic and microscopic identification.

Decaying wood samples around Uşak University 1 Eylül Campus were collected for the isolation of the species reported in the study (Figure 1).

The collected *Pinus nigra* Arnold. *subsp. Pallasiana* (Lamb.) Holmboe wood samples were diluted with distilled water. Rose Bengal Agar (RBA) and Potato Dextrose Agar (PDA) were inoculated by the samples and left to incubate for a week in the dark environment at 25 °C. At the end of one week incubation, colonies were selected and stored in pure culture at + 4℃ in RBA. Macroscopic and microscopic descriptions of the isolated fungi were made. Fungi were cultured on CDA and MEA at 25 °C in 14 days for identification. In macroscopic identification of fungi, colony growth pattern, surface topography surface texture smell, pigmentation, mycelium and sporulation pattern were assessed. In microscopic identification, measurements were made from slide preparations stained with lactophenol-aniline blue. Microscopic identification is based on the branching shape of conidiophores, the shape and emergence of phialids, as well the shape, color and wall characteristics of the conidia (Hasenekoğlu, 1991*;* Singh, 2014).

Penicillium toxicarium I. Miyake ex C. Ramírez, Manual and Atlas of the *Penicillia* (Amsterdam): 125 (1982) Specimen examined: Colonies on CDA were 40 mm in diameter.

Moderately deep, dense and velutinous to lanose, radially sulcate and often centrally wrinkled; the margins were narrow with mycelium that were white; sporulation moderate, deep blue or blue-green in color; without exudates; soluble pigment pale amber; without odor; a reverse coloration of salmon pale.

Figure 1**:** Uşak University 1 Eylül Campus where *Pinus nigra* Arnold*. subsp. pallasiana* decaying wood sample has been collected (from Google maps)

Colonies on MEA were 20~25 mm in diameter, dense and velutinous, plane to radially sulcate; margins entire or ırregular; mycelium white, becoming yellow or green; sporulation moderate to danse; exudates yellow; soluble pigment brown (Figure 2). Conidiophores monoverticillate, stipes delicate 50~10 ×2.0~2.5 µm, smooth walled; phialides phialides ampulliform (7.5~10.0)×(2.3~3.5) µm, in verticils of 3~6,; Conidia, globose 2~2.5 µm, slightly rough, borne in short disordered chains (Figure 3).

PCR reaction was carried out with Solis Biodyne (Estonia) FIREPol® and DNA Polymerase taq polymerase enzyme. After PCR, a single band was obtained in agarose gel using 100 bp DNA Ladder Ready toLoad (SolisBioDyne) marker, and it was observed that the PCR process was successful. Colony characteristics and micro-morphology of the fungus were similar to thedescription of P. toxicarium (Pitt,1979)

Molecular identification has been performed by culturing of the isolated fungus on potato dextrose agar (PDA). The EurXGene MATRIX Plant and Fungi DNA isolation kit was used for DNA isolation from fungus (https://eurx.com.pl/docs/manuals/en/e3595.pdf.). Spectrophotometric measurement (Thermo Scientific Nanodrop 2000 USA) was carried out in order to control the amount and purity of DNA obtained after DNA isolation.

In the PCR study, gene regions targeted for species identification were amplified using fungal specifically universal primers ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) (Gardes and Bruns, 1993).

PCR reaction was carried out with Solis Biodyne (Estonia) FIREPol® and DNA Polymerase taq polymerase enzyme. After PCR, a single band was obtained in agarose gel using 100 bp DNA Ladder Ready toLoad (SolisBioDyne) marker, and it was observed that the PCR process was successful.

ExoSAP-IT ™ for single band samples was obtained in the PCR product purification stage. The PCR product was purified using a PCR Product Clean up Reagent (Thermo Fisher Scientific, USA) and was performed according to the procedures of the kit (www.thermofisher.com). For Sanger sequencing, the ABI 3730XL Sanger sequencer (Applied Biosystems, Foster City, CA) and Big Dye Terminator v3.1 Cyclesequencing kit have been used. Reads obtained with the ITS primers were contiguous to form a consensus sequence. CAP contig assembly in BioEdit software to perform this operation algorithm is used.

Species (OK037182) determination result according to the nearest species *Penicillium toxicarium* on NCBI Showed the following data:

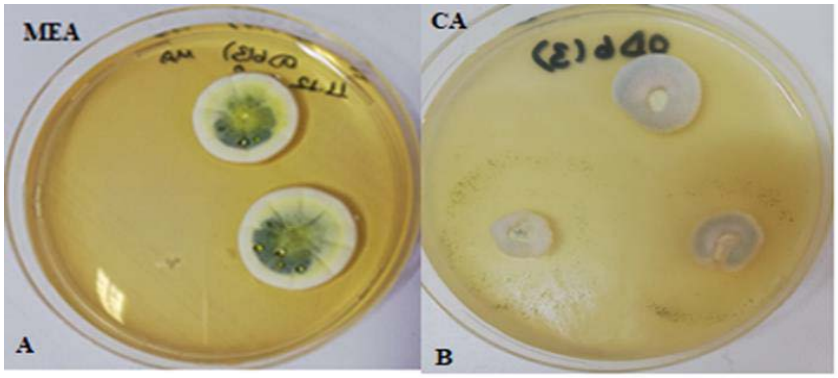

Figure 2: Colonies appearance of *P.toxicarium* on (A). MEA and (B) on CDA (Korcan vd, 2021)

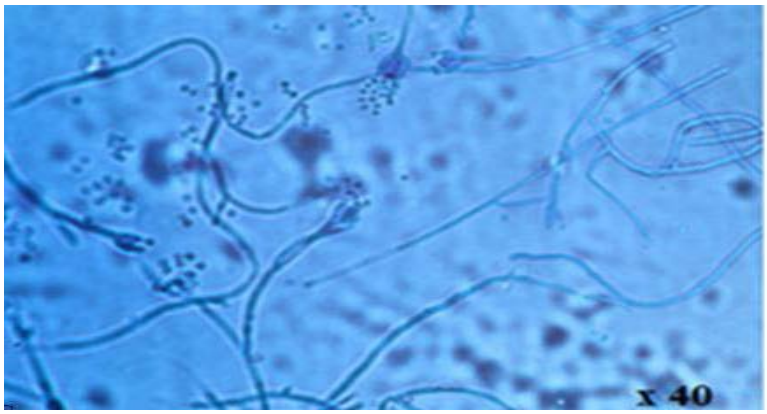

Figure 3: Microscope images of *P. Toxicarium* under High Power Objective Lens (40x) (Korcan et al., 2021)

Penicillium toxicarium

Total Base Number: 532

SimilarityScore: 983

SequenceMatch Rate: 100%

Similarity Rate: 100% (Korcan et al., 2012).

The FASTA format revealed the following data:

>OK037182.1 *Penicillium toxicarium* strain KJ173540.1 internaltranscribedspacer 1, partialsequence; 5.8S ribosomal RNA gene andinternaltranscribedspacer 2, completesequence; andlargesubunitribosomal RNA gene, partialsequence

GGTCACCTCCCACCCGTGTTTATCGTACCTTGTTGCT
TCGGCGGGCCCGCCGCAAGGCCGCCGGGGGGCA
TCTGCCCTCTGGCCCGCGCCCGCCGAAGACACCATTG
AACGCTGTCTGAAGATTGCAGTCTGAGCAATTA

GTTAAATAACTTAAAACTTTCAACAACGGATCTCTTGGTTC
CGGCATCGATGAAGAACGCAGCGAAATGC
GATACGTAATGTGAATTGCAGAATTCAGTGAATCATCGAGT
CTTTGAACGCACATTGCGCCCCCTGGTAT
TCCGGGGGGCATGCCTGTCCGAGCGTCATTGCTGCCCTCAA
GCACGGCTTGTGTGTTGGGCTCCGTCCTC
CTTCCGGGGGACGGGCCCGAAAGGCAGCGGCGGCACCGCGT
CCGGTCCTCGAGCGTATGGGGCTTCGTCA
CCCGCTCTGCAGGCCCGGCCGGCGCTTGCCGACACATCAAT
CTTTTTTCCAGGTTGACCTCGGATCAGGTAGGGATACCCGC
TGAACTTAAGCATATCAATAAGCGGAGGAA

Methods

Obtained sequences have been matched to the ID region presenting the Genbankdata base using the BLAST application of the GenBank NCBI data base. Sequences generated from materials in this study and extracted from GenBank were initially aligned by clustal W using the MegaX program and the phylogenetic tree was created using the neighbor joining, UPGMA and Neighbor-Joining phylogenetic analysis (Saitou and Nei, 1987).

Results

It is widely used in species identification and interspecies relations in taxonomic studies. ITSs regions have become an accepted official molecular barcode in the taxonomy of fungi,

In this study, phylogenetic trees were drawn by including the ITS region analyzed. Accession Numbers of

DNA sequences used in constructing phylogenetic trees are given in the table 1.

The phylogenetic tree deduced from the ID region showed similarity to *P. toxicarium* 100% *Penicillium toxicarium* KJ173540.1, *Penicillium toxicarium* strain EIODSF017, *Penicillium toxicarium* strain CNU 06007, *Penicillium toxicarium* strain NRRL 6172, *Penicillium toxicarium* strain NRRL 29751 and *Penicillium toxicarium* strain NRRL 2047 (Figure 4-6).

Discussion:

Penicillium is a genus that often found and isolated from different habitats like, soil ,food ,indoor habitat, and decaying wood .Being as decompose ragent making this fungi play an important role ecologically (Pitt, 1979; Visagie et al., 2014; Peterson, Bayer et al., 2004).

Penicillium species have been isolated as endophytes of large variety of plants (Nicoletti et al., 2014). It has been found that through living as endophytes, *Penicillium* species can provide plants protection against biotic stresses and pathogen attacks as well enhance their growth (Waqas, 2015; Hassan, 2017). Many endophytic *Penicillium* sp. Have been distinguished as biocatalysts, promoters of plant growth, phytoremediators, and producers of enzyme (Toghueo and Boyom, 2020). (Kim et al., 2008) recognized many species of *Penicillium*, through is work on the diversity of endophytic fungi from needles of pine trees in Korea, Two species which were new to Korea has been identified in this study and *Penicillium toxicarium* was one of them (Kim et al., 2008).

Recent technologies have showed characters, which were earlier not indicated in some species (Paterson et al., 2003). Analyses of .Rdna sequence revealed that the subgenus *Penicillium* is mainly monophyletic, and present species may be diversities (Peterson, 2000a).

So depending on the novel molecular techniques as well morphological characteristics, has been participated in the isolation of new species of *Penicillium* which have not been reported in certain regions before (Deng et al., 2012).

(Sugiura et al., 2020) stated that *Penicillium citreonigrum* NBRC 4692 was originally isolated as the toxigenic fungus responsible for the yellow rice incident in Japan in 1937 and named *Penicillium toxicarium* by I. Miyake, which was considered invalid due to the lack of a Latin diagnosis. Later *P. toxicarium*, was validated by C. Ramírez in 1982 with a Latin diagnosis and type designation. But *P. toxicarium* was assigned to *Penicillium trzebinskii* by Houbraken et al. in 2014. Later *Penicillium toxicarium* was treated as synonym of *Penicillium citreosulfuratum* based on the conclusion of molecular phylogenetic analysis by Visagie et al. 2016.

(Sugiura et al.2021) discovered the taxonomic and nomenclatural short communication (in Japanese) by I. Miyake in 1947 on *P. toxicarium* sp. nov. with its Latin description and four illustrations but lacking the type designation.

They determined that phylogenetic analysis revealed that the NBRC strain belongs to a unique clade, different from the clade comprising *P. citreosulfuratum* strains. Consequently, *P. toxicarium* I. Miyake (1947) was reinstated as a correct name with the lectotype designation by Sugiura et al. 2020.

Many articles regarding flora studies of mycobiota in Turkey have been reported. In 2000 a check list documented most of these articles since 1914 and reported about 159 of *Penicillium* species (Asan, 2000).

In 2004 another check list swhich was updated in 2015, added 66 species to the earlier list, increasing the number of isolated in Turkey to 225 (Asan, 2004).Up to date more than this number are found since reporting of new record of *Penicillium* species is in continue (Çakır and Maden, 2015, Kolanlarli et al., 2019).

Recent check list about certain fungi found in Turkey has been also published in 2020 (Sesli et al., 2020).

Despite of all these articles and the huge number of *Penicillim* species isolated in Turkey, up to the writing of this paper we couldn't find one published article reporting the isolation of *Penicillium toxicrium* in Turkey. So were corded for the first time the isolation of *Penicillium toxicarium* in Turkey.

Table 1 BLAST analysis results by ITS gene region

Species Accession Number	Matching species	Matching species rate (MI)	Matching species Accession Number
OK037182.1	*Penicillium toxicarium* strain EIODSF017	532/532(100%)	KJ173540.1
OK037182.1	Fungal sp. strain Xmf132	532/532(100%)	KX098096.1
OK037182.1	*Penicillium citreosulfuratum*	528/528(100%)	NR153252.1
OK037182.1	*Penicillium toxicarium* strain NZD-mf65	527/530(99%)	KM278076.1
OK037182.1	*Penicillium sp.*isolate MG-09 i	532/533(99%)	MK788347.1
OK037182.1	*Penicillium toxicarium* strain NZD-mf144	527/527(100%)	KM278008.1
OK037182.1	*Penicillium toxicarium* strain	532/532(100%)	KJ173540.1
OK037182.1	*Eurotiales sp.*	516/516(100%)	MG437224.1
OK037182.1	*Penicillium fundyense* strain KAS 2174	524/527(99%)	KT887853.1
OK037182.1	*Phialocephala fortinii* strain PPE5	525/527(99%)	KM042212.1
OK037182.1	Penicillium sp. CBS 140612	525/528(99%)	KX961207.1
OK037182.1	*Penicillium toxicarium* strain S4-M-3-10	528/528(100%)	KP216896.1
OK037182.1	*Penicillium toxicarium* strain CNU 060075	528/528(100%)	FJ557247.1
OK037182.1	*Penicillium toxicarium* strain NRRL 6172	528/528(100%)	EF198650.1
OK037182.1	*Penicillium toxicarium* strain NRRL 29751	528/528(100%)	EF198654.1
OK037182.1	*Penicillium toxicarium* strain NRRL 2047	528/528(100%)	EF198648.1
OK037182.1	Penicillium sp. strain Eef-3	527/527(100%)	MK120856.1
OK037182.1	*Penicillium toxicarium* strain NRRL 35628	527/528(99%)	EF198662.1
OK037182.1	*Penicillium toxicarium* strain NRRL 29679	528/529(99%)	EF198652.1
OK037182.1	*Penicillium toxicarium* strain R6-1-1	527/529(99%)	HM042308.1
OK037182.1	*Penicillium toxicarium* isolate 97	528/531(99%)	KP794065.1
OK0371821	*Penicillium sp. strain MG-02*	503/524(96%)	MK788349.1

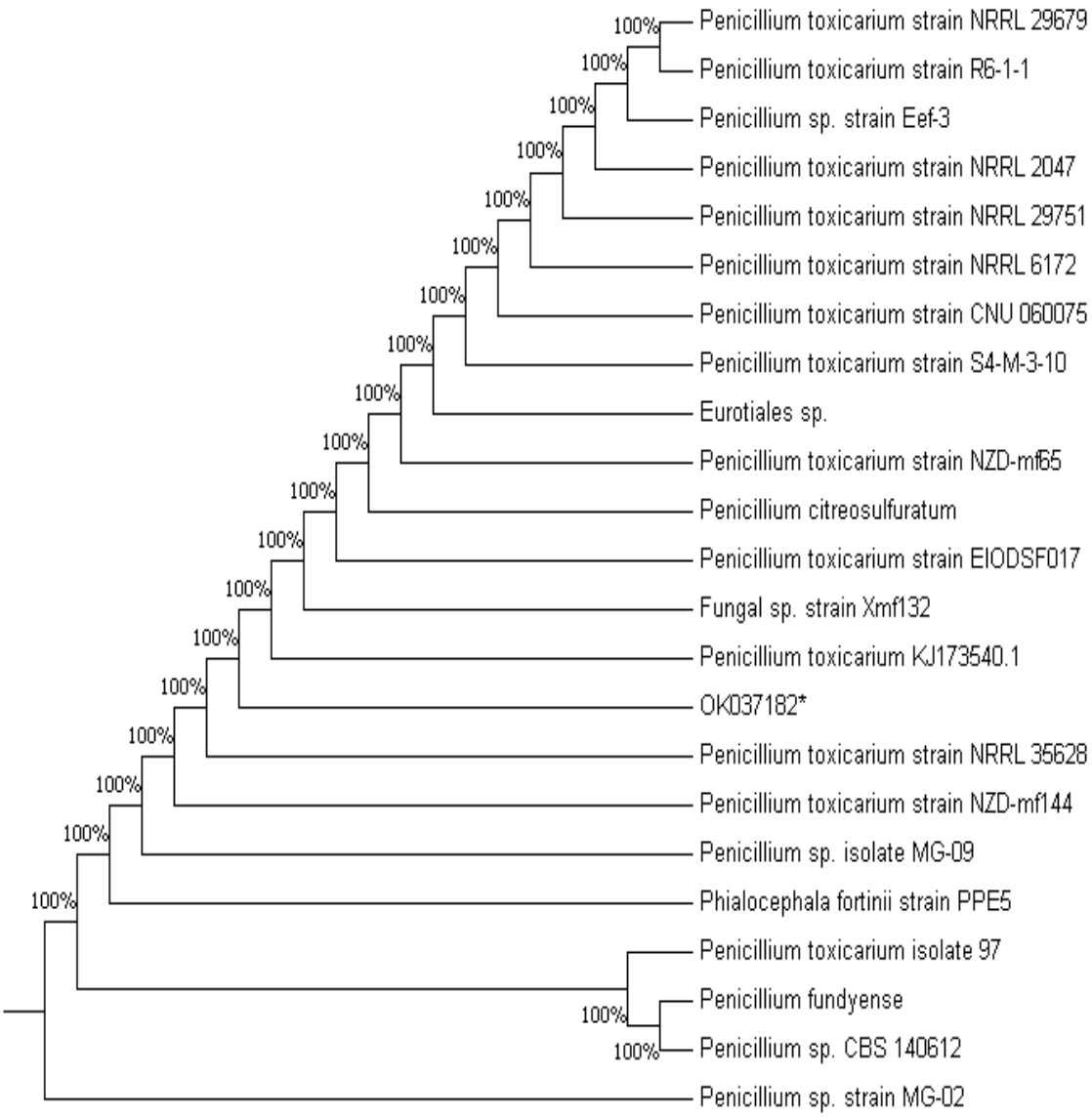

Figure 4: UPGMA phylogenetic analysis of OK037182.1

UPGMA tree shows that isolated strain in our study belongs to *P. toxicarium*. Branching of *Penicillum* species, which were molecularly identified according to ITS gene region, in the UPGMA phylogenetic tree Reference strain sequences were obtained from NCBI Genkbank.

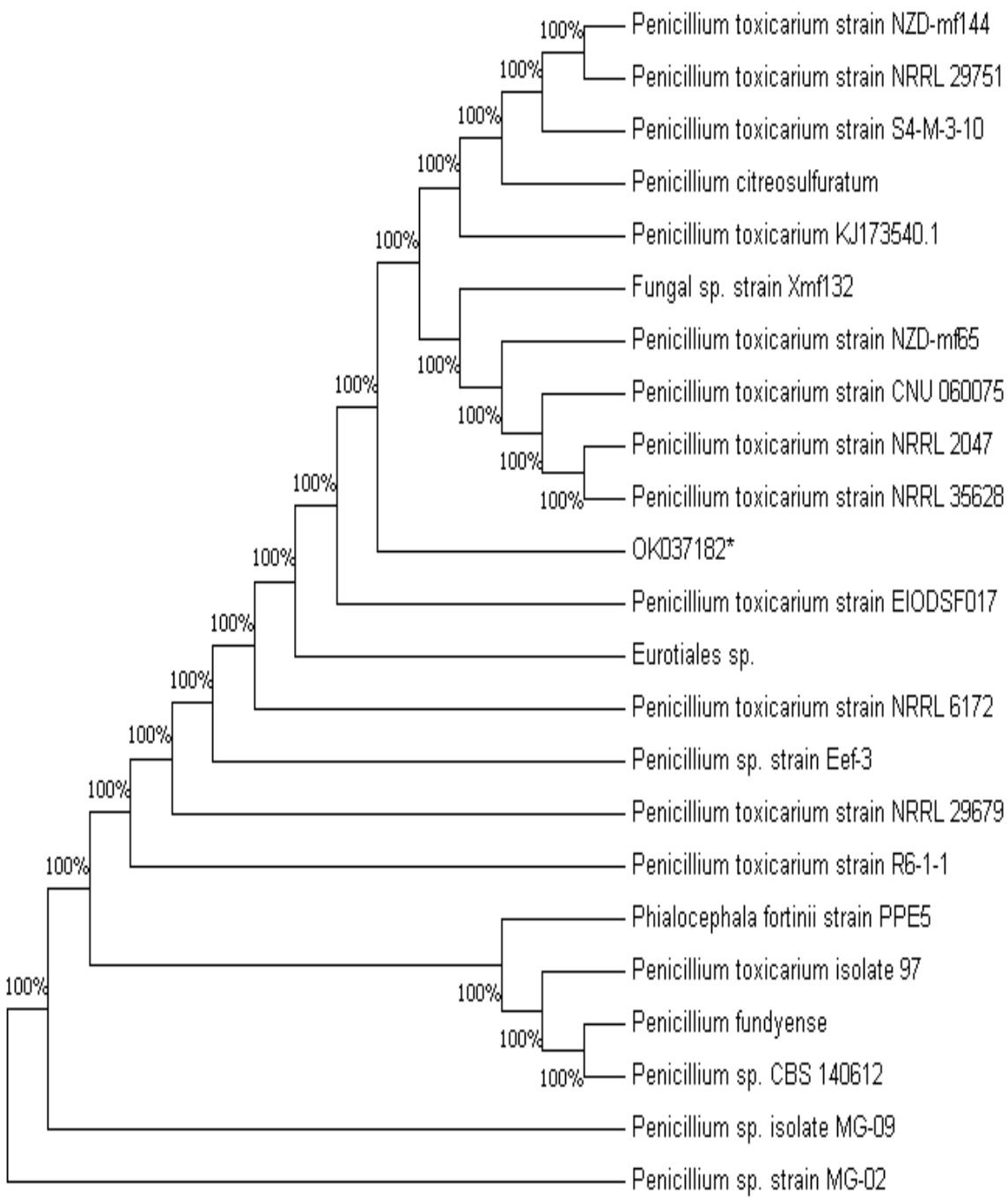

Figure 5 Neighbor-Joining phylogenetic analysis of OK037182.1

The phylogenetic tree deduced from the ID region showed 100% similarity to *P. toxicarium* Neighbor-Joining phylogenetic analysis of *Penicillium* species diagnosed molecularly according to ITS gene region. Reference strain sequences from NCBI Genkbank taken.

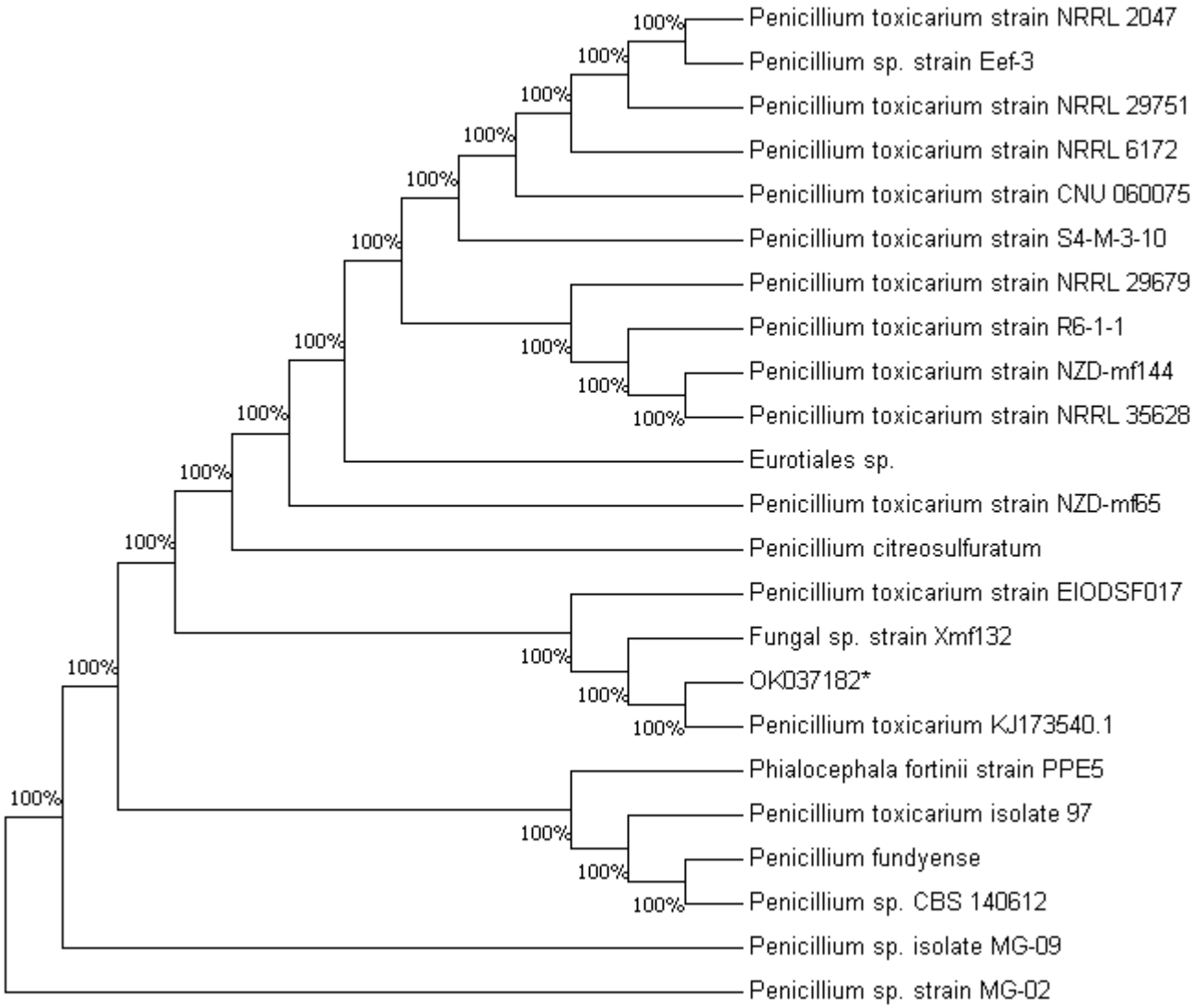

Figure 6. Maximum Likelihood phylogenetic analysis of OK037182.1

The presence of this species in Turkey opens the door for further studies to discover the facts behind the appearance of this unfamiliar species in the ecology of Turkey. Dispersal of this species to other regions of Turkey must also be studied and founding weather this species is originally one of the mycobiota of Turkey or being transported from other regions especially that the isolation of this species in this study was conducted in university campus area. Habitat so ther than decaying wood, more morphological and biochemical studies as well environmental, physiochemical and other factors should all be investigated in future studies.

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