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Exploring yeast diversity of dry-salted naturally black olives from Greek retail outlets with culture dependent and independent molecular methods

Zoe Gounari[a], Stamatoula Bonatsou[a], Ilario Ferrocino[b], Luca Cocolin[b], Olga S. Papadopoulou[c], Efstathios Z. Panagou[a,*]

[a] Agricultural University of Athens, Department of Food Science and Human Nutrition, School of Food and Nutritional Sciences, Laboratory of Microbiology and Biotechnology of Foods, Iera Odos 75, Athens 11855, Greece

[b] University of Turin, Department of Agricultural, Forestry and Food Sciences, Largo Paolo Braccini 2, 10095 Grugliasco, Torino, Italy

[c] Institute of Technology of Agricultural Products, Hellenic Agricultural Organization DIMITRA, S. Venizelou 1, Lycovrissi 14123, Attiki, Greece

Corresponding author: E.Z. Panagou, E-mail: stathispanagou@aua.gr

23 **Abstract**

24 In the present study, the physicochemical (pH, water activity, moisture content,
25 salt concentration) classical plate counts (total viable counts, yeasts, lactic acid
26 bacteria, *Staphylococcus aureus*, *Pseudomonas* spp., *Enterobacteriaceae*) and
27 amplicon sequencing of naturally black dry-salted olives obtained from different retail
28 outlets of the Greek market were investigated. According to the results, the values of
29 the physicochemical characteristics presented great variability among the samples.
30 Specifically, pH and water activity (a_w) values ranged between 4.0 and 5.0, as well as
31 between 0.58 and 0.91, respectively. Moisture content varied between 17.3 and 56.7%
32 (g H_2O/100 g of olive pulp), whereas salt concentration ranged from 5.26 to 9.07% (g
33 NaCl/100 g of olive pulp). No lactic acid bacteria, *S. aureus*, *Pseudomonas* spp. and
34 *Enterobacteriaceae* were detected. The mycobiota consisted of yeasts that were
35 further characterized and identified by culture-dependent (rep-PCR, ITS-PCR, and
36 RFLP) and amplicon target sequencing (ATS). *Pichia membranifaciens*, *Candida*
37 *sorbosivorans*, *Citeromyces nyonsensis*, *Candida etchelsii*, *Wickerhamomyces*
38 *subpelliculosus*, *Candida apicola*, *Wickerhamomyces anomalus*, *Torulaspora*
39 *delbrueckii* and *Candida versatilis* were the dominant species according to ITS
40 sequencing (culture-dependent), while ATS revealed the dominance of *C. etchelsii*,
41 *Pichia triangularis*, *P. membranifaciens,* and *C. versatilis* among samples. The results
42 of this study demonstrated considerable variability in quality attributes among the
43 different commercial samples of dry-salted olives, reflecting a lack of standardization
44 in the processing of this commercial style. However, the majority of the samples were
45 characterized by satisfactory microbiological and hygienic quality and complied with
46 the requirements of the trade standard for table olives of the International Olive
47 Council (IOC) for this processing style in terms of salt concentration. In addition, the
48 diversity of yeast species was elucidated for the first time in commercially available
49 products, increasing our knowledge on the microbial ecology of this traditional food.
50 Further investigation into the technological and multifunctional traits of the dominant
51 yeast species may result in better control during dry-salting and enhance the quality
52 and shelf-life of the final product.

53

54 **Keywords:** black olives, dry-salted olives, yeasts, rep-PCR, RFLP, amplicon target
55 sequencing

56 **1. Introduction**

57 Naturally black dry-salted olives are traditionally produced in the island of
58 Thassos in Northern Greece, using the olives of a local table olive cultivar (cv.
59 Thassos), although any other cultivar of black or alkali treated green olives can be
60 processed in the same way to produce dry-salted olives (Brenes et al., 2022). Olives
61 are harvested when fully mature (the surface color of the drupes is black) or overripe
62 and subsequently placed in concrete tanks in layers with coarse salt (Panagou et al.,
63 2002). Under these conditions, practically no fermentation takes place and for this
64 reason dry salting is characterized as a ‘‘curing’’ or ‘‘desiccation’’ process (Panagou,
65 2006; Ramirez et al., 2013). Due to the high osmotic pressure exerted by the salt,
66 water and water-soluble components, such as oleuropein, are gradually removed from
67 the mesocarp, resulting in gradual debittering of olives that are ready for consumption
68 after 30-40 days (Değirmencioğlu et al., 2014). From the organoleptic point of view,
69 dry-salted olives are considered to have a slight bitter and sweet taste, which is well
70 accepted by consumers, but the high salt content needed for the preservation of the
71 final product makes it unattractive to international markets. According to the CXS 66-
72 1981 standard of table olives of the Codex Alimentarius (FAO, 1981) that was revised
73 in 2013, the minimum salt content for this trade preparation of olives should be 8%
74 (w/v). Consequently, the shelf-life of the final product is ensured by the high salt
75 concentration and the corresponding low water activity (a_w) of dry-salted olives. It has
76 been reported that the a_w level of the final product ranges between 0.75 and 0.85,
77 depending on salt concentration during the dry-salting process and therefore only salt-
78 tolerant microorganisms, such as yeasts, could become the dominant microbiota
79 (Panagou, 2006; Panagou et al., 2002; Papagora et al., 2013). Among the pathogenic
80 microorganisms, the presence of *Staphylococcus aureus* should be taken into serious
81 consideration, because of its ability to grow in low a_w levels (a_w<0.86), where it
82 presents a generation time of 300 min (Adams and Moss, 2008).

83 Taxonomic identification of microbial species is a difficult process that requires
84 the application of several genetic markers to be sequenced. Several studies related to
85 the microbial diversity of fermented products have employed amplicon sequencing
86 techniques (Bokulich and Mills, 2012; Cocolin et al., 2013; Ercolini et al., 2012,
87 2013; Jampaphaeng et al., 2017; Mota-Gutierrez et al., 2019). However, new
88 approaches to explore diversity using next generation sequencing technologies (NGS)

89 have revealed the importance of the reliability of PCR primers targeting a specific
90 genetic marker (Bokulich et al., 2013; Mota-Gutierrez et al., 2019). Restriction
91 endonucleases are enzymes that are able to recognize a specific sequence motif of the
92 DNA known as restriction site. This taxonomic method can create a restriction map
93 through the enzymatic digestion of DNA into smaller fragments, which are then
94 separated by electrophoresis based on the composition of DNA and the % G+C
95 content. One of the most common methods based on the enzymatic reaction of these
96 restriction enzymes is Restriction Fragment Length Polymorphism (RFLP) used in the
97 classification of both bacteria and yeasts (Nadin-Davis et al., 2007). The identification
98 occurs after the digestion of the regions of the unidentified sample compared to
99 reference strains and the discriminant ability is increasing with the implementation of
100 multiple enzymes. The gene targets for the sequencing of 26S rRNA gene using the
101 Sanger method are the variables ITS1 and ITS2. However, because of the limitations
102 of these regions for yeast identification, D1/D2 of 26S rRNA gene in the large subunit
103 of eukaryotic ribosomes is commonly used (Kurtzman and Robnett, 1997). It needs to
104 be noted that Sanger method could be insufficient due to the complexity of bacterial
105 diversity and therefore Next Generation Sequencing (NGS) or "massively parallel
106 sequencing" can overcome these limitations, not only by promoting millions of
107 parallel sequencing reactions, but also permitting the sequence of uncultured and
108 unpurified samples. To avoid bias, it is important to compare different targeting
109 regions and molecular techniques to explore the microbial populations on a food
110 matrix.

111 The objectives of this work were (i) to characterize the physicochemical and
112 microbiological profile of commercial naturally black dry-salted olives, and (ii) to
113 elucidate the yeast diversity of the olives through culture dependent and independent
114 molecular approaches.

115

116 **2. Materials and Methods**

117 *2.1 Olive samples*

118 Nine different commercially available samples of naturally black dry-salted
119 olives cv. Thassos were studied. Each sample was obtained from a different
120 supermarket in the wider area of Athens. Olives were displayed in bulk in retail

121 outlets and none of them was subjected to thermal treatment (pasteurization) or
122 preserved by the use of authorized preservatives (information provided by suppliers).
123 Instead, their preservation was based exclusively on the physicochemical
124 characteristics attained during the dry-salting process, namely salt concentration. The
125 average size of each sample was ca. 500 g.

126

127 *2.2 Microbiological and physicochemical analyses*

128 Lactic acid bacteria (LAB), yeasts, *Enterobacteriaceae*, *Pseudomonas* spp., total
129 mesophilic counts and *Staphylococcus aureus* were determined for each sample in
130 duplicate. Twenty-five grams (25 g) of olive pulp were aseptically added in 225 mL
131 sterile 1⁄4 Ringer's solution and homogenized in a Stomacher device (LabBlender,
132 Seward Medical, London, UK) for 60 s at room temperature. The resulting suspension
133 was serially diluted in the same diluent and 1.0 or 0.1 mL of the appropriate dilutions
134 were mixed or spread on the following non-selective and selective media: (i) de Man-
135 Rogosa-Sharpe (MRS; 401728, Biolife, Milan, Italy) for LAB, adjusted to pH 5.7 and
136 supplemented with 0.05% (w/v) cycloheximide (AppliChem GmbH, Darmstadt,
137 Germany), incubated at 30 °C for 48-72 h; (ii) Rose Bengal Chloramphenicol agar
138 (RBC; LAB036, LAB M, Lancashire, UK) for yeasts/molds, supplemented with
139 selective supplement X009 (Bury, United Kingdom), incubated at 25 °C for 48-72 h;
140 (iii) Violet Red Bile Glucose agar (VRBGA; 4021882, Biolife) for
141 *Enterobacteriaceae*, incubated at 37 °C for 24 h; (iv) Pseudomonas agar base (CFC;
142 LAB108, LAB M)) for *Pseudomonas* spp., supplemented with CFC supplement
143 (X108), incubated at 25°C for 48-72 h; (v) Baird-Parker agar (BP; LAB285, LAB M))
144 for *S. aureus,* supplemented with egg yolk (X075), incubated at 37 °C for 48 h, and
145 (vi) total viable counts on Plate Count Agar (PCA; Biolife)) incubated at 25 °C for
146 48-72 h. Each sample was microbiologically analyzed in duplicate and the results
147 were expressed as log values of colony forming units per gram (log CFU/g) of olives
148 ± standard deviation.

149 Determinations of pH, water activity (a_w), salt and moisture content in dry-salted olive
150 pulp were performed as detailed elsewhere (Garrido-Fernández et al., 1997; Panagou
151 et al., 2002; Argyri et al., 2015). Specifically, the pH of the olives was determined in a
152 sample (50 g) of olive mesocarp that was subjected to homogenization at room

153 temperature using an Ultra Turrax T25 blender (IKA Labortecknik, Staufen,
154 Germany) in 50 mL of distilled water. The pH was determined by immersing the
155 electrode of the digital pH-meter (Orion 940, Orion Research Inc., Boston, MA, USA)
156 in the olive paste using three independent samples of olive paste. Olive moisture was
157 determined by oven drying five portions of the homogenized paste (ca. 5 g each) at
158 105 °C until constant weight. The water activity (a_w) of the olives was measured with
159 an Aqualab 4TE apparatus (Meter Group Inc., Pullman, WA, USA). Four to five
160 pieces of olive pulp were placed in the disposable cap of the instrument and a_w was
161 determined in triplicate. For the determination of sodium chloride content, 10 g of
162 olive paste were diluted in 90 mL of distilled water. The suspension was filtered
163 through filter paper and 10 mL of the filtrate were titrated using a standardized
164 solution of 0.05 N $AgNO_3$ with potassium chromate solution as indicator and
165 expressed as % NaCl per 100 g olive pulp. Finally, the values of salt content in olive
166 juice were determined according to the method described by García-Serrano et al.
167 (2023) taking into account the humidity of the samples and expressed as % NaCl per
168 100 mL of olive juice. This was considered necessary, as international regulatory
169 standards for table olives express salt content per 100 mL of olive juice and not 100
170 mL of olive pulp. Sodium chloride determinations were performed in triplicate using
171 three independent samples of olive paste. In all physicochemical determinations,
172 results were expressed as mean values ± standard deviation.

173

174 *2.3 Yeasts characterization using culture dependent and independent molecular*
175 *techniques*

176 *2.3.1 Yeasts isolation, samples preparation and DNA extraction*

177 Twenty percent (20%) of the colonies were randomly selected from Plate Count
178 Agar (PCA) plates from each sample of dry-salted olives according to Harrigan
179 (1998). The isolated colonies were purified by successive streaking on Yeast Mold
180 Agar (YM) medium consisting of 0.5% Bacteriological Peptone (Biolife), 0.3% Yeast
181 extract (LAB M, Lancashire, UK), 0.3% Malt extract (LAB M), and 1% Dextrose
182 (Merck, Darmstadt, Germany), incubated at 25 °C for 48 h. After incubation, the
183 colonies were subjected to visual inspection, catalase and oxidase assays, Gram-
184 staining and microscopic observation. Pure cultures were maintained at -80 °C in YM

185 broth medium supplemented with 20% glycerol. A total of 180 colonies (20 colonies
186 selected per sample × 9 samples) were subjected to culture dependent molecular
187 analysis. Yeast isolates were numerically coded from 1 to 180.

188 For culture independent analysis, 10 g of olives were placed into a filtered
189 stomacher bag and 20 mL of sterilized Ringer's solution was added to wash the
190 olives. After 2 min of mild malaxation, the solution was discarded to remove the
191 non/loosely attached cells on olive epidermis. Then, 40 mL of the same diluent were
192 added and a second mild malaxation followed to detach the biofilms from the olives.
193 Finally, the suspension was subjected to centrifugation for 10 min (5000 × *g* at 4 ^{o}C)
194 to collect the pellet.

195 DNA extraction was performed for the total number of the 180 purified isolates
196 and the nine samples of olives according to Bonatsou et al. (2018) with an extra step
197 of DNA re-suspension in elution buffer containing 1 μL RNase followed by
198 incubation at 40 ^{o}C for 30 min before quantification.

199

200 *2.3.2 Culture dependent molecular analysis*

201 *2.3.2.1 Repetitive DNA amplification (rep-PCR)*

202 Genotypic diversity was assessed in a final volume of 25 μL containing 1.5 μL
203 mM $MgCl_2$, 0.5 μL $(GTG)_5$ primer, 0.2 μLTaq polymerase (KAPA Taq PCR kit,
204 KAPA Biosystems, United States), 0.5 μL dNTP's and 2 μL DNA of template DNA
205 (100 ng/μL). Amplification was carried out in a thermocycler (ProFlex PCR System,
206 Applied Biosystems, Milan, Italy) under the following conditions: initial denaturation
207 at 95 °C for 5 min; 30 cycles at 95 °C for 30 s, 40 °C for 1 min, and 65 °C for 8 min;
208 and a final extension at 65 °C for 16 min. All PCR products were separated by gel
209 electrophoresis in 2% agarose (Biorad, Madrid, Spain) in TBE 1X at 120 V for 2 h
210 and gels were scanned in UVIpro Platinum (Uvitec) using the software UV
211 proplatinum 1.1 (Eppendorf, Hamburg, Germany). Analysis was performed with
212 Bionumerics ver. 6.1 software (Applied Maths, Sint-Martens-Latem, Belgium) using
213 the Dice coefficient and the unweighted pair group method with arithmetic mean
214 (UPGMA) cluster analysis. Isolates with identification percentage higher than 90%
215 were considered to belong to the same cluster.

216

217 *2.3.2.2 Internal Transcribed Spacer Regions (SSU) PCR*

218 From each group derived by cluster analysis with rep-PCR, one representative
219 isolate was selected for ITS PCR amplification, using ITS_1 (5'–
220 TCCGTAGGTGAACCTGCGG–3') and ITS_4 (5' – TCCTCCGCTTATTGATATGC
221 – 3') as primers (White et al., 1990). Amplification of ITS regions was performed in
222 a final volume of 50 μL containing 3 μL $MgCl_2$, 1 μL of each primer, 0.3 μL Taq
223 polymerase (Applied Biosystems) 1μL dNTP's and 75 ng of template DNA.
224 Amplification was carried out under the following conditions: initial denaturation at
225 95 °C for 5 min; 35 cycles of 95 °C for 1 min, 53 °C for 1 min, and 72 °C for 1 min;
226 and a final extension at 72 °C for 1 min (Esteve-Zarzoso et al., 1999) Amplicons were
227 separated in 1.5% (w/v) agarose in TBE 1X at 100 V for 20 min. For each DNA
228 amplicon, the RFLP method was performed for further classification, as described
229 below.

230

231 *2.3.2.3 Restriction Fragment Length Polymorphism (RFLP)*

232 RFLP was performed according to Esteve-Zarzoso et al. (1999) using three
233 restrictive endonucleases namely, Hinf I, Hae III, and Cfo I. For each enzymatic
234 reaction with a total volume of 15 μL, 10 μL from each amplicon of ITS-PCR and 0.5
235 μL of the enzymes were used, followed by incubation at 37 °C for 90 min in a
236 thermocycler ProFlex PCR System (Applied Biosystems) and electrophoresis in 1.5%
237 agarose gel in TBE 1X at 120 V for 80 min. The analysis and comparison of the
238 electrophoretic profiles of the isolates after ITS-amplification and RFLP, resulted in
239 classification of the isolates to further groups. One representative isolate from each
240 group was selected for sequencing after 26S RNA amplification. Amplicons were sent
241 for sequencing to GATC-Biotech (Cologne, Germany). To determine the closest
242 known relatives of the 26S rRNA gene sequences obtained, searches were performed
243 in public data libraries (GenBank) with the BLAST search program
244 (http://www.ncbi.nlm.nih.gov/blast/).

245

246 *2.3.3 Culture independent molecular methods*

247 *2.3.3.1 Next Generation Sequencing (NGS)*

248 For the implementation of NGS, amplification and sequencing of the D1/D2
249 domain of 26S rRNA gene was performed according to Mota-Gutierrez et al. (2019),
250 using primers LS2 and NL4MS Amplicons were then purified and tagged by using the
251 nextera XT index following the illumina metagenomic pipeline. Sequencing was then
252 performed on a MiSeq platform (2X250bp). After sequencing raw.fastq files were
253 first joined by FLASH software and then quality filtered using QIIME 1.9 according
254 to the pipeline of Mota-Gutierrez et al. (2019). Taxonomic assignment was performed
255 against SILVA database. BlastN tool (http://www.ncbi.nlm.nih.gov/blast/) was used
256 to confirm the taxonomy assignment. OTU tables (clustered at 97% of similarity)
257 were rarefied at the lowest number of sequences per sample and filtered for OTUs
258 occurring at 1% of the relative abundance in at least 2 samples.

259

260 *2.4 Statistical analysis*

261 Data were analyzed by one-way analysis of variance (ANOVA) using the
262 Statgraphics Centurion XVII ver. 17.2.00 (Statgraphics Technologies Inc., The Plains,
263 Virginia). The Tuckey's HSD (Honest Significant Difference) test was employed to
264 compare mean values and significant differences were determined at $p < 0.05$. In
265 addition, Principal Components Analysis (PCA) was employed as an exploratory
266 unsupervised approach to investigate any association between the abundance (%) of
267 yeast species defined by culture dependent molecular analysis and the
268 physicochemical characteristics of the nine samples of dry-salted olives. PCA was
269 performed with Statistica software version 7.0 (Statsoft Inc., Tulsa, OK, USA).

270

271 **3. Results and discussion**

272 *3.1 Microbiological analyses*

273 The results of the microbiological analysis showed that in the majority of the
274 examined samples, yeasts were the dominant microorganisms, with variations in their
275 populations between samples (Table 1). Specifically, sample S6 showed the highest
276 population level with counts up to 6.4 log CFU/g, while samples S1, S4 and S7 had

277 population levels of approximately 5.4-5.7 log CFU/g. Yeast population from samples
278 S2, S5, S8 and S9 was found close to 3.4-3.9 log CFU/g, depending on the sample. In
279 contrast, in sample S3 yeasts were below the detection limit of the enumeration
280 method (< 2.0 log CFU/g). It needs to be noted that total viable counts (TVC)
281 represented the dominant microbiota in each sample and the counts were comparable
282 with the yeast counts, indicating the dominance of yeasts in the dry-salting process in
283 8 out of the 9 samples. However, for sample S3, where no yeasts were enumerated,
284 total viable counts were close to 4.4 log CFU/g. No *Enterobacteriaceae, S. aureus*
285 and *Pseudomonas* spp. were detected in any of the 9 examined dry-salted olive
286 samples. Finally, no LAB could be detected in any sample, with the exception of
287 sample S6, where a high population of 6.0 log CFU/g was detected. The results
288 obtained in this work were in agreement with a previously published work (Panagou,
289 2006), where the impact of dry-salting process was assessed on the physicochemical
290 and microbiological profile of cv. Thassos naturally dry-salted black olives under
291 different packaging conditions followed by storage at 4 and 20 °C. Before dry salting
292 the identified microorganisms were LAB, yeasts, *Enterobacteriaceae* and
293 *Pseudomonas* spp., whereas at the end of the process only yeasts could be
294 enumerated, due to the low a_w (0.761) combined with the high salt concentration
295 (7.4%). In another work (Mantzouridou and Tsimidou, 2011) performed on hot air-
296 dried black olives of the same cultivar, the initial microbiota of the raw material
297 consisted of LAB and yeasts, but in the end of the process the dominant microbiota
298 was yeasts. In the same work, no undesirable microorganisms were detected
299 (*Enterobacteriaceae, Bacillus* spp., *Clostridium* spp., *Staphylococcus aureus*) on
300 olives during storage at 4 and 20 °C, under different packaging conditions for 180
301 days of storage. These authors also reported that the low a_w (0.893), the naturally
302 occurring phenolic compounds and the free fatty acids were inhibitory for the growth
303 of the above microorganisms. Finally, the results obtained in this work were in line
304 with a recently published work (García-Serrano et al., 2023), assessing the
305 microbiological and physicochemical profile of 20 commercially available samples of
306 dehydrated olives using different processing methods from different countries. The
307 authors reported high yeast populations in 4 of these samples, with counts ranging
308 from 3.5 log CFU/g to 4.1 CFU/g. In addition, no LAB and *Enterobacteriaceae* were
309 enumerated on any sample of dehydrated olives with the exception of one sample,
310 where the latter microorganisms were detected in 3.4 log CFU/g that could be

311 attributed to the high pH (6.10) and a_w (0.91) of the specific sample. In total, all of the
312 aforementioned works, as well as the current work, highlighted the high presence of
313 yeasts in such products, a fact that can be attributed to their physicochemical
314 characteristics attained during the dry-salting process.

315

316 *3.2 Physicochemical analyses*

317 Naturally black dry-salted olives are a ready-to-eat food containing at least 10%
318 (w/v) NaCl, when these products are preserved by the specific chemical
319 characteristics attained during the dry-salting process, according to the specifications
320 laid out in the trade standard applying to table olives of the International Olive
321 Council (IOC, 2004). On the contrary, no limits are defined for the maximum pH and
322 the minimum acidity expressed in % lactic acid (w/v) of this product, yet a GMP
323 (Good Manufacturing Practice) must be employed throughout the dry-salting process.
324 However, this high salt concentration has been reconsidered in the revised standard
325 for table olives of the Codex Alimentarius (FAO, 1981), providing a reduced
326 minimum NaCl concentration of 8% (w/v) in the final product.
327 The physicochemical characteristics of the commercial naturally black dry-salted
328 olives are presented in Table 2. The average pH value of the samples was 4.7 (range
329 4.0-5.0) in agreement with the average pH value reported recently for dehydrated
330 black olives surveyed from 9 countries (García-Serrano et al., 2023) and slightly
331 lower than 5.0-5.2 units reported previously for black dry-salted olives cv. Thassos
332 (Panagou, 2006; Panagou et al., 2002). It needs to be noted that in a previous market
333 survey (Panagou et al., 2006), the pH value of dry-salted olives cv. Thassos obtained
334 from retail outlets was 4.6, which is in line with the mean pH value reported in this
335 work. However, in the same work, dry-salted olives cv. Conservolea presented lower
336 pH values (3.84) indicating that the olives were subjected to fermentation prior to dry-
337 salting (Panagou et al., 2006). It is worth noting that 5 out 9 samples presented pH
338 values higher than 4.6, which is considered a limit for the growth of *Clostridium*
339 *botulinum* (Raatjes and Smelt, 1979) and thus additional hurdles should be taken into
340 consideration to ensure the safety of the final product. The additional hurdle in this
341 trade preparation is the high salt concentration of the olives. Indeed, the average value
342 of salt content was 6.9 g NaCl/100 g olive pulp. It must be underlined that the IOC

343 trade standard for table olives (IOC, 2004) defines a minimum salt concentration of
344 10% (w/v) for this type of olives. However, the trade standard indicates that the salt
345 content should be determined in the olive juice and not in the olive pulp, which is the
346 standard method of analysis (Değirmencioğlu et al., 2014). For this reason, the
347 concentration of salt in the olives was expressed as % NaCl per 100 mL of olive juice
348 taking into consideration the moisture content of the olives and based on the
349 assumption that the salt is concentrated in the aqueous phase of the olive (García-
350 Serrano et al., 2023). Thus, only sample S6 presented NaCl content in the olive juice
351 less than 10% (w/v) and could not meet the specifications of the trade standard of the
352 IOC. Another important parameter is water activity (a_w). It has been reported that a_w
353 ranges from 0.99-0.98 in raw olive drupes to 0.75-0.92 in the final dry-salted product
354 (Panagou, 2006; Ramírez et al., 2013). In this work, the average a_w was 0.75, which is
355 in good agreement with previously published values for Greek dry-salted olives
356 (García-Serrano et al., 2023; Panagou, 2006). It should also be noted that a_w ranged
357 between 0.58 and 0.91 reflecting a lack of standardization in the dry-salting process
358 among the different processors. Another noteworthy observation is that the highest a_w
359 value corresponded to sample S6 that presented also and lowest salt concentration and
360 the highest yeast population (6.4 log CFU/g) along with the presence of LAB in high
361 numbers (6.0 log CFU/g). Finally, the average moisture of the samples was 29.5%,
362 which is in good agreement with the moisture content reported recently for packaged
363 dehydrated Greek dry-salted olives (García-Serrano et al., 2023). However, sample S6
364 presented a high moisture content close to 57% that could support the growth of fungi
365 during the shelf-life of the product, unless preservatives (e.g., sorbic acid in the form
366 of potassium sorbate) or pasteurization can be applied to inhibit the growth of yeasts
367 and moulds (Casado et al., 2010). As a consequence of high moisture content, sample
368 S6 presented also a high a_w value that when combined with the low salt content of the
369 olives may jeopardize the safety of the product, a fact that was also evident from the
370 microbiological analysis for this sample.

371

372 *3.3 Culture dependent and culture independent molecular identification*

373 *3.3.1 Repetitive DNA amplification (rep-PCR)*

374 After rep-PCR, cluster analysis of the profiles of the 178 yeast isolates was
375 performed using Bionumerics software (Supplementary Fig. 1). The dendrogram

376 obtained after cumulative image analysis of Rep-PCR patterns resulted in 36 different
377 clusters. The prevalence of the different isolates within each of the 36 clusters is
378 summarized in Table 3. Most isolates (43) were included in Cluster 5 and were
379 recovered from samples S1, S2, S3, S5, and S8 (data not shown). The rest of the
380 isolates were distributed among the remaining clusters without showing any specific
381 trend.

382

383 *3.3.2 Internal Transcribed Spacer Regions (SSU) PCR and Restriction Fragment*
384 *Length Polymorphism*

385 One representative isolate of the 36 groups from rep-PCR was selected and
386 consequently subjected to ITS-PCR and RFLP for further clustering. Comparison of
387 the electrophoretic profiles obtained from RFLP resulted in further classification of
388 the isolates in a final number of 20 clusters (Table 3). One isolate of each cluster was
389 subjected to sequencing and taxonomic assessment was performed via BLAST
390 software leading to the final classification of the representative isolates at species
391 level (Table 4). According to Tables 3 and 4, the total number of isolates belonged to
392 9 species, namely *Candida versatilis*, *Wickerhamomyces subpelliculosus*, *Candida*
393 *apicola*, *Wickerhamomyces anomalus*, *Torulaspora delbrueckii*, *Citeromyces*
394 *nyonsensis*, *Candida etchelsii*, *Pichia membranifaciens* and *Candida sorbosivorans*. 4
395 isolates belonging to the same cluster (cluster 3) after the final grouping could not be
396 identified. The identification percentage of the yeast species isolated and identified for
397 each of the nine samples with culture dependent techniques is shown in Figure 1.
398 *Candida etchelsii* was identified in most of the samples, except S4, S6, and S9.
399 Specifically, 100% isolation frequency was observed in sample S3, whereas
400 coexistence with *Candida versatilis* was noticeable for samples S1 (5.0%-95.0%), S7
401 (50.0%-50.0%), and S8 (65.0%-35.0%), followed by *Citeromyces nyonsensis* for
402 sample S5 (73.7%-21.1%-5.3%). *Pichia membranifaciens* was dominant in samples
403 S6 (90.0%) and S9 (60.0%), followed by *Candida sorbosivorans* with 10.0% and
404 40.0%, respectively. *Candida etchelsii* was also identified in sample S2 (45.0%)
405 followed by *Wickerhamomyces subpelliculosus* (30.0%) and *Candida apicola* (5.0%).
406 *Wickerhamomyces subpelliculosus* was the dominant species in sample S4 (84.2%)
407 followed by *Torulaspora delbrueckii* (10.5%) and *Wickerhamomyces anomalus*

408 (5.3%). *Citeromyces nyonsensis* was also identified at a low percentage close to 5%
409 for sample S5, while 20% of the isolates (5 isolates) in sample S2 were characterized
410 as non-identified. The results obtained are in accordance with previous studies, since
411 all identified species have been also isolated from different fermented food matrices
412 and/or environments with low a_w and high salt concentrations. More specifically, *C.*
413 *etchelsii* and *C. versatilis* which are salt tolerant yeasts are commonly isolated from
414 miso and soy fermentations and are important in flavour enhancing through the
415 production of 4-ethyl guaiacol (4EG) and 4-ethyl phenol (4EP), which are
416 characteristic flavours of soy sauce and miso (Feng et al. 2012; Suezawa et al., 2006).
417 Both species have been previously isolated from bloaters of fermented green table
418 olives during storage (Asehraou et al., 2000). *Candida apicola*, *Pichia*
419 *membranifaciens* and *Wickerhamomyces anomalus* are well known species related to
420 table olive fermentations (Arroyo-López et al., 2006; Bautista-Gallego et al., 2011;
421 Bleve et al., 2014, 2015; Nisiotou et al., 2010). *Wickerhamomyces subpelliculosus* is a
422 salt-tolerant microorganism previously isolated from fermented cucumbers (Kurtzman
423 et al., 1998) and is considered as an alternative to baker's yeast (Zhou et al., 2017).
424 *Citeromyces nyonensis* is a yeast previously isolated from fermented black olive
425 brines and olive oil (Casaregola et al., 2013), while *Torulaspora delbrueckii* is a well-
426 known yeast presenting high resistance to osmotic stress, as well as biotechnological
427 potential due to aroma-enhancing properties and therefore is widely employed in
428 wine, beer and bread dough fermentations (Fernandes et al., 2021). *T. delbrueckii* has
429 been previously isolated from black olives (Kotzekidou, 1997). *Candida*
430 *sorbosivorans* is a salt tolerant yeast previously isolated from honey (Carvalho et al.,
431 2010) and more recently isolated during the production of Algerian Smen, a
432 traditional fermented butter (Boussekine et al., 2022).

433

434 *3.3.3 Amplicons target sequencing*

435 The yeast species identified with amplicons sequencing are illustrated in Figure
436 2. Due to the high number of yeasts identified, only OTUs with a relative abundance
437 higher than 5% are displayed. For the nine samples of dry-salted olives the dominant
438 yeast species were *Candida etchelsii*, *C. versatilis, P. membranifaciens, C. apicola, P.*
439 *triangularis*, *Wickerhamomyces anomalus, Aureobasidium pullulans*, *Ogataea*

440 *boidinii* and *Hortaea werneckii*. *Candida etchelsii* and *C. versatilis* were the dominant
441 species in most samples with relative abundances ranging from 23.0% to 55.3%,
442 followed by *W. anomalus* (13.4%), *O. boidinii* (7.9%) and *C. apicola* (6.9%) for
443 sample S4, *O. boidinii* (5.5%) for sample S8, *A. pullulans* (11.3%) and *C. apicola*
444 (5.5%) for sample S9. In addition, in samples S2 and S5 the dominant species were *P.*
445 *triangularis* with relative abundances of 41.0% and 31.0%, respectively, followed by
446 *C. etchelsii* (33.1% and 30.9%, respectively), *C. versatilis* (8.1% and 27.1%,
447 respectively) and *Hortaea werneckii* (7.4%) for sample S2. These results are in
448 accordance with the results obtained from culture dependent techniques in this study
449 since the dominant identified species were common among the two techniques with
450 slight deviations. Specifically, *P. triangularis,* that was identified only with amplicon
451 sequencing, has been previously isolated from environments with high salt
452 concentrations, such as natural fermented Italian sausages (Rantsiou and Cocolin,
453 2006), soy sauce, sea water and fermented mushrooms (Elbandy et al., 2008; Romero
454 et al., 2012), while *A. pullulans* and *O. boidinii* (or *C. boidinii*) have been previously
455 isolated from natural black olive fermentation (Bonatsou et al., 2018; Coton et al.,
456 2006; Nisiotou et al., 2010). Finally, *H. werneckii* is a black yeast with remarkable
457 tolerance to salt with both pathogenic and spoilage potential that is usually isolated
458 from hypersaline environments (Zalar et al., 2019). Despite the fact that several
459 studies have previously studied microbial populations and physicochemical
460 characteristics of dry-salted olives (Brenes et al., 2022; García-Serrano et al., 2023;
461 Panagou, 2006; Ramírez et al., 2013), it is the first-time microbial diversity of this
462 product is identified using molecular techniques.

463 In order to clarify the relationship between the physicochemical parameters and
464 the yeast species identification after ITS PCR analysis of dry-salted naturally black
465 olives samples, PCA was performed, and results are presented in Fig. 3. The plot of
466 the PCs enables the investigation of correlation between the variables in the input
467 dataset. The projection of the variables in the subspace of the first two PCs explained
468 57.8% of the information included in the raw data and allowed the segregation of
469 identified yeast species in different clusters. In this way, *P. membranifaciens* and *C.*
470 *sorbosivorans* were positively correlated with the vector of moisture content and a_w
471 (Fig. 3A) and they were also associated with sample S6 (Fig. 3B) that presented the
472 highest moisture (56.7%) and a_w (0.91) from all samples analyzed. Moreover, *C.*

473 *etchelsi*i and *C. nyonsensis* were associated with the vectors of salt level in the pulp
474 and olive juice and they were related to samples S3 and S5 that presented the highest
475 levels of salt (Table 3). *C. versatilis* was in the same quadrant as a_w and it was
476 correlated with samples S1, S7 and S8 that presented a_w values of 0.77 and 0.79.
477 These samples were located close to each other demonstrating that there is a positive
478 correlation between them and a negative correlation with samples S2 and S4 located
479 on the diagonally opposite quadrant. Finally, sample S4 was associated with *T.*
480 *delbrueckii* and *W. anomalus*, whereas sample S2 was correlated with *W.*
481 *subpelliculosus* and *C. apicola*. Both samples were positioned in diagonally opposed
482 quadrants with the vector for a_w, meaning that they had the lowest a_w values. This is
483 more evident for sample S2 that presented the lowest a_w (0.58) among all samples
484 analyzed. Concerning the distribution of samples in the plot of loadings (Fig. 3B),
485 PC1 could be associated with the parameter of a_w, since there is a transition from
486 lower (a_w<0.72, left side) to higher (a_w>0.77, right side) a_w values.

487

488 **4. Conclusion**

489 Nine samples of naturally black dry-salted olives from the Greek retail market
490 were analyzed for the determination of their physicochemical characteristics and
491 microbial diversity, using both culture dependent and independent molecular
492 techniques. The values of the physicochemical characteristics showed great variability
493 reflecting the lack of standardization of the dry-salting process. Moreover, the
494 diversity of yeasts was elucidated in final products obtained from the market. *Pichia*
495 *membranifaciens*, *Candida sorbosivorans*, *Citeromyces nyonsensis*, *Candida*
496 *etchelsii*, *Wickerhamomyces subpelliculosus*, *Candida apicola*, *Wickerhamomyces*
497 *anomalus*, *Torulaspora delbrueckii* and *Candida versatilis* were the dominant yeast
498 species identified with ITS sequencing, while NGS revealed higher yeast diversity
499 including *Candida etchelsii, Candida versatilis, Pichia membranifaciens, Candida*
500 *apicola, Pichia triangularis, Wickerhamomyces anomalus, Pichia membranifaciens,*
501 *Aureobasidium pullulans, Ogataea boidinii and Hortaea werneckii.* The genotypic
502 profile obtained from culture dependent techniques is similar to the profile obtained
503 by NGS, indicating that despite the discrimination power of NGS, validation with the
504 traditional sequencing methods is required for the classification of the microbial

505 diversity. Finally, this study provides a comparison among culture dependent and
506 independent molecular techniques for the identification of the microbial diversity of
507 dry-salted olives. The obtained results are important, since they enhance our
508 knowledge on the microbial ecology of naturally black dry-salted olives and
509 specifically contribute to a better understanding of the microbial diversity present due
510 to the different physicochemical parameters among samples. Further research into the
511 evaluation of the technological characteristics and multifunctional potential of the
512 dominant yeast species may result in better control of the dry-salting process, improve
513 the nutritional value and enhance the quality and shelf-life of the final product.

514

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520

521 Declaration of competing interest

522 The authors declare that they have no known competing financial interests or personal
523 relationships that could have appeared to influence the work reported in this paper.

524

525 **References**

526 Adams, M.R., Moss, M.O., 2008. Food Microbiology. 3rd Edition, The Royal Society
527 of Chemistry, Cambridge, UK.
528 Arroyo-López, F.N., Durán-Quintana, M.C., Ruiz-Barba, J.L., Querol, A., Garrido-
529 Fernández, A., 2006. Use of molecular methods for the identification of yeast
530 associated with table olives. Food Microbiol. 23, 791–796.
531 Argyri, A.A., Nisiotou, A.A., Pramateftaki, P., Doulgeraki, A.I., Panagou, E.Z.,
532 Tassou, C.C., 2015. Preservation of green table olives fermented with lactic acid
533 bacteria with probiotic potential under modified atmosphere packaging. LWT-
534 Food Sci. Technol. 62, 783-790.

535 Asehraou, A., Peres, C., Brito, D., Faid, M., Serhrouchni, M., 2000. Characterization
536 of yeast strains isolated from bloaters of fermented green table olives during
537 storage. Grasas y Aceites 51, 225-229.
538 Bautista-Gallego, J., Rodríguez-Gómez, F., Barrio, E., Querol, A., Garrido-
539 Fernández, A., Arroyo-López, F.N., 2011. Exploring the yeast biodiversity of
540 green table olive industrial fermentations for technological applications. Int. J.
541 Food Microbiol. 147, 89–96.
542 Bleve, G., Tufariello, M., Durante, M., Perbellini, E., Ramires, F.A., Grieco, F., 2014.
543 Physico-chemical and microbiological characterization of spontaneous
544 fermentation of Cellina di Nardò and Leccino table olives. Front. Microbiol. 5:570.
545 Bleve, G., Tufariello, M., Durante, M., Grieco, F., Ramires, F.A., Mita, G., 2015.
546 Physico-chemical characterization of natural fermentation process of Conservolea
547 and Kalamata table olives and development of a protocol for the pre-selection of
548 fermentation starters. Food Microbiol. 46, 368–382.
549 Bokulich, N.A., Mills, D.A., 2013. Improved selection of internal transcribed spacer-
550 specific primers enables quantitative, ultra-high-throughput profiling of fungal
551 communities. Appl. Environ. Microbiol. 79, 2519–2526.
552 Bokulich, N.A., Bamforth, C.W., Mills, D.A., 2012. Brewhouse-resident microbiota
553 are responsible for multi-stage fermentation of American coolship ale. PLoS One
554 7(4): e35507.
555 Bonatsou, S., Paramithiotis, S., Panagou, E.Z., 2018. Evolution of yeast consortia
556 during the fermentation of Kalamata natural black olives upon two initial
557 acidification treatments. Front. Microbiol. 8:2673.
558 Boussekine, R., Bekhouche, F., Debaets, S., Thierry, A., Maillard, M.B., Falentin, H.,
559 Pawtowski, A., Barkat, M., Coton, M., Mounier, A., 2022. Deciphering the
560 microbiota and volatile profiles of Algerian *Smen*, a traditional fermented butter.
561 Microorganisms 10, 736.
562 Brenes, M., García-Serrano, P., Brenes-Álvarez, M., Medina, E., García-García, P.,
563 Romero, C., 2022. Dehydrated black olives from unfermented and alkali treated
564 green olives. LWT – Food Sci. Technol. 172, 114177.
565 Carvalho, C.M., Meirinho, S., Estevinho, M.L.F., Choupina, A., 2010. Yeast species
566 associated with honey: Different identification methods. Arch. de Zootec. 59, 103-
567 113.

568 Casado, F.J., Sánchez, A., Rejano, L., de Castro, A., Montaño, A., 2010. Stability of
569 sorbic and ascorbic acids in packed green table olives during long-term storage as
570 affected by different packing conditions, and its influence on quality parameters.
571 Food Chemistry, 122 , pp. 812-818
572 Casaregola, S., Jacques, N., Louis-Mondesir, C., Coton, M., Coton, E., 2013.
573 *Citeromyces nyonsensis* sp. nov., a novel yeast species isolated from black olive
574 brine. Int. J. Syst. Evol. Microbiol. 63, 3086-3090.
575 Cocolin, L., Alessandria, V., Botta, C., Gorra, R., De Filippis, F., Ercolini, D.,
576 Rantsiou, K., 2013. NaOH-debittering induces changes in bacterial ecology during
577 table olives fermentation. PLoS One 8: e69074.
578 Coton, E., Coton, M., Levert, D., Casaregola, S., Sohier, D, 2006. Yeast ecology in
579 French cider and black olive natural fermentations. Int J Food Microbiol.
580 15;108(1): 130-5.
581 Değirmencioğlu, N., Gürbüz, O., Değirmencioğlu, A., Yildiz, S., 2014. Effect of
582 pretreatments on microbial growth and sensory properties of dry-salted olives. J.
583 Food Prot. 77, 1527-1537.
584 Elbandy, M., Shinde, P.B., Dang, H.T., Hong, J., Bae, K.S., Jung, J.H., 2008. Furan
585 metabolites from the sponge-derived yeast *Pichia membranifaciens*. J. Nat. Prod.
586 71, 869-872.
587 Ercolini, D., 2013. High-throughput sequencing and metagenomics: moving forward
588 in the culture-independent analysis of food microbial ecology. Appl. Environ.
589 Microbiol. 79, 3148–3155.
590 Ercolini, D., De Filippis, F., La Storia, A., Iacono, M., 2012. "Remake" by high-
591 throughput sequencing of the microbiota involved in the production of water
592 buffalo mozzarella cheese. Appl. Environ. Microbiol. 78, 8142-8145.
593 Esteve-Zarzoso, B., Belloch, C., Uruburu, F., Querol, A., 1999. Identification of
594 yeasts by RFLP analysis of the 5.8S rRNA gene and the two ribosomal internal
595 transcribed spacers. Int. J. Syst. Bacteriol. 49, 329–337.
596 FAO (1981) Standard for table olives. CXS 66-1981 (available at:
597 https://www.fao.org/fao-who-codexalimentarius/sh-
598 proxy/en/?lnk=1&url=https%253A%252F%252Fworkspace.fao.org%252Fsites%2
599 52Fcodex%252FStandards%252FCXS%2B66-1981%252FCXS_066e.pdf)

600 Fernandes, T., Silva-Sousa, F., Pereira, F., Rito, T., Soares, P., Franco-Duarte, R.,
601 Sousa, M.J., 2021. Biotechnological importance of *Torulaspora delbrueckii*: From
602 the obscurity to the spotlight. J. Fungi 7, 712.
603 García-Serrano, P., Brenes-Álvarez, M., Romero, C., Medina, M., García-García, P.,
604 Brenes, M., 2023. Physicochemical and microbiological assessment of commercial
605 dehydrated black olives. Food Control 145, 109417.
606 Garrido-Fernández, A., González Pellisó, F., González Cancho, F., Sánchez Roldan,
607 F., Rejano Navarro, L., Cordón Casanueva, J.L., Fernández Diez, M.J., 1997.
608 Modificaciones de los procesos de elaboración y envasado de aceitunas verdes de
609 mesa en relación con la eliminación y reuso de salmueras. Grasas y Aceites 28,
610 267-285.
611 Feng, J., Zhan, X.B., Wang, D., 2012. Identification and analysis of the metabolic
612 functions of a high – salt – tolerant halophilic aromatic yeast *Candida etchellsii* for
613 soy sauce production. World J. Microbiol. Biotechnol. 28, 1451- 1458.
614 Harrigan, W.F., 1998. Laboratory Methods in Food Microbiology. 3rd Edition,
615 Academic Press, London, UK.
616 International Olive Council (IOC), 2004. Trade standard applying to table olives.
617 RES-2/91-IV/04. IOOC, Madrid.
618 Jampaphaeng, K., Cocolin, L., Maneerat, S., 2017. Selection and evaluation of
619 functional characteristics of autochthonous lactic acid bacteria isolated from
620 traditional fermented stinky bean (Sataw-Dong). Ann. Microbiol. 67, 25-36.
621 Kailis, S., Harris, D., 2007. Producing table olives. CSIRO Publishing, Australia.
622 Kotzekidou, P., 1997. Identification of yeasts from black olives in rapid system
623 microtiter plates. Food Microbiol. 14, 609-616.
624 Kurtzman, C.P., Robnett, C.J., 1997. Identification of clinically important
625 Ascomycetous yeasts based on nucleotide divergence in the 5′ end of the large-
626 subunit (26S) ribosomal DNA gene. J. Clin. Microbiol. 35, 1216-1223.
627 Mantzouridou, F., Tsimidou, M., 2010. Microbiological quality and biophenol content
628 of hot air – dried Thassos cv. table olives upon storage. Eur. J. Lipid Sci. Technol.
629 113, 786-795.
630 Mota-Gutierrez, J., Ferrocino, I., Rantsiou, K., Cocolin, L., 2019. Metataxonomic
631 comparison between internal transcribed spacer and 26S ribosomal large subunit
632 (LSU) rDNA gene. Int. J. Food Microbiol. 290, 132-140.

633 Nadin-Davis, S.A., 2007. Molecular epidemiology. In: Jackson, A.C., Wunner, W.H.
634 (Eds.), Rabies. 2nd Edition, Academic Press, Amsterdam, pp. 69–122.
635 Nisiotou, A.A., Chorianopoulos, N., Nychas, G.-J.E., Panagou, E.Z., 2010. Yeast
636 heterogeneity during spontaneous fermentation of black Conservolea olives in
637 different brine solutions. J. Appl. Microbiol. 108, 396–405.
638 Panagou, E., Tassou, C.C., Katsaboxakis, K.Z., 2002. Microbiological,
639 physicochemical and organoleptic changes in dry-salted olives of Thassos variety
640 stored under different modified atmospheres at 4 and 20°C. Int. J. Food Sci.
641 Technol. 37, 635-641.
642 Panagou, E., 2006. Greek dry-salted olives: monitoring the dry salting process and
643 subsequent physicochemical and microbiological profile during storage under
644 different packing conditions at 4 and 20 °C. LWT – Food Sci. Technol. 39, 323-
645 330.
646 Panagou, E., Tassou, C.C., Skandamis, P.N., 2006. Physicochemical, microbiological,
647 and organoleptic profiles of Greek table olives from retail outlets. J. Food Prot. 69,
648 1732-1738.
649 Papagora, C., Roukas, T., Kotzekidou, P., 2013. Optimization of extracellular lipase
650 production by *Debaryomyces hansenii* isolates from dry-salted olives using
651 response surface methodology. Food Bioprod. Process. 91, 413-420.
652 Raatjes, G.J., Smelt, J.P., 1979. *Clostridium botulinum* can grow and form toxin at pH
653 values lower than 4.6. Nature 281, 398-399.
654 Ramírez, E., García-García, P., de Castro, A., Romero, C., Brenes, M., 2013.
655 Debittering of black dry-salted olives. Eur. J. Lipid Sci. Technol. 115, 1319–1324.
656 Rantsiou, K., Cocolin, L., 2006. New developments in the study of the microbiota of
657 naturally fermented sausages as determined by molecular methods: a review. Int. J.
658 Food Microbiol. 108, 255-267.
659 Romero, A.M., Mateo, J.J., Maicas, S., 2012. Characterization of an ethanol – tolerant
660 1,4 – β – xylosidase produced by *Pichia membranifaciens*. Lett. App. Microbiol.
661 55, 354-361.
662 Suezawa, Y., Kimura, I., Inoue, M., Gohda, N., Suzuki, M., 2006. Identification and
663 typing of miso and soy sauce fermentation yeasts *Candida etchellsii* and *C.*
664 *versatilis* based on sequence analyses of the D1D2 domain of the 26S ribosomal
665 RNA gene, and the region of internal transcribed spacer 1, 5.8S ribosomal RNA
666 gene and internal transcribed spacer 2. Biosci. Biotechnol. Biochem. 70, 348-354.

667 White, T.J., Bruns, T., Lee, S., Taylor, J., 1990. Amplification and direct sequencing
668 of fungi ribosomal RNA genes for phylogenetics. In: Innis, M.A., Gelfand, D.H.,
669 Sninsky, J.J., White, T.J. (Eds.), PCR Protocols, a Guide to Methods and
670 Applications. Academic Press, San Diego, pp. 315–322.
671 Zalar, P., Zupančič, J., Gostinčar, C., 2019. The extremely halotolerant black yeast
672 *Hortaea werneckii* - a model for intraspecific hybridization in clonal fungi. IMA
673 Fungus 10:10.
674 Zhou, N., Schifferdecker, A.J., Lluna, A.G., Compagno, C., 2017. *Kazachstania*
675 *gamospora* and *Wickerhamomyces subpelliculosus*: Two alternative baker's yeasts
676 in the modern bakery. Int. J. Food Microbiol. 250, 45-58.

677 **Table 1.** Population of yeasts, Total Viable Counts (TVC), *Enterobacteriaceae,*
678 *Staphylococcus aureus, Pseudomonas* spp. and Lactic Acid Bacteria (LAB) (log CFU/g ±
679 standard deviation) of naturally black dry-salted olive samples from retail outlets.

Sample	Yeasts	TVC	*Enterobacteriaceae*	*S. aureus*	*Pseudomonas spp.*	LAB
S1	5.4 ± 0.2a	5.3 ± 0.1a	< 1.0	< 2.0	< 2.0	< 1.0
S2	3.9 ± 0.1b	4.2 ± 0.1b	< 1.0	< 2.0	< 2.0	< 1.0
S3	< 2.0c	4.4 ± 0.7b	< 1.0	< 2.0	< 2.0	< 1.0
S4	5.7 ± 0.3d	5.8 ± 0.4c	< 1.0	< 2.0	< 2.0	< 1.0
S5	3.2 ± 0.1e	3.4 ± 0.2d	< 1.0	< 2.0	< 2.0	< 1.0
S6	6.4 ± 0.1f	6.4 ± 0.1e	< 1.0	< 2.0	< 2.0	6.0 ± 0.4
S7	5.7 ± 0.2d	6.0 ± 0.1c	< 1.0	< 2.0	< 2.0	< 1.0
S8	3.4 ± 0.6e	4.9 ± 0.2f	< 1.0	< 2.0	< 2.0	< 1.0
S9	3.4 ± 0.1e	4.9 ± 0.1f	< 1.0	< 2.0	< 2.0	< 1.0

680 For each microbial group, values followed by a different lowercase letter in the same column define
681 statistically significant differences according to Tuckey's test ($p < 0.05$).

682 **Table 2.** Physicochemical characteristics of naturally black dry-salted olive samples from
683 retail outlets.

Sample	pH[(a)]	a_w[(b)]	Moisture (%)	NaCl (%)[(c)]	NaCl (%)[(d)]
S1	4.4a	0.79a	28.21±2.13a	8.22±0.13a	18.69±0.31a
S2	4.9b	0.58b	17.33±1.89c	6.18±0.1b	20.92±0.34b
S3	5.0c	0.70c	26.83±1.38ab	7.90±0.14c	18.67±0.33a
S4	4.8b	0.70c	27.77±1.01a	7.03±0.26d	16.18±0.60c
S5	5.0c	0.72d	28.05±2.16a	9.15±0.16e	20.81±0.37b
S6	4.6e	0.91e	56.65±2.16d	5.26±0.14f	7.33±0.20e
S7	4.1f	0.77f	27.69±1.77a	6.43±0.12g	14.84±0.28d
S8	4.9b	0.77f	27.12±1.82ab	6.44±0.01g	15.08±0.05cd
S9	4.0g	0.83g	24.67±2.35b	5.61±0.01h	14.18±0.04d

684 [(a),(b)] Standard deviation values for pH and a_w were < 0.01 and 0.001, respectively; [(c)] g NaCl/100 g of
685 olive pulp; [(d)] g NaCl/100 mL juice
686 For each physicochemical parameter, values followed by a different lowercase letter define statistically
687 significant difference according to Tuckey's test ($p < 0.05$)

688 **Table 3.** Clustering of yeast isolates from dry-salted olives according to repetitive extragenic
689 palindromic DNA sequence-based PCR (REP-PCR) and subsequent clustering of
690 representative isolates using RFLP.

Clusters after REP-PCR	Isolates within each cluster	Clusters after RFLP	Isolates within each cluster
1	6	1	3
2	1	2	3
3	9	3	2
4	9	4	1
5	43	5	1
6	3	6	2
7	2	7	3
8	1	8	2
9	12	9	3
10	1	10	2
11	1	11	1
12	1	12	2
13	1	13	2
14	1	14	1
15	7	15	1
16	7	16	1
17	2	17	3
18	1	18	1
19	17	19	1
20	4	20	1
21	1		
22	1		
23	5		
24	1		
25	1		
26	3		
27	1		
28	1		
29	1		
30	1		
31	5		
32	9		
33	1		
34	10		
35	7		
36	1		

691 .

692

693 **Table 4.** Species identification of yeast isolates according to the RFLP clustering (20 clusters)

Cluster number	Species (ITS PCR)	Number of isolates [a]
1 and 17	*Candida versatilis*	40
2	*Wickerhamomyces subpelliculosus*	7
4	*Candida apicola*	1
5 and 7	*Wickerhamomyces anomalus*	16
6	*Torulaspora delbrueckii*	2
8	*Citeromyces nyonsensis*	1
9, 13, 16, 18, and 19	*Candida etchelsii*	78
10 and 11	*Pichia membranifaciens*	18
12, 14, 15, and 20	*Candida sorbosivorans*	11
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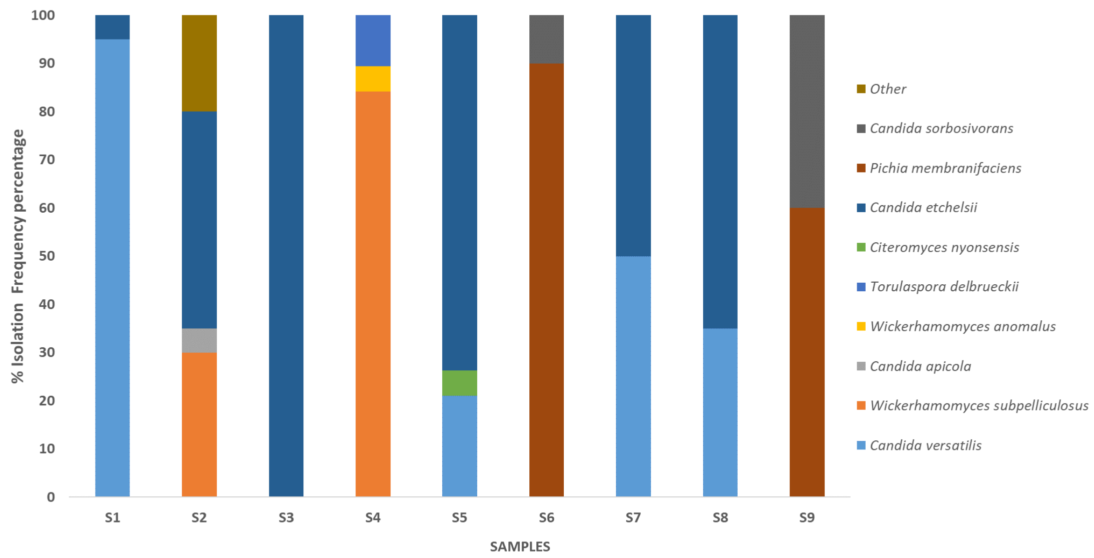
694 * Isolate from cluster 3 was not identified.

695 [a]: abundance of isolates in each cluster based on Table 3



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697 **Fig. 1.** Isolation frequency (%) of the yeast species for the nine samples of dry-salted olives

698 with culture dependent techniques.

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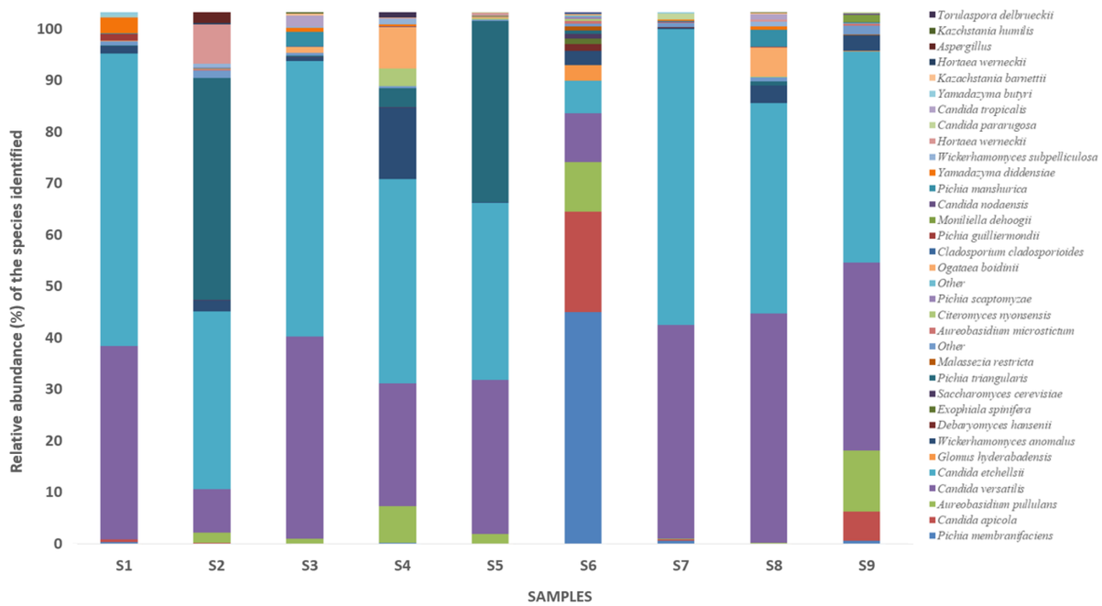

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702 **Fig. 2.** Relative abundance (%) of yeast species for the nine samples of dry-salted olives

703 derived by amplicon sequencing.

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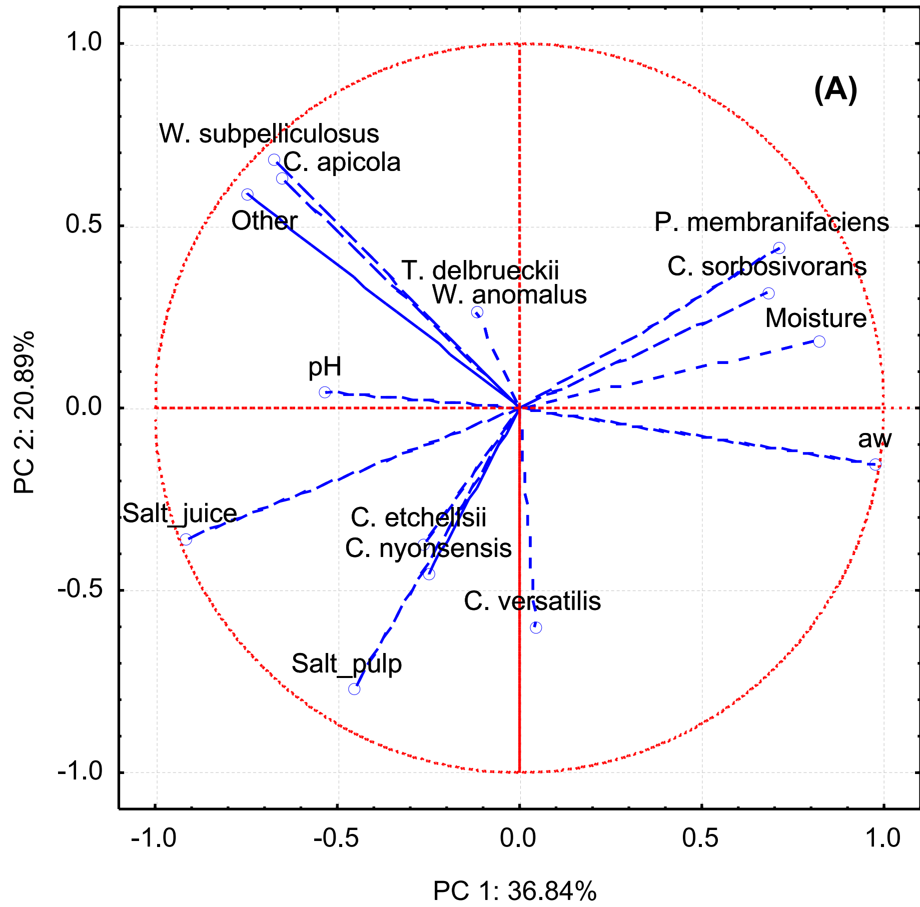

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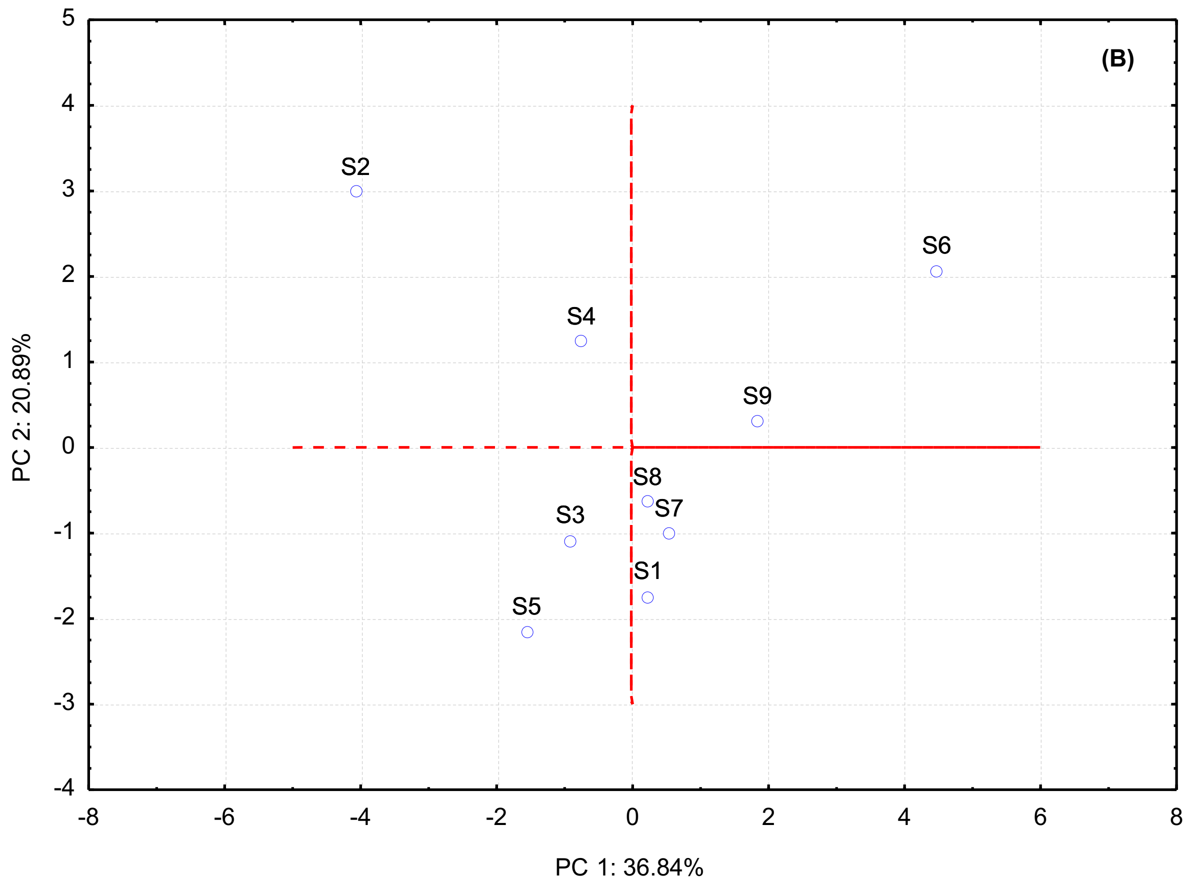
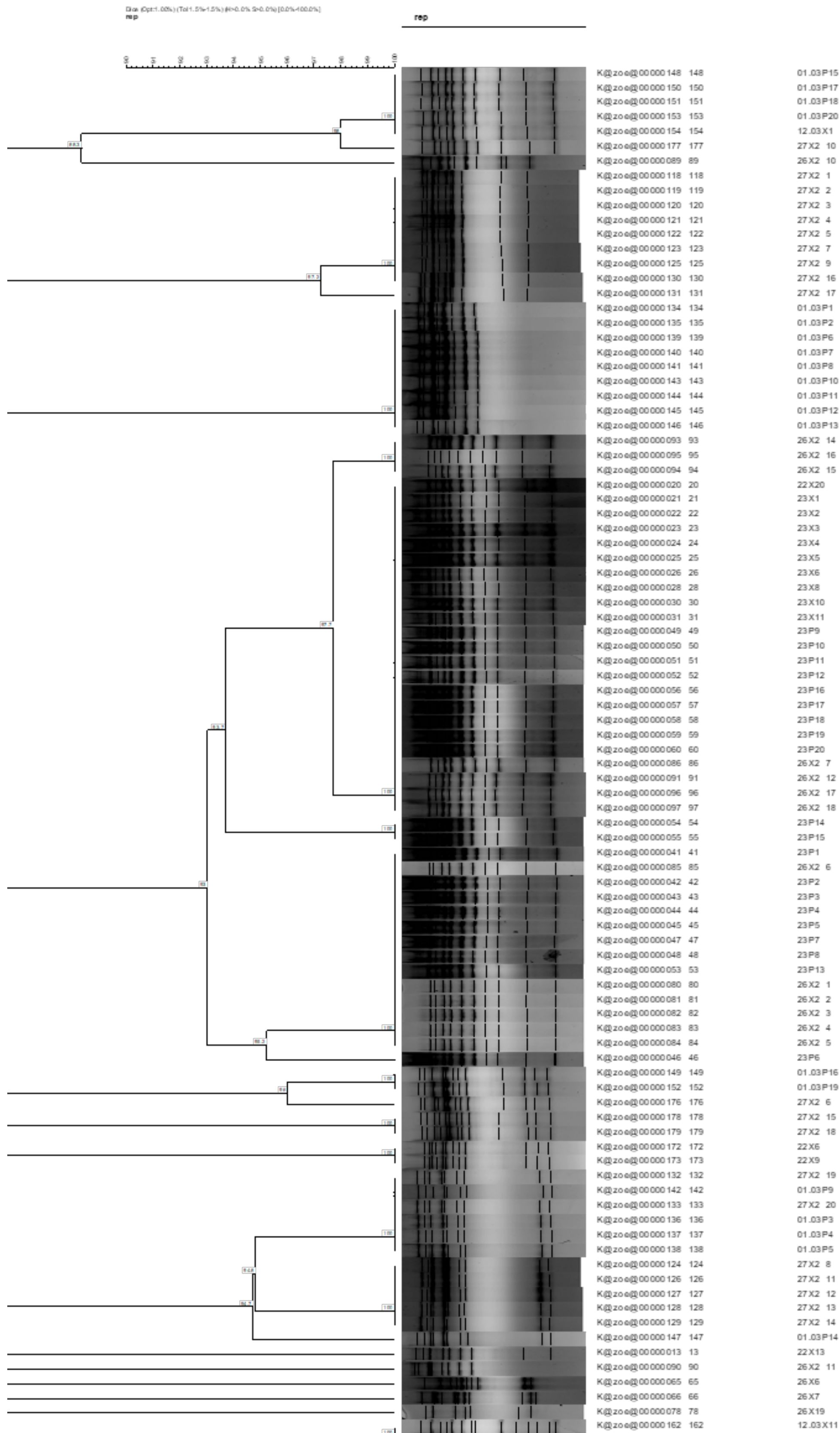


735 **Fig. 3.** PCA plot of loadings (A) (abundance of yeasts and physicochemical parameters) and
736 scores (B) (samples of dry salted olives) of the first two principal components (PCs).

737 **Supplementary Material**



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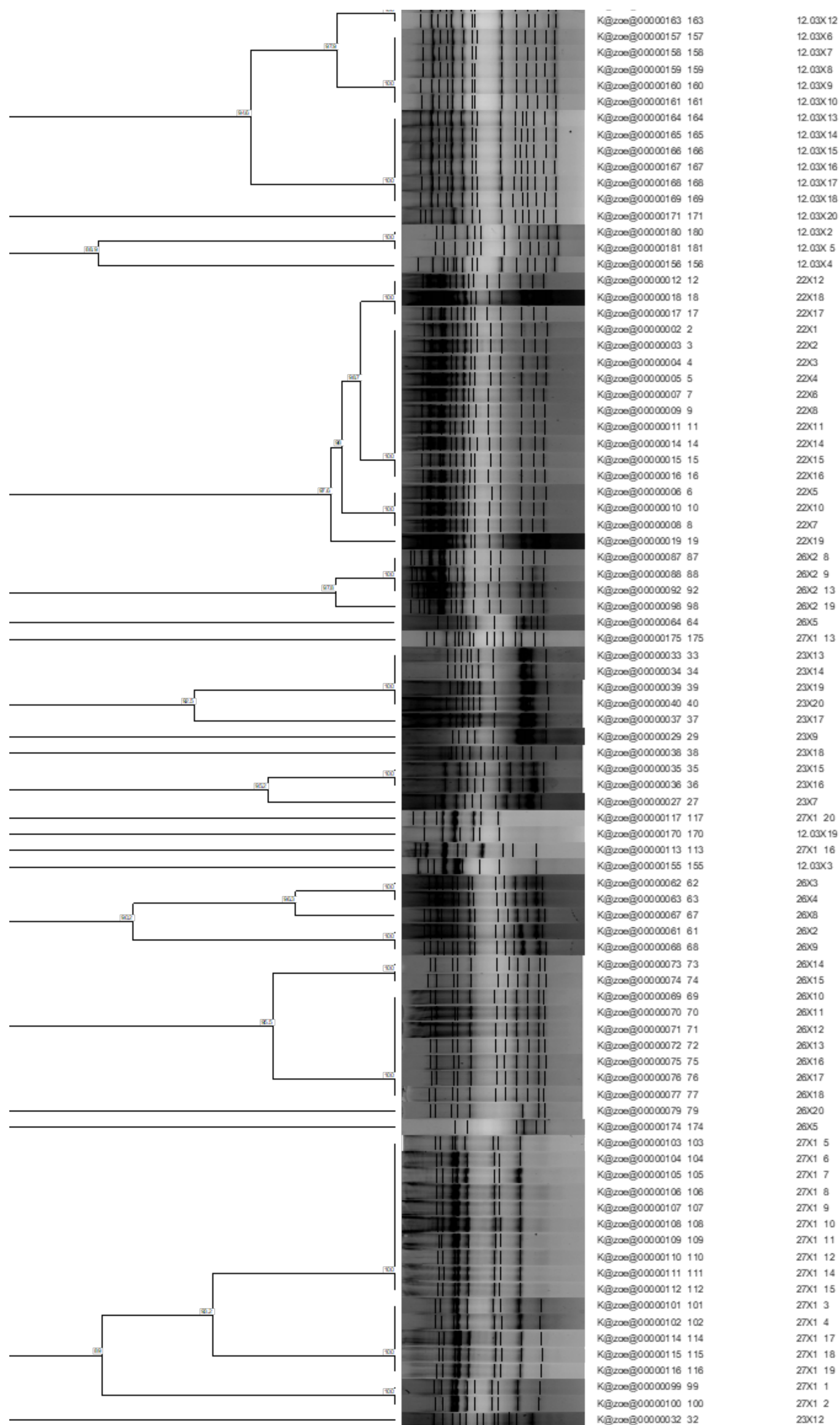

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742 **Supplementary Fig. 1.** Cluster analysis of yeast isolates after rep-PCR with $(GTG)_5$ primer.