

## FUNGI ASSOCIATED WITH FREE-LIVING SOIL NEMATODES IN TURKEY

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**Abstract:** Free-living soil nematodes have successfully adapted world-wide to nearly all soil types from the highest to the lowest of elevations. In the current study, nematodes were isolated from soil samples and fungi associated with these free-living soil nematodes were determined. Large subunit (LSU) rDNAs of nematode-associated fungi were amplified and sequenced to construct phylogenetic trees. Nematode-associated fungi were observed in six nematode strains belonging to *Acroboloides*, *Steinernema* and *Cephalobus* genera in different habitats. *Malassezia* and *Cladosporium* fungal strains indicated an association with *Acroboloides* and *Cephalobus* nematodes, while *Alternaria* strains demonstrated an association with the *Steinernema* strain. Interactions between fungi and free-living nematodes in soil are discussed. We suggest that nematodes act as vectors for fungi.

**Key words:** Nematode-fungi association; *Malassezia*; *Cladosporium*; *Alternaria*; phylogeny.

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### INTRODUCTION

Nematodes are the most common, abundant and genetically diverse metazoans found in many habitats, particularly soils and sediments, even in the most extreme environments (Baldwin et al., 1999; Derycke et al., 2008). Free-living forms of the Rhabditida order display different feeding habits (Abolafia and Pena-Santiago, 2007). The free-living soil nematodes play an important role in the nutrient cycling in terms of food web enrichment, environmental perturbation, and recovery (Ferris et al., 1997; Yeates and Wardle, 1996; Wang et al., 2004). They also contribute to environmental pollution and soil quality studies as bio-indicators (Levi et al., 2012).

Fungus-nematode interactions vary depending on the fungi and nematode group in the soil. Nematode-fungi relationships were reported by some researchers (Renker et al., 2003; Adam et al., 2014).

This relationship is likely to change from beneficial to harmful (Renker et al., 2003).

*Malassezia*, *Cladosporium* and *Alternaria* include important fungal species showing common distribution and association with different substances. *Malassezia* is a distinct fungal genus within the Basidiomycota and are known to be components of the microflora of human skin and other warm-blooded animals (Renker et al., 2003; Talaei et al., 2014). *Malassezia* species can become pathogenic under certain predisposing factors, such as alterations in skin condition and changes in host defenses (Talaei et al., 2014), and they are also associated with a variety of diseases such as pityriasis versicolor in hot and humid climates (Gupta et al., 2002; Khosravi et al., 2009). They can grow in both yeast (mainly prevalent) and mycelial phases on non-affected skins (Ashbee and Evans 2002; Renker et al., 2003).

*Cladosporium* is an endophytic fungal genus belonging to Ascomycota. This is a cosmopolitan genus and found in association with different substrata (Brown et al., 1998; Schubert et al., 2007; Rosa et al., 2010). *Cladosporium* species are abundantly found on fading or dead leaves of herbaceous and woody plants, as a secondary invader on necrotic leaf spots. They have frequently been isolated from air, soil, foodstuffs, paints, textiles, humans and numerous other substrates (Domsch et al., 1980; Samson et al., 2000; Schubert et al., 2007).

The other cosmopolitan genus *Alternaria* includes saprophytic, endophytic and pathogenic species. Plant pathogenic species of *Alternaria* infect a number of economically important plants, such as tangerine, apple, pear, tomato and potato. Many of the pathogenic species produce host-specific toxins, having pathogenicity factors in diseases (Johnson et al., 2000, Peever et al., 2004). *Alternaria* are also known as a common allergen and cause hay fever or hypersensitivity reactions in humans (Potkar and Jadhav, 2012)

Nuclear ribosomal DNA (rDNA) is mainly used in fungal phylogenetic and systematic studies (James et al., 2006; Hibbett et al., 2007; Porter and Golding, 2012). Species-level characterization of most fungi can be accomplished by analyzing rDNA regions, including the small subunit (SSU, 18S), internal transcribed spacer (ITS, ITS1+5.8S+ITS2), and large subunit (LSU, 25-28S). Although the ITS region has been suggested as the official fungal 'barcode', there are some cases where a large subunit may particularly be targeted with or without the adjacent internal transcribed spacer region in amplicon-based sequencing studies. LSU can be aligned across the diverse range of fungi recovered from various fields (Porter and Golding, 2012). In the present study, 28s rDNA regions of nematode-associated fungi were screened through the PCR method and sequenced for constructing phylogenetic trees.

## MATERIALS AND METHODS

### Isolation of nematodes

Soil samples were obtained from various locations and habitats in the eastern Mediterranean region of Turkey (Karabörklü, 2012; Karabörklü et al., 2015). Samples were baited with last instar *Galleria mellonella* L. (Lepidoptera: Pyralidae) larvae (Bedding and Akhurst, 1975) during the screening of entomopathogenic nematodes in previous studies (Karabörklü, 2012). A great number of free-living rhabditid nematodes were also detected and harvested from dead *G. mellonella* larvae, which were individually placed into modified White traps (Kaya and Stock, 1997). Harvested nematodes were washed in distilled H<sub>2</sub>O and stored at 10°C (Karabörklü, 2012; Karabörklü et al., 2015).

### DNA extraction and PCR amplification

Nematode-associated fungi were detected in studies during DNA isolation from free-living soil nematodes. LSU (28S) rDNA genes of nematode-associated fungi were amplified using the PCR method. The steps for DNA extraction and PCR have been provided in detail in previous studies (Karabörklü, 2012; Karabörklü et al., 2015). In PCR amplification, a 2.5-µL DNA suspension and primer pairs specific to 28S rDNA region were used. Five µL of PCR product was loaded on a 1% agarose gel and visualized (Karabörklü, 2012; Karabörklü et al., 2015).

### Phylogenetic analysis

PCR products of nematode-associated fungi were purified using a purification kit (Fermentas, K0513) and sequenced in the RefGen Biotechnology Laboratory (METU, Turkey). Alignments were displayed using the National Center for Biological Information's (NCBI) Basic Local Alignment Search Tool (BLAST) to match the sequence data with known sequences. Phylogenetic analyses were carried out using MEGA version 5 software (Tamura et al., 2011). Phylogenetic trees were created using the neighbor-joining (NJ)

and bootstrap tree (BT) methods (according to 1000 bootstrap replications) of the same program. Alignment gaps and missing data were removed in pairwise sequence comparisons (Yılmaz et al., 2012).

## RESULTS

Nematode-associated fungi were observed in six strains among 17 nematode isolates (35.3%). Of the isolates, SK-16 was obtained from grasslands (Adana), SK-17 and SK-34 from horticultural fields (Adana), and SK-20, SK-32 and SK-51 from agronomical fields (Adana and Osmaniye). According to phylogenetic analysis, the nematode species were identified as *Acrobeloides* sp. (SK-16, SK-32 and SK-34), *Steinernema affine* (SK-17) and *Cephalobus* sp. (SK-20 and SK-51) (Karabörklü, 2012; Karabörklü et al., 2015). LSU (28S) rDNA regions of the fungi were purified from the gel and sequenced. Isolates were clustered at the genus level. Similarity rates and BTs of nematode-associated fungi are given in Table 1 and Figs. 1-6. SK-16-2 and SK-32-2 fungi were associated with *Acrobeloides* sp., and indicated 96 and 94% identity with *Malassezia globosa* strain MPS3 and *Malassezia globosa* strain 160.1, respectively (Figs. 1 and 4). Another fungus (SK-34-2) associated with *Acrobeloides* sp. was found to indicate 92% similarity with *Cladosporium langeronii* strain CBS:101880 (Fig. 5). The fungal isolate SK-17-2 associated with *Steinernema affine* displayed 95% identity with *Alternaria tenuissima* strain CZ494-3 (Fig. 2). The *Cephalobus* sp.-associated isolate SK-20-2 resembled the *Malassezia globosa* strain NCCPF 127007 (Fig. 3) at a level of 91%. Another fungal isolate, SK-51-2, associated with *Cephalobus* sp., displayed 94% identity with *Cladosporium* sp. CBG\_III3CCH (Fig. 6).

## DISCUSSION

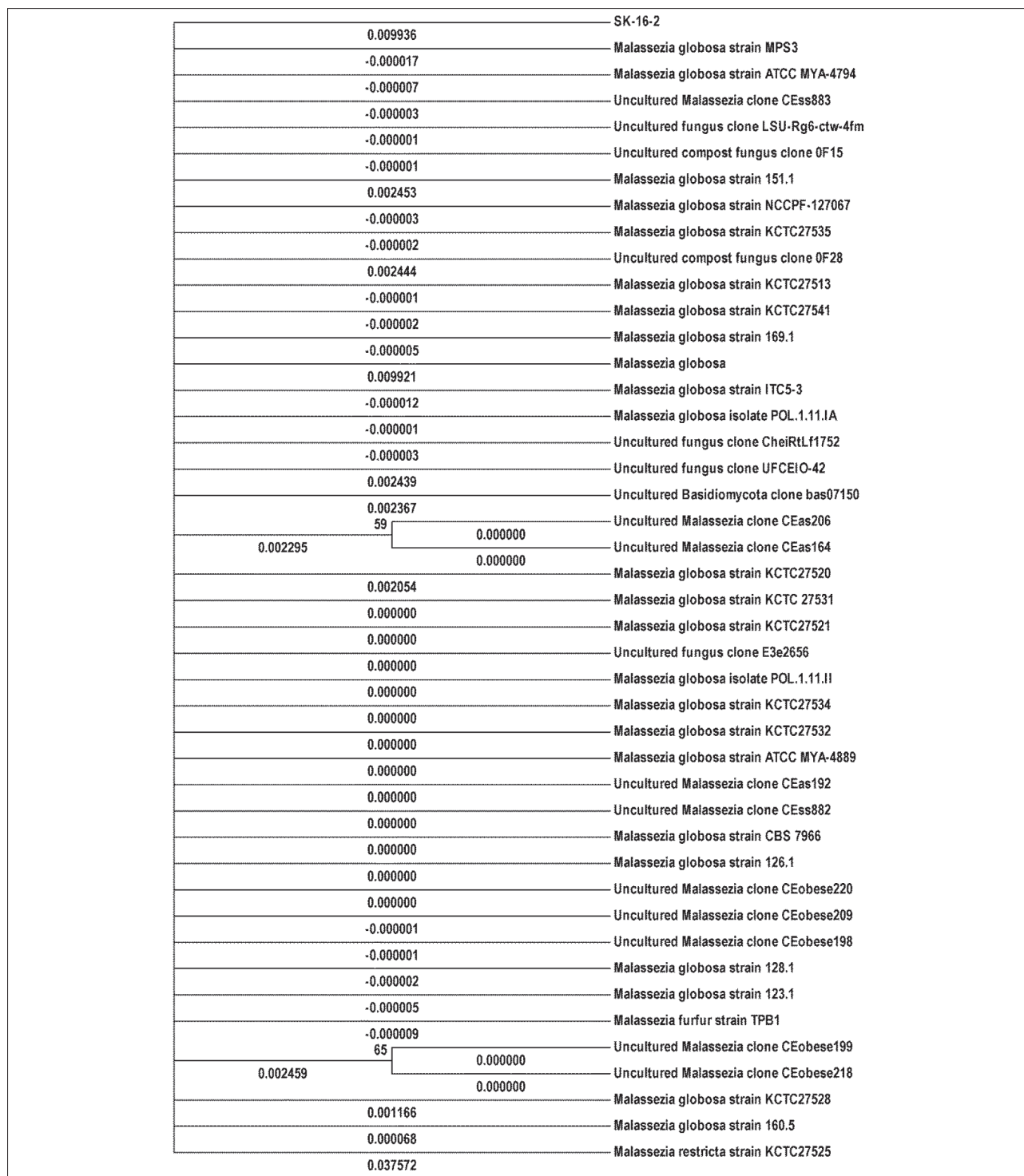
The contributions of free-living soil nematodes to nutrient cycling, nitrogen mineralization and distribution are very important (Neher, 2001). Free-living soil nematodes also have entomopathogenic forms (*Steinernematidae* and *Heterorhabditidae*), which are used as excellent biocontrol agents for many insect pests (Grewal et al., 2005).

In the present study, nematode-associated fungi were determined in six nematode strains belonging to *Acrobeloides*, *Steinernema* and *Cephalobus* genera from different habitats in temperate regions of Turkey. Phylogenetic analyses indicated that the fungi associated with these nematodes were *Malassezia*, *Alternaria* and *Cladosporium* strains. *Malassezia*, *Alternaria* and *Cladosporium* genera have been reported to include cosmopolitan species in temperate regions (Renker et al., 2003; Peever et al., 2004; Schubert et al., 2007). SK-16-2, SK-32-2 and SK-34-2 isolates associated with *Acrobeloides* sp. strains were identified as *Malassezia globosa*, *Malassezia* sp. and *Cladosporium* sp. The fungus isolate SK-17-2 associated with *Steinernema affine* strain was determined as *Alternaria tenuissima*. Also, both *Malassezia* sp. (SK-20-2) and *Cladosporium* sp. (SK-51-2) were observed in *Cephalobus* strains.

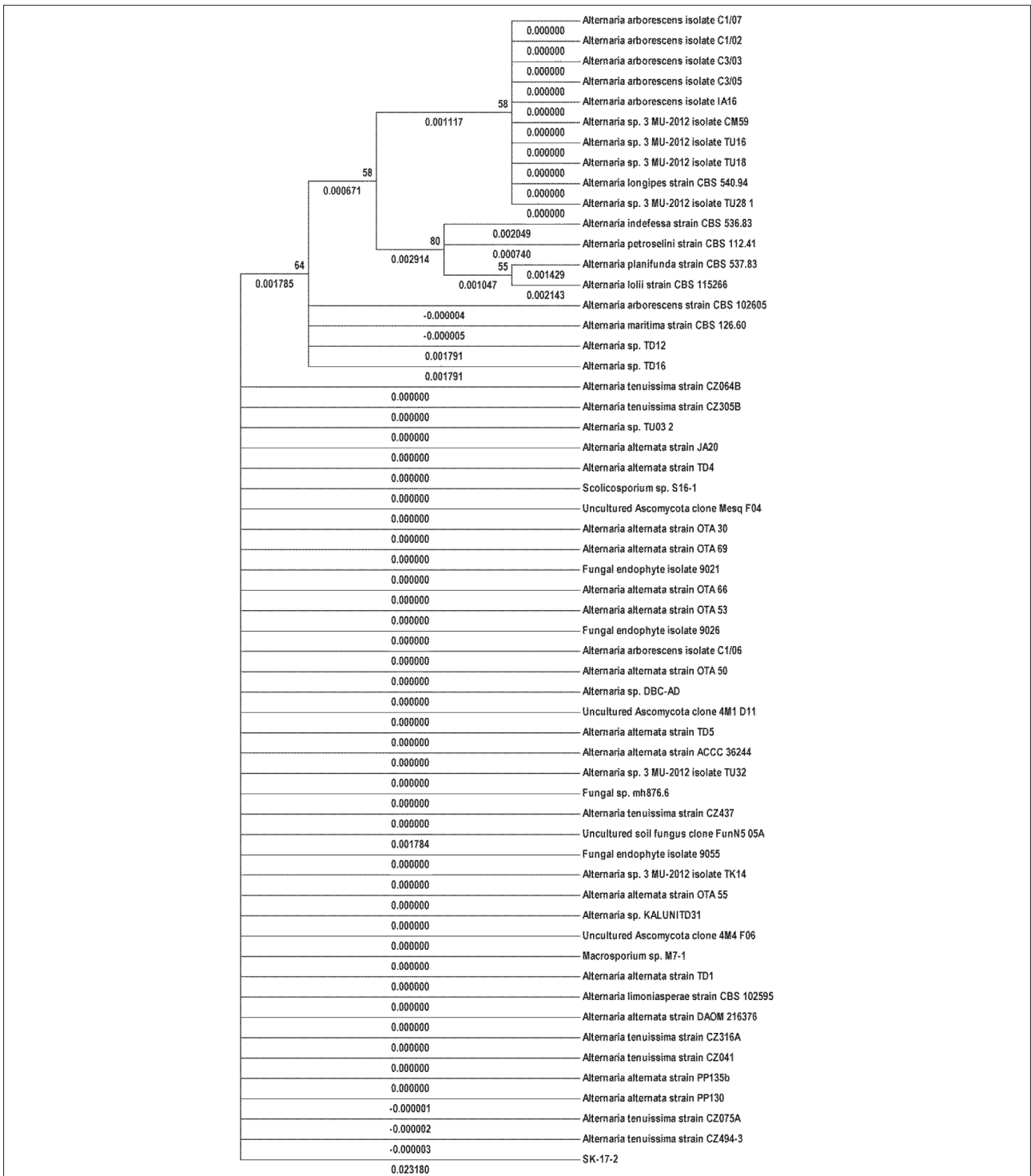
In the present study, *Malassezia* and *Cladosporium* strains were found in association with *Acrobeloides* and *Cephalobus* strains. On the other hand, *Alternaria tenuissima* had an association only with *Steinernema*. Renker et al. (2003) revealed that *Malassezia* species were associated with *Malenchus* sp. and *Tylolaimophorus typicus*. The attachment of *Malassezia* and *Cladosporium* strains to the cuticle of the root-knot nematode *Meloidogyne hapla* has also been

**Table 1.** Similarity rates of nematode-associated fungi with their accession numbers (AN)

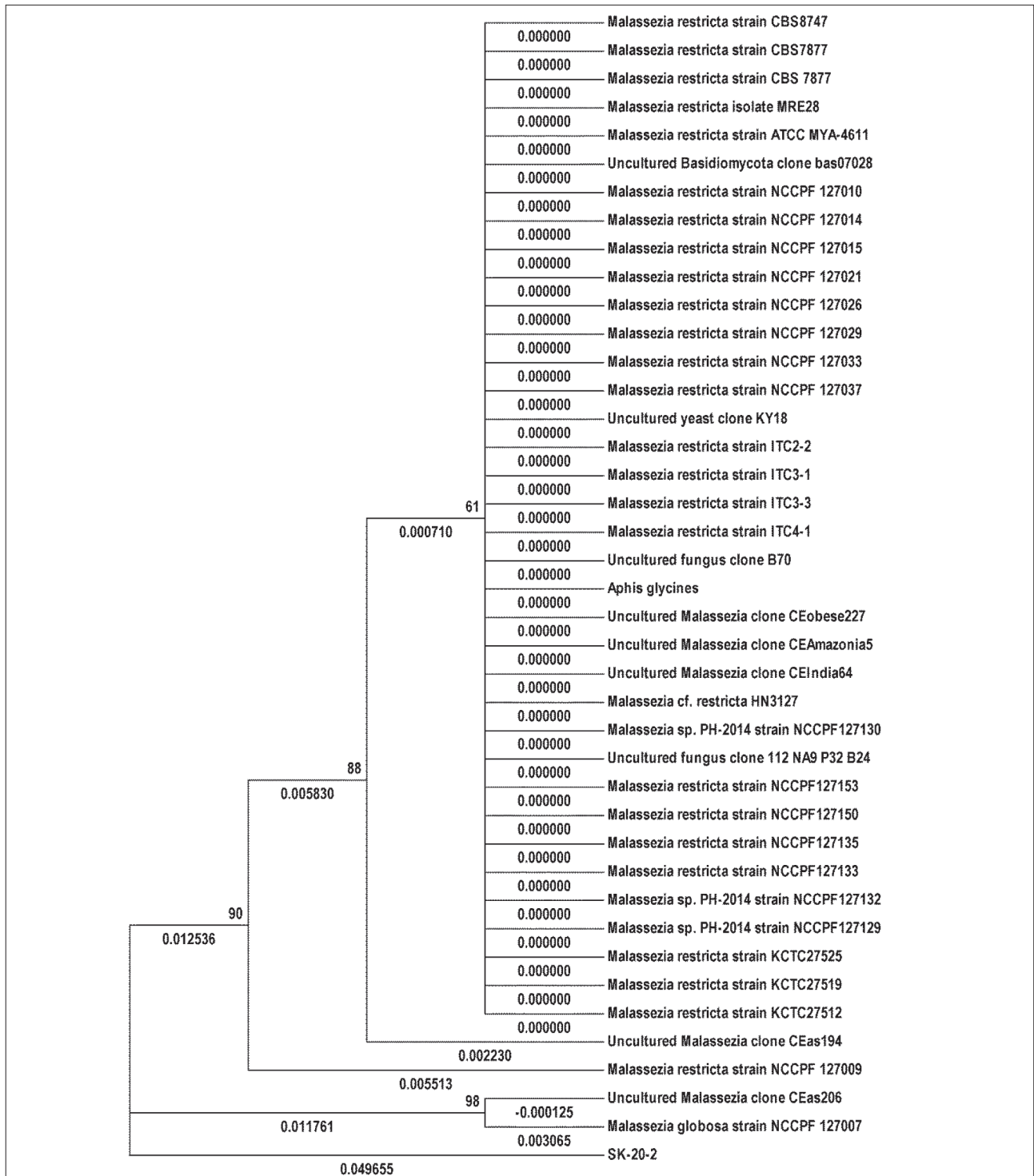
Isolates (AN)	Source organism (AN)	Similarity (%)
SK-16-2 (KP658357)	<i>Malassezia globosa</i> strain MPS3 (AY387231)	96
SK-17-2 (KP658356)	<i>Alternaria tenuissima</i> strain CZ494-3 (FJ755195)	95
SK-20-2 (KP658358)	<i>Malassezia globosa</i> strain NCCPF 127007 (JN651941)	91
SK-32-2 (KP658353)	<i>Malassezia globosa</i> strain 160.1 (KM269164)	94
SK-34-2 (KP658354)	<i>Cladosporium langeronii</i> strain CBS:101880 (DQ780380)	92
SK-51-2 (KP658355)	<i>Cladosporium</i> sp. CBG_III3CCH (HQ026794)	94



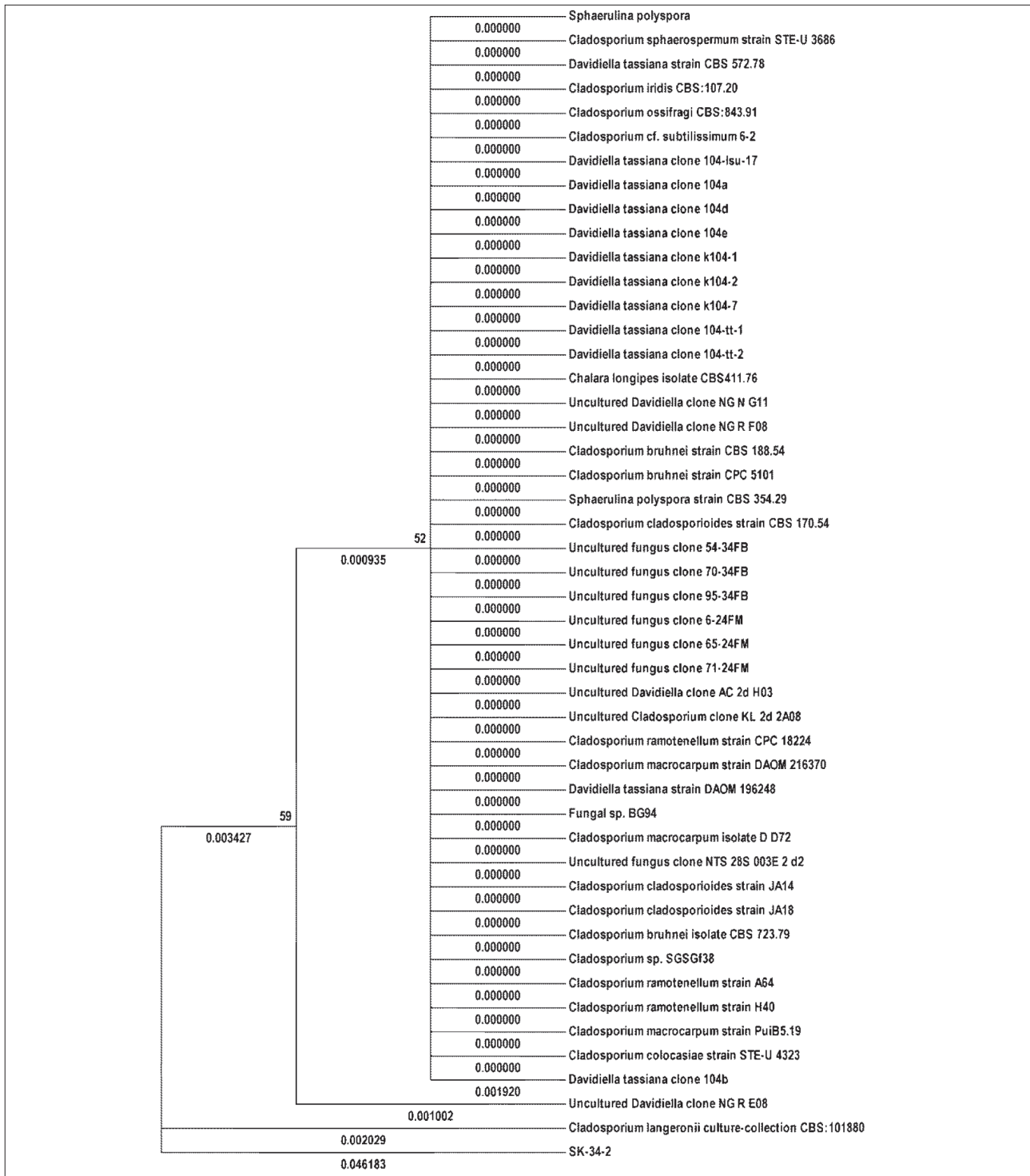
**Fig. 1.** Phylogenetic relationships of different fungal species with nematode-associated fungus (SK-16-2). Phylogeny obtained from the alignment of the 587bp of LSU rDNA region. Tree was created by the neighbor-joining method. Bootstrap values higher than 50% are indicated, alignment gaps and missing data were removed in pairwise sequence comparisons. The horizontal bar shows 0.005% differences in nucleotide identities.



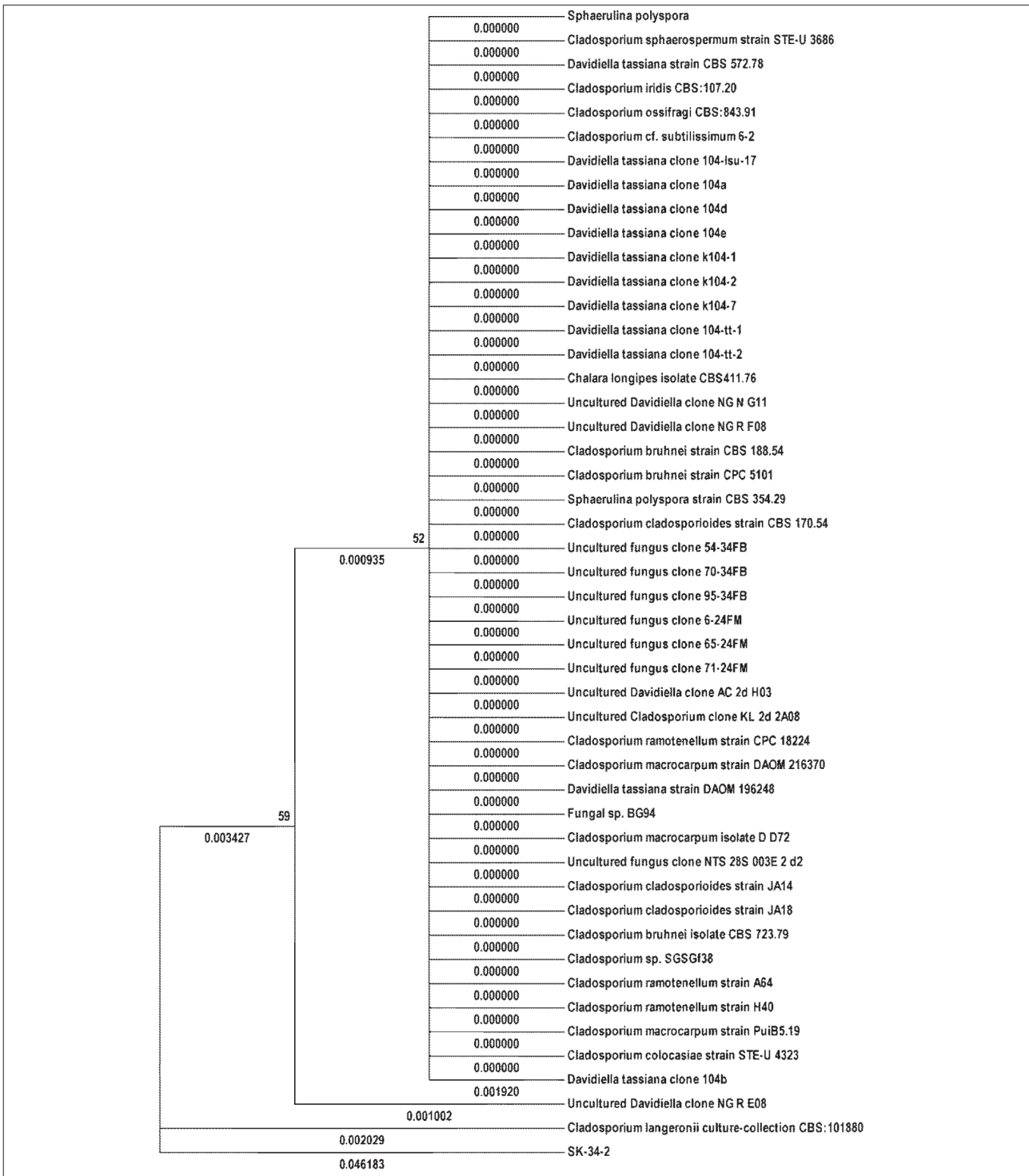
**Fig. 2.** Phylogenetic relationships of different fungus species with nematode-associated fungus (SK-17-2). Phylogeny obtained from the alignment of the 587bp of LSU rDNA region. Tree was created by the neighbor-joining method. Bootstrap values higher than 50% are indicated, alignment gaps and missing data were removed in pairwise sequence comparisons. The horizontal bar shows 0.005% differences in nucleotide identities.



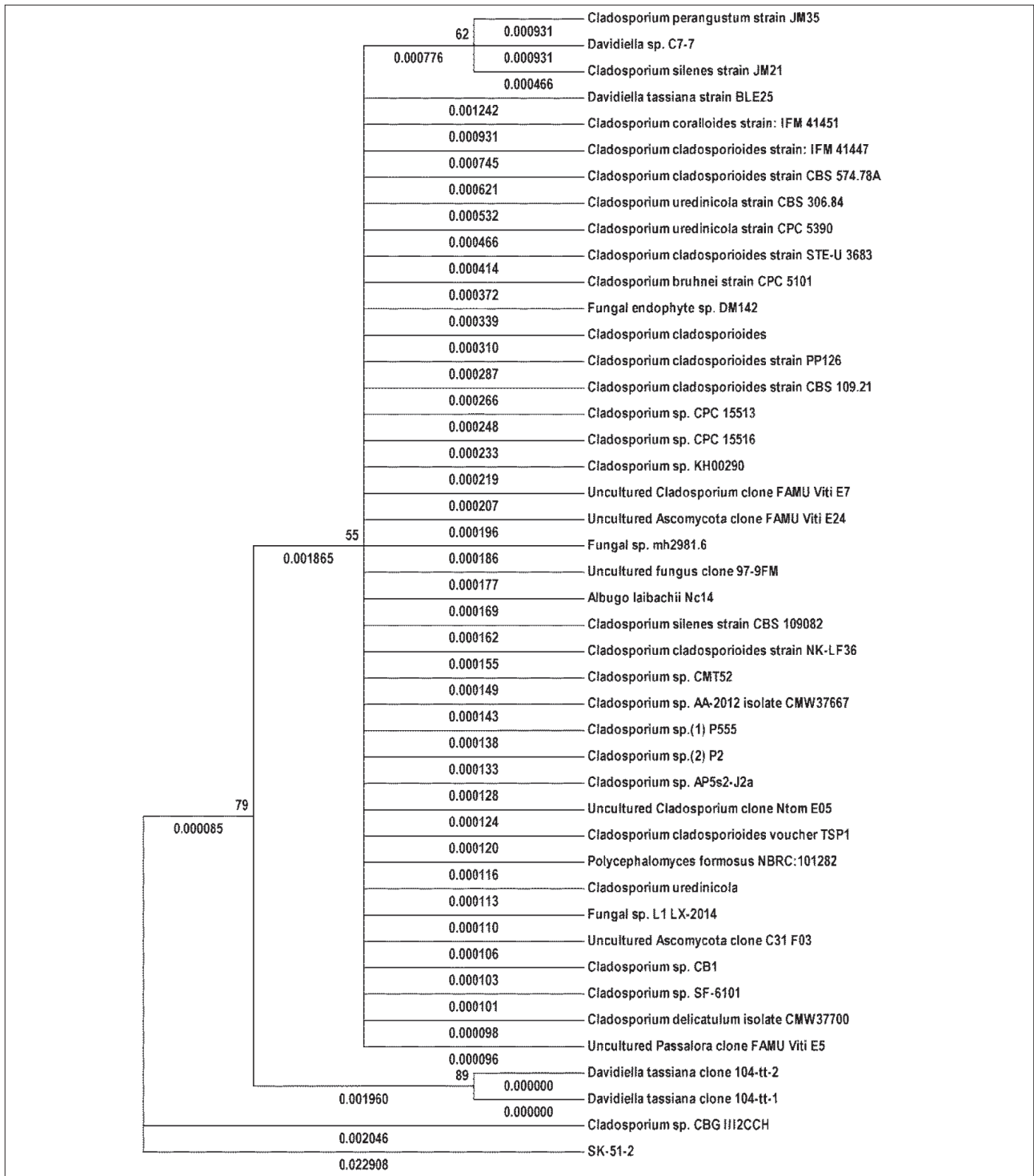
**Fig. 3.** Phylogenetic relationships of different fungus species with nematode-associated fungus (SK-20-2). Phylogeny obtained from the alignment of the 461bp of LSU rDNA region. Tree was created by the neighbor-joining method. Bootstrap values higher than 50% are indicated, alignment gaps and missing data were removed in pairwise sequence comparisons. The horizontal bar shows 0.005% differences in nucleotide identities.



**Fig. 4.** Phylogenetic relationships of different fungal species with nematode-associated fungus (SK-32-2). Phylogeny obtained from the alignment of the 612bp of LSU rDNA region. Tree was created by the neighbor-joining method. Bootstrap values higher than 50% are indicated, alignment gaps and missing data were removed in pairwise sequence comparisons. The horizontal bar shows 0.005% differences in nucleotide identities.



**Fig. 5.** Phylogenetic relationships of different fungal species with nematode-associated fungus (SK-34-2). Phylogeny obtained from the alignment of the 584bp of LSU rDNA region. Tree was created by the neighbor-joining method. Bootstrap values higher than 50% are indicated, alignment gaps and missing data were removed in pairwise sequence comparisons. The horizontal bar shows 0.005% differences in nucleotide identities.



**Fig. 6.** Phylogenetic relationships of different fungal species with nematode-associated fungus (SK-51-2). Phylogeny obtained from the alignment of the 583bp of LSU rDNA region. Tree was created by the neighbor-joining method. Bootstrap values higher than 50% are indicated, alignment gaps and missing data were removed in pairwise sequence comparisons. The horizontal bar shows 0.005% differences in nucleotide identities.

reported (Adam et al., 2014). Different interactions were reported between fungi and nematodes in soils (Renker et al., 2003). The interactions between nematodes and fungi can be categorized into four groups: nematodes as predators of fungi, fungi as predators or parasites of nematodes, nematodes and fungi as partners of plant disease complexes, and nematodes as vectors for fungal spores (Mendoza de Gives et al., 1999; Viaene and Abawi 2000; Bakhtiar et al., 2001; Zahid et al., 2002; Renker et al., 2003). Fungi in the genera *Malassezia*, *Alternaria* and *Cladosporium* were detected in free-living nematodes, which play important roles in nutrient cycling. The presence of fungi together with free-living nematodes supports the hypothesis that nematodes act as vectors for fungi. A similar assumption for *Malassezia* species was also suggested by Renker et al. (2003).

To conclude, it was found that fungal species of *Malassezia*, *Alternaria* and *Cladosporium* were in association with free-living soil nematodes. However, further studies should be conducted to clarify the relationships between these fungi and nematodes.

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**Authors' contributions:** Studies were planned by SK and AA; sample collection was carried out by SK; isolation, molecular studies and phylogenetic analyses were carried out by SK, SY and UA. Preparation of the manuscript, interpretation of the results and discussions were performed by all authors.

**Conflict of interest disclosure:** The authors declare that they have no competing interests.

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