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Comparison of rDNA regions (ITS, LSU, and SSU) of some *Aspergillus, Penicillium,* and *Talaromyces* spp.

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Abstract: *Aspergillus, Penicillium,* and *Talaromyces* spp. are commonly found worldwide and include industrially and medicinally important members. These genera are deeply related with life from soil to health hazards via their industrial products, surface proteins, biodiversity, food spoilage, mycotoxins, etc. These three genera progressively encounter important taxonomic revisions, such as the one fungus-one name concept, and various methods are available to establish their evolutionary and phylogenetic relationships. Besides morphological, microscopic, and extrolite analyses, nucleotide sequence analysis is the most preferred method. In particular, rDNA regions, such as the internal transcribed spacer (ITS), large subunit (LSU), and small subunit (SSU), are commonly used for taxonomic and phylogenetic studies. Although the ITS locus is accepted as the barcode gene for fungal identification, it has some limitations. Therefore, the aim of the present study was to compare ITS, LSU, and SSU loci in terms of ease of application, discrimination of species, and phylogeny by using 43 strains belonging to the above-mentioned three genera of fungi. The results indicated that the ITS and LSU loci were the most effective, exhibiting identical topology and species discrimination, while the SSU locus was the least effective, demonstrating lower species discrimination and the worst topology. Furthermore, the long and high-quality ITS, LSU, and SSU sequences obtained in the present study, which belong to the above-mentioned three genera of fungi, have been deposited in the NCBI database.

Key words: ITS, LSU, SSU, *Aspergillus, Penicillium, Talaromyces*

1. Introduction

Aspergillus and *Penicillium* spp. are well known and the most common fungi found in a range of habitats, such as soil, indoor and outdoor air, and various food products (Krijgsheld et al., 2013; Houbraken et al., 2014; Samson et al., 2014; Visagie et al., 2014). In addition, these two genera have worldwide distribution and cause major economic and health effects on human, animal, and plant life (Asan, 2004; Demirel et al., 2013). They have many functions, including decomposition of organic materials, pre- and postharvest pathogenesis of food products, production of important mycotoxins (Demirel and Sariozlu, 2014), and causing some health effects, such as aspergillosis (Samson et al., 2014; Visagie et al., 2014). Owing to their numerous positive and negative effects, studies on the identification of these genera are extremely important.

In 1955, the genus *Talaromyces* was described by Benjamin as a sexual stage of *Penicillium*. Subsequently, based on polyphasic species studies and the one fungus-one name concept, *Penicillium* subgenus *Biverticillum* was reassigned to this genus because it forms a monophyletic clade with *Talaromyces* (Samson et al., 2011; Yılmaz et al., 2014). The genus *Talaromyces* includes medically important members, fungal antagonists, and enzyme and soluble pigment producers. Following the recent development in this genus, particularly through multilocus gene sequencing and phylogenetic studies, taxonomy has gained more importance in mycology (Yılmaz et al., 2014).

To achieve accurate and quick results, various identification strategies are available that consider morphological properties and molecular structures of extrolites. Morphological identification techniques are one of the traditional identification strategies based on the investigation of morphological and microscopic properties of the fungi on different culture media and conditions. However, the findings obtained through these techniques are affected by the experiences and decisions of the researchers (Pitt, 2000; Klich, 2002; Samson et al., 2010). Extrolite profile analyses are based on the determination of secondary metabolites and are very useful with ultrahigh-performance equipment and well-developed databases. Although these analyses, together with morphological

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identification studies, have been very successful, identification purely based on secondary metabolites is not feasible (Frisvad et al., 2007; Visagie et al., 2014). Although many biochemical and physiological techniques provide useful information for the identification of fungi, the nucleotide sequence is the most accurate and sensitive identification tool to discriminate between organisms and accurately determine their evolutionary and phylogenetic relationships (White et al., 1990; Hibbet et al., 2007; Samson et al., 2010).

Numerous gene regions, such as rDNA regions, mitochondrial regions, and some protein-expressing genes, can be used for sequencing and phylogenetic analyses (White et al., 1990; Hibbet et al., 2007; Peterson, 2012; Schoch et al., 2012). For molecular identification based on sequencing, rDNA regions, such as the internal transcribed spacer (ITS), large subunit (LSU), and small subunit (SSU) regions, are very effective. In particular, the ITS locus is considered to be the official DNA barcode for fungi (Seifert, 2009; Schoch et al., 2012; Yılmaz et al., 2014; Das and Deb, 2015). Therefore, the purpose of the present study was to compare the performance of ITS, LSU, and SSU loci of the genera *Aspergillus*, *Penicillium*, and *Talaromyces* in terms of ease of application, species discrimination, and phylogeny using maximum likelihood (ML) analysis.

2. Material and methods

2.1. Fungal strains

A total of 43 strains belonging to *Aspergillus*, *Penicillium*, and *Talaromyces* were used in this study. The strains were obtained from 25 different cereal-based flour samples (12 wheats, 3 whole wheats, 3 corns, 3 rusks, 1 rice, 1 soy, 1 gluten-free, and 1 rye) from Thrace (Tekirdağ, three samples), Northwest Anatolia (İstanbul, six samples; Bilecik, two samples), West Anatolia (Aydın, five samples; İzmir, one sample), and Central Anatolia (Ankara, six samples; Konya, two samples) of Turkey. Additional information on these and related strains can be found elsewhere (Demirel and Sariozlu, 2014). The strains were maintained at 4 °C on malt extract agar (MEA).

2.2. DNA extraction, PCR amplification, and sequencing

DNA extraction was performed with strains grown on MEA for 7 days at 25 °C using the Ultraclean Microbial DNA Isolation Kit (MoBio, Solana Beach, CA, USA) following the manufacturer's protocols, and the extracted DNA was stored at –20 °C. To study the phylogenetic relationships among the three genera of fungi, the ITS, LSU, and SSU regions of the rDNA were amplified using the following primerpairs: V9G-F, 5'-TTACGTCCCTGCCCTTTGTA-3' and LS266-R, 5'-GCATTCCCAAACAACTCGACTC-3'; LR0R, 5'-ACCCGCTGAACTTAAGC-3' and LR5,5'-TCCTGAGGGAAACTTCG-3'; and NS1, 5'-GTAGTCATATGCTTGTCTC-3' and NS4, 5'-CTTCCGTCAATTCCTTTAAG-3' (Samson et al., 2010), respectively.

PCRs were performed using a Veriti 96-Well Thermal Cycler (Applied Biosystems, Foster City, CA, USA) in 25 mL of solution containing 1 µL of genomic DNA, 2.5 µL of 2.5 µM forward and reverse primers, 2.5 µL of Taq buffer + KCl-$MgCl_2$ (Fermentas), 2.5 µL of 25 mM $MgCl_2$ (Fermentas), 2 µL of 2.5 mM dNTP mix, 0.25 µL of 5 U/µL Taq DNA polymerase (Fermentas), and 11.75 µL of sterile deionized water. The amplification conditions consisted of denaturation at 94 °C for 5 min (ITS)/10 min (LSU and SSU), followed by 35 cycles at 94 °C for 45 s (ITS)/15 s (LSU and SSU), 56 °C for 30 s (ITS)/48 °C for 30 s (LSU)/52 °C for 30 s (SSU), and 72 °C for 2 min (ITS)/1.5 min (LSU and SSU) and a final extension at 72 °C for 6 min (ITS)/7 min (LSU and SSU) (Samson et al., 2010). The PCR products were separated by agarose gel electrophoresis (1% w/v in 1X TAE), visualized by GelRed staining, cleaned using EXOSAP-IT (Affymetrix, Santa Clara, CA, USA), and used for sequencing.

Fragments containing the ITS locus, which were amplified using the primers V9G and LS266, were sequenced using ITS1– 5'-TCCGTAGGTGAACCTGCGG-3' and ITS4–5'-TCCTCCGCTTATTGATATGC-3' (White et al., 1990). Similarly, fragments containing the LSU locus were sequenced using the LR0R and LR5 primers, while those containing the SSU locus were sequenced using the NR1 and NR4 primers. All the sequencing reactions were performed using the CEQ DTCS Quick Start Kit (Beckman Coulter, Brea, CA, USA) with the CEQ 8000 Genetic Analysis System.

2.3. Data analyses

For the DNA sequence analyses, all the sequences were compared with GenBank sequences using BLAST, and alignments were performed using Muscle in the MEGA 6.0 software package, together with the barcode genes for ITS and the closest BLAST results for LSU and SSU (Tamura et al., 2013). The aligned datasets were examined using ML analysis based on the Tamura–Nei model (Tamura and Nei, 1993) as implemented in MEGA 6.0 with 1000 bootstrap replications. All the positions containing gaps and missing data were eliminated. *Paecilomyces variotii* (GU968674, JN938878 and JN939029), not a member of the investigated three genera, was used as the outgroup. The obtained sequence data were deposited in GenBank with accession numbers.

3. Results and discussion

The phylogenetic relationships among the strains belonging to *Aspergillus*, *Penicillium*, and *Talaromyces* were studied through sequencing of three loci, ITS, LSU, and SSU. A total of 43 isolates belonging to 32 taxa were included in the analysis. The lengths of the alignments of the ITS, LSU, and SSU loci were 700 base pairs and position 166, 850 base pairs and position 177, and 1080 base pairs and position 422, respectively.

Figure 1 shows that the members of the genera *Aspergillus*, *Penicillium*, and *Talaromyces* have almost identical topology with respect to the ITS locus. A phylogenetic tree based on the ITS region was constructed at higher divergence levels. For *Aspergillus* spp., nine sections, namely *Candidi*, *Circumdati*, *Clavati*, *Eurotium*, *Flavi*, *Fumigati*, *Niger*, *Versicolor*, and *Terreus*, could be clearly noted (Samson et al., 2014). For *Penicillium* spp., which are mainly closely related to terverticillates, identical positions and eight sections for specific clades such as *Aspergilloides*, *Chrysogena*, *Citrina*, *Exilicaulis*, *Fasciculate, Lanata-Divaricata*, *Penicillium*, and *Ramosa* were found (Houbraken et al., 2014). The genus *Talaromyces* occurred in two main clades and two clear sections, namely *Talaromyces* and *Islandici*, were noted. However, no indication of saturation or loss of phylogenetic signal in the ITS-1/5.8S/ITS-2 sequence data was observed even at higher divergences. Although the ITS locus has been considered as the barcode gene for fungal identification (Peterson, 2012; Schoch et al., 2012), it has some limitations. For example, only 16 of 51 isolates of section *Aspergilloides* (31.3%) could be identified by using the ITS sequence; therefore, the ITS locus cannot be used for species-level identification of this group (Houbraken et al., 2014).

With regard to the performance of LSU and SSU loci, both were found to be efficient in the identification of *Aspergillus* and *Talaromyces*. Figures 2 and 3 show the members of these two genera separated by clades using phylogeny based on LSU and SSU loci. In contrast, when considering the topology of the phylogenetic trees obtained based on these two loci, LSU and SSU phylogenies presented low resolution and poor efficiency when compared with the ITS locus. The phylogeny based on the LSU locus showed the same topology as that obtained based on the ITS locus (Figure 2). Furthermore, although the separation of sections based on the LSU locus was clear for *Aspergillus* and *Talaromyces*, a poor result was obtained for the genus *Penicillium*. In addition, the LR0R and LR5 primers used for the LSU locus produced longer sequences when compared with other large subunit primers, such as D1 and D2 regions. When these longer sequences produced by LR0R and LR5 primers were compared by BLAST, reference sequences with poor coverage occurred because of shorter parts or low-quality sequences belonging to the LSU locus in GenBank. A more diverse and high-quality coverage training set, such as the ITS region GenBank data, may be particularly useful for classifying different phyla (Liu et al., 2011). Therefore, development of a LSU gene sequence database needs to be accomplished with diverse taxa. The LSU training sets obtained in the present study, belonging to *Aspergillus*, *Penicillium*, and *Talaromyces*, have been deposited in the NCBI database (Table). As the LSU locus presented virtually no amplification, sequencing, alignment, or editing problems, similar to the ITS locus, it is considered as the second preferred phylogenetic marker (Schoch et al., 2012) after the ITS locus and can be used along with the ITS locus.

Phylogeny based on the SSU region showed a different topology when compared with that based on the ITS and LSU regions (Figures 3, 1, and 2, respectively). Taxonomic revision studies have indicated the main phylogenetic positions of *Aspergillus*, *Penicillium*, and *Talaromyces* spp.; in particular, the genera *Talaromyces* and *Penicillium* have been accepted to be different (Houbraken and Samson, 2011; Yılmaz et al., 2014). However, Figure 3 shows *Talaromyces* and *Penicillium* as monophyletic. The phylogenetic training set of the SSU locus included the 422 position, which is higher than those of the ITS and LSU training sets, indicating that the SSU locus has very poor efficiency in determining the phylogeny of *Aspergillus*, *Penicillium*, and *Talaromyces*. Considering identification, differentiation, and phylogeny of species, the SSU locus was found to be the worst marker with the lowest species discrimination (Schoch et al., 2012). In addition, another disadvantage with regard to the phylogenetic use of the SSU locus is the length of the reference sequences and their quality in the databases. In the NCBI database, there are only some SSU sequences available, such as those of *Penicillium allii* and *Penicillium implicatum*. The 43 sequences obtained in the present study, which belong to the members of *Aspergillus, Penicillium* and *Talaromyces*, have been deposited in the NCBI database (Table).

The findings of this study demonstrated the efficiency of rDNA regions and phylogenetic analyses in taxonomic studies of members belonging to the genera *Aspergillus*, *Penicillium*, and *Talaromyces*. The results of the present study showed the phylogenetic performances of ITS, LSU, and SSU loci. In particular, the ITS and LSU regions were found to be the preferred regions because of their high performance with regard to ease of application, topology, identification, and discrimination, whereas the SSU locus was found to be a poor marker because of its lower species discrimination and worst topology results. The long and

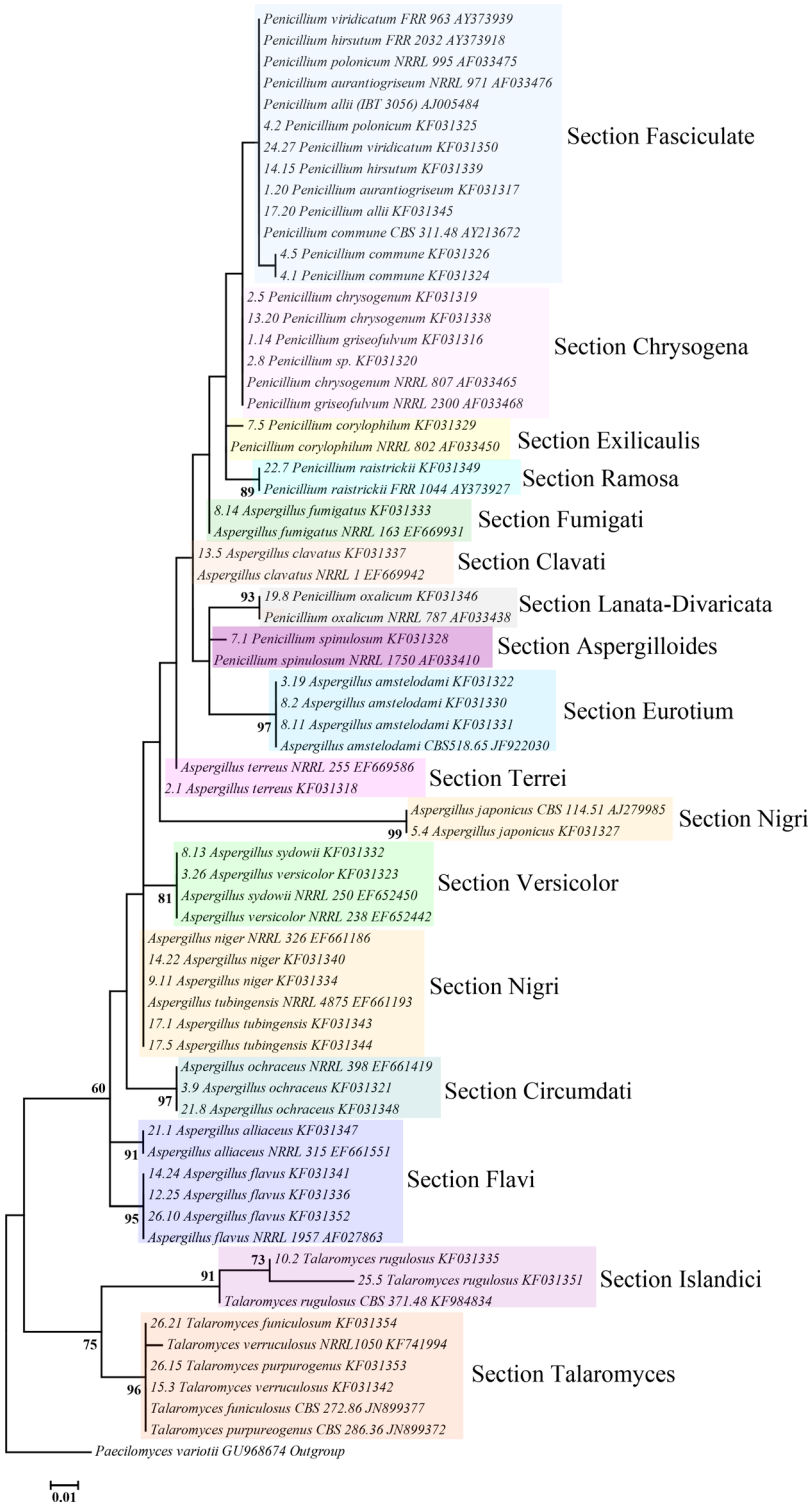

Figure 1. Best-scoring maximum likelihood tree based on the Tamura–Nei model calculated using MEGA 6.0 based on ITS sequences showing the relationships of the newly generated sequences in this study with previously known taxa in the NCBI GenBank. The scale bar denotes 0.01 substitutions per position. The tree with the highest log likelihood (–674.8079) is shown. Initial tree(s) for the heuristic search were obtained by applying the neighbor-joining method to a matrix of pairwise distances estimated using the maximum composite likelihood approach. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 68 nucleotide sequences. All positions with less than 50% site coverage, containing gaps, or missing data were eliminated. There were a total of 166 positions in the final dataset. The tree is rooted with *Paecilomyces variotii* (GU968674) (bootstrap 1000).

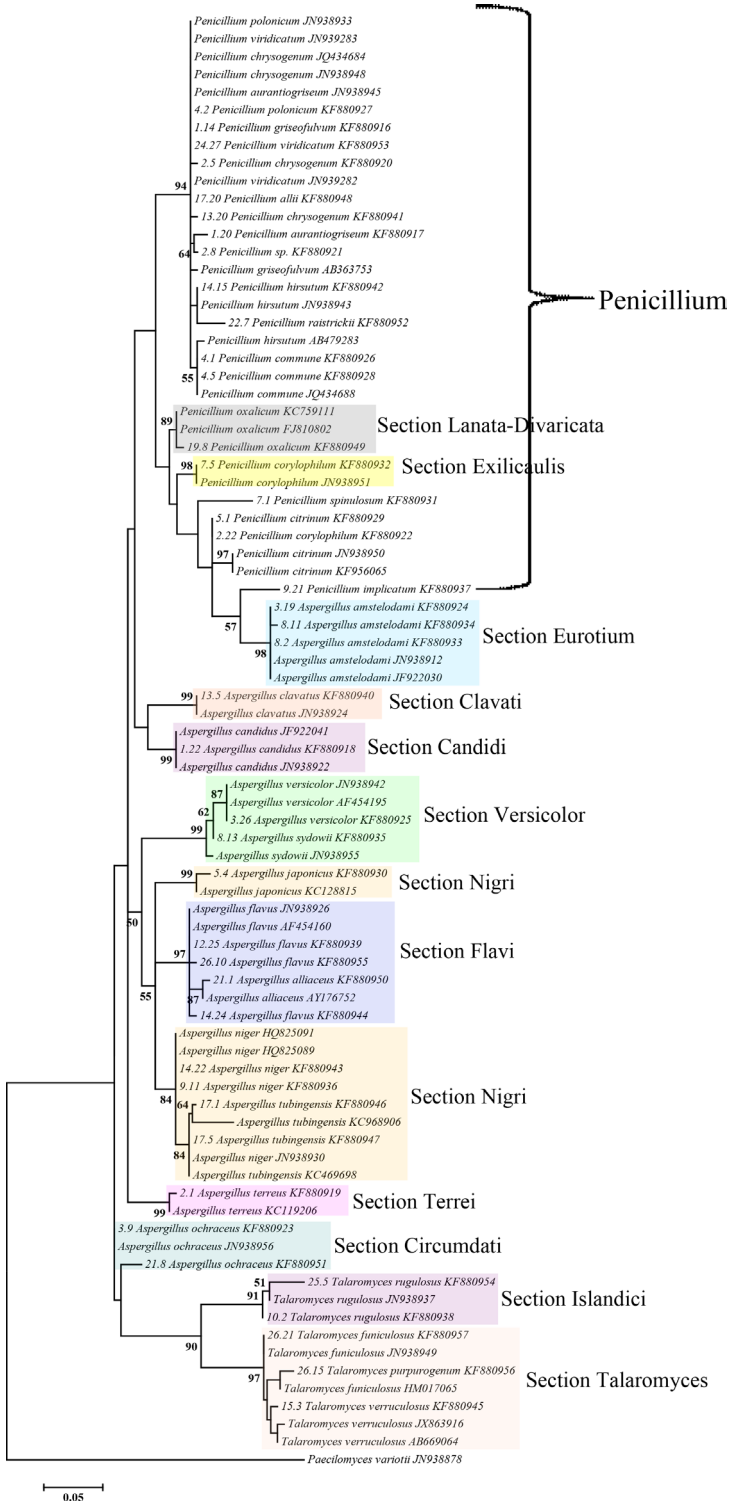

Figure 2. Best-scoring maximum likelihood tree based on the Tamura–Nei model calculated using MEGA 6.0 based on LSU sequences showing the relationships of the newly generated sequences in this study with previously known taxa in the NCBI GenBank. The scale bar denotes 0.05 substitutions per position. The tree with the highest log likelihood (–1384.5811) is shown. Initial tree(s) for the heuristic search were obtained automatically by applying neighbor-joining and BioNJ algorithms to a matrix of pairwise distances estimated using the maximum composite likelihood approach and then selecting the topology with superior log likelihood value. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 82 nucleotide sequences. All positions with less than 50% site coverage, containing gaps, or missing data were eliminated. There were a total of 177 positions in the final dataset. The tree is rooted with *Paecilomyces variotii* (JN938878) (bootstrap 1000).

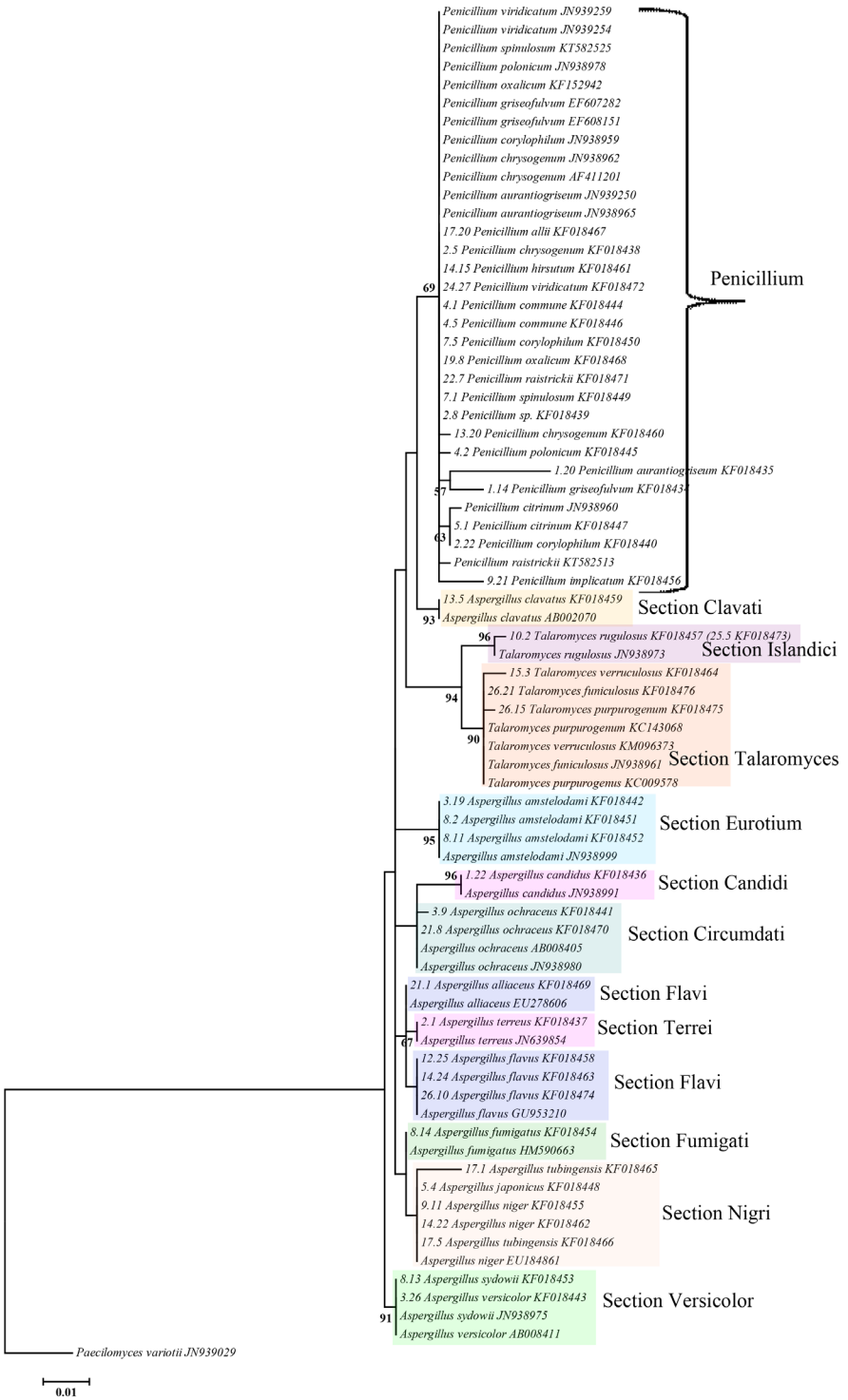

Figure 3. Best-scoring maximum likelihood tree based on the Tamura–Nei model calculated using MEGA 6.0 based on SSU sequences showing the relationships of the newly generated sequences in this study with previously known taxa in the NCBI GenBank. The scale bar denotes 0.01 substitutions per position. The tree with the highest log likelihood (–1192.7854) is shown. Initial tree(s) for the heuristic search were obtained automatically by applying neighbor-joining and BioNJ algorithms to a matrix of pairwise distances estimated using the maximum composite likelihood approach and then selecting the topology with superior log likelihood value. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 74 nucleotide sequences. All positions with less than 50% site coverage, containing gaps, or missing data were eliminated. There were a total of 422 positions in the final dataset. The tree is rooted with *Paecilomyces variotii* (JN939029) (bootstrap 1000).

Table. Newly generated ITS, LSU, and SSU sequences with their GenBank accession numbers.

Species	Culture number	GenBank acc. number (ITS)	GenBank acc. number (LSU)	GenBank acc. number (SSU)
Aspergillus alliaceus Thom & Church	21.1	KF031347	KF880950	KF018469
Aspergillus amstelodami Thom & Church	3.19, 8.2, 8.11	KF031322, KF031330, KF031331	KF880924, KF880933, KF880934	KF018442, KF018451, KF018452
Aspergillus candidus Link	1.22		KF880918	KF018436
Aspergillus clavatus Desm.	13.5	KF031337	KF880940	KF018459
Aspergillus flavus Link	12.25, 14.24, 26.10	KF031336, KF031341, KF031352	KF880939, KF880944, KF880955	KF018458, KF018463, KF018474
Aspergillus fumigatus Fresen	8.14	KF031333		KF018454
Aspergillus japonicus Saito	5.4	KF031327	KF880930	KF018448
Aspergillus niger Tiegh	9.11, 14.22	KF031334, KF031340	KF880936, KF880943	KF018455, KF018462
Aspergillus ochraceus G. Wilh.	3.9, 21.8	KF031321, KF031348	KF880923, KF880951	KF018441, KF018470
Aspergillus sydowii (Bainier & Sartory) Thom & Church	8.13	KF031332	KF880935	KF018453
Aspergillus terreus Thom	2.1	KF03131op8	KF880919	KF018437
Aspergillus tubingensis Mosseray	17.1, 17.5	KF031343, KF031344	KF880946, KF880947	KF018465, KF018466
Aspergillus versicolor (Vuill.) Tirab.	3.26	KF031323	KF880925	KF018443
Penicillium allii Vincent & Pitt	17.20	KF031345	KF880948	KF018467
Penicillium aurantiogriseum Dierckx	1.20	KF031317	KF880917	KF018435
Penicillium chrysogenum Thom	2.5, 13.20	KF031319, KF031338	KF880920, KF880941	KF018438, KF018460
Penicillium hirsutum Dierckx	14.15	KF031339	KF880942	KF018461
Penicillium viridicatum Westling, Ark. Bot.	24.27	KF031350	KF880953	KF018472
Penicillium citrinum Thom	5.1		KF880929	KF018447
Penicillium commune Thom	4.1, 4.5	KF031324, KF031326	KF880926, KF880928	KF018444, KF018446
Penicillium corylophilum Dierckx	2.22, 7.5	KF031329	KF880922, KF880932	KF018440, KF018450
Penicillium griseofulvum Dierckx	1.14	KF031316	KF880916	KF018434
Penicillium implicatum Biourge	9.21		KF880937	KF018456
Penicillium oxalicum Currie & Thom	19.8	KF031346	KF880949	KF018468
Penicillium polonicum K.M. Zaleski	4.2	KF031325	KF880927	KF018445
Penicillium raistrickii G. Sm.	22.7	KF031349	KF880952	KF018471
Penicillium spinulosum Thom	7.1	KF031328	KF880931	KF018449
Penicillium waksmanii K.M. Zalessky	2.8	KF031320	KF880921	KF018439
Talaromyces funiculosus (Thom) Samson, N. Yilmaz, Frisvad & Seifert	26.21	KF031354	KF880957	KF018476
Talaromyces purpurogenus Samson, Yilmaz, Houbraken, Spierenb., Seifert, Peterson, Varga & Frisvad	26.15	KF031353	KF880956	KF018475
Talaromyces rugulosus (Thom) Samson, N. Yilmaz, Frisvad & Seifert	10.2, 25.5	KF031335, KF031351	KF880938, KF880954	KF018457, KF018473
Talaromyces verruculosus (Peyronel) Samson, N. Yilmaz, Frisvad & Seifert	15.3	KF031342	KF880945	KF018464

high-quality sequence sets of the ITS, LSU, and SSU loci obtained in the present study, some of which are novel, particularly those belonging to some members of the genus *Penicillium*, have been deposited in the NCBI database.

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