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Non-*Saccharomyces* yeasts diversity on grape berries and molecular characterization of *Starmerella bacillaris* yeasts strains having high invertase activity

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The diversity of yeast species on grape berries changes depending on various factors. Determination of indigenous yeast intensity and diversity on grape berries is important for increasing the sensory characteristics of wine as well as fermentation efficiency. The natural yeast biota of grape berries affects wine flavour and quality by producing some secondary metabolites and hydrolytic enzymes. Despite the application of different nonconventional yeasts in food and fermentation industries, many significant researches are conducted in finding and improving the new strains having industrially important enzyme activities. Invertase enzyme has a vital role in the food industry in which it increases the sweetness of food without crystallizing them. Here, we studied yeast diversity in grape berries from selected localities and also their invertase activity. We collected grape berries from Alphonse, Kınalı Yapıncak, Çavuş, Efes Karası, Cinsaut, Atasarısı and Isabella grape varieties cultivated in Bozcaada island and Gelibolu peninsula. Twenty-one yeast species belonging to seven genera were identified. The yeast strains having high invertase activities were identified with 5.8S-ITS rDNA sequencing technique. The diversity of yeast biota on berries collected from Gelibolu was greater than that of Bozcaada. *Metschnikowia pulcherrima*, *Cryptococcus laurentii and Rhodotorula glutinis* yeast species were dominant yeast species on grape berries. A total of 294 sucrose grown yeast strains showed growth on sucrose, and 19 of them exhibit the highest invertase activity that is not glucose repressible. These 19 yeast strains were identified as *Starmerella bacillaris* using 5.8S ITS rDNA region and the phylogenetic analysis was inferred with the Maximum Parsimony method.

Keywords: 5.8S rDNA, *Vitis vinifera* cultivars, Wine fermentation

Yeast can be found in many different habitats in nature. In specialized habitats, various yeast species occupy the same habitat and coexist together mutually or competitively. Grape berry surface is a good example to these kinds of habitats. The indigenous yeast population on grape berries that is important for wine fermentation, can be affected by many different factors such as grape variety, geographical location of vineyard and climatological conditions, pesticide handling[1-5]. In addition, yeast diversity and intensity can be changeable within the bunch of grapes depending on the degree of maturity at harvest and damage to grape berries[6]. Therefore, identification of indigenous yeast diversity on grape berries is important for wine fermentation efficiency and the developing of the wine organoleptic profile[7-9]. Studies on the diversity of non-*Saccharomyces* yeasts associated with grape varieties and wine fermentation stages are not uncommon[10-12]. It is well known that during the fermentation process some non-*Saccharomyces* yeast species decrease ethanol production and increase fermentative efficiency than *Saccharomyces cerevisiae*[13]. In addition, different enzymes produced by non-*Saccharomyces* yeast can develop the wine sensory quality[14-16].

Non-*Saccharomyces* yeasts have hydrolytic enzyme activities such as glucosidases, lipases, proteases that cause to formation of volatile and non-volatile by-products, also important for food technology and other industries[16-18]. One of the major industrial enzymes derived from yeast is invertase (EC3.2.1.26) which catalyzes the breakdown of sucrose into glucose and fructose, inverted sugar syrup. *Saccharomyces cerevisiae* is the main yeast species used for the production of invertase commercially. In *S. cerevisiae*, the *SUC2* gene encoded for invertase is repressed in the presence of glucose (i.e. glucose repression) and derepressed when glucose is exhausted (i.e. glucose derepression)[19]. The released glucose during the breakdown of sucrose or raffinose, surprisingly, can cause glucose repression in *S. cerevisiae*. In this context, non-*Saccharomyces* yeast species having high unrepressible invertase activity gain importance.

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Here, we examined yeast biota on grape berry surfaces and tried to identify the yeast species having high invertase activities. The yeast diversity on five grape varieties collected from two different vineyards were determined and 19 yeast strains showed high invertase activity that is not repressible.

Materials and Methods

Grape sampling

Grape (*Vitis vinifera* L.) samples were collected from five vineyards located in two different districts in Çanakkale, Turkey, during harvest (between August and October) in 2009 vintage. Alphonse, Kınalı Yapıncak, Çavuş, Efes Karası, and Cinsaut grape varieties were collected from 5 different vineyards in Bozcaada island (39° 50' 6" N 26° 4' 10" E). Isabella, Atasarısı, and Çavuş grape varieties were collected from the same vineyard in Gelibolu peninsula (40° 51' 50" N 26° 37' 20" E). From each vineyard, 50 g of healthy grape samples were randomly and aseptically collected. Samples were transported in cold boxes to the laboratory and analyzed within 24 h of harvest from the vineyard.

Yeast isolation, selection, and identification of non-*Saccharomyces* yeast isolates

About 10 g of each grape sample was aseptically homogenized in 100 mL of distilled water. Homogenates were serially diluted with sterile distilled water and 100 μL from each dilution was plated in duplicate on YGC medium (5 g/L yeast extract, 20 g/L glucose, 0.1 g/L chloramphenicol, 14.9 g/L agar) supplemented with 0.1% sodium propionate. After incubation at 30 °C for 3 days, the colonies were counted out in duplicates. According to colony morphology and frequency, yeast colonies were isolated and restreaked on YPD (10 g/L yeast extract, 20 g/L peptone, 20 g/L glucose) medium for purification. All isolated yeast strains were stored at 4°C on YPD slants and also −80°C for future analysis.

For differentiation of the non-*Saccharomyces* yeasts strains, all yeast isolates were streaked on lysine medium (Oxoid, Hampshire, UK) and incubated at 30°C for 3 days. The yeast isolates showing growth in lysine medium were assigned as non-*Saccharomyces*[20]. API ID 32 C yeast identification kit system (BioMe´rieux, France) and ID 32 C V3.0 database were used to identify non-*Saccharomyces* yeasts strains depending on the manufacturer's suggestions.

Screening of sucrose positive yeasts and determining invertase activities

The ability of non-*Saccharomyces* yeast isolates to hydrolyze sucrose was tested using YP medium (10 g/L yeast extract, 20 g/L peptone) supplemented with either 20 g/L sucrose or 20 g/L raffinose. Sucrose positive phenotypes were evidenced by growth on both YP-Suc and YP-Raf plates. *Kluyveromyces lactis* (ATCC8585) and *Saccharomyces cerevisiae* (BY4741) yeast strains were used as the standard control strains in our experiments.

The secreted invertase activities of all sucrose positive yeast isolates were pre-cultured and grown up on a fresh YPD medium as described previously[21]. After that, the secreted invertase activities of the sucrose positive yeast were determined using whole cells as previously suggested by Rothe and Lehle[22]. The released glucose was measured with a glucose oxidase assay kit (BIOCON, Germany). Invertase activities were expressed in µmoles of glucose liberated per minute per 100 mg dry wt. of yeast cells at 37°C[21]. All experiments were repeated three times under the same conditions and standard deviations were less than 10%.

DNA extraction and PCR amplification

The genomic DNA of 19 yeast strains having high invertase activity was isolated according to the protocol described elsewhere and stored at -20°C until further use[23]. The DNA amplifications were performed in BIO-RAD Thermal Cycler. The PCR reactions were carried out in a 25 µL reaction volume containing a Master Mix of PCR Amplification kit (Fermentas, K0171). For amplification of the 5.8S-ITS rDNA region, ITS1 (5′-TCCGTAGGTGAACCT GCGG-3′) and ITS4 (5′-TCCTCCGCTTATTGATAT GC-3′) primers were used[24]. Gel-Pro Analyzer was used for estimating fragment lengths corresponding to the bands of the lane.

Sequencing and data analysis

The PCR products of 5.8S ITS rDNA regions were sequenced using ITS1 or ITS4 primers after being purified with a PCR purification kit (Fermentas, China). Applied Biotechnologies 3500xl Genetic Analyzer (Applied Biosystems, Foster City, USA) was used for sequencing and editing DNA sequences. The obtained ITS1-5.8S-ITS2 sequences were compared with sequences presented in the GenBank database available at the National Center for Biotechnology Information (NCBI) using the basic local alignment search tool (BLAST). Sequences with

98% nucleotide identity or higher were considered to represent the same species.

The haplotype analysis of ITS1-5.8S-ITS2 rDNA gene sequences was performed using the DnaSP v6.12.01 program. Two *S. bacillaris* strains (CBS: 2799, Accession No: KY102526.1 and CBS: 9494, Accession No: KY102524.1) from Westerdijk Fungal Bio-Diversity Institute CBS-KNAW (Holland) yeast collection were used in analyzes as a reference yeast strains[25]. All sequences of *S. bacillaris* yeast strains were aligned according to the ClustalW 1.6 parameter found in the MEGA X program[26]. ITS1-5.8S-ITS2 rDNA gene sequence of *S. bacillaris* (CBS: 2799) yeast strain was used for trimming the leading and trailing characters of the sequences, and trimmed sequences were recorded as mega files.

Polymorphic regions (S), nucleotide (π) and haplotype diversity (h), and nucleotide differences of the sequences were calculated using the DnaSP program[27-29]. To infer phylogenetic relationships among *S. bacillaris* yeast taxa, Maximum Parsimony (MP) method was performed by using MEGA X[26]. *S. cerevisiae* (Accession No: MF375633.1) and *Meyerozyma* (*Pichia*) *guilliermondii* (Accession No: AF022717.1) yeast species were used as outgroups. The bootstrap consensus tree inferred from 1000 replicates is taken to represent the evolutionary history. Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches[30]. The MP tree was obtained using the Subtree-Prunning-Regrafting (SPR) algorithm with search level 1 in which the initial trees were obtained by the random addition of sequences (10 replicates)[31].

Nucleotide sequence accession numbers

The partial ITS1-5.8S-ITS2 sequences were submitted to GenBank under accession numbers: KU950227 (B-19), KU950228 (B-21B), KU950229 (B-23C), KU950236 (B-36A), KU950237 (B-51A), KU950230 (B-62), KU950231 (B-68A), KU950232 (B-69A), KU950238 (B-82A), KU950233 (FGB-163), KU950234 (FGB-169A), KU950239 (FGB-169B), KU950240 (FGC-1), KU950241 (FGC-4), KU950242 (FGC-5), KU950243 (FGC-6), KU950244 (FGC-10), KU950235 (FGC-15) and KU950245 (FGO-18B).

Results and Discussion

Yeast population on wine grapes

From five vineyards located in two different districts (Bozcaada: B and Gelibolu: G) eight grape varieties, Alphonse, Kınalı Yapıncak, Çavuş (B), Efes Karası, Cinsaut, Çavuş (G), Isabella and Atasarısı, were used for isolation of yeast strains. The number of yeasts isolated from grape varieties changed from 1.3×10^5 to 1.5×10^7 CFU/mL. The yeast populations of grapes are roughly comprised of between 10^2 and 10^7 cells/g[32]. The high population size counted on grape berries may be explained by the sampling of ripened grapes, having high sugar content, during the harvest sessions. In addition, this wide range of values may be explained, at least partially, by bunch sampling without accurate separation of damaged berries, as we do in our research, which gives rise to smaller variations in total yeast counts. When the total counts obtained by surveys where the samples were separated according to the health status of the grape, were analyzed the damage induces at least an increase of one log cycle. The availability of high sugar concentrations explains the higher populations of damaged grape berries[33].

A total of 691 yeast isolates, seven yeast strains, isolated from Gelibolu grape varieties, were not grown on Lysine medium and so, they were separated as *Saccharomyces* species. The distribution of 684 non-*Saccharomyces* yeast isolates on grape varieties were: 23 isolates from Alphonse (B1), 53 isolates from Kınalı Yapıncak (B2), 47 isolates from Çavuş (B3), 36 isolates from Efes Karası (B4), 32 isolates from Cinsaut (B5), 156 isolates from Çavuş (G1), 175 isolates from Isabella (G2) and 162 isolates from Atasarısı (G3). The number of non-*Saccharomyces* yeast species associated with wine grapes in Gelibolu peninsula (72.1%) was nearly three times more than in Bozcaada island (27.9%). Alphonse, Kınalı Yapıncak, Efes Karası, Cinsaut and Isabella grape varieties have red-black berries while Çavuş and Atasarısı grape varieties have green-yellow berries. The non-*Saccharomyces* yeast communities on red-black berries (46.6%) were found less than that of green-yellow berries (53.4%).

Diversity of yeast species

Of a total of 684 non-*Saccharomyces* yeast isolates, 359 isolates were identified by the API ID 32C kit system while 325 isolates were not identified with this system. Among the unidentified yeast isolates 119 strains gave a low discrimination profile, 25 strains

showed a susceptible profile, 97 strains had an unacceptable profile and 84 strains gave no identification result on API ID 32C strips. All these identified and unidentified isolates were stored for future molecular identifications. The number of identified yeast species from Bozcaada and Gelibolu grape varieties was 138 (out of 191) and 221 (out of 493), respectively. The percentages of ascomycetous and basidiomycetous yeasts on grape varieties collected from Bozcaada and Gelibolu districts were different. Ascomycetous yeast population on Bozcaada grapes (39.1%) was lower than on Gelibolu grapes (71.9%). Meanwhile, the number of basidiomycetous yeasts on grape varieties isolated from Gelibolu (28.1%) was lower than Bozcaada district (60.9%).

The distribution of identified indigenous yeast species present on wine grape berry surfaces was given in Table 1. A total of 21 different yeast species belonging to seven different genera were found by kit system identification, except *S. bacillaris* which was identified by analyzing its 5.8S rDNA nucleotide sequences. All isolates of *S. bacillaris* yeast species were identified as *Candida holmii* (84.4% perfect profile**)** according to API ID 32 C (Version 3.0) kit system and as *S. bacillaris* (syn. *Candida zemplinina*) according to molecular techniques[34]. *S. bacillaris* is an important yeast species because of its ability to grow at high glucose and ethanol concentrations and its fructophilic character[9,35-37]. Due to its antifungal activity against *Botrytis cinerea*, *S. bacillaris* is a potential biocontrol agent for grapes and other fruits[38].

Table 1 — Distribution of identified yeast species on grape varieties and their sampling locations

Yeast Species	Sampling location and grape varieties								Total
	Bozcaada (B)					Gelibolu (G)			
	B1	B2	B3	B4	B5	G1	G2	G3	
Candida famata	1	2							3
C. globosa						1			1
C. inconspicua							2		2
C. intermedia							1		1
C. membranifaciens							1		1
C. norvegica						1			1
C. rugosa						1			1
C. sake			1			6	2		9
C. valida							3		3
C. zeylanoides					1				1
Cryptococcus albidus								1	1
C. humicola							1		1
C. laurentii	4	12	12	1	3		32	1	65
C. neoformans			2	1	2	2	13	6	26
Kloeckera apiculata		2			1	11	17	13	44
K. japonica						1	3	2	6
Lachancea thermotolerans								1	1
Metschnikowia pulcherrima	1	23	6	2	5	28	37	18	120
Rhodotorula glutinis	6	4	14	15	8		2	1	50
R. mucilaginosa							2	1	3
Starmerella bacilliaris	3	1	3	1	1	6	3	1	19
Total	15	44	38	20	21	57	119	45	359

[B1, Alphonse; B2, Kınalı Yapıncak; B3, Çavuş; B4, Efes Karası; & B5, Cinsaut; and G1, Çavuş; G2, Isabella; & G3, Atasarısı]

While the yeast species belonging to the genera *Candida*, *Cryptococcus*, and *Rhodotorula* are the predominant species on unripe grape berries, the yeast species belonging to *Candida*, *Cryptococcus*, *Debaryomyces*, *Hansenula*, *Issatchenkia*, *Kluyveromyces*, *Metschnikowia*, *Pichia*, *Rhodotorula*, *Kloeckera/Hanseniaspora*, *Saccharomyces*, *Torulaspora* and *Zygosaccharomyces* genera are common on mature grape berries[39].

In our research undamaged, mature grape berries were collected and the yeast species belonging to *Metschnikowia*, *Cryptococcus*, *Rhodotorula*, *Kloeckera*, *Candida* and *Starmerella* genera were dominant. The diversity of the yeast biota on grapes changes according to geographical location, grape variety, distance to the sea, temperature, precipitation, soil quality, pesticide handling, and also sampling methods[1-6,39]. In our research grape berries were collected from two different geographical locations and seven different grape varieties. Therefore, the diversity of yeast species and their numbers were found different between the two sampling locations. Nine different yeast species belonging to 6 different genera were found on samples collected from Bozcaada, and 19 different yeast species belonging to 7 genera were found on grape berry surfaces sampled from Gelibolu. The diversity and number of non-*Saccharomyces* yeast species associated with wine grapes in Gelibolu were nearly two times more than in Bozcaada. The predominant yeast species in both sampling locations were *Metschnikowia pulcherrima* (33.4%), *Cryptococcus laurentii* (18.1%), *Rhodotorula glutinis* (13.9%), *Hanseniaspora uvarum* (anamorph *Kloeckera apiculata*) (12.3%), *Cryptococcus neoformans* (7.2%), *S. bacillaris* (5.3%) and *Candida sake* (2.5%) which were 92.7% of total identified yeast species. The proportion of other indigenous yeast species was less than 2% in both sampling locations. *Candida famata* and *Candida zeylanoides* yeast species were found only on grape samples collected from Bozcaada. Among the 21 different yeast species, 19

yeast species were isolated and identified only on grape berries collected from Gelibolu.

The percent distribution of 359 identified yeast species on wine grape varieties were found as: 33.2% on Isabella (G2), 15.9% on Çavuş (G1), 12.5% on Atasarısı (G3), 12.3% on Kınalı Yapıncak (B2), 10.6% on Çavuş (B3), 5.9% on Cinsaut (B5), 5.6% on Efes Karası (B4) and 4.2% on Alphonse (B1). The number of indigenous yeast species on Isabella grape variety was much more than the other grape varieties. Also, the diversity of yeast species on Isabella grape berry surfaces (14 sp.) was greater than other wine grape berries which were Atasarısı (10 sp.), Gelibolu Çavuş (9 sp.), Cinsaut (7 sp.), Kınalı Yapıncak (6 sp.), Bozcaada Çavuş (6 sp.), Efes Karası (5 sp.) and Alphonse (5 sp.) (Table 1). The number and diversity of indigenous yeast species on grape varieties collected from Gelibolu district were found two times more than Bozcaada district. Of a total of identified yeasts, 219 yeast species (61%) were isolated from red-black berries and 140 yeast species (39%) were isolated from green-yellow berries.

Invertase activities of yeast species

The ability of identified non-*Saccharomyces* yeast isolates to utilize sucrose was tested by growing on YEP medium supplemented with 2% sucrose or 2% raffinose as a carbon source. A total of 294 yeast strains belong to *Candida famata*, *C. inconspicua*, *C. intermedia*, *C. membranifaciens*, *C. valida*, *Cryptococcus humicola*, *C. laurentii*, *C. neoformans*, *M. pulcherrima*, *R. glutinis*, *R. mucilaginosa*, and *S. bacillaris* yeast species showed growth on both sucrose and raffinose, so labelled as sucrose positive. The yeast strains of *Candida globosa*, *C. norvegica*, *C. rugosa*, *C. norvegensis*, *C. sake*, *C. zeylanoides*, *Kloeckera apiculata*, *K. japonica*, and *Lachancea thermotolerans* species cannot show growth on sucrose and raffinose, so labeled as sucrose negative.

Three randomly selected yeast strains from each sucrose positive yeast species were used for the determination of invertase activities. If there are not enough individuals representing the yeast species, all yeast strains were used for the enzyme assay. The secreted invertase activities of sucrose positive yeast strains were determined in derepress (0.05% dextrose) conditions. The released glucose was measured and given as an enzyme unit. One unit of enzyme equals the amount of invertase that catalyzes the liberation of one µmol glucose per minute per 100 mg dry wt. of yeast cells. All sucrose positive yeast species, except *S. bacillaris*, showed very low invertase activity changing from 6.04±2.5 to 143.2±27.3 units (data not given). The highest invertase activity was determined (as 3039.6±225.2 unit) in *S. bacillaris* yeast species. Therefore, it was determined whether invertase activity is glucose repressed or not in all strains of *S. bacillaris* yeast species.

All members of *S. bacillaris* yeast species, 19 yeast strains, were grown in YP culture including 2% glucose as a repress condition. Then yeast cells were harvested, washed, and transferred to YP medium supplemented with 0.05% glucose as derepress conditions. The yeast cells were further incubated at 30°C for 2 h and then harvested to determine invertase activity. All *S. bacillaris* yeast strains, except the B-62 strain, were displayed the nearly the same level of enzyme activity both in repress and derepress conditions (Table 2). Three yeast strains, B-36-A, B-69-A, and FGC15, had low invertase activities under a derepressed environment but they were not glucose repressible. In addition, the yeast strain B-62 (7826.32±398 unit) showed the highest invertase activity which is not repressed by glucose. The invertase activities of other *S. bacillaris* yeast strains were also not glucose repressible.

Due to its fructophilic, psychrotolerant, osmotolerant, and acidogenic properties, *S. bacilliaris*

Table 2 — Invertase activities of *Starmerella bacillaris* yeast species both in repressed and derepressed conditions

Yeast strains	Invertase activity*	
	Repress	Derepress
B-19-D	2514.2±126	3258.2±235
B-21-B	1547.2±265	2512.1±237
B-23-C	2271.7±248	3256.2±229
B-36-A	2474.4±128	2348.3±226
B-51-A	3247.6±228	4523.2±392
B-62	2324.8±278	7826.3±398
B-68-A	2519.6±281	3658.7±232
B-69-A	3837.5±167	3495.7±179
B-82-A	2040.8±257	3241.3±215
FGB163	2245.7±243	3254.3±234
FGB169-A	2965.6±239	3695.5±218
FGB169-B	3515.149±	4263.2±238
FGC1	1865.7±217	2367.2±263
FGC4	1887.8±195	2854.3±352
FGC5	3170.5±325	3456.8±231
FGC6	2215.1±165	2968.8±191
FGC10	2405.6±284	3254.2±237
FGC15	2450.6±211	1969.4±1551
FGO-18-B	3351.5±328	3358.2±127
K. lactis (ATCC85)	149.5±26	326.7±57
S. cerevisiae (BY4741)	12.3±2,4	1428.4±129.6

[*invertase activities were given in micromoles of glucose deliberated per min per 100 mg dry wt. of cells (±Standard deviations)]

has become an industrially remarkable yeast species in recent years[35,40,41]. The sequential inoculation of *S. bacilliaris* with *S. cerevisiae* decreases the acetic acid content in sweet wines due to the growing ability of the *S. bacilliaris* at high sugar concentrations[41]. In our study, the non-repressible invertase activity of *S. bacilliaris* yeast strains shows that *S. bacilliaris* can use other sugars besides glucose during the fermentation process.

Phylogeny analysis of *S. bacillaris* yeast strains

The genomic DNA of all *S. bacillaris* yeast strains was extracted and used for amplification and sequencing of the 5.8S-ITS rDNA region. The PCR products of ITS1-5.8S-ITS2 regions were around 450 bp-500 bp in length. All nineteen yeast strains were identified as *S. bacillaris* depending on sequence analysis. The sequences of yeast strains were submitted to GenBank and their accession numbers were given.

As a result of haplotype analysis with DnaSP v6.12.01 program, 19 *S. bacillaris* yeast strains showed 11 different haplotype profiles (H1 (B-19D), H2 (B-21B), H3 (B-23C), H4 (B-36A, B-51A, B-82A, FGB-169B, FGC-1, FGC-5, FGC-6, FGC-10 and FGO-18B) H5 (B-62), H6 (B-68A), H7 (B-69), H8 (FGB-163), H9 (FGB-169A), H10 (FGC4) and H11 (FGC-15)). The haplotype diversity (Hd) was found as 0.789. All *S. bacillaris* yeast strains showed different haplotype distribution except B-36A, B-51A, B-82A, FGB-169B, FGC-1, FGC-5, FGC-6, FGC-10 and FGO-18B yeast strains having same haplotype profile, H4. Nucleotide diversity (Pi) per site was 0.3094 and the average number of nucleotide differences (k) was 19.491. G+C content in polymorphic sites was found as 0.463. The total number of sites excluding sites with gaps or missing data was 321 and the sites with alignment gaps or missing data was 168. The total number of mutations was 74. The number of variable (polymorphic) and invariable (monomorphic) sites were found as 63 and 258, respectively. Singleton variable sites with two variants were 21 in site positions of 98, 99, 101, 104, 126, 131, 132, 216, 274, 287, 288, 307, 339, 340, 341, 342, 350, 379, 404, 405 and 427. Parsimony informative sites were 31 with two variants (site positions: 196, 201, 228, 260, 334, 335, 337, 344, 375, 396, 397, 428, 443, 447, 448, 453, 455, 456, 458, 459, 460, 463, 464, 476, 477, 478, 480, 482, 484, 485 and 486) and 11 with three variants (site positions: 202, 333, 348, 409, 445, 446, 454, 467, 481, 483 and 487) (Fig. 1).

The evolutionary history of 19 *S. bacillaris* yeast strains was inferred using the Maximum Parsimony method. *S. cerevisiae* and *M. guilliermondii* yeast species were used as outgroups, and two S. *bacillaris* yeast strains from CBS collection were also included in MP analysis. MP tree produced two clades: clade A is composed of 9 yeast strains having private haplotypes (H1, H2, H3, H9, H5, H6, H7, H8, H11). However, clade B is composed of 10 yeast strains having the same haplotype (H4), except FGC4 (H10) (Fig. 2). Two S. *bacillaris* yeast strains from the CBS collection are localized as a subclade in clade B.

Conclusion

The biodiversity of grape-associated yeasts has been studied in different geographical regions and grape varieties. The indigenous yeast populations on grapes are important for wine-makers to improve the wine quality. Therefore, in our study, 684 non-*Saccharomyces* yeast strains were isolated from eight grape cultivars located in two different geographical locations. While 359 non-*Saccharomyces* yeast isolates were identified with the kit system, 325 isolates were not identified. In future studies, it would be appropriate to identify all isolated yeast strains by molecular methods and to determine their intraspecific variations. Twenty-one different yeast species belonging to seven genera were identified on the grape samples, including, *Candida famata*,

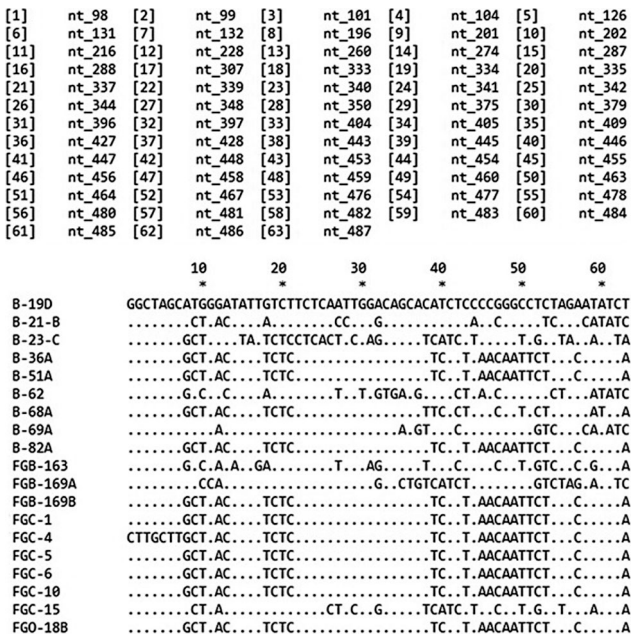

Fig. 1 — Nucleotide polymorphism determined in ITS1-5.8S-ITS2 regions of *Starmerella bacillaris* yeasts species

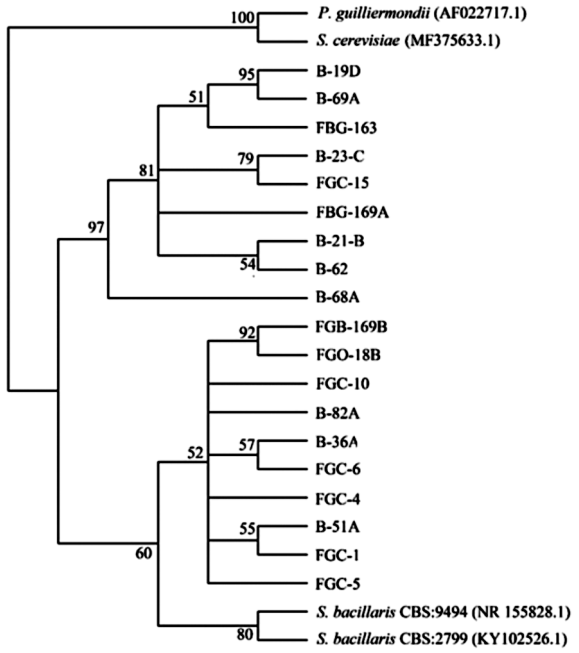

Fig. 2 — Maximum-parsimony phylogenetic tree of *S. bacillaris* species obtained with sequences of ITS1-5.8S-ITS2 regions

C. globosa, *C. inconspicua*, *C. intermedia*, *C. membranifaciens*, *C. norvegica*, *C. rugosa*, *C. sake*, *C. valida*, *C. zeylanoides*, *Cryptococcus albidus*, *C. humicola*, *C. laurentii*, *C. neoformans*, *Kloeckera apiculata*, *K. japonica*, *Lachancea thermotolerans*, *Metschnikowia pulcherrima*, *Rhodotorula glutinis*, *R. mucilaginosa* and *Starmerella bacilliaris*. *M. pulcherrima* was the dominant species present on the grape berries. The diversity and number of non-*Saccharomyces* yeast species associated with grapes collected from Gelibolu were nearly twice that of Bozcaada. The diversity and number of indigenous yeast species on Isabella grape variety were much more than the other grape varieties. This is the first broad-spectrum study in Turkey to describe the yeast species from berries of different grape cultivars (not in fermentation stages) and different geographical locations. In addition, in our study, it was determined that only one of the 21 yeast species had industrially important non-repressible invertase activity.

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Conflict of Interest

Author declares no competing interests.

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