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Genetic diversity and population structure of *Penicillium roqueforti* isolates from Turkish blue cheeses

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ABSTRACT

Blue cheeses, including renowned mold-ripened varieties such as Roquefort (France), Gorgonzola (Italy), Stilton (UK), Danablue (Denmark), and Cabrales (Spain), owe their distinct blue-green color and unique flavor to the fungal species *Penicillium roqueforti*. In Turkey, traditional cheeses similar to blue cheeses, namely mold-ripened Tulum and Civil, employ production techniques distinct from their European counterparts. Notably, mold-ripening in Turkish cheeses is spontaneous and does not involve starter cultures. Despite *P. roqueforti* being recognized for its distinct genetic populations sourced from various blue cheeses and non-cheese origins globally, the characteristics of the *P. roqueforti* population within Turkish cheeses remain unexplored. This study aimed to unravel the genetic characteristics and population structure of *P. roqueforti* from Turkish mold-ripened cheeses. Analysis of mold-ripened Civil ($n = 22$) and Tulum ($n = 8$) samples revealed 66 *P. roqueforti* isolates (76.6 % of total fungal isolates). Subsequently, these isolates ($n = 66$) and those from previous studies (Tulum $n = 53$, Golot $n = 1$) were used to assess genetic characteristics and mating genotypes. All 120 isolates harbored horizontal transfer regions (*Wallaby* and *CheesyTer*) and predominantly possessed the *MAT1-2* mating genotype, similar to global blue cheese populations. However, most lacked the *mpaC* deletion associated with such populations. Analysis of the population with three polymorphic microsatellite markers revealed 36 haplotypes (HTs). Some cheeses contained isolates with different HTs or opposite mating genotypes, aligning with spontaneous fungal growth. Tulum and Civil isolates exhibited similar population diversity without forming distinct subgroups. Phylogenetic analysis of 20 selected isolates showed 75 % aligning with global blue cheese isolates, while 25 % formed unique clades. Overall, Turkish *P. roqueforti* isolates share genetic similarities with global populations but exhibit unique characteristics, suggesting potential new clades deserving further investigation. This research illuminates the characteristics of *P. roqueforti* isolates from Turkish cheeses, contributing to the knowledge of the global intraspecific diversity of the *P. roqueforti* species.

1. Introduction

Blue cheeses are well-known for their distinctive blue-green color attributed to the filamentous fungus *Penicillium roqueforti* (Cantor et al., 2004). Various countries produce blue cheeses, each with a unique size and texture, employing different production techniques leading to different products such as Roquefort, Danablue, Gorgonzola, Cabrales, and Stilton (Cantor et al., 2004; Ardö, 2011). Roquefort is produced using raw ewe milk, while Gorgonzola, Stilton, and Danablue utilize pasteurized or thermized cow milk (Desmasures, 2014; Ardö, 2011). On the other hand, Cabrales is made using a mixture of raw cow, sheep, and goat milk (Morales et al., 2018). After coagulation, the curd is molded and drained without external pressure, then salted and ripened for 6 weeks to 6 months at 8–15 °C and 85–95 % humidity (Ardö, 2011). Piercing the curd facilitates air passage, allowing *P. roqueforti* spores to grow within channels, forming characteristic blue veins (Gripon, 2002; Desmasures, 2014). In cases like Cabrales, environmental strains may drive ripening without a specific starter strain (Morales et al., 2018). The presence of *P. roqueforti* not only influences color and texture but also contributes to the cheese flavor through enzymatic activities during ripening (Gripon, 2002).

Turkey also has traditional mold-ripened cheeses similar to blue

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cheeses, notably Tulum and Civil cheeses (Kirtil et al., 2021; Metin, 2018). These cheeses are traditionally encased in goat skins in artisanal facilities or plastic barrels in larger production plants. Civil cheese, renowned for its fibrous texture, originates from eastern Turkey, particularly in Erzurum and nearby regions (Cakmakci et al., 2014; Yildiz et al., 2021). Erzurum Civil cheese, granted Protected Geographical Indication (PGI) status by the Turkish Patent Institute (TPI) (TPI, 2009), is produced using raw skimmed cow milk (Cakmakci et al., 2014). The curd is stirred and heated up to 60–65 °C, kneaded, pulled and hung to form fibers (Cakmakci et al., 2012). Civil cheese, either alone or combined with 25–30 % whey cheese, is salted, placed in barrels, and ripened to produce Kuflu (mold-ripened) Civil cheese (Fig. 1A), also bearing a TPI PGI label (TPI, 2012). The barrels are stored at 8–12 °C for at least 60 days until the spontaneous fungal growth results in the distinctive blue-green color, facilitated by tiny air pockets among the fibers. To date most filamentous fungi isolated from Erzurum Kuflu Civil cheese were identified as *P. roqueforti* (Cakmakci et al., 2012).

The second most commonly enjoyed mold-ripened cheese in Turkey is Tulum cheese. Kuflu Tulum Cheese (Fig. 1B) is produced using raw sheep or goat milk, predominantly in the central Anatolia region, including Konya and Karaman (Hayaloglu and Kirbag, 2007). Unlike Civil cheese, Tulum cheese is processed by gathering the curd in a fabric bag after coagulation, breaking it into pea-sized pieces, salting, and pre-ripening over ten days in the bag (Hayaloglu et al., 2008). Subsequently, the curd is tightly packed into goat skin bags or plastic barrels, and ripened for approximately three months at 6–12 °C with 80–90 % relative humidity. The bags or barrels are then opened, and the cheese is divided into 5–6 cm blocks to encourage natural fungal growth, predominantly *Penicillium* species, including *P. roqueforti* (Hayaloglu and Kirbag, 2007; Kirtil et al., 2021; Seri and Metin, 2021).

Population genetic studies have recently been conducted on *P. roqueforti* isolates, covering various blue cheeses and non-cheese substrates like wood, silage, and human-related environments (Ropars

Fig. 1. Turkish mold-ripened cheese varieties. A. Kuflu Civil cheese. B. Konya Kuflu Tulum cheese. C. Geographical distribution of *Penicillium roqueforti* isolates. Isolate numbers are indicated in parentheses.

et al., 2014; Gillot et al., 2015; Dumas et al., 2020; Crequer et al., 2023). These investigations unveiled five distinct populations: three featuring cheese-originating isolates and two non-cheese populations. Two of the cheese-originating populations, one comprising blue cheese isolates from different countries (the Non-Roquefort population) and another specific to Termignon cheese, harbored totally or partially specific horizontally transferred regions. These regions with as high as ~100 % sequence identity across various *Penicillium* species were uncovered in the genomes of cheese-associated *Penicillium* species, including those used as starters (i.e., *P. roqueforti* and *P. camemberti*), and contaminants (i.e., *P. solitum* and *P. biforme*) in cheese (Cheeseman et al., 2014; Ropars et al., 2015). Notably, the *Wallaby* region spans over 500 kb with more than 250 genes, while *CheesyTer* is approximately 80 kb, encompassing around 40 genes (Cheeseman et al., 2014; Ropars et al., 2015). The remarkable similarity of these regions across species and their distinct nucleotide composition compared to the rest of the genomes strongly suggests recent horizontal transfers of these genomic segments (Cheeseman et al., 2014). Experimental studies have confirmed that the *Wallaby* and *CheesyTer* regions confer faster growth ability and a competitive advantage on cheese (Ropars et al., 2015). The third cheese population in the *P. roqueforti* species primarily includes Roquefort cheese isolates, with neither this population nor the non-cheese populations possessing the mentioned horizontally transferred regions, *Wallaby* and *CheesyTer* (W – C –). Additionally, the populations exhibit variations in allele distribution at the mating type (*MAT*) locus, governing sexual reproduction (Ropars et al., 2014). This locus comprises two alleles or idiomorphs, *MAT1-1* and *MAT1-2*, allowing sexual reproduction between strains with different alleles at the *MAT* locus (Ropars et al., 2012). The W + C+ population predominantly carried *MAT1-2* alleles (91 %), while W – C – isolates were primarily *MAT1-1* (82 %) (Ropars et al., 2014). Furthermore, the *Wallaby* and *CheesyTer* regions were associated with a 174 bp deletion in the polyketide synthase gene, *mpaC*, responsible for mycophenolic acid production, correlating with low or negligible mycophenolic acid production in the isolates (Gillot et al., 2017; Coton et al., 2020; Metin, 2023).

The genetic characteristics and population structure of *P. roqueforti* in Turkish mold-ripened cheeses have not been previously determined. The presence of horizontally transferred regions, such as *Wallaby* and *CheesyTer*, or the *mpaC* gene deletion within Turkish *P. roqueforti* isolates is unknown. Additionally, the *MAT* allele distribution remains unexplored. Given the distinct production practices of Turkish mold-ripened cheeses, which differ from European blue cheeses and do not involve starter cultures, the Turkish *P. roqueforti* population may contribute to global diversity. Thus, this study aimed to identify the mycobiota in Turkish mold-ripened cheeses, including Tulum and Civil varieties; to determine the genetic characteristics, including the presence of *Wallaby* and *CheesyTer*, *MAT* allele distribution, and the potential *mpaC* deletion; to analyze population diversity using microsatellites; and to compare Turkish isolates with global populations through phylogenetic analyses.

2. Material and methods

2.1. Samples

Cheese samples ($n = 30$), including Kuflu Civil from Erzurum ($n = 19$) and Kars (n = 3) and Kuflu Tulum from Konya ($n = 5$), Karaman (n = 1), Divle (n = 1), and Sivas-Zara (n = 1) were collected from local cheesemakers and village bazaars between October 2019 and January 2021. The samples were transferred to the laboratory in a cold chain and analyzed the same day.

2.2. Isolation of fungi

Ten g of each cheese sample was aseptically transferred to stomacher bags and 90 mL of 2 % sodium citrate (Merck, Darmstadt, Germany) buffer was added (Seri and Metin, 2021). The mixture was homogenized for 2 min (Bagmixer 400, Interscience, Saint Nom, France). Serial dilutions were obtained from the cheese homogenates in 1/4 Ringer's solution (Merck) and inoculated on potato dextrose agar (PDA, Merck) plates. The plates were incubated at 25 °C for 5 days (Albenzio et al., 2001). For each cheese sample, at least two isolates with different macroscopic characteristics (e.g., color and texture) were selected. In total, 86 isolates were obtained from 30 cheese samples. Culture stocks were prepared in yeast extract peptone dextrose broth (YPD; 10 g/L yeast extract [Sigma-Aldrich, Saint Louis, Missouri, USA], 20 g/L peptone [Merck], and 20 g/L dextrose [Sigma-Aldrich]) containing 20 % glycerol (Merck) and were kept at −80 °C for long-term storage (Metin, 2020). In addition, 54 *Penicillium roqueforti* isolates previously described (Metin, 2020; Seri and Metin, 2021) were used in this work.

2.3. DNA extraction

Genomic DNA extraction from the isolates was performed as described in Seri and Metin (2021). The purity and quantitation of isolated DNAs were detected with a BioSpec Nanospectrophotometer (Shimadzu, Kyoto, Japan).

2.4. Fingerprinting analysis

To group isolates, fingerprinting analysis was conducted using (GTG) 5 and M13F primers (Table S1, Miot-Sertier and Lonvaud-Funel, 2007; Versalovic et al., 1994). Preparation of the polymerase chain reaction (PCR) mix and the conditions used for PCR were described in Seri and Metin (2021). Amplicons were migrated using the Wide Mini-Sub Cell GT gel electrophoresis (Biorad, California, USA) at 35 V for five h on a 0.8 % agarose gel with Red Safe (Intron Biotechnology, Gyeonggi, South Korea) nucleic acid staining solution and visualized using Azure c300 imaging system (Azure Biosystems, Dublin, CA, USA).

2.5. PCR and sequencing

For species identification, *benA* (beta-tubulin gene) and/or internal transcribed spacer (ITS) region was amplified using the primers Bt2A and Bt2B, and ITS1 and ITS4, respectively (Table S1, White et al., 1990; Glass and Donaldson, 1995). The PCR mix consisted of 2.5 μL of 10× Taq Buffer (Thermo Fisher Scientific, Massachusetts, USA), 2.5 mM of $MgCl_2$ (Thermo Fisher Scientific), 0.2 mM of dNTP mix (Thermo Fisher Scientific), 1 μL of 10 mM forward primer, 1 μL of 10 mM reverse primer, ~50 ng template DNA, 2 U Dream Taq DNA polymerase (Thermo Fisher Scientific), and water added to a volume of 25 μL. PCR amplicons were run on a 1 % agarose gel at 85 V for 45 min. The amplicons were purified using the Invitrogen PureLink PCR purification kit (Thermo Fisher Scientific) and submitted to MedSantek company (Istanbul, Turkey) for Sanger sequencing analysis. Sequences were analyzed using NCBI BLAST (Altschul et al., 1990).

The presence of the *Wallaby* and *CheesyTer* regions was investigated using PCR with the primers given in Table S1, according to Ropars et al. (2015). The mating genotype of the *P. roqueforti* isolates was determined with the PCR primers (MAT1-1F and MAT1-1R, and MAT1-2F and MAT1-2R) designed using the Primer 3 software (Table S1, Untergasser et al., 2012) to amplify either *MAT1-1* or *MAT1-2*, PCR was conducted as explained for species identification. The 174 bp gene deletion in the *mpaC* gene of the *P. roqueforti* isolates, PCR was performed using the MPACDEL_F and MPACDEL_R primers (Table S1) as described in Gillot et al. (2017). Using these primers, the mentioned deletion results in a 67 bp PCR product, whereas a 241 bp PCR product is obtained when there is no deletion.

Diversity of *P. roqueforti* isolates was determined using three microsatellite regions (*proq16*, *proq01_3*, and *proq02_2*) for all *P. roqueforti* isolates and five polymorphic loci (*benA*, *cmd*, *proq235*, *proq631*, *proq845*) for 20 selected isolates. These loci were chosen based on the study by Gillot et al. (2015). These regions were amplified by PCR, as

Table 1
Filamentous fungal species identified in Turkish mold-ripened cheeses.

Cheese type	Region	Number of cheese samples	Fungal species
Kuflu Civil	Erzurum	19	*Penicillium roqueforti* (*n* = 42) *Penicillium paneum* (*n* = 2) *Penicillium biforme* (n = 1) *Penicillium brevicompactum* (n = 1) *Alternaria* sp. (n = 1) *Talaromyces* sp. (n = 1)
	Kars	3	*Penicillium roqueforti* (n = 8)
Kuflu Tulum	Sivas-Zara	1	*Penicillium roqueforti* (n = 2) *Albifimbria verrucaria* (n = 1) *Cladosporium sphaerospermum* (n = 1)
	Konya	5	*Penicillium roqueforti* (*n* = 10) *Penicillium crustosum* (*n* = 3) *Penicillium biforme* (n = 1)
	Karaman	1	*Penicillium roqueforti* (n = 2) *Penicillium roseomaculatum* (n = 1)
	Divle	1	*Penicillium roqueforti* (n = 2) *Penicillium nordicum* (n = 2) *Penicillium biforme* (n = 1)

explained in the species identification section with the primers given in Table S1.

2.6. Population genetic analyses

Microsatellite (*proq01_3*, *proq02_2*, *proq16*) genotypes were analyzed using GenAlEx (version 6.502) (Peakall and Smouse, 2012) to determine the diversity measures, allele frequencies, the effective number of alleles, and unbiased genotypic diversity (h_u) (Nei, 1978). To assess allelic associations, the index of association (I_A) was determined as described by Maynard Smith et al. (1993). If there is no association between loci, I_A is expected to be 0 (Xu, 2006). Whether I_A is significantly different from 0 (error rate: 0.01) was determined using a randomized data set with 999 permutations using GenAlEx.

Microsatellites data were used to analyze the population structure using a Bayesian clustering approach in Structure (version 2.3.4) software (Pritchard et al., 2000). Ten independent analyses were performed for each number of clusters (from K = 1 to K = 10), with 50,000 step preparation times and 500,000 MCMC iterations. The best K value was determined by uploading the results to the Structure Harvester (version v0.6.94, http://taylor0.biology.ucla.edu/structureHarvester/) using the Evanno method (Earl and vonHoldt, 2012; Evanno et al., 2005). The population structure was also analyzed in Splitstree (version 4.18.3) (Huson and Bryant, 2006) using the distance matrix created by GenAlEx based on the microsatellite data.

2.7. Phylogenetic analyses

Twenty representative isolates were chosen from the population for phylogenetic analysis using the Splitstree diagram. Sequences from five polymorphic loci (*cmd, benA*, *proq235*, *proq631*, and *proq845*) were aligned with corresponding sequences from the NCBI database using Clustal W. Maximum likelihood method and Kimura 2-parameter model were employed for phylogenetic analyses in Mega X (version 10.2.6) software (Kimura, 1980; Kumar et al., 2018). A concatenated tree was generated using *proq235*, *proq631* and *proq845 loci*, as *benA* and *cmd* sequences were excluded due to significant incongruence with *proq235*, *proq631* and *proq845* according to the incongruence length difference (ILD) test (Gillot et al., 2015).

3. Results and discussion

3.1. Filamentous mycobiota of Turkish traditional mold-ripened cheeses

Eighty-six fungal isolates were obtained from 30 cheese samples across Anatolian regions (Tables 1, S2). Initial grouping utilized (GTG)5 and M13F primers for fingerprinting analysis. Different banding patterns led to further identification using beta-tubulin (*benA*) sequencing, with additional ITS sequencing for certain species like *Cladosporium* and *Alternaria* isolates (Table S2). Both primer sets exhibited consistent species-specific banding patterns, aiding preliminary grouping. Predominantly, *P. roqueforti* comprised 66 isolates (76.6 %), with other *Penicillium* species including *P. biforme* (3.5 %), *P. crustosum* (3.5 %), *P. paneum* (2.3 %), *P. nordicum* (2.3 %), *P. brevicompactum* (1.2 %), and *P. roseomaculatum* (1.2 %) (Tables 1, S2). Additional identified fungi corresponded to *Cladosporium sphaerospermum* (1.2 %), *Albifimbria verrucaria* (1.2 %), *Alternaria* sp. (1.2 %), and a *Talaromyces* sp. (1.2 %). The latter is potentially a new species as a 94.2 % *benA* identity was found (Tables 1, S2). Identified yeast species included *Kluyveromyces lactis* (2.3 %) and the filamentous yeast *Geotrichum silvicola* (2.3 %).

In Turkey, mold-ripened cheeses are produced without the use of fungal starter cultures. Therefore, the fungal microbiota is spontaneous and arises from the environment where the cheeses are ripened. While *P. roqueforti* stands out as the predominant filamentous fungal species in Turkish mold-ripened cheeses, some other *Penicillium* species, including *P. biforme*, *P. crustosum*, *P. expansum*, *P. verrucosum*, *P. polonicum*, *P. chrysogenum*, *P. solitum*, *P. corylophilum*, *P. spinulosum*, *P. rubens*, and *P. brevicompactum* have also been identified (Hayaloglu and Kirbag, 2007; Sagdic et al., 2008; Cakmakci et al., 2012; Ozturkoglu-Budak et al., 2016; Metin, 2020; Ertas-Onmaz et al., 2021; Kirtil et al., 2021; Seri and Metin, 2021). Filamentous fungal genera other than *Penicillium* have also been observed in these cheeses, such as *Mucor*, *Scopulariopsis*, *Rhizopus*, *Alternaria*, *Aspergillus*, *Trichoderma*, and *Cladosporium* (Hayaloglu and Kirbag, 2007; Ozturkoglu-Budak et al., 2016; Seri and Metin, 2021).

3.2. Genetic characteristics and diversity of the *Penicillium roqueforti* isolates from Turkish mold-ripened cheeses

To analyze the characteristics and diversity of *P. roqueforti* isolates from Turkish mold-ripened cheeses, we used 120 isolates: 66 from this study, 53 from Konya Kuflu Tulum cheese samples (Seri and Metin, 2021), and one from Kuflu Golot cheese (Rize-Ardesen) (Metin, 2020). All isolates are listed in Table S3, and their geographical distribution is shown in Fig. 1C.

3.2.1. Genetic characteristics of the *P. roqueforti* isolates

The horizontal gene transfer regions, *Wallaby* and *CheesyTer*, were found in all 120 *P. roqueforti* isolates from Turkish mold-ripened cheeses, with 95 % of isolates carrying the *MAT1-2* allele (Table S3). These characteristics align the Turkish isolates with the W + C+ *P. roqueforti* population observed in blue cheeses (Dumas et al., 2020; Ropars et al.,

Table 2
Diversity measures of the *P. roqueforti* population isolated from Turkish mold-ripened cheeses.

Population	Locus	N[1]	N_a[2]	N_e[3]	h_u[4]
Overall	Proq01_3	120	6	1.505	0.338
	Proq02_2	120	19	5.517	0.826
	Proq16	120	7	2.510	0.607
	Mean ± standard error		10.7 ± 4.2	3.177 ± 1.205	0.590 ± 0.141
Kuflu Tulum	Proq01_3	69	6	1.498	0.337
	Proq02_2	69	16	6.425	0.857
	Proq16	69	7	3.058	0.683
	Mean ± standard error		9.667 ± 3.180^{A}	3.660 ± 1.454^{A}	0.626 ± 0.153^{A}
Kuflu Civil	Proq01_3	50	5	1.460	0.322
	Proq02_2	50	14	3.994	0.765
	Proq16	50	3	1.753	0.438
	Mean ± standard error		8.500 ± 1.979^{A}	3.031 ± 0.794^{A}	0.567 ± 0.094^{A}

The superscript letters represent whether the values are significantly different, as determined by a Student *t*-test.

[1] N = Sample size.

[2] N_a = Number of different alleles.

[3] N_e = Effective number of alleles (1/ [Σ p_i^2]), p_i = the frequency of allele i.

[4] h_u = Unbiased genetic diversity = (N/ (N-1)) * (1 - Σ p_i^2).

2014). However, unlike the blue cheese isolates, most Turkish isolates (114/120) did not exhibit the *mpaC* gene deletion associated with *Wallaby* and *CheesyTer* presence in the non-Roquefort population (Gillot et al., 2017; Crequer et al., 2023). The remaining six isolates did not lead to a PCR product, possibly due to polymorphisms in the primer binding sites or the absence of the *mpaC* gene in those isolates. Harboring the *Wallaby* and *CheesyTer* regions but lacking the 174 bp deletion in the *mpaC* gene, Turkey isolates differ from the blue cheese isolate group studied by Gillot et al. (2017) but resemble the Termignon isolates (Crequer et al., 2023).

An interesting finding is that both *MAT1-1* and *MAT1-2* isolates coexisted in three cheese samples—Erzurum Kuflu Civil-13, Sivas-Zara Kuflu Tulum-19, and Konya Kuflu Tulum-29— (Table S3). Similar findings were reported by Ropars et al. (2012), who isolated both mating types from a single cheese originating from various countries. The presence of opposite mating types within these cheeses suggests the potential for sexual reproduction of *P. roqueforti* in the cheese environment. Although neither Ropars et al. (2012) nor our study observed sexual structures on the cheese samples, recent research has shown that *P. roqueforti* is capable of sexual reproduction (Ropars et al., 2014). The coexistence of different mating types on cheese indicates that, at the very least, conditions conducive to bringing different mating types together exist during cheese production.

3.2.2. Population structure of P. roqueforti

Turkish *P. roqueforti* isolates were assessed for population structure using three microsatellites—*proq01_3*, *proq02_2*, and *proq16*—developed by Gillot et al. (2015). Genetic diversity measures, including the number of different alleles, effective alleles, and Nei's unbiased genetic diversity (Nei, 1978; Xu, 2006), were calculated using GenAlEx 6.5 (Peakall and Smouse, 2012) (Table 2). The highest genetic diversity (h_u = 0.826) was observed at the *proq02_2* locus, as well as the number of alleles (N_a = 19) and the number of effective alleles (N_e = 5.517). The number of effective alleles changed between 1.505 and 5.517. Considering all three loci, the mean genetic diversity was found to be 0.590 (±0.141) (Table 2). Analysis of *P. roqueforti* isolates from Kuflu Tulum and Kuflu Civil cheeses separately revealed no significant differences in allele counts, effective alleles, or unbiased genetic diversity, as confirmed by Student *t*-tests at a 95 % confidence interval (t-values: 0.54, 0.66, and 0.60, respectively).

Each microsatellite allele combination represents a specific haplotype (HT) (Table S3). Notably, multiple HTs of *P. roqueforti* were often observed within the same cheese. For instance, Erzurum Kuflu Civil cheese sample 1 harbored strains with two different HTs, HT4 and HT22, while nineteen cheese samples contained isolates of two different HTs (Table S3). Interestingly, some samples, such as Erzurum Kuflu Civil cheese samples 7, 13, and 14, and Konya Kuflu cheese sample 27, contained strains with three different HTs (Table S3). This diversity reflects the natural introduction of fungi from the environment into cheese ripening places, as Turkish mold-ripened cheeses typically rely on spontaneous fungal growth rather than starter cultures. Consequently, various strains thrive on the cheeses, with some samples even containing strains with opposite mating genotypes.

The microsatellite analysis identified 36 HTs among 120 *P. roqueforti* isolates (Table S3). HT2 was the most prevalent, representing 35 % of the population with 42 isolates, followed by HT10 (10 %), HT5 (7 %), HT1 (5 %), and HT21 (3 %) (Table S3). Together, these five HTs represented 60 % of all isolates, while 19 HTs comprised only one isolate each (Table S3). Gillot et al. (2015) detected 28 HTs among 164 *P. roqueforti* isolates originating from different blue cheeses and non-cheese samples using four microsatellites (*proq01_3*, *proq02_2*, and *proq16*, the ones used in the present study, and *proq17*). While only 13 HTs were identified using 140 blue cheese isolates, 15 HTs were detected in 24 non-cheese samples. The greater number of HTs in the present study could be because *P. roqueforti* starter cultures are not used in Turkish mold-ripened cheeses.

To evaluate the population structure, microsatellite allele profiles were analyzed using Structure software, running ten independent analyses with varying cluster numbers (K) from 1 to 10. The optimal K value, determined using Structure Harvester, was K = 2 (Fig. 2A), indicating two genetic clusters based on allele profiles (Fig. 2B). All *six MAT1-1* isolates clustered in group 1, while 66.0 % of Kuflu Civil isolates and 66.7 % of Kuflu Tulum isolates, along with the Golot cheese isolate, were grouped in cluster 1. However, distinct clustering based on cheese type was not evident.

Although Tulum and Civil cheeses exhibit significant differences in processing technology, texture, areas of production, and raw milk sources, genetic diversity measures and HT distribution of the *P. roqueforti* isolates from these cheese types did not indicate a clear distinction. This might be due to the introduction of strains from common sources to the raw materials, such as milk, and to the production and ripening locations. Additionally, the similarity of environmental conditions, including humidity and temperature, might cause similar strains to thrive on the cheeses. The strains might also be exchanged during transportation and trading.

To further investigate the population structure, a distance matrix was generated using microsatellite data via GenAlEx 6.5. This matrix was then utilized to construct a diagram using the Splitstree program (Huson and Bryant, 2006) (Fig. 3). The two clusters obtained using Structure also stand out in the Splitstree diagram. Notably, a primarily clonal structure is observed, especially evident in the clonal branches formed by prevalent HTs like HT2 and HT10. However, regions resembling

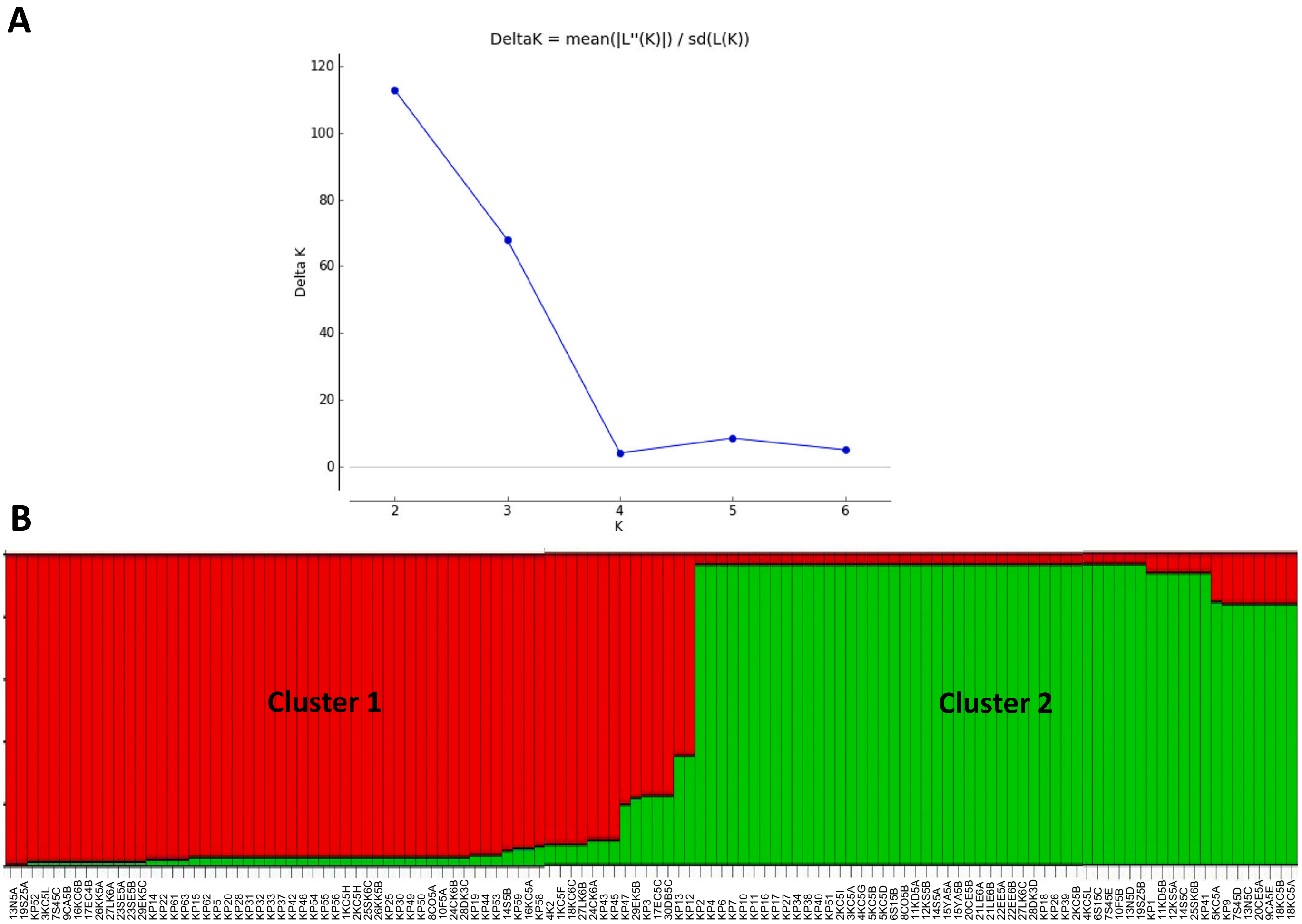

Fig. 2. The population structure of *Penicillium roqueforti* isolates originating from Turkey. (A) The optimal number of clusters (K value) determined using Structure Harvester, according to Evanno's method. (B) The population structure of *Penicillium roqueforti* isolates originating from Turkey inferred using a Bayesian clustering approach in the Structure software with isolate names.

spider webs suggest limited recombination (Fig. 3).

In haploid microorganisms, detecting recombination and clonality in natural populations involves examining the association between alleles at different loci. The index of association (I_A) is a common parameter used for this purpose in haploid organisms. An I_A of 0 indicates no linkage between loci (Xu, 2006), but in the Turkish *P. roqueforti* population, the I_A was calculated to be 0.563, significantly different from 0 (error rate: 0.01). This suggests that the population significantly deviates from random mating. Since fungi can reproduce asexually via mitosis, a clonal structure is expected in natural populations (Xu, 2006), particularly in medically or industrially important fungi.

3.2.3. Phylogenetic analyses of the P. roqueforti strains

To compare the *P. roqueforti* isolates of Turkey origin with the world population; first, 20 isolates highlighted in red in the Splitstree diagram (Fig. 3) prioritizing the most commonly observed HTs were selected. Then, the sequences of the widely used fungal identification markers, *benA*, and the calmodulin gene, *cmd*, as well as a set of three polymorphic markers defined for *P. roqueforti*, *proq235*, *proq631*, and *proq845* (Gillot et al., 2015), were used in the phylogenetic analyses. *BenA* and *cmd* phylogenetic trees were initially constructed with all available sequences in the NCBI database. Then, to simplify, the trees were presented with a reduced number of isolates, comprising those from Turkey ($n = 20$) and those with available sequences for the loci *proq235*, *proq631*, and *proq845* ($n = 21$) (Fig. 5). The remaining isolates were denoted in each phylogenetic group with the respective number of isolates in that specific group. The presence or absence of *Wallaby* and *CheesyTer*, the mating genotype, and the *mpaC* deletion status were also indicated whenever this information was available. The sequence IDs for the isolates from Turkey submitted to NCBI and the NCBI IDs of other isolates worldwide used for the phylogenetic analyses are provided in Tables S4 and S5, respectively.

Using *benA* sequences of 20 selected Turkish isolates and those from the NCBI database ($n = 275$), a phylogenetic tree divides *P. roqueforti* isolates into three main groups (Fig. 4A). One group (clade 2, marked in green) comprised solely non-cheese isolates, except for isolate 26KK5A from Karaman Tulum cheese. The latter strain might originate from a non-cheese source and was transmitted to the cheese sample from Karaman. Clades 1 (blue) and 3 (yellow) included isolates from both cheese and non-cheese substrates (Fig. 4A). Turkish isolates, excluding 26KK5A, were nested in clades 1 and 3. The two most common HTs, HT2 and HT10, were found in clade 3.

In the phylogenetic tree constructed using *cmd* sequences, isolates from the NCBI database ($n = 196$) formed four groups (Fig. 4B). Three groups (clades 1, 2, and 4) include isolates from both cheese and non-cheese sources, while clade 3 only contains non-cheese isolates. The Turkish isolates were placed in clades 1 and 2. Notably, 26KK5A was positioned near clade 2 but is distinct, as evidenced by its long branch (Fig. 4B).

The markers *proq235*, *proq631*, and *proq845* demonstrated higher polymorphism compared to *benA* and *cmd*, with *proq631* and *proq845* distinctly separating non-cheese isolates. Phylogenetic analysis of

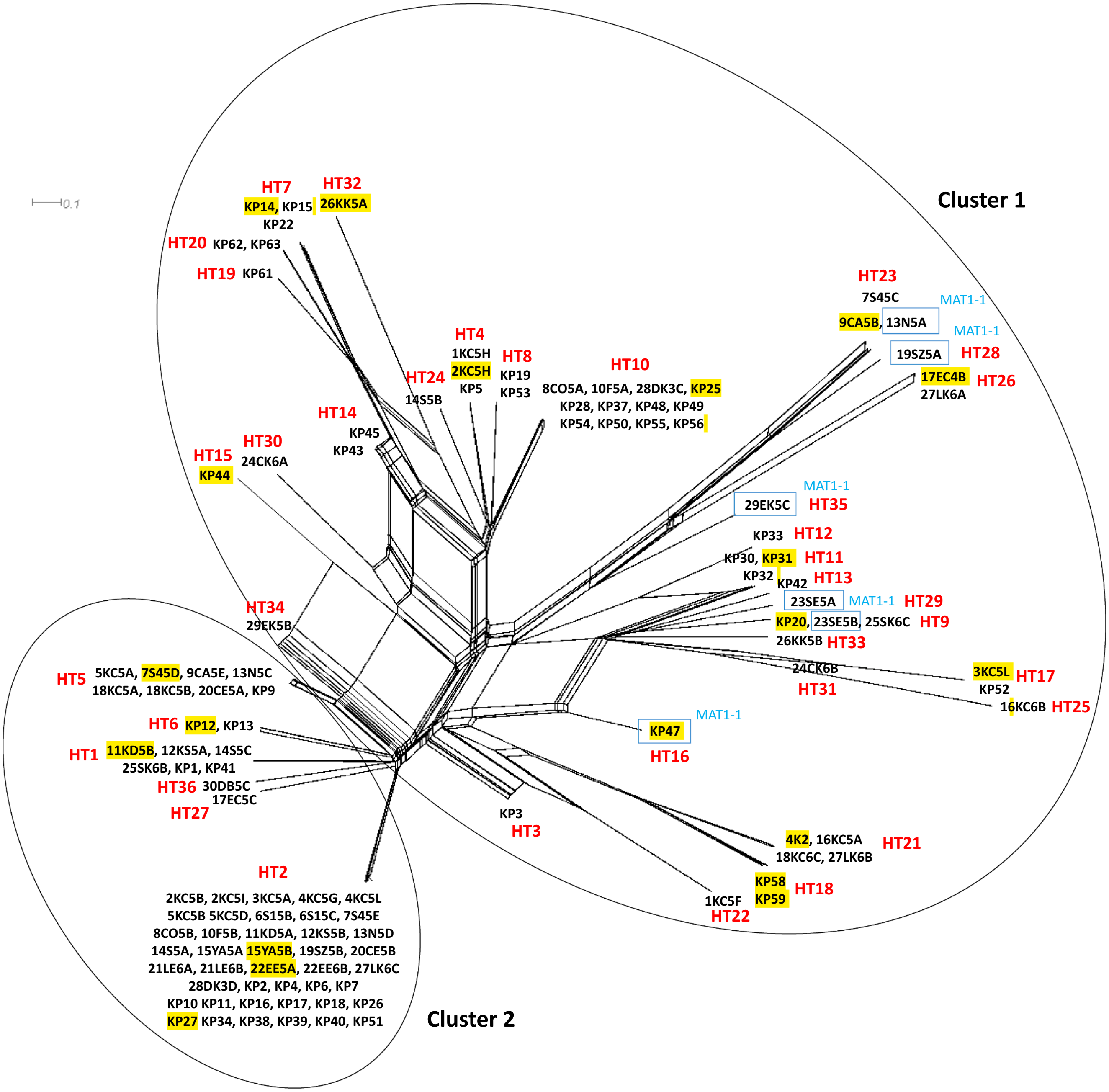

Fig. 3. The Splitstree diagram illustrating *Penicillium roqueforti* isolates from Turkey ($n = 120$). The 36 haplotypes (HTs) are highlighted in red, while rare *MAT1-1* isolates are enclosed in blue rectangles. Two clusters, obtained using Structure software, are indicated by big ovals. Isolates highlighted in yellow were selected for further phylogenetic analyses.

concatenated sequences revealed two main groups, each with subgroups (Fig. 5). Turkish blue cheese isolates were dispersed across three clades (1A, 1C, and 2A), with no discernible correlation to cheese type, Tulum or Civil. According to whole-genome analysis by Crequer et al. (2023), *P. roqueforti* populations were classified into five populations: Non-Roquefort, consisting of strains isolated from blue cheeses in various countries; Termignon, comprising isolates obtained from Termignon blue cheese produced through spontaneous fungal colonization; Roquefort, predominantly Roquefort isolates; and two non-cheese populations, silage/food spoilers and wood/food spoilers. Asterisks in Fig. 5 denote groups aligning with those Crequer et al. (2023) defined. Using the markers *proq235*, *proq631*, and *proq845*, Termignon isolates did not form a separate clade; instead, three of the four analyzed isolates aligned with the Non-Roquefort group (1A) which may be explained by the difference of methods used (microsatellites vs whole genome single nucleotide polymorphism).

Most Turkish isolates, including common HTs like HT2 and HT10, clustered in clade 1A, aligning with the Non-Roquefort and Termignon groups. All clade 1A isolates harbor one or both horizontally transferred regions, with a predominant *MAT1-2* mating type. Turkish isolates were similar to the Termignon isolates in that they did not bear the *mpaC* gene deletion in their genome, unlike nearly all other blue cheese isolates in the same clade, where this deletion is present in all but one isolate (F36-1). This difference may stem from the use a reduced number of starter in various countries due to commercial access.

Three Turkish cheese isolates were assigned to clade 1C (Fig. 5) along with one Termignon cheese isolate, while the sister clade, clade 1B, included two W—C— non-cheese isolates with a different origin

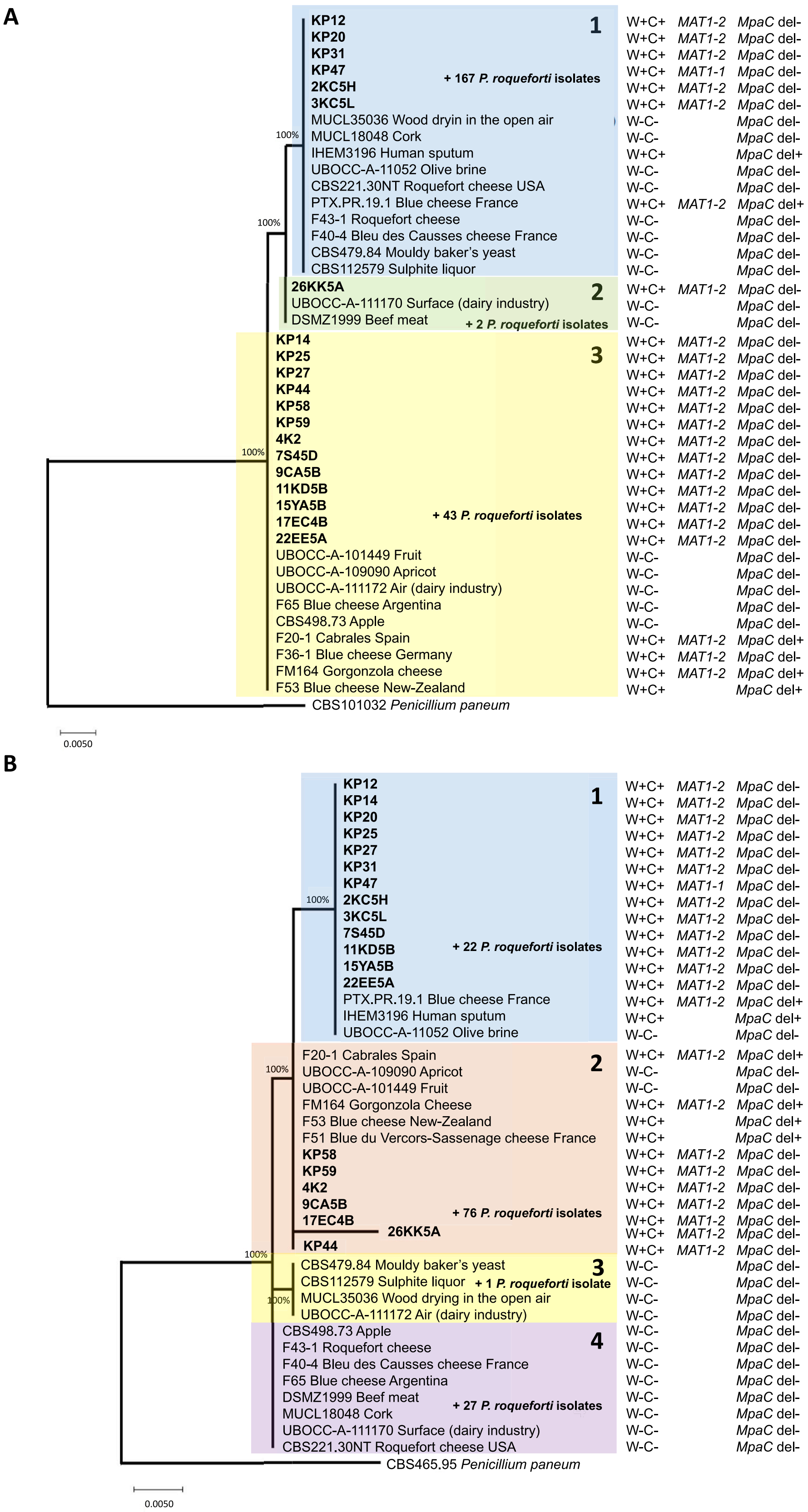

Fig. 4. Maximum likelihood phylogenetic analysis of the *Penicillium roqueforti* isolates using *benA* (A) and *cmd* (B) sequences. (A) The tree with the highest log likelihood (−673.52) for *benA* analysis is displayed, covering 393 positions in the final dataset. P*enicillium paneum* CBS101032 (KM503670.1) was used as the root. (B) For *cmd* sequences, the tree with the highest log likelihood (−791.75) is presented, comprising 465 positions in the final dataset. *Penicillium paneum* CBS465.95 (HQ442334.1) was used as the root. W + C+/W-C-: presence/absence of *Wallaby* and *CheesyTer* loci.

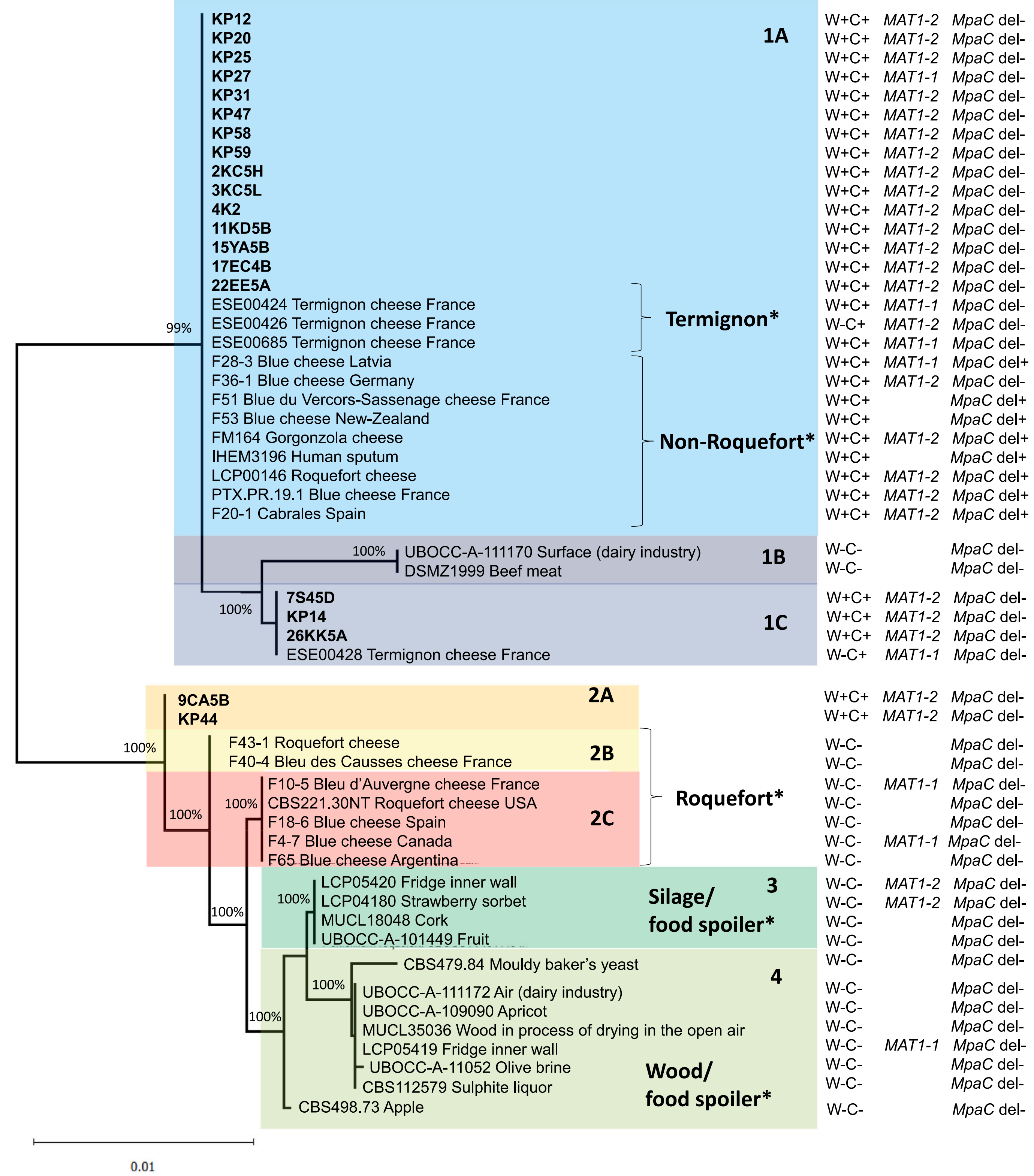

Fig. 5. Maximum likelihood phylogenetic analysis of *Penicillium roqueforti* isolates using the concatenated dataset of *proq235*, *proq631*, and *proq845* loci. The tree with the highest log likelihood (−4829.14) is displayed, encompassing a total of 2947 positions in the final dataset. W + C+/W-C-: presence/absence of *Wallaby* and *CheesyTer* loci. Asterisks indicate the groups defined by Crequer et al. (2023).

compared to other clade 1 subgroups. Whether Turkish isolates in clade1A and 1C correspond to the Termignon cheese group or form distinct groups needs further research with a more comprehensive comparison using whole genomes.

In the second main group in Fig. 5, the clades 2B and 2C constituted the Roquefort group, while clades 3 and 4 corresponded to the non-cheese groups, namely the silage/food spoiler, and wood/food spoiler groups, respectively. Isolates 9CA5B and KP44 formed a separate clade, different from the Roquefort and food spoiler groups in that they are W + C+, while the others are W – C –. These isolates apparently form a separate clade, unlike other groups.

4. Conclusion

This study aimed to determine the genetic characteristics and population structure of *P. roqueforti* isolates from Turkish mold-ripened cheeses, with a focus on the predominant varieties, Tulum and Civil. Using 8 Tulum and 22 Civil cheese samples, we explored the fungal diversity, with *P. roqueforti* comprising 76.6 % of isolates. Next, we determined the genetic characteristics and population structure of Turkey-originating *P. roqueforti* using these isolates ($n = 66$) and strains that we previously isolated from Konya Kuflu Tulum ($n = 53$) and Golot cheese ($n = 1$) samples. All 120 isolates harbored horizontal transfer regions *Wallaby* and *CheeseTer*. The primary mating genotype was *MAT1-2* (95 %). In three cheese samples, isolates with opposite mating genotypes coexisted, demonstrating that the first requirement for sexual reproduction—opposite mating types coming in close proximity—naturally occurs in cheeses produced in Turkey using spontaneous mold-ripening. Thirty-six haplotypes (HT) were identified, often coexisting within the same cheese sample, suggesting spontaneous ripening. The genetic diversity measures and HT distribution were not significantly different between isolates from Tulum and Civil cheeses, indicating Tulum and Civil cheeses do not represent distinct subpopulations. Maximum likelihood phylogenetic analysis of the concatenated sequences of the most polymorphic loci—*proq235*, *proq631*, and *proq845*—separated the Turkish isolates into three clades, with one clade shared with Non-Roquefort and Termignon blue cheese samples. The Turkish isolates might be distinct from other known populations of *P. roqueforti*, a possibility that could be elucidated in future studies by more comprehensive analyses using whole genomes. To our knowledge, this study is the first to analyze the genetic diversity and population structure of *P. roqueforti* isolates originating from Turkish mold-ripened cheeses and contributes to the information on the global diversity of *P. roqueforti*.

CRediT authorship contribution statement

Hatice Ebrar Kirtil: Writing – original draft, Visualization, Investigation, Formal analysis, Data curation. **Aysenur Orakci:** Investigation. **Muhammet Arici:** Writing – review & editing. **Banu Metin:** Writing – review & editing, Supervision, Project administration, Methodology, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no conflicts of interest.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijfoodmicro.2024.110801.

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