Filamentous Fungi Associated with the Golot Cheese of the Rize-Ardesen Highlands in Turkey

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Abstract

The aim of this study was to identify the filamentous fungi associated with the Golot cheese produced in Ardesen, Rize Province, East Black Sea region, Turkey by using molecular methods. Sixteen fungi were isolated from seven cheese samples and subjected to sequencing analysis using different fungal DNA barcodes. The first marker used, internal transcribed spacer (ITS), allowed identification of Geotrichum candidum and Penicillium roqueforti. The other isolates, defined as Penicillium and Cladosporium, could be identified only at the genus level using ITS; therefore, the beta-tubulin (benA) gene and the microsatellite PC4 markers were used to define the remaining Penicillium isolates as P. biforme and P. solitum. The Cladosporium isolate was identified as C. macrocarpum by analysis using benA and translation elongation factor 1-α (tef1) genes. The most common species associated with Golot cheeses were determined to be P. biforme (56%) and G. candidum (25%). Previously unknown sequences of P. solitum PC4 and C. macrocarpum tef1, and a longer version of C. macrocarpum benA were submitted to GenBank.

Keywords: Golot cheese, Mycobiota, Fungal identification markers, Penicillium, Cladosporium.

Türkiye'de Rize-Ardeşen Yaylalarının Golot Peyniri ile İlişkili Filamentli Funguslar

Öz

Bu çalışmamın amacı, Doğu Karadeniz Bölgesi, Rize İli, Ardeşen'de üretilen Golot peyniri ile ilişkili filamentli fungusların moleküler yöntemler kullanılarak belirlenmesidir. Yedi peynir örneğinden on altı fungus izole edilmiş ve farklı fungal DNA barkodları kullanılarak dizilim belirlenecektir. İzole edilen funguslar için ilk olarak beta-tubulin (benA) veなければ mikrosatellit PC4 genleri kullanılarak tanımlanmış, daha sonra P. biforme ve P. solitum izolatları için tef1 genleri kullanılarak analiz edilerek C. macrocarpum olarak tanımlanmıştır. 

Anahtar Kelimeler: Golot peyniri, Mikobiyota, Fungal identifikasyon markörleri, Penicillium, Cladosporium.

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1. Introduction

Golot cheese of the East Black Sea region of Turkey is a local type of more than 100 of Turkey’s reported cheeses (Cakmakci, 2011; Kamber and Terzi, 2008). Golot is a type of Kashar cheese produced by traditional methods in houses or small factories to be consumed especially as a fondue called “muhlama”, a popular dish made from cheese, cornmeal, and butter (Kamber and Terzi, 2008; Unsal, 1997). In the traditional production method, after fat separation, the low-fat milk is kept at room temperature for 12–24 h according to environmental temperature to acidify using its own microbiota (Balcan, 2017; Unsal, 1997). The curd is then cooked until elastic drained using a fabric bag. After draining, when the temperature is still high enough to allow shaping, the curd is separated into pieces that are shaped to resemble a pita bread. The resulting cheese is consumed either fresh or salted and ripened in wooden barrels in a cool environment for approximately 3 months (Balcan, 2017; Unsal, 1997; Kamber and Terzi, 2008). Because the pita bread of the region is called “Kolet” or “Kolot”, the cheese having a similar shape is called “Golot”, “Kolot”, “Koilet” or “Koleti” based on the specific neighborhood in which it is produced (Tuncurturk and Ozdemir, 2005).

Cheese ripening involves complex glycolytic, lipolytic and proteolytic biochemical processes (Fox et al., 1993; McSweeney, 2004). The enzymes of filamentous fungi growing inside some cheeses, such as the blue-vein cheeses, and on the rind of some, such as the surface mold–ripened cheeses, contribute to the ripening process and are responsible for the formation of characteristic flavors and textures (Gripon, 1993; Metin, 2018). Penicillium species, especially P. roqueforti and P. camemberti (Gripon, 1993; Spinnler and Gripon, 2004), and Fusarium domesticum (Bachmann et al., 2005), Scopulariopsis (Chabalier et al., 1995), Sporendonema (Ratomahenina et al., 1994; Ropars et al., 2012), and Mucor species (Werner et al., 1999) have been described from various mold-ripened cheeses. On the other hand, molds might also be involved in undesirable changes, such as spoilage or mycotoxin contamination (Lund et al., 1995; Hymery et al., 2014). Investigation of the fungal communities sets the basis for understanding the contribution of filamentous fungi to the ripening process and to identify possible undesirable effects that are relevant in cheese production, such as excessive enzymatic activities resulting in off flavors, ropy textures, or mycotoxin production.

There are few studies that describe the main chemical, biochemical, and microbiological properties of Golot cheese (Caglar et al., 1998; Tuncurturk and Ozdemir, 2005; Yazici and Dervisoglu, 2002). The aim of our study was to use molecular methods to determine the filamentous fungi associated with ripened Golot cheese. The fungal isolates were identified molecularly using a multilocus sequencing approach.

2. Materials and Methods

2.1. Cheese Samples

Seven cheese samples were obtained from local stores in the Ardesen village of Rize, Turkey. The samples were vacuum packaged and stored under refrigeration until analyses, which were conducted within 1 week.

2.2. Isolation of Filamentous Fungi

To isolate the filamentous fungi, 10 g cheese sample taken from the rind was homogenized in 90 mL 2% sodium citrate (Sigma-Aldrich, , St. Louis, MO, USA) using a Stomacher (Bagmixer 400, Interscience, Saint Nom, France). Serial dilutions were prepared from the homogenate with 1/4 Ringer’s solution (Merk KGaA, Darmstadt, Germany) and inoculated onto potato dextrose agar (PDA, Merck) in duplicate (Harrigan, 1998). After incubating the plates at 25 °C for 5 days, morphologically different filamentous fungi grown on each plate were transferred to new PDA plates and purified by two subsequent inoculations of single colonies. The isolates were assigned XKY, where X was cheese sample number, and Y was the isolate number from each cheese sample. The resulting 16 fungi were stored at -80 °C in yeast extract peptone dextrose broth (YPD, Merck) containing 20% glycerol for long-term storage.

2.3. DNA Extraction and Polymerase Chain Reaction

The fungi were grown in 10 mL YPD at 25 °C for 1 day, and DNA was extracted as described by Turin et al. (2000). The concentration and purity of the DNA samples were measured using the BioSpec Nano spectrophotometer (Shimadzu, Kyoto, Japan). Different amplification reactions were conducted to identify different isolates (Table 1)—the internal transcribed spacer (ITS) region with universal primers ITS1 and ITS4 (White et al., 1990), the microsatellite locus PC4 with PC4-F and PC4-R (Giraud et al., 2010), the β-tubulin gene (benA) with Bt2a and Bt2b (Glass and Donaldson, 1995), and the elongation factor-1α gene (tefl) with EF6 and EF1α (Peterson, 2004). Polymerase chain reactions (PCR) were conducted in 50 µL reaction mixture containing 1X buffer, 0.2 mM dNTP mix, 2.0 µL 10 mM forward primer, 2.0 µL10 mM reverse primer, ~50 ng template DNA and 2.5 U µL Dream Taq DNA polymerase (Thermo Fisher Scientific, St. Louis, MO, USA) using the T100 Thermal Cycler (Bio-Rad Laboratories, Hercules, CA, USA). The PCR conditions were as follows: denaturation at 94 °C for 1 min followed by 34 cycles of denaturation at 94 °C for 30 s; annealing at 48 °C for PC4, benA, and tefl; 52 °C for ITS for 30 s; extension at 72 °C for 1 min; and final chain elongation at 72 °C for 10 min. The resulting PCR products were purified using the GeneJET PCR Purification Kit (Thermo Fisher Scientific) and subjected to Sanger sequencing analysis using the primers used in PCR. Sequences were visualized using CLC Main Workbench (Qiagen, Hilden, Germany) and compared to the previously published sequences using BLAST in the National Center for Biotechnology Information (NCBI) website.
2.4. Phylogenetic Analyses

Phylogenetic analyses were conducted using MEGA X (Kumar et al., 2018) and the maximum likelihood method and Tamura-Nei model (Tamura and Nei, 1993). The trees were drawn to scale, with branch lengths measured by the number of substitutions per site.

3. Results and Discussion

3.1. Isolation of Fungi

Seven Golot cheese samples were used in isolating the fungi. Although filamentous fungal growth was not observed in 2 of the cheese samples, 16 strains were isolated from the remaining 5 samples.

3.2. Universal Fungal Barcode: ITS

The isolates were first screened by sequencing ITS, the universal DNA barcode for fungi, to identify the fungi (Schoch et al., 2012). ITS sequencing was helpful in defining *G. candidum* (1K1, 2K3, 4K1, and 5K1) and *P. roqueforti* (4K2); however, it was not helpful for differentiating other *Penicillium* species and identifying the *Cladosporium* isolate (Table 1).

Table 1. Isolates and the Regions used for Identification

| Number | Isolate | Identified species          | Regions used for identification |
|--------|---------|----------------------------|---------------------------------|
| 1      | 1K1     | *Geotrichum candidum*      | ITS MH443758                   |
| 2      | 1K3     | *Penicillium biforme*      | GU726757 FJ930944              |
| 3      | 1K4     | *Penicillium solitum*      | MT160803* AY674355             |
| 4      | 1K6     | *Penicillium biforme*      | GU726757 FJ930944              |
| 5      | 2K1     | *Cladosporium macrocarpum* | MN543047* MN543048*            |
| 6      | 2K2     | *Penicillium biforme*      | GU726757 FJ930944              |
| 7      | 2K3     | *Geotrichum candidum*      | MH443758                       |
| 8      | 4K1     | *Geotrichum candidum*      | MH443758                       |
| 9      | 4K2     | *Penicillium roqueforti*   | KM115117                       |
| 10     | 4K3     | *Penicillium biforme*      | GU726757 FJ930944              |
| 11     | 4K4     | *Penicillium biforme*      | GU726757 FJ930944              |
| 12     | 5K1     | *Geotrichum candidum*      | MH443758                       |
| 13     | 6K1     | *Penicillium biforme*      | GU726757 FJ930944              |
| 14     | 6K2     | *Penicillium biforme*      | GU726757 FJ930944              |
| 15     | 6K3     | *Penicillium biforme*      | GU726757 FJ930944              |
| 16     | 6K4     | *Penicillium biforme*      | GU726757 FJ930944              |

*Sequences submitted to GenBank in this study

ITS sequencing identified 4 isolates among the 16 as *Geotrichum candidum* (25%). The filamentous yeast *G. candidum* is an important contributor to ripening in many cheeses, including soft cheeses, such as Camembert, and semihard cheeses, such as, Saint Nectaire and Reblochon (Boutrou and Guéguen, 2005). This yeast grows on the rind and the actions of its lipolytic and proteolytic enzymes contribute to flavor formation; therefore, *G. candidum* is used as a starter culture in the cheese industry (Boutrou and Guéguen, 2005). In addition to being part of raw-milk microbiota, *G. candidum* can be found in various environments, such as soil, plants, grass, and silage as well as in humans and other mammals (Boutrou and Guéguen, 2005; Marcellino and Benso, 2013). Because Golot cheese is cooked, the origins of the fungi detected are expected to be environmental rather than milk.

The other species detected by ITS, *P. roqueforti*, was observed in one cheese sample. *Penicillium roqueforti* is the ripening culture in blue cheeses, such as Roquefort and Gorgonzola, but it can be a contaminant in other cheese types (Hymery et al., 2014) and potentially produce several mycotoxins, such as roquefortine C, PR toxin, ergot alkaloids (isofumigaclavines, festuclavine and agroclavine), and mycophenolic acid (Hymery et al., 2014). PR toxin, the most potent of these, is converted to less toxic derivatives in the cheese environment, while roquefortine C is not produced at high enough quantities to pose a health risk (Martin and Liras, 2017).

3.3. Beta-Tubulin (BenA) and the Microsatellite PC4 used to Identify *P. biforme* and *P. solitum*

The *Penicillium* isolates other than 4K2 (*P. roqueforti*) could not be differentiated by ITS; therefore, they were further analyzed using both *benA* (Fig. 1) and the PC4 microsatellite marker (Fig. 2) that can be used to differentiate between *P. camemberti*-related *Penicillium* species (Giraud et al., 2010). *BenA* and PC4 sequencing showed that most of the remaining *Penicillium* isolates were *P.
biforme (1K3, 1K6, 2K2, 4K3, 4K4, 6K1, 6K2, 6K3, and 6K4), while one (1K4) was \( P. \) solitum. Because the PC4 sequence of \( P. \) solitum was not available from NCBI, the sequence was submitted to GenBank (accession number: MT160803).

Figure 1. Phylogenetic tree generated using the \( \beta \)-tubulin (\textit{benA}) sequences of the isolates in the section \textit{Viridicata} of the genus \textit{Penicillium}. The tree with the highest log likelihood (-622.09) is shown. This analysis involved 19 nucleotide sequences. There were 334 positions in the final dataset. The reference strains and their accession numbers are as follows: \( P. \) biforme CBS 297.48 (FJ930944), \( P. \) paltians CBS 101031 (AY674362), \( P. \) camemberti CBS 299.48 (FJ930954), \( P. \) commune MUCL34882 (FJ930974), \( P. \) fuscoglaucum NRRL892 (FJ930977), \( P. \) echinulatum CBS 31748 (AY674341), \( P. \) discolor CBS 47484 (AY674348), \( P. \) solitum CBS 14786 (AY674355), and \( P. \) crustosum CBS 313.48 (FJ930937).

Figure 2. Phylogenetic tree generated using the microsatellite PC4 sequences of the isolates in the section \textit{Viridicata} of the genus \textit{Penicillium}. The tree with the highest log likelihood (-350.99) is shown. The analysis involved 16 nucleotide sequences. There were 157 positions in the final dataset. The reference strains and their accession numbers are as follows: \( P. \) biforme CBS 297.48 (GU726757), \( P. \) paltians CBS 101031 (EU003130), \( P. \) camemberti CBS 299.48 (EU003152), \( P. \) commune MUCL34882 (EU003165), \( P. \) fuscoglaucum NRRL892 (GU726764), and \( P. \) crustosum CBS 313.48 (EU003136).
Penicillium biforme, the most common filamentous fungus that we isolated from Golot cheese (56 %), is a cheese contaminant closely related to P. camemberti, a fungal starter used in the production of Camembert cheese (Ropars et al., 2012); however, they differ in their ability to produce certain secondary metabolites. For example, although P. biforme can produce the ergot alkaloids rugulovasine A and B, P. camemberti cannot (Fabian et al., 2018); however, it should be noted that in the cheese environment, P. biforme cannot produce those ergot alkaloids (Fabian et al., 2018). Although there are no other reports on P. biforme mycotoxins, closely related species P. camemberti and P. commune are known to produce cyclopiazonic acid (Frisvad and Samson, 2004). Older reports have accepted P. biforme and P. fuscoglaucum as synonyms of P. camemberti and P. commune, respectively (Frisvad and Samson, 2004); however, it has been shown that the multilocus sequence analysis of various P. commune strains from culture collections correspond to those of P. biforme and P. fuscoglaucum (Giraud et al., 2010). Therefore, both the taxonomic position and the mycotoxin profile of P. biforme must be clarified in future studies.

Penicillium solitum was also detected in the Golot cheese samples in our study. Although P. solitum inhabits mainly fruits (Frisvad and Samson, 2004), the species has also been detected in various cheeses (Anelli et al., 2019; Decontardi et al., 2018; Ramos-Pereira et al., 2019) and meat products (Merla et al., 2018, Pleadin et al., 2017; Scaramuzza et al., 2015). This species has not only been isolated from the product, but also from the air itself in the production plants (Scaramuzza et al., 2015), which indicates that air is one of the contamination routes. Although P. solitum produces a number of secondary metabolites, such as cholesterol-lowering agent compactin and cyclopeptin- and viridicatin-related alkaloids, it is not known to produce mycotoxins (Frisvad et al., 2004).

3.1. BenA and Translation Elongation Factor 1-α (Tef1) used to Identify Cladosporium macrocarpum

We were not able to differentiate among Cladosporium species using the ITS sequence of 2K1. Consistent with this finding, the resolution power for ITS was weak for discriminating among Cladosporium species (Bensch et al., 2012); therefore, in phylogenetic studies, multilocus approaches have been used, such as actin (ACT) and tef1 (Bensch et al., 2018), ITS, ACT and tef1 (Bensch et al., 2012) and ITS, ACT, tef1, calmodulin (CAL) and histone H3 (Schubert et al., 2007). In this study, we sequenced benA and tef1 regions of the isolate 2K1. Tef1 was able to differentiate C. macrocarpum from the most closely related species, C. herbarum and others (Fig. 3). Although the tef1 sequence of 2K1 is one base different from that of the closest strains (CBS 299.67 and CBS 121623), because 2K1 was clustered together with the C. macrocarpum strains, we identified the isolate accordingly. Because the tef1 sequences of C. macrocarpum isolates in the NCBI database are ~440 bp long (e.g., EF679450), the newly generated longer tef1 sequence of 2K1 (737 bp) was submitted to GenBank (MN543048). In addition, because the benA sequence for C. macrocarpum was not available, it was submitted under accession number MN543047.

![Phylogenetic tree generated using tef1 sequences of Cladosporium macrocarpum–related strains and the isolate 2K1.](image)

Figure 3. Phylogenetic tree generated using tef1 sequences of Cladosporium macrocarpum–related strains and the isolate 2K1. The tree with the highest log likelihood (-972.61) is shown. The analysis involved 11 nucleotide sequences. There were 421 positions in the final dataset. The reference strains and their accession numbers used to construct the tree were as follows: C. macrocarpum CBS 175.82 (EF679448), C. macrocarpum CBS 223.31 (EF679449), C. macrocarpum CBS 299.67 (EF679450), C. macrocarpum CBS 121623 (EF679453), C. herbarum CBS 121621 (EF679440), C. herbarum CBS 300.49 (EF679434), C. herbarum CBS 111.82 (EF679433), C. herbaroides CBS 121626 (EF679432), C. variabile CBS 121635 (EF679481), and C. phlei CBS 358.69 (JN906991).

Cladosporium is a common genus found in indoor environments as well as in dairy products (Amrouche et al., 2020; Bensch et al., 2018; Costanzo et al., 2018; Hymery et al., 2014; Panelli et al., 2012); however, some species, such as C. sphaerospermum and C. cladosporioides, were also reported as opportunist human pathogens (Batra et al., 2019; Gu et al., 2016). Cladosporium macrocarpum has also been reported in some clinical cases (Cuétara et al., 2009; Laluzza et al., 2011). In cheeses, C. macrocarpum can be a spoilage agent, although it is not as common as other Cladosporium species, such as C. herbarum and C. cladosporioides (Garnier et al., 2017; Hocking and Faedo, 1992; Marín et al., 2015; Pitt and Hocking, 2009). No mycotoxins have been reported for C. macrocarpum (Pitt and Hocking, 2009).
4. Conclusions and Recommendations

This current study presents the first research conducted to identify the filamentous fungi associated with Golot cheeses. We used a multilocus sequencing approach to precisely identify 16 fungal isolates. Although the universal fungal barcode, ITS, helped to define G. candidum and P. roqueforti, benA and the microsatellite PC4 sequences were used to identify P. biforme and P. solitum. The Cladosporium isolate that could not be identified at species level using ITS was identified as C. macrocarpum using benA and tef1 sequences. The previously unavailable PC4 sequence of P. solitum and the benA sequence of C. macrocarpum and a longer version of C. macrocarpum tef1 were submitted to GenBank. Our analysis conducted with seven cheese samples indicated that the most common species in Golot cheeses is P. biforme (56%). Future studies with larger sample sets will provide more information on the mycobiota of Golot cheeses as well as on other artisanal cheeses of the East Black Sea region of Turkey.

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