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Assessment of Multi Fragment Melting Analysis System (MFMAS) for the Identification of Food-Borne Yeasts

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Abstract
Multi Fragment Melting Analysis System (MFMAS) is a novel approach that was developed for the species-level identification of microorganisms. It is a software-assisted system that performs concurrent melting analysis of 8 different DNA fragments to obtain a fingerprint of each strain analyzed. The identification is performed according to the comparison of these fingerprints with the fingerprints of known yeast species recorded in a database to obtain the best possible match. In this study, applicability of the yeast version of the MFMAS (MFMAS-yeast) was evaluated for the identification of food-associated yeast species. For this purpose, in this study, a total of 145 yeast strains originated from foods and beverages and 19 standard yeast strains were tested. The DNAs isolated from these yeast strains were analyzed by the MFMAS, and their species were successfully identified with a similarity rate of 95% or higher. It was shown that the strains belonged to 43 different yeast species that are widely found in the foods. A clear discrimination was also observed in the phylogenetically related species. In conclusion, it might be suggested that the MFMAS-yeast seems to be a highly promising approach for a rapid, accurate, and one-step identification of the yeasts isolated from food products and/or their processing environments.

Introduction

Yeasts are one of the most versatile microorganisms in food microbiota. The main impact of the yeasts in food industry is associated with their essential role in the production of fermented foods and beverages such as bread, beer, and wine [1] Additionally, yeasts and yeast extracts are recognized as an important source of food ingredients like colorants, vitamins, antioxidants, and nutraceutical or health-promoting supplements [2]. On the other hand, yeasts are considered as a potential spoilage organism that can grow in a wide range of substrates and may cause decomposition resulting in undesirable changes [3]. Yeast-borne spoilage is a widespread problem for the food and beverage industries that leads to enormous economic losses [4]. Moreover, the contamination of foods with opportunistic strains may also lead to yeast infections that might become an increasingly serious threat to public health [5, 6].

The accurate identification of yeast species in the food ecosystem allows us to understand their metabolic activities and the physiological properties associated with process efficiency and product quality/safety as well [7, 8]. Traditional yeast identification methods based on morphological, physiological, and biochemical characteristics are usually time consuming and often lead to misidentification and for this reason these methods are no longer considered acceptable for an accurate identification. It has been revealed that many yeast strains even in reputed culture collections have been misidentified and several new species have not been recognized by traditional taxonomic methods [9]. In the last decades, DNA-based molecular techniques have revolutionized the identification and characterization of yeast species. Especially, several PCR-based methods have been applied to identify species- even at the strain-level-discrimination of the yeast isolates [10]. Sequence analysis of complete or partial ribosomal RNA genes and their internal transcribed spacers (ITS) is widely accepted

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as identification method due to its high reliability and efficiency [11]. However, the number of nucleotide differences in a single DNA sequence may not always be enough to discriminate closely related species [3, 10]. Additionally, high analysis cost and the requirement for expensive instrument are the major drawbacks of this method [12]. Other PCR-based molecular techniques that are frequently used in the characterization of yeasts are restriction fragment length polymorphism (PCR–RFLP) [13, 14], random amplified polymorphic DNA (PCR–RAPD) [15], amplified fragment length polymorphism (AFLP) [16], and repetitive element sequence-based PCR (rep-PCR) techniques [17]. However, none of them considered as direct identification methods, and they require time-consuming and troublesome post-PCR procedures. Moreover, the lack of a standardized universal procedure for the application of these methods often makes it difficult to obtain reproducible results. Over the past two decades, novel nucleic acid-based molecular techniques such as nucleic acid sequence-based amplification (NASBA) [18], fluorescent capillary electrophoresis [19], peptide nucleic acid (PNA)-based fluorescent in situ hybridization (PNA/FISH) [20], and PCR coupled with electrospray ionization mass spectrometry (PCR/ESI-MS) [21], were applied for identification of yeast species. However, most of these studies focused primarily on identification of a single or narrow yeast genus that have clinical importance.

Recently, a novel approach based on the melting profile of PCR-amplified DNA fragments showed potential for the characterization and discrimination of microorganisms. The melting behavior is characteristic for a particular DNA fragment and depends on the sequence length and GC content [22, 23]. This approach is called DNA melting curve analysis, and its next-generation application, High Resolution Melting (HRM) analysis, has become very popular in the last decade for the detection of genetic mutations or the genetic variation in any population. A number of studies have evaluated the potential of HRM analysis and suggested that it is a new approach which is a rapid and efficient method for the differentiation of microorganism species. For example, HRM analysis has been applied to *Mycobacterium* species [24], lactobacilli [25, 26] acetic acid bacteria [27], catalase-positive cocci [28], yeast isolates from clinical samples [29, 30], and fowl adenovirus serotypes [31], and to a certain degree, successful discrimination has been obtained among these species. Just recently, we developed Multi Fragment Melting Analysis System (MFMAS) for yeast as a one-step identification tool based on the characteristic melting behavior of unknown yeast isolates. This system consists of a ready-to-use plate and a computer software used for the outcome analysis. In this system, the MFMAS plate is designed for the simultaneous analysis of 8 different DNA regions that have interspecific sequence heterogeneity. In each well, target DNA fragments are firstly amplified and then subjected to melting analysis. Thanks to the MFMAS software, the melting curves are visualized, analyzed, compared, and then stored. The identification is performed according to the similarity rate between the curves obtained for each DNA region of the unknown species, and those of the known species that are registered in the database (Fig. 1).

Consequently, a rapid and reliable method for the identification of yeast species occurring in the food ecosystem that will contribute to the accurate evaluation of product quality

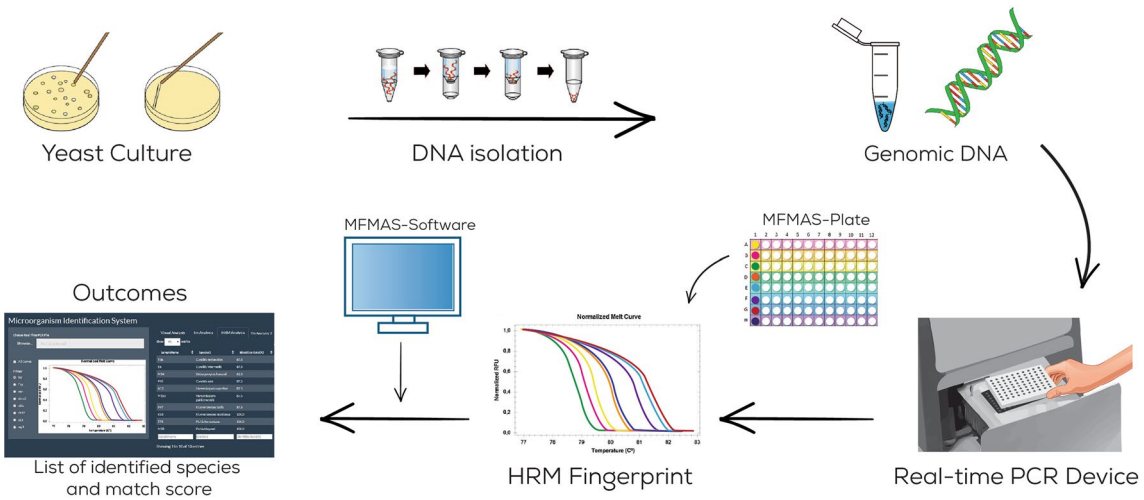

Fig. 1 Schematic representation of identification principle of MFMAS

and improve the control of the production processes are needed. We expect that the MFMAS-yeast could be a potential identification method developed to meet this necessity. For this reason, this study aimed to evaluate the applicability of the MFMAS-yeast system for a fast and accurate detection of the yeasts originating from various foods, beverages, and/or their process environment.

Materials and Methods

Yeast Isolates and Standard Strains

A total of 145 yeast strains were obtained from the culture collection of Erciyes University Food Engineering Department (EUCC). All of the EUCC strains were previously recovered from various foods and beverages, and they were identified by using API® ID 32 C (bioMérieux, Nürtingen, Germany) and sequence analysis of the D1/D2 and ITS regions. Additionally, 19 standard yeast strains were supplied from the American Type Culture Collection (ATCC) and Agricultural Research Service Culture Collection (NRRL) (Table 1). All of the strains were stored in malt extract broth containing 15% glycerol at −80 °C until use and were recovered on Dichloran Rose Bengal Chloramphenicol Agar (DRBC) at 30 °C.

DNA Isolation from Yeast Cells

The suspension of yeast cells to be used for DNA extraction was prepared according to [17]. DNA isolation was carried out using a commercial DNA isolation kit (QIAamp® DNA Mini Kit, Qiagen) by following the user's protocol. The concentration of eluted DNA was measured using a microvolume UV–Vis spectrophotometer (Quawell Q5000, San Jose, USA) and adjusted to 50 ng/µl with DNase-free H_2O.

Identification of Yeast Species with MFMAS-Yeast

Multi Fragment Melting Analysis System (MFMAS) is a novel culture-dependent identification system utilizing the pure culture of microorganisms. The MFMAS consists of a ready-to-use plate and a software compatible with real-time PCR instruments. The plate is a 8 × 12 well plate in which occurs amplification of target DNA fragments and subsequently melting analysis. In this system, 8 different DNA regions of each yeast sample are analyzed simultaneously on a single plate. In the first column of the plate, DNase-free water is analyzed as a negative control while the second column is dedicated to the positive control. The reactions of unknown yeast isolates are performed in the 3rd–12th column. Each of the target DNA regions is analyzed on a different line of the MFMAS plate. The target DNA fragments are highly variable regions among the yeast that amplified

Table 1 The specifications about target DNA regions and primers

The row in plate	Target DNA region	Amplicon			Primers			
		Approximate length (bp)	GC content (%)	T_m (°C)[a]	Direction	Length (bp)	GC content (%)	T_m (°C)[a]
A	26S rRNA gene	246	49	95.6	F	24	42	60.3
					R	22	50	61.2
B	26S rRNA gene	260	50	96.2	F	20	38	58.1
					R	20	55	61.0
C	26S rRNA gene	187	49	94.6	F	23	39	59.2
					R	20	55	60.5
D	26S rRNA gene	199	55	97.3	F	20	50	58.4
					R	24	46	60.6
E	26S rRNA gene	140	56	96.0	F	20	50	58.4
					R	21	50	61.0
F	26S rRNA gene	185	45	93.0	F	25	40	60.5
					R	20	55	60.5
G	18S rRNA gene	212	44	93.1	F	24	33	57.3
					R	24	33	57.1
H	Chromosome XII sequence	243	48	95.2	F	22	45	60.1
					R	22	45	60.0

[a]Salt adjusted melting temperature (T_m) calculations (http://biotools.nubic.northwestern.edu/OligoCalc.html)

F forward, *R* reverse

with the constant sequences of the primers (Table 1). All of the primers were designed from the conserved universal sequences of all major Ascomycota and Basidiomycota lineages that contain most of the known yeast species. The each well of ready-to-use plate contains real-time PCR master mix with HRM dye, forward and reverse primers, and all of the reactions in the plate are carried out in a total volume of 20 µl. Identification is performed approximately in 3 h/10 samples (in 3rd–12th column) after DNA purification step.

In this study, 100 ng template for each strain analyzed was pipetted to all wells in any column between 3 and 12 and analysis was performed using a real-time PCR device (Light Cycler 480 Roche Applied Science, Germany). A universal temperature program was applied for the amplification of all target DNA fragments starting the first denaturation at 94 °C for 10 min and the following denaturation at 94 °C for 45 s, annealing at 52 °C for 30 s, and extension at 72 °C for 45 for 30 cycles. The melting analysis was performed after the last extension at 72 °C for 10 min. When the temperature was increased from 60 to 95 °C at 0.03 °C/s, the fluorescence accumulation was measured and the melting curves were obtained.

Software Analysis

We developed MFMAS-yeast software that concurrently processed the raw melting curve data of multiple DNA fragments and compared the processed data with those in reference and known yeast species in the database. The software of the MFMAS-yeast uses R programming language and has two main components, a web module and a database module. The web module supports the real-time PCR data markup language (RDML) file (.rdml), which is a universal qPCR data export/import format, to export melting curves. In the web module, the normalized melting curves and their difference plots constructed by subtracting melting curves of each sample from a single reference strain (a selected standard strain) can be visualized, digitized, and analyzed. It uses several mathematical algorithms to compare the melting curves and the difference plots of unknown species with those of known species that were found in the database and the comparison results expressed as a similarity rate. The comparison process is repeated for each of the targeted DNA fragments. The database module uses the Microsoft SQL Database Server, and it stores reference and known species. Additionally, the software can generate a dendrogram for the strains analyzed using the numerical data of the melting curve analysis of 8 different target DNA fragments. The grouping of the melting fingerprints is performed by using the unweighted pair group method with the arithmetic averages (UPGMA) clustering algorithm.

Results and Discussion

In this study, the applicability of the MFMAS was evaluated for a total of 164 yeast strains belonging to 43 different species (Table 2). A one-step identification procedure was performed without the need of an additional time-consuming and laborious method. The outcomes of the analysis were observed as text and image and displayed on the computer screen. All of the strains analyzed were confirmed by similarity rate of 95% or higher with the known species in the MFMAS database. Again, a clear differentiation was also obtained between the phylogenetically related species.

A total of 35 strains belonging to 10 different species in the genus *Candida* and 33 strains that represent 3 different species in the genus *Pichia* were analyzed. The analyzed *Candida* species are frequently isolated from foods, and some of them can be regarded as opportunistic pathogens. An application example for *Candida* species is given in Fig. 2. In the genus *Pichia*, besides the strains of *P. fermentans* that originated from olive, cream, yoghurt, and pickle, the strains belonging to the *P. kluyveri* and *P. membranifaciens* species were also accurately identified with a 100% similarity rate. It was also observed that the system can clearly differentiate phylogenetically closely related species including *H. valbyensis–H. uvarum, K. marxianus–K. lactis, M. caribbica–M. guilliermondii*, and *W. anomalus–W. subpelliculosa*. Additionally, all of the standard strains from the international culture collections were confirmed by the MFMAS with a similarity rate of 95% or higher. On the other hand, the MFMAS dendrogram produced by combining the melting curve data of all the target fragments provided successful discrimination and grouping among the yeast strains analyzed and offered a new perspective to understand the phylogenetic relationship between them (Fig. 3).

The MFMAS is based on the analysis of 8 different DNA fragments which have high interspecies and low intraspecies variability. The interspecies variability of the target fragments was assessed according to the resolution between closely related species, while the intraspecies variability of the target regions was determined by analyzing the variation among many individuals from the same species. Although the discrimination ability of each target region is different in each species analyzed, a successful discrimination is obtained when 8 different regions are evaluated simultaneously. Thus, a melting fingerprint

Table 2 The results of MFMAS analysis of yeast strains

Analyzed species	Origin	Number of isolates	MFMAS results	
			Identified species	The average similarity rate of the strains (%)
Aureobasidium pullulans	Pickle	2	*A. pullulans*	100
Candida deformans	Salami	4	*C. deformans*	100
Candida diversa	Brine	3	*C. diversa*	100
Candida ethanolica	Vinegar	2	*C. ethanolica*	100
Candida glabrata	NRRL Y-65	1	*C. glabrata*	100
Candida intermedia	Meat	2	*C. intermedia*	97.5
Candida krusei (*Issatchenkia orientalis*)	Pickle	2	*C. krusei*	97.5
	Brine	2	*C. krusei*	100
Candida membranifaciens	Fruit	3	*C.membranifaciens*	97.5
	NRRL Y-2089	1	*C.membranifaciens*	100
Candida sake	Cheese	2	*C. sake*	100
	NRRL Y-17808	1	*C. sake*	100
Candida tropicalis	NRRL Y-607	1	*C. tropicalis*	100
	NRRL Y-12968	1	*C. tropicalis*	100
Candida zeylanoides	Cheese	4	*C. zeylanoides*	100
	Pickle	4	*C. zeylanoides*	100
	ATCC 36768	1	*C. tropicalis*	99
Debaryomyces hansenii	Cheese	3	*D. hansenii*	100
	Meat	4	*D. hansenii*	100
Dekkera anomala	Vinegar	3	*Dek. anomala*	99
Dekkera bruxellensis	NRRL Y-12961	1	*Dek. bruxellensis*	99
Hanseniaspora uvarum	Fruit	6	*H. uvarum*	100
	NRRL Y-1380	1	*H.uvarum*	97.5
Hanseniaspora valbyensis	NRRL Y-1626	1	*H. valbyensis*	100
Kazachstania exigua	Meat	3	*Kaz. exigua*	95
Kazachstania naganishii	Cheese	3	*Kaz. naganishii*	100
Kluyveromyces lactis	Cream	4	*K. lactis*	100
	NRRL Y-8279	1	*K. lactis*	100
Kluyveromyces marxianus	Cream	4	*K.marxianus*	100
	Yoghurt	3	*K.marxianus*	100
	Meat	3	*K.marxianus*	100
Kregervanrija fluxuum	Vinegar	2	*Kr. fluxuum*	95
Meyerozyma caribbica	Fruit	3	*M. caribbica*	97.5
Meyerozyma guilliermondii	Brine	3	*M. guilliermondii*	99
	Fruit	3	*M. guilliermondii*	100
Pichia fermentans	Olive	2	*P. fermentans*	100
	Cream	2	*P. fermentans*	97.5
	Fruit	4	*P. fermentans*	99
	Yoghurt	2	*P. fermentans*	100
	Pickle	3	*P. fermentans*	100
	ATCC 28483	1	*P. fermentans*	100
Pichia kluyveri	Olive	4	*P. kluyveri*	100
	Fruit	4	*P. kluyveri*	100
	NRRL Y-17724	1	*P. kluyveri*	99
Pichia membranifaciens	Tomato paste	4	*P. membranifaciens*	100
	Fruit	5	*P. membranifaciens*	97.5
	ATCC 58072	1	*P. membranifaciens*	97.5

Table 2 (continued)

Analyzed species	Origin	Number of isolates	MFMAS results	
			Identified species	The average similarity rate of the strains (%)
Rhodotorula glutinis	NRRL Y-2502	1	*R. glutinis*	100
Rhodotorula mucilaginosa	Turkish sausage	5	*R. mucilaginosa*	100
	NRRL Y-2510	1	*R. mucilaginosa*	100
Saccharomyces cerevisiae	Yoghurt	*3*	*S. cerevisiae*	100
Saccharomycopsis schoenii	Fruit	2	*Sacc. schoenii*	97.5
Saccharomycopsis vini	Fruit	3	*Sacc. vini*	100
Schizosaccharomyces pombe	NRRL Y-9	1	*Sc. pombe*	100
Schwanniomyces pseudopolymorphus	Brine	2	*Sch. pseudopolymorphus*	100
Starmerella bacillaris	Fruit	2	*St. bacillaris*	100
Trichosporon asahii	Salami	3	*Tr. asahii*	100
Torulaspora delbrueckii	Fruit	3	*T. delbrueckii*	99
	Cheese	2	*T. delbrueckii*	100
Ustilago hordei	Pickle	4	*U.hordei*	100
Wickerhamomyces anomalus	Fruit	3	*W. anomalus*	99
Wickerhamomyces subpelliculosa	Tomato paste	*3*	*W.subpelliculosa*	100
Yarrowia lipolytica	Cream	3	*Y. lipolytica*	100
	ATCC 8662	1	*Y. lipolytica*	99
Zygosaccharomyces bailii	Fruit	2	*Z.bailii*	100
Zygosaccharomyces bisporus	NRRL Y-7558	1	*Z. bisporus*	100
Zygosaccharomyces lentus	NRRL Y-27275	1	*Z. lentus*	100
Total		164		

MFMAS Multi Fragment Melting Analysis System, *NRRL* Agricultural Research Service Culture Collection, *ATCC* American Type Culture Collection

is produced for each yeast strain analyzed by combining the melting data of all the target fragments. The MFMAS-yeast software compares the characteristic melting data with the database and determines the yeast species with the highest similarity. In this system, species identification is performed by assigning the yeast species with the highest similarity rate with an unknown strain at the end of the database search. Using the software of the MFMAS-yeast, the melting curves are visualized, digitized, analyzed, and compared with the database. Species-level identification is performed according to similarity rate of the yeast isolate that was analyzed with the data of known species recorded in the database. The MFMAS software uses the difference plots of the melting data between the reference strain and unknown isolates for each DNA fragment analyzed, instead of direct use of the raw data from the melting curve. The design of the database is flexible, and so it can be modified to store the characteristics of new species. Additionally, the software of the MFMAS-yeast has an extendible platform and so new algorithms and features can still be included into the software.

DNA sequencing is too expensive and time consuming to apply for large number of isolates and it requires specialized equipment that is not available in all molecular biology laboratories. Additionally, in PCR-based sequencing methods, only one of the partial or complete sequences of 18S rDNA, the D1/D2 variable domains of 26S rDNA or the ITS1–5.8S–ITS2 regions for which sequence databases are available for many yeast species are targeted. However, identification of yeast taxa based solely on the number of nucleotide differences in a single gene is not always reliable [3]. However, the MFMAS-based identification is finished approximately in 3 h after DNA isolation. The plates used in MFMAS are ready-to-use and only template DNAs are added to the plate before analysis. Ten target regions are simultaneously analyzed in the same plate. Therefore, MFMAS-yeast is a time-saving identification method when compared to sequence analysis. A sophisticated preparation procedure is not required for MFMAS, and DNA purification and measurement of DNA concentration are no different from those in other PCR-based molecular analyses. As, eight different DNA regions showing high interspecies

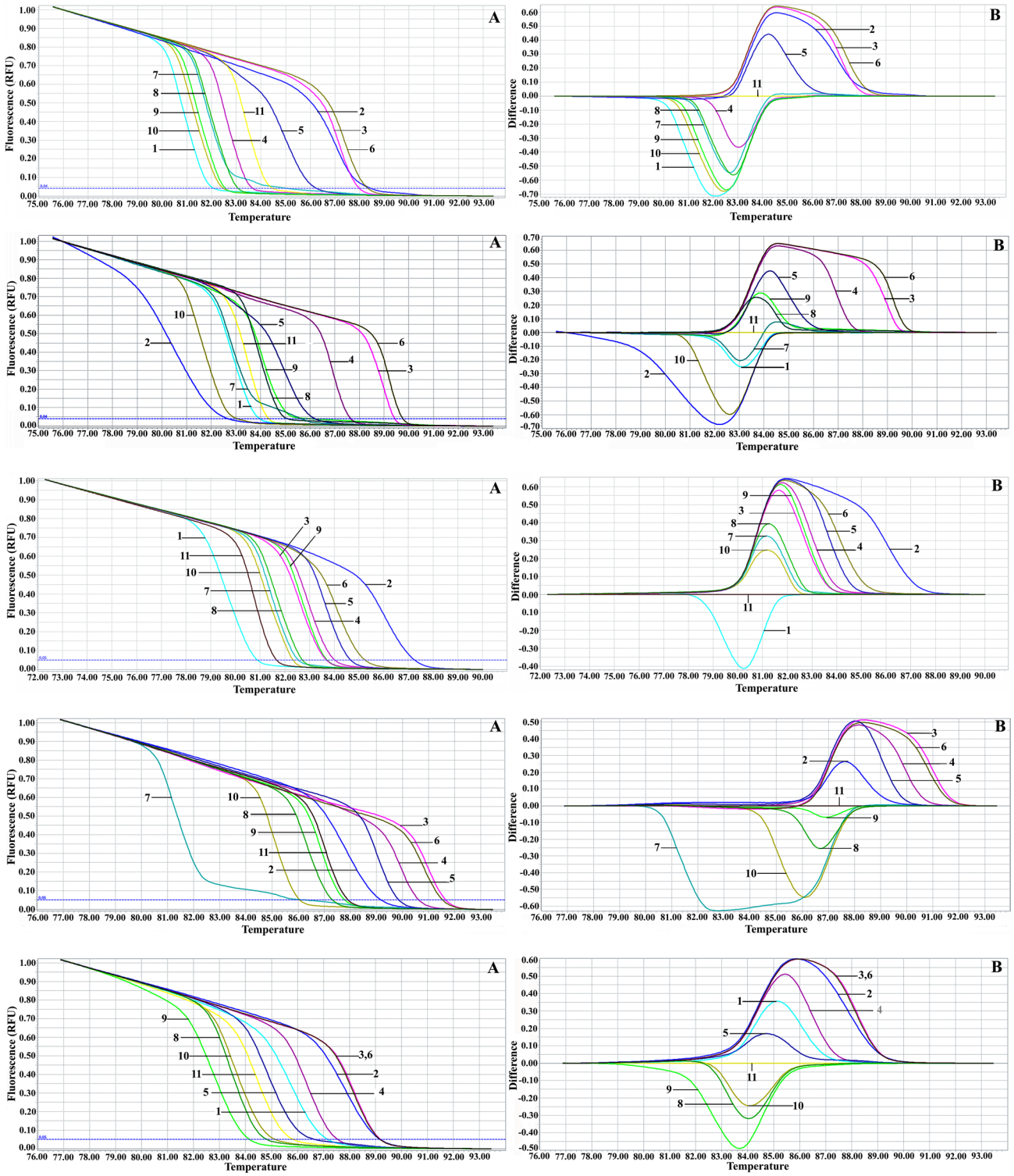

Fig. 2 An application example for *Candida* species (1) *C.deformans;* (2) *C. diversa;* (3) *C. ethanolica;* (4) *C. glabrata;* (5) *C. intermedia;* (6) *C. krusei;* (7) *C. membranifaciens;* (8) *C. sake;* (9) *C. tropicalis;* (10) *C. zeylanoides;* (11) *S. cerevisiae* (positive control)

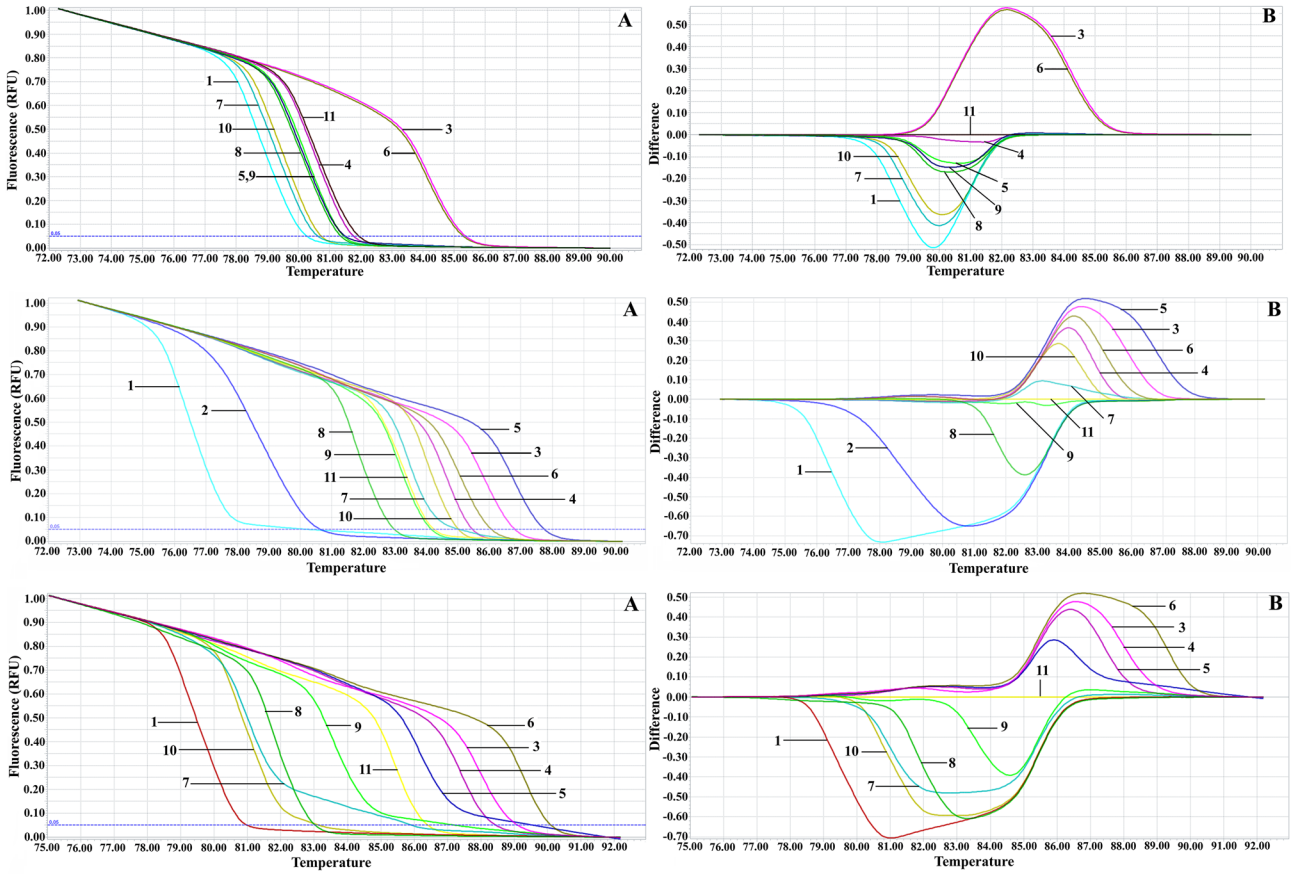

Fig. 2 (continued)

heterogeneity are simultaneously analyzed, an accurate and highly reliable identification is obtained. Additionally, the MFMAS dendrogram produced by combining the melting curve data of all target fragments can help to understand the phylogenetic relationships and the placement of species that cannot be 100% identified. In comparison, as mentioned in the conclusion of the manuscript, the database of the system needs to be constantly updated by registering newly defined yeast species. This is a major limitation of this system.

In this study, the possibility of using the MFMAS-yeast for the identification of yeast species was investigated in first time. For this purpose, a total of 145 food-related yeast isolates and 19 standard yeast strains were tested by the MFMAS. All the analyzed strains were successfully identified with a similarity rate of 95–100%, and MFMAS provided a one-step, rapid, and accurate identification of the yeast species analyzed. In conclusion, the MFMAS-yeast seems to be a promising method for species-level identification of food-borne yeasts. It is believed that this system might be a solution in a certain degree for the inadequacies and the challenges posed by the current methods used in yeast identification. It is also thought that this system will contribute to the prevention of yeast-borne spoilages by providing accurate and reliable detection of the species causing deterioration in foods, and it will also be beneficial to monitor and/or control the processes in yeast fermentations. Additionally, the MFMAS-yeast is also a suitable method not only for the identification of food-borne yeasts, but also for isolates from various clinical and environmental sources such as soil, water, and feed. On the other hand, the database of the system needs to be constantly updated by registering newly defined yeast species from all the sources.

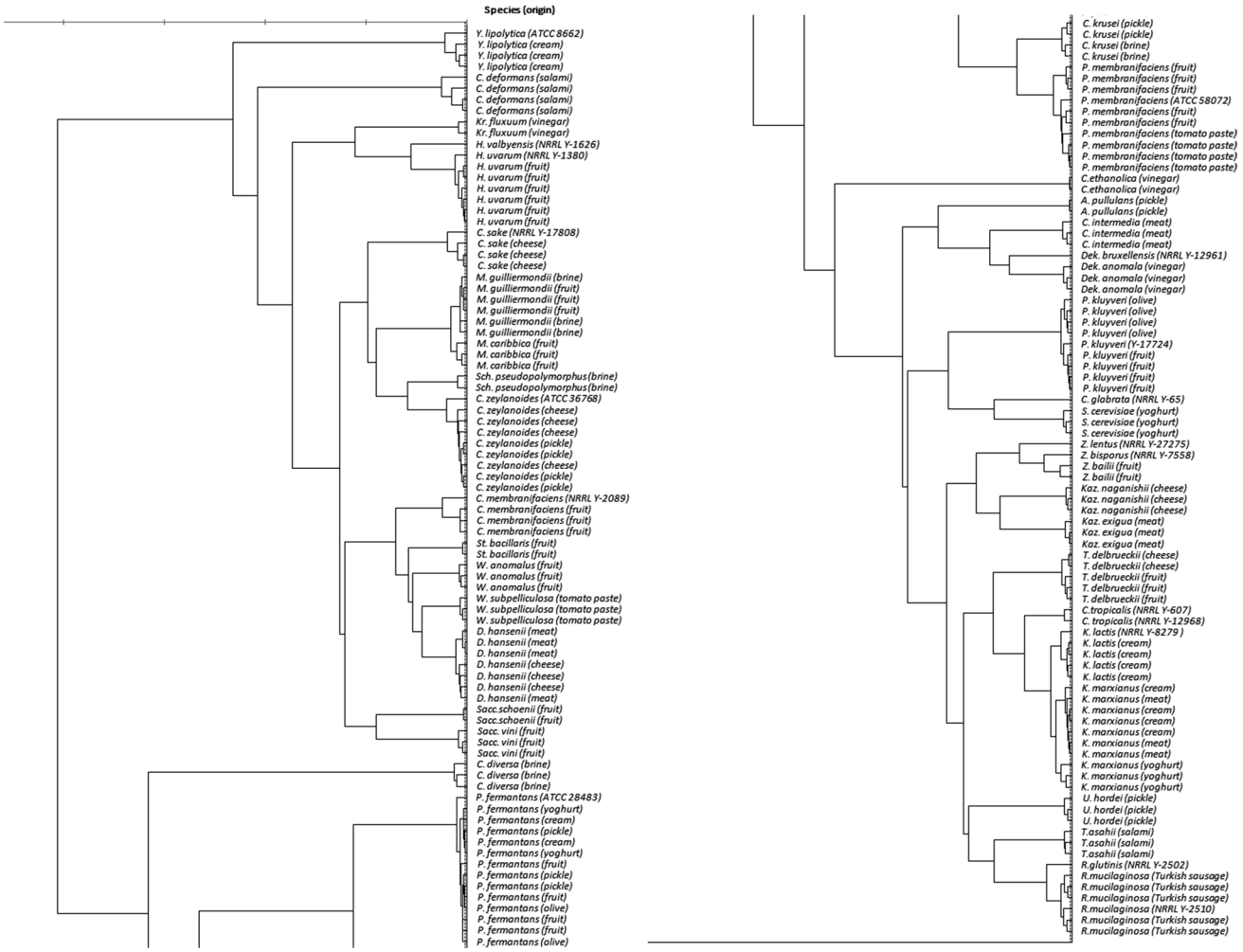

Fig. 3 The dendrogram of cluster analysis of the yeast strains constructed using MFMAS fingerprint patterns

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Compliance with Ethical Standards

Conflict of interest No conflict of interest declared.

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