

IDENTIFICATION OF FILAMENTOUS FUNGI IN TURKISH MOLD-RIPENED CHEESES AND SCREENING OF MYCOTOXIN GENES OF *PENICILLIUM ROQUEFORTI* ISOLATES

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ABSTRACT

Turkish mold-ripened cheese varieties are traditionally produced by spontaneous fungal growth during the ripening process in cellars or caves. In this study, forty strains of filamentous fungi were isolated from mold-ripened cheeses of different regions. Internal transcribed spacer (ITS) sequencing identified the most common species as *Penicillium roqueforti* (52%). The two key genes, *roqA/rds* and *mpaC*, involved in the biosynthesis of roquefortine C and mycophenolic acid, respectively, were screened by PCR. The presence of fragments of these genes in all *P. roqueforti* isolates indicates the potential of the isolates for production of these metabolites. Four selected strains of *P. roqueforti* produced roquefortine C, but mycophenolic acid was detected in only two strains. Variability in the production of the metabolites might indicate the presence of polymorphisms outside of the region amplified or in other genes or their regulatory regions in the biosynthetic pathway.

Keywords: Mold-ripened cheeses, filamentous fungal diversity, *P. roqueforti*, roquefortine C, mycophenolic acid

INTRODUCTION

Mold-ripened cheese products are popular cheese types consumed worldwide. They are composed of two main groups: blue-veined cheeses, such as Roquefort, Danish Blue, Cabrales, Gorgonzola, and Stilton; and surface-ripened cheeses, including but not limited to Camembert-type soft cheeses and semi-hard ones, e.g., Tomme, Cantal, Tilsit and Ossau Iraty (Fox et al., 2016; Metin, 2018). Mold ripened cheese products are produced either by using fungal secondary starters, such as *Penicillium roqueforti* and *Penicillium camemberti*, or left for spontaneous fermentation in artisanal production facilities (Metin, 2018). Turkey also has traditional mold-ripened cheese varieties, most of which can be described as blue cheeses. The most famous of these are Erzurum Kufllu Civil and Karaman Divle Cave Tulum cheeses, which have geographical certification marks. Other varieties include but are not limited to Konya Kufllu (Green) cheese, mold-ripened Hatay Surk, Kup and Tomas (Cokelek, Serto, Dorak) cheeses (Çakmakçı, 2011; TPE, 2019). Most of these varieties are produced by spontaneous fermentation in caves, cellars, or in store-houses without using a mold starter.

Fungal secondary metabolites, including mycotoxins, are substances that are not vital for fungal growth but are believed to confer a competitive advantage to the producer fungus (Hymery et al., 2014). They are generally bioactive compounds. Some (e.g. agroclavines, isofumigaclavines, ochratoxins, citrinin) are harmful to human health due to their toxic properties. Others, such as andrastin A and mycophenolic acid are important for pharmaceutical industry due to antitumor, antibacterial, or immunosuppressive effects (Rojas-Aedo et al., 2017). *Penicillium roqueforti*, the principal fungal species of blue cheeses, is known to produce PR toxin, roquefortine C and D, mycophenolic acid, isofumigaclavine A (roquefortine A), festuclavine, agroclavine, eremofortines, andrastins and citreo isocoumarin (Nielsen et al., 2006; O'Brien et al., 2006; Visagie et al., 2014; Fontaine et al., 2015; Del-Cid et al., 2016; Garcia-Estrada & Martin, 2016; Gillot et al., 2017a). Roquefortine C, mycophenolic acid and PR toxin are the most commonly reported among these metabolites (Gillot et al., 2017b; Garcia-Estrada & Martin, 2016). Although PR toxin is of great concern toxicologically, due to the amino acid composition of the cheese and the microaerophilic conditions, it is not stable in cheese and is reduced to PR imine that has lower toxicity or turn into other metabolites such as PR amide and PR acid (Scott & Kanhere, 1979; Cheng et al., 2003; Dubey et al., 2018). Roquefortines have antibacterial activity against Gram-positive bacteria and can be useful in controlling the population of bacteria during the cheese ripening

process (Frisvad et al., 2007; Perrone & Susca, 2017). However, roquefortine C is a mycotoxin with a low level of neurotoxic and cytotoxic effects and its presence might create a health risk (Kosalkova et al., 2015; Garcia-Estrada & Martin, 2016). Mycophenolic acid, on the other hand, has antimicrobial, antitumor, and antipsoriasis activities. Specifically, because of its immunosuppressive activity, it is used in the formulations of drugs used to prevent organ rejection in kidney, liver and heart transplant patients (Del-Cid et al., 2016; Gillot et al., 2017a).

Fungal secondary metabolites are generally synthesized by the action of a group of enzymes, the genes of which are linked together in the genome and organized in clusters (Lind et al., 2017). Based on the chemical structure of the secondary metabolite, the clusters might harbor genes that code for a terpene synthase, a polyketide synthase or a nonribosomal peptide synthase (Brown et al., 2011). For example, roquefortine C is an indole alkaloid synthesized from precursor amino acids that are condensed by a non-ribosomal peptide synthetase coded by the *rds/roqA* gene (Garcia-Estrada & Martin, 2016). In addition to *rds/roqA*, the roquefortine C biosynthesis gene cluster harbors *rdh/roqR*, *rpt/roqD* and the residual pseudogene *gmt/roqN*. *Rdh/roqR* and *rpt/roqD* are a cytochrome P450 oxidoreductase and a prenyl transferase, respectively. They have roles in later steps in roquefortine D and roquefortine C biosynthesis (Ali et al., 2013; Kosalkova et al., 2015; Garcia-Estrada & Martin, 2016). Likewise, *P. roqueforti* has seven genes (*mpaA*, *mpaB*, *mpaC*, *mpaDE*, *mpaF*, *mpaG*, and *mpaH*) in the gene cluster of mycophenolic acid biosynthesis (24.4 kbp) (Del-Cid et al., 2016; Garcia-Estrada & Martin, 2016). Mycophenolic acid is an organic acid, and its backbone is formed as the first step in the biosynthetic pathway by the action of the putative polyketide synthase encoded by *mpaC* (Del-Cid et al., 2016; Garcia-Estrada & Martin, 2016).

Molecular methods show that secondary metabolite gene clusters can be polymorphic among isolates; single nucleotide polymorphisms (SNPs)/short indels, gene loss/gains, and entire cluster loss/gains can be observed, or the location of the cluster on the chromosome might differ (Lind et al., 2017). These kinds of polymorphisms were reported for the secondary metabolite gene clusters of several fungi, including *Aspergillus fumigatus*, *Aspergillus flavus*, *Fusarium fujikuroi*, and *Cochliobolus carbonum*, in addition to *P. roqueforti* (Ahn & Walton, 1996; Chang et al., 2005; Wiemann et al., 2013; Chiara et al., 2015; Lind et al., 2017; Gillot et al., 2017a).

In this study, we aimed to identify the filamentous fungi isolated from traditional mold-ripened cheeses of Turkey by molecular methods. In addition, we screened *P. roqueforti* isolates for the presence of the *roqA/rds* and *mpaC* genes, the

products of which play roles in the biosynthesis of roquefortine C and mycophenolic acid, respectively. This screening was performed to determine if whole-gene losses were present among the isolates. Selected *P. roqueforti* isolates were also analyzed by Q-TOF LC/MS for the production of roquefortine C and mycophenolic acid.

MATERIAL AND METHODS

Cheese samples

A total of 17 cheese samples including eleven Konya Kuflu Tulum (samples 1-11), three Divle Cave Tulum (samples 12, 13, 17) and three Erzurum Kuflu Civil (samples 14-16), were collected from local markets and were stored at +4°C until the analysis.

Mold isolation from cheese samples

Ten g from each cheese sample was aseptically transferred to sterile bags and 90 mL of 0.1% peptone water was added. Then the mixture was homogenized for 2 min (Stomacher Blender SJIA-04C, China). Dilutions were prepared and inoculated on potato dextrose agar (PDA) plates. All plates were incubated at 25°C for 5 days. Twenty-two molds were isolated from Konya Kuflu Tulum, Divle Cave Tulum, and Erzurum Kuflu Civil cheeses. Another 18 molds previously isolated (from Cecil (Chechil) cheese from the Kars district, Tomas cheese from the Elazig-Bingol district, Surk cheese from the Hatay district, and Tulum cheese from the Mediterranean region) were also used in this study. In total, 40 mold isolates were purified on PDA (Florez & Mayo, 2006; Çakmakçı et al., 2012; Fontaine et al., 2015).

To prepare stock cultures, purified isolates were grown on PDA at 25°C for 5 days. The mycelia collected were transferred with a sterile needle into cryo tubes containing 500 µL of 40% sterile glycerol solution (Kosalkova et al., 2015). Culture stocks were kept at -80°C. For short-term storage, the prepared PDA agar slants were stored at +4°C (Florez & Mayo, 2006; Çakmakçı et al., 2012).

Morphological examination

Macro-morphological features of the isolates, such as colony colors, forms, and exudate production, were examined on PDA and malt extract agar (MEA) plates. For microscopic examination, the selected isolates were cultivated on PDA and incubated at 25°C for three days. Mycelium structures (coniphore, conidia, and branching type) were investigated on light microscope (Olympus BX53, DP27, Japan) by staining with lactophenol cotton blue (LPCB) and on a scanning electron microscope (SEM, Fei Quanta FEG250, USA) by zooming 2,500; 5,000; 10,000; and 20,000 times (Rosana et al., 2014).

Isolation of genomic DNA

Fungal DNA isolation was performed by phenol-chloroform-isoamyl alcohol (PCI) extraction after cell lysis (Florez & Mayo, 2006; Umesha et al., 2016). First, the mycelia of the fungal cultures grown on plates were collected with a sterile needle and transferred to microcentrifuge tubes containing 450 µL of 1X Tris-EDTA buffer. After gentle mixing to resuspend the mycelia in the buffer solution, 50 µL of 10% SDS and 2 µL proteinase K (1 mg/mL) were added to the mixture, and the tubes were mixed well. Then, the mixture was incubated first at 37°C for 60 min and next at 65°C for 10 min. Then, 500 µL of PCI solution (25:24:1) (Acros, USA) was added to the tubes and mixed well. After 5 min of incubation at room temperature (25°C), the tubes were centrifuged at 12,000 rpm at 4°C for 5 min. The supernatant was transferred to new tubes and PCI extraction was repeated. After that, 50 µL of 5M sodium acetate (pH 5.2) and 1 mL of isopropanol were added and the tubes were centrifuged at 10,000 rpm for 5 min. The supernatant was removed and the pellet was washed with 500 µL of 70% ethanol. After centrifugation, the pellet was dried at 37°C for 5 min and resuspended in 100 µL of 1X Tris-EDTA buffer (pH 8.0). The quality of the DNA extracts was measured with a BioSpec Nano spectrophotometer (Shimadzu, Japan). DNA samples having an OD₂₆₀/OD₂₈₀ (optical density) value between 1.7-2.0 were considered acceptable for further use. DNA isolates were stored at -20°C (Florez & Mayo, 2006; Florez et al., 2007).

PCR amplification

For identification purposes, internal transcribed spacer (ITS) regions was used. The ITS region was amplified by using the forward ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') primer and the reverse ITS4 (5'-TCCTCCGCTTATTGATATGC-3') primer (White et al., 1990). PCR mix (25 µL) consisted of 12.5 µL 2X PCR Master Mix (*i*-Taq) solution (Intron, South Korea), 7.5 µL of dH₂O, 2 µL of 12.5 mM forward and 2 µL of 12.5 mM reverse primers, and 1 µL (~50 ng) of template DNA. For no-DNA control, water was used instead of DNA. PCR was carried out using a thermal cycler (BioRad, T100, USA) with the following conditions: initial denaturation at 94°C for 1 min, 34 cycles of denaturation at 94°C for 30 sec, annealing at 52°C for 30 sec, extension

at 72°C for 1 min, and a final extension at 72°C for 10 min was used (White et al., 1990; Çakmakçı et al., 2012; Panelli et al., 2012).

For the detection of the mycotoxin genes of *P. roqueforti* isolates, the dipeptide synthetase gene, *rds/roqA* (GenBank accession number: KP970559.1), which is involved in the production of roquefortine C, and the polyketide synthase gene, *mpaC* (KU234530.1), which is responsible for the biosynthesis of mycophenolic acid were selected. Primers were designed by Primer 3 software using the *rds/roqA* and *mpaC* gene sequences (Untergasser et al., 2012). The *rds/roqA* gene was amplified by PCR using the forward RoqAF (5'-ACTACACCGCCATTGACTCC-3') and the reverse RoqAR (5'-CTCAATCTCGTGACCTCAA-3') primers. For the *mpaC* gene, a forward MpaCF (5'-TCTGTCAAGGCAGACTGGTG-3') primer and a reverse MpaCR (5'-TCGTCCGATAGCTCAGTGTG-3') primer were used. PCR conditions for *rds/roqA* and *mpaC* were the same as for ITS PCR except that the annealing temperature was 50°C for *rds/roqA* and 54°C for *mpaC*. Selected PCR products obtained were sequenced to confirm the specificity of the amplicons.

The PCR amplicons were electrophoresed on 1.2% agarose gel in 0.5X Tris-Borate-EDTA buffer at 120 V for 90 min using OWL A2 gel electrophoresis system (Thermo Fisher Scientific, St. Louis, MO, USA) (Panelli et al., 2012; Çakmakçı et al., 2012). For electrophoresis, samples were mixed with 6X loading dye (Thermo Fisher Scientific) and loaded with 100 bp DNA ladder (Thermo Fisher Scientific). Visualization was performed using the Gel-Doc XR+ gel imaging system (Bio-Rad Laboratories, Hercules, CA, USA). The PCR products were purified with a Vivantis GF-1 PCR Clean-up Kit (Vivantis, Malaysia) according to manufacturer's instructions for sequencing purposes.

Sequencing and phylogenetic analysis

Sequencing of the PCR amplicons was performed by a commercial company (MedsanTek, Istanbul, Turkey) using BigDye® Direct Cycle Sequencing Kit (Thermo Fisher Scientific) and Applied Biosystems 24-capillary 3500xL Genetic Analyzer (Thermo Fisher Scientific). From the NCBI BLAST database, the identity of the isolates was determined with 100% sequence identity. Sequence chromatograms were visualized using CLC Main Workbench 7 (Qiagen, Germany). Phylogenetic trees were generated by MEGA X software using an alignment produced by ClustalW (Kumar et al., 2018). The sequences of reference (type) strains used in alignment were as follows: *Penicillium roqueforti* CBS 221.30 (NR_103621), *Penicillium chrysogenum* CBS 306.48 (NR_077145), *Penicillium corylophilum* NRRL 802 (NR_121236), *Penicillium bifforme* CBS 297.48 (NR_138325), *Penicillium camemberti* CBS 299.48 (AB479314), *Penicillium crustosum* FRR 1669 (NR_077153), *Penicillium rubens* CBS 129667 (NR_111815) and *Penicillium spinulosum* FRR 1750 (NR_077158). The evolutionary history was inferred by using the Maximum Likelihood method and the Kimura 2-parameter model, the best-fit model determined using MEGA X, with 1000 bootstrap replicates (Kimura, 1980). 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) 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.

Secondary metabolite production of selected *P. roqueforti* isolates

Mycotoxin production levels of four randomly selected *Penicillium roqueforti* isolates were determined on YES medium (Yeast Extract Sucrose Agar) with Q-TOF LC/MS (Agilent 6530 Accurate-Mass Quadrupole Time-of-flight, USA). First, the isolates were inoculated on PDA plates and incubated at 25°C for 5 days. Then, they were cultivated on YES medium and incubated in the dark at 25°C for 14 days (Frisvad & Samson, 2004; Gillot et al., 2017b). Afterwards, the YES cultures were homogenized for 1 min using an Ultraturax T18 (IKA, Heidelberg, Germany). Then, 4 g of the sample was transferred into a 100-mL bottle, and 25 mL of acetonitrile containing 0.1% formic acid was added. The mixtures were vortexed for 30 s and kept in an ultrasonic water bath (Selecta, Spain) for 15 min. Then, the samples were centrifuged at 5000 g for 10 min at 4°C. The supernatant was collected and stored at -20°C until analysis (Gillot et al., 2017b).

For chromatography, the extracts were filtered through a 0.2 µm PTFE membrane syringe 4 mm filter and 1 mL of each of the samples were transferred to vials. The extracts were analyzed for the presence of roquefortine C and mycophenolic acid by a Q-TOF LC/MS system with ESI+ (Electrospray Ionization) Scan mode using the method developed by Gillot et al. (2017b), with two replicates. In HPLC, a ZORBAX Extend-C-18 (2.1 x 50 mm and 1.8 µm, 600 bar) column was used. The starting temperature of the column was 35°C, and the mobile phase flow rate was 0.3 mL/min. As mobile phases, solvent A (Milli-Q water + 0.1% LC/MS grade formic acid (v/v) + 0.1% ammonium formate (v/v)) and solvent B (100% LC/MS grade acetonitrile) were used. Solvent B was maintained at 10% for 4 min, and a gradient flow of 10-100% was provided for 36 min. Eventually, solvent B was held at 100% for 10 min. Mass spectrometer conditions were set as follows: Ion range from 100 to 1000 m/z; capillary

voltage, 4 kV; source temperature, 325°C; drying gas, 12 L/min, and nebulizer pressure, 50 psig.

RESULTS

Species diversity of the Turkish mold-ripened cheeses

For genotypic identification, ITS1-5.8S-ITS2 rRNA (ITS) region was used. ITS sequencing detected 6 different species, namely, *P. roqueforti* (52%, 21 isolates), *Penicillium corylophilum* (14%, 6 isolates), *Penicillium bifforme* (13%, 5 isolates), *Penicillium crustosum* (8%, 3 isolates), *Penicillium spinulosum* (8%, 3 isolates) and *Penicillium rubens* (5%, 2 isolates) (Table 1). Among these, *P. roqueforti* was the most prominent species with 21 isolates. The phylogenetic tree based on the ITS region is given in Figure 1. The phylogenetic analysis grouped the isolates into 6 different branches according to the differences in their ITS region. Phylogenetic analysis showed that ITS sequence diversity was sufficient to discriminate between closely related species, such as *P. rubens* from *P. chrysogenum*, or *P. bifforme* from *P. camemberti*. The cheese isolates were grouped into well-supported (>97%) clades together with the type strain of each species verifying their identity.

Conidiophore branching patterns were used in taxonomy in the past, but are still considered important in identification (Visagie et al., 2014). The isolates randomly chosen from each species were visualized by scanning electron microscopy (SEM) (Figure 2). Conidiophore patterns varies from simple, such as the monoverticillate pattern of *P. spinulosum* (Figure 2H) having phialides directly connected to the stipe and the biverticillate pattern of *P. corylophilum* (Figure 2G), which has metulae between the stipe and the phialides to more complex patterns (Houbraken et al., 2014). *P. roqueforti* (Figure 2A, 2B, and 2C), *P. crustosum* (Figure 2D) and *P. bifforme* (Figure 2E) have terverticillate patterns including one more branch between the stipe and the metulae. In addition, the warty structure of the stipes and the branches were noted as described previously (Frisvad & Samson, 2004). *Penicillium rubens* (Figure 2F) has quaterverticillate structure having an extra branch beyond the terverticillate pattern (Frisvad & Samson, 2004).

Table 1 Identification of *Penicillium* strains isolated from Turkish traditional mold-ripened cheeses

Isolate Code	ITS Sequencing	Isolation Source
1Y5D*	<i>Penicillium roqueforti</i>	Konya Moldy Tulum Cheese
2Y5F	<i>Penicillium roqueforti</i>	Konya Moldy Tulum Cheese
3Y5A	<i>Penicillium roqueforti</i>	Konya Moldy Tulum Cheese
4Y5A	<i>Penicillium roqueforti</i>	Konya Moldy Tulum Cheese
4Y5B*	<i>Penicillium roqueforti</i>	Konya Moldy Tulum Cheese
4Y5D*	<i>Penicillium crustosum</i>	Konya Moldy Tulum Cheese
5B	<i>Penicillium bifforme</i>	Other**
5Y5A	<i>Penicillium roqueforti</i>	Konya Moldy Tulum Cheese
5Y5C	<i>Penicillium crustosum</i>	Konya Moldy Tulum Cheese
6	<i>Penicillium corylophilum</i>	Other
6Y5B	<i>Penicillium roqueforti</i>	Konya Moldy Tulum Cheese
6Y5C	<i>Penicillium roqueforti</i>	Konya Moldy Tulum Cheese
6Y5E	<i>Penicillium roqueforti</i>	Konya Moldy Tulum Cheese
7Y5E	<i>Penicillium roqueforti</i>	Konya Moldy Tulum Cheese
8Y5B	<i>Penicillium roqueforti</i>	Konya Moldy Tulum Cheese
9Y5B	<i>Penicillium roqueforti</i>	Konya Moldy Tulum Cheese
10D	<i>Penicillium spinulosum</i>	Other
10Y5C	<i>Penicillium roqueforti</i>	Konya Moldy Tulum Cheese
11C	<i>Penicillium bifforme</i>	Other
11Y5B	<i>Penicillium roqueforti</i>	Konya Moldy Tulum Cheese
12D	<i>Penicillium bifforme</i>	Other
12F*	<i>Penicillium roqueforti</i>	Other
12Y5D	<i>Penicillium crustosum</i>	Divle-Cave Moldy Tulum Cheese

13B*	<i>Penicillium corylophilum</i>	Other
13Y6E*	<i>Penicillium rubens</i>	Divle-Cave Moldy Tulum Cheese
14Y5B	<i>Penicillium roqueforti</i>	Erzurum Moldy Civil Cheese
16Y4B	<i>Penicillium roqueforti</i>	Erzurum Moldy Civil Cheese
17Y5C	<i>Penicillium roqueforti</i>	Divle-Cave Moldy Tulum Cheese
17Y5D*	<i>Penicillium roqueforti</i>	Divle-Cave Moldy Tulum Cheese
21C	<i>Penicillium corylophilum</i>	Other
22E	<i>Penicillium bifforme</i>	Other
23A	<i>Penicillium corylophilum</i>	Other
23B	<i>Penicillium corylophilum</i>	Other
23C	<i>Penicillium roqueforti</i>	Other
25C	<i>Penicillium corylophilum</i>	Other
25D	<i>Penicillium rubens</i>	Other
29D	<i>Penicillium roqueforti</i>	Other
30C*	<i>Penicillium spinulosum</i>	Other
30D	<i>Penicillium spinulosum</i>	Other
31A*	<i>Penicillium bifforme</i>	Other

*Species selected to be analyzed by SEM.

**Other: Previously isolated molds from Chechil Cheese from the Kars district, Tomas Cheese from the Elazig-Bingol district, Surk Cheese from the Hatay district, and Tulum cheese from the Mediterranean region.

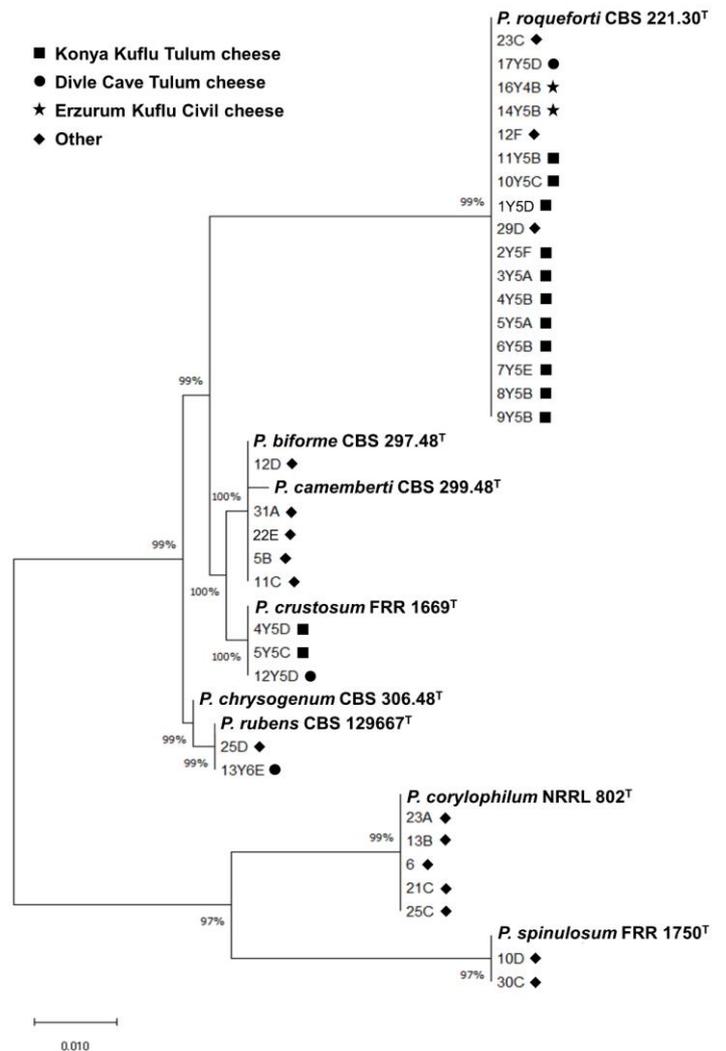


Figure 1 Phylogenetic tree of the isolates generated by the Maximum Likelihood approach using the ITS1-5.8S-ITS2 rRNA (ITS) region. The tree with the highest log likelihood (-831.52) is shown. In total, there were 380 positions in the final dataset. Isolates with identical sequences obtained from the same cheese sample were excluded from the analysis (4Y5A, 6Y5C, 6Y5E, 17Y5C, 23B, and 30D).

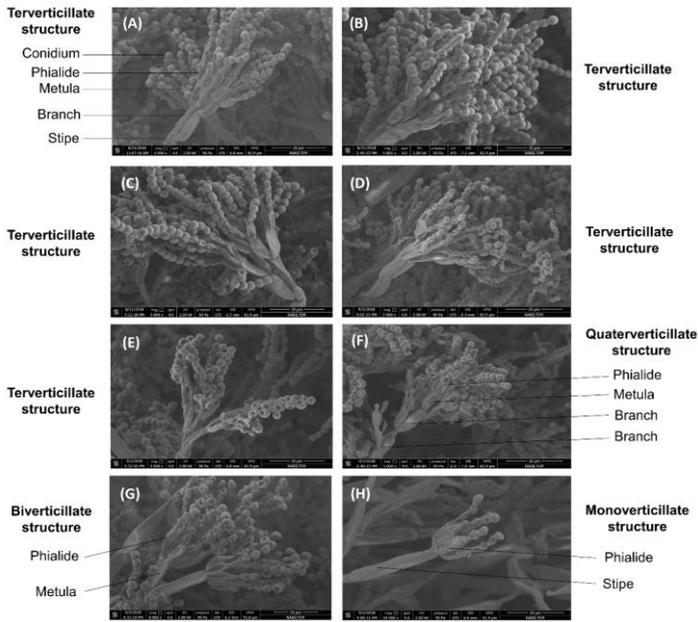


Figure 2 SEM Images of *P. roqueforti* 12F (A), 1Y5D (B) and 4Y5B (C), *P. crustosum* 4Y5D (D), *P. bifforme* 31A (E) and *P. rubens* 13Y6E (F), *P. corylophilum* 13B (G) and *P. spinulosum* 30C (H) isolates.

PCR screening indicates the presence of *rds/roqA* and *mpaC* genes in all *P. roqueforti* isolates

PCR screening showed that all *P. roqueforti* isolates (21 strains), harbored 360-bp and 590-bp products of the *rds/roqA* and *mpaC* genes, respectively. The PCR

products of two isolates were sequenced to confirm the specificity of the PCR reaction. The *rds/roqA* and *mpaC* genes are responsible for roquefortine C and mycophenolic acid production, respectively. An agarose gel image of the PCR products of four representative isolates is shown in Figure 3.

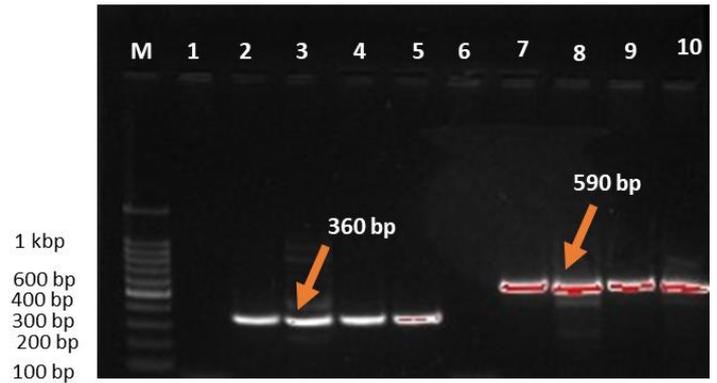


Figure 3 Agarose gel image of PCR products of mycotoxin genes; M: DNA marker (100 bp-1kbp), 1: *roqA* negative control, 2: 1Y5D, 3: 12F, 4: 4Y5B, 5: 17Y5D, 6: *mpaC* negative control, 7: 1Y5D, 8: 12F, 9: 4Y5B, 10: 17Y5D

Determination of the secondary metabolites of selected *Penicillium roqueforti* isolates by Q-TOF LC/MS

While roquefortine C was determined in the all extracts of the four selected *P. roqueforti* isolates, mycophenolic acid could only be detected in two (1Y5D and 17Y5D) (Figure 4).

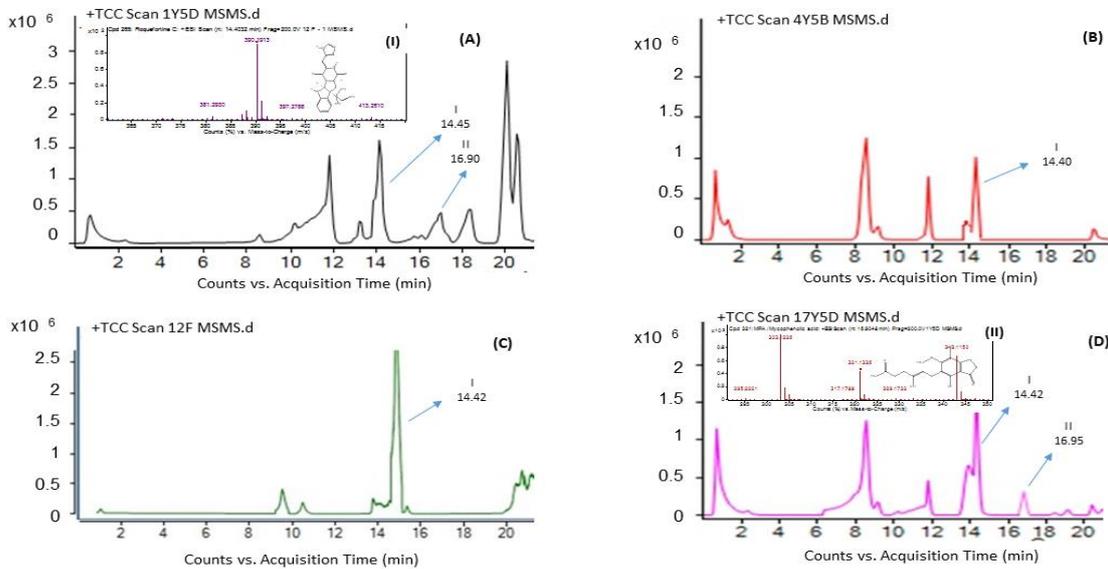


Figure 4 Total chemical chromatograms (TCC) of the extracts of the *P. roqueforti* isolates 1Y5D (A), 4Y5B (B), 12F (C) and 17Y5D (D). Peaks of identified metabolites are shown as I: Roquefortine C, II: Mycophenolic acid. Roquefortine C ($C_{22}H_{23}N_5O_2$) was determined in ESI+ mode with the addition of $[H+]$ ion in the mass spectrum (B1) for all four isolates with a retention time of 14.40-14.45 min. Similarly, mycophenolic acid ($C_{17}H_{20}O_6$) (A1) was determined in ESI+ mode (retention time of 16.90-16.95), but in only two of the isolates (1Y5D and 17Y5D).

DISCUSSION

In blue cheeses worldwide, *P. roqueforti* is the dominant species (Florez & Mayo, 2006; Gillot et al., 2017b; Ropars et al., 2017; Metin, 2018). Studies conducted on Turkish mold-ripened cheeses demonstrated similar findings, but they involve mostly morphological identification techniques. For example, in moldy Civil cheese, 165 of 186 isolates were identified as *P. roqueforti*, and the remaining 21 isolates were identified as *Penicillium* spp. (Çakmakçı et al., 2012). In a study conducted in Konya and its vicinity, 140 mold-ripened cheese samples were examined; 86% of the isolates were identified at the genus level as *Penicillium*, and the remaining 12% were identified as *Aspergillus* (Özkalp &

Durak, 1998). In another study, 158 mold isolates were identified using morphological characteristics, and 70% of these were described as *Penicillium* species harboring *P. commune*, *P. roqueforti*, *P. verrucosum*, *P. expansum*, and *P. chrysogenum* (Hayaloglu & Kirbag, 2007). In another study, 21 mold-ripened cheese samples from Konya, Mersin, Nevsehir, and Nigde were analyzed, and eight different species were identified morphologically. *P. roqueforti* was the most commonly isolated mold, followed by *P. chrysogenum* (Sağdıç et al., 2008). Twelve of the 16 mold species isolated from the moldy Tulum cheese sold in Erzurum and its vicinity were identified based on morphology as *P. roqueforti* (Erdogan et al., 2003). In a study analyzing the microbial diversity of Divle cave cheese, among the 101 filamentous fungi isolated, *Penicillium* was the dominant

genus and the most predominant species were *P. polonicum*, *P. bifforme*, *P. roqueforti*, and *P. chrysogenum* (Öztürkoglu-Budak et al., 2016). Similar to these findings, we identified all 40 strains isolated from various Turkish mold-ripened cheeses as *Penicillium*; the most common species was *P. roqueforti*.

In our study we aimed to determine roquefortine C and mycophenolic acid production of four selected *P. roqueforti* isolates on YES medium. While we detected roquefortine C in all four isolates using Q-TOF LC/MS, we could observe mycophenolic acid production in only two of them. In previous studies, mycophenolic acid production level was observed to be lower than that of roquefortine C (Gillot et al., 2017b); therefore we may not be able reach the detection limit of mycophenolic acid. Another reason might be that the strains selected were not mycophenolic acid producers. Consistent with that, previous studies show that mycotoxin production varied among different isolates of *P. roqueforti*. For example, Larsen et al. (2002) indicated the presence of roquefortine A (isofumigaclavine A) and roquefortine C; however, mycophenolic acid or PR toxin could not be detected. In another study (Nielsen et al., 2006), while roquefortine C was found in all 9 isolates, mycophenolic acid was detected in eight of them. O'Brien et al. (2006) analyzed 79 *P. roqueforti* isolates, and roquefortine C and mycophenolic acid production were detected in 96% and 85% of the isolates, respectively. In another study, while roquefortine C was detected in 96% of the isolates, mycophenolic acid was found in 87% of 55 *P. roqueforti* isolates (Gillot et al., 2017b).

The variability in the production of fungal secondary metabolites among strains of the same species might be directly linked to polymorphisms in the gene cluster involved in the production of the secondary metabolite or to the regulatory network directing the expression of this cluster. For example, recent studies have shown that secondary metabolite gene clusters are highly divergent because of some variations such as single nucleotide polymorphisms (SNPs), gene and gene cluster gains and losses, and idiomorph and genomic location polymorphisms (Lind et al., 2017). For instance, four out of 66 isolates of *Aspergillus fumigatus* lacked the non-ribosomal peptide synthetase (NRPS) gene in addition to two other genes in a secondary metabolite cluster (Lind et al., 2017). In addition, some strains completely lack whole clusters of certain secondary metabolites. (Lind et al., 2017). A similar study on non-aflatoxinogenic *Aspergillus flavus* isolates shows that these isolates have different types of deletions in the aflatoxin gene cluster (Chang et al., 2005). For example, while some of these strains are devoid of nearly half of the aflatoxin cluster, others completely lack the cluster. Similar findings were also reported for *Fusarium fujikuroi* and *Cochliobolus carbonum* (Chiara et al., 2015; Wiemann et al., 2013; Ahn & Walton, 1996). Similar to these studies, Gillot et al. (2017a) demonstrated that 19 out of 55 *P. roqueforti* isolates have a 174-bp deletion in their *mpaC* gene, coding for the polyketide synthase involved in mycophenolic acid synthesis, and this deletion was in correlation with no or limited production of mycophenolic acid. In our study, we show that all *P. roqueforti* isolates harbor the *rds/roqA* and *mpaC* genes detected by PCR, and no major change (such as large indels) is present within the regions amplified. Our amplification region does not span the 174-bp region detected previously; therefore, polymorphisms can be present in this region. In addition, there might be other polymorphisms in the genes that can only be detected by sequencing. In addition, possible gain/loss events in the other genes in the clusters can only be detected by screening of the other genes as well, or ideally, by whole genome sequencing (WGS).

Regulatory pathways are also important in secondary metabolite production. For example, a global regulator, putative methyltransferase *LaeA* has been shown to affect secondary metabolic gene cluster expression in many fungi, including *Penicillium* species (Zhang et al., 2016). The study by Soliman et al. (2015) shows that, although a number of *Penicillium* species studied all had an *idh* gene responsible for patulin production, transcription of *idh* was only observed in *P. expansum*. Similarly, Bogs et al. (2006) showed that a polyketide synthase gene involved in ochratoxin A biosynthesis is present in both *P. nordicum* and *P. nalgiovense*. It is transcribed only in *P. nordicum*, which shows the importance of regulatory factors. Variation of secondary metabolite production would also be linked to regulatory networks. WGS or several longer PCR reactions to retrieve the entire cluster would also shed light on the mutations that might be present in the regulatory regions of the genes.

CONCLUSION

In this study, we isolated and molecularly identified the filamentous fungi of traditional Turkish mold-ripened cheeses. Fourty fungal strains included six different *Penicillium* species with the most prevalent being *P. roqueforti* (52%), concordant with the previous studies. Other species consist of *P. rubens*, *P. bifforme*, *P. crustosum*, *P. corylophilum*, and *P. spinulosum*. *P. roqueforti* isolates were screened for the presence of the *roqA/rds* and *mpaC* genes, the products of which play roles in the biosynthesis of roquefortine C and mycophenolic acid, respectively. All *P. roqueforti* isolates were observed by PCR to harbor the *rds/roqA* and *mpaC* gene fragments. This showed the absence of large deletion events in the corresponding genes and the potential of the isolates to produce these metabolites. When four selected *P. roqueforti* isolates were analyzed by Q-TOF LC/MS, all were observed to produce roquefortine C. Meanwhile, mycophenolic acid was detected in two isolates. Variability in the secondary

metabolite production of the isolates might be related to various kinds of polymorphisms in the biosynthetic pathway genes. Future studies might involve metabolite detection of a larger number of isolates. In addition, WGS could be helpful in detecting all kinds of variations in secondary metabolite biosynthetic gene clusters. Determination of the effect of these metabolites on cheese production would be helpful in the search for a fungal starter culture that can be used in the production of healthier products.

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