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MOLECULAR CHARACTERIZATION OF YEAST STRAINS ISOLATED FROM DIFFERENT SOURCES BY RESTRICTION FRAGMENT LENGTH POLYMORPHISM

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Abstract

Various molecular techniques like analysis of the amplified rDNA internal transcribed spacers (ITS), intragenic spacers and total ITS region analysis by restriction fragment length polymorphism (RFLP) has been introduced for yeast identification but there are limited databases to identify yeast species on the basis of 5.8S rDNA. In this study, twenty nine yeast strains from various sources including spoiled fruits, vegetables, foodstuffs, and concentrated juices were characterized by PCR-RFLP. PCR-RFLP has been used to characterize yeasts present in different spoiled food samples after isolation of the yeasts. By using this technique, the isolated yeast strains were characterized by direct 5.8S-ITS rDNA region amplification. RFLP analysis was applied to each of the amplification products (varied from 400bp to 800bp) detected, and the corresponding yeast identifications were made according to each specific restriction patterns obtained after treatment with two endonucleases *Taq*I and *Hae*III which yielded a specific banding pattern for each species. For further confirmation amplified products of eleven selected isolates were sequenced and blast on NCBI. Both RFLP and sequence analyses of the strains with accession nos. KF472163, KF472164, KF472165, KF472166, KF472167, KF472168, KF472169, KF472170, KF472171, KF472172, KF472173 gave significantly similar results. The isolates were found to belong five different yeast species including; *Candida* spp., *Pichia* spp., *Kluyveromyces* spp., *Clavispora* spp. and *Hanseniaspora* spp. This method provides a fast, easy, reliable and authentic way for determining yeast population present in different type of samples, as compared to traditional characterization technique

Key words: Yeast; RFLP; 5.8S-ITS*; Candida*; *Pichia*: *Clavispora*.

Introduction

Yeast is pervasive in the environment but is commonly obtained from fruits, berries and exudates (sap) of plants which are high in sugar content. The definition of yeast is unicellular fungi whose vegetative reproduction is either by budding or by fission which involves a vast variety of organisms including *Ascomycetes, Basidiomycetes* and *Imperfect yeasts* (Fungi imperfecti) (Moiina & Raspor, 1997; Tikka *et al.*, 2013). The useful aspects in working with yeast include their easy growth in the laboratory, genetically easy to control and utilization as a model system in the study of eukaryotic cellular processes (Brar *et al.*, 2012).

Yeast has contributed a lot in fermentation of bread, alcoholic beverages, cheeses and for production of biologically important products like insulin, enzyme, vitamins and antibiotics (Furman, 2011; Passoth *et al.*, 2013). Rapid and advanced methodologies like cell morphology, conidiogenesis, G + C content, DNA-DNA hybridization (Libkind *et al.*, 2011; Naumov *et al.*, 2013; Torok *et al.*, 1993), karyotyping (Nadal *et al.*, 1996; Tofalo *et al.*, 2013; Vaughan-Martini & Martini, 1995), microsatellite (ssr) analysis (Baleiras Couto *et al.*, 1994), amplification of interdelta regions (Legras & Karst, 2003) and PCR-based procedures including species-specific PCR (Josepa *et al.*, 2000), RFLP of chromosomal DNA (Fernandez-Espinar *et al.*, 2001; Versavaud & Hallet, 1995), and restriction fragment length polymorphism of mitochondria1 DNA (Araujo *et al.*, 2007; Hidalgo *et al.*, 2013) should be adopted for detection of yeast in food which will aid in reduction of food spoilage and economic loss.

The purpose of the recent study is the identification, differentiation and characterization of yeasts isolated from various sources including spoiled fruits, vegetables, foodstuffs, and concentrated juices by using PCR-RFLP. In this technique, the restriction profile yielded from the sequences across the internal transcribed spacers (ITS4 & ITS1) and 5.8S rRNA gene referred to as 5.8S-ITS region, was utilized to characterize a total number of 30 yeast isolates. In this case, amplified products and their restriction patterns, obtained by cutting with two restriction enzymes like *Taq*I and *Hae*III which were specific for each species, were achieved.

Materials and Methods

Isolation and purification of yeast isolates: Samples from different sources such as rotten fruits and vegetable, commercial yeasts, milk, wine, yogurt, preserved food, garden soil from different areas of Lahore and Faisalabad , were collected and stored at 4ºC in airtight bags. Serial dilutions up to 10^{-3} of each 1% sample were prepared and 100 µl from each dilution was spread on YEPD (Kurtzmann & Fell, 1998). The plates were incubated for overnight at 30^{o}C. Colonies appeared on plates after incubation were selected and streaked on specific medium Eosin-Methylene Blue Differential (EMBD) medium contained 0.3%Peptone, 1%Glucose, 0.05% Chloramphenicol, 0.0065% Methylene blue, 0.01% Triphylterazolium, 0.04% Eosin, 0.3% Malt extract and 2% Agar (Deák, 2007). Different colored colonies appeared on EMBD plates were selected and streaked on new YEPD plates and incubated at the same condition as before to get pure single colonies. Purified cultures were preserved as 30% glycerol stocks at -80ºC for further use.

Morphological characteristics of isolated yeast: The purified yeast strains were morphologically identified by wet mount method at 40X and 100X magnification of compound microscope and budding yeasts of different shapes and sizes were observed.

DNA isolation from different yeast strains: DNA from yeast strains was isolated by modified CTAB method (Doyle, 1990; Shahzadi *et al.*, 2010). Overnight grown yeast cultures in YEPD broth were centrifuged at maximum speed. About 10mg of yeast cells for each strain were taken and pre warmed 200 µl of solution I at 65°C containing 1.4M NaCl, 2% CTAB, 20mM EDTA (pH 8.0), 0.2% β-mercaptoethanol and 100mM TrisHCl (pH 8.0) was introduced, mixed well and incubated at 65°C for 15-20 minutes in water bath. After incubation, all tubes were cooled for 3-5 minutes and same volume of solution II (Chloroform: Isoamyl alcohol, 24:1) was added, mixed thoroughly and centrifuged at 14,000 rpm for 10 minutes at room temperature. Aqueous phase (upper) were taken from each eppendorf separately and 3M Na acetate (1/10) was introduced in each eppendorf along with equal volume of cold iso-propanol or double volume of cold absolute ethanol, mixed it gently and placed on ice for 10 minutes. All tubes after incubation were centrifuged at 12000 rpm at 4°C for 15 minutes and supernatant was disposed off. Five hundred microlitre of chilled 70% ethanol (solution III) was added directly for washing pellet and then centrifuged at 14000 at 4°C for 2 minutes. The pellet was air dried after discarding supernatant from each tube. The pellet was resuspended in 50µl double deionized water or TE-buffer to store at –20°C. The yield of DNA was quantified by Spectrophotometer (Sambrook *et al*., 2004*)*.

Amplification of 5.8S-ITS region by polymerase chain reaction: Amplification of 5.8S-ITS region of rRNA gene was done by using ITS1(F) 5'TCCGTAGGTGAACCTGCGG3' and ITS4 (R) 5'TCCGTAGGTGAACCTGCGG3' primers (White *et al.*, 1990) in thermocycler (Bioerxp cycler). The reaction mixture contained 100ng DNA, 5µl of 10pmol each oligonucleotide primer, 3µl of 25mM $MgCl_2$, 3µl of 250mM dNTPs mixture and Taq DNA polymerase (5units) in a total volume of 50 µl. PCR conditions were as follow: 3 min. at 94C^o followed by 35 cycles (45 sec. at 94^{o}C, 45 sec. at 55^{o}C (annealing temperature), 1 min. at 72^{o}C^o and final extension for 7 min. at 72^{o}C. The amplified product was checked by running on 0.8% agarose gel and visualized by using UV illuminator and photographed.

Amplified ITS region analysis by RFLP: PCR products of partially amplified-ITS region were subjected to restriction fragment length polymorphism (RFLP) for two restriction endonucleases *Taq*I and *Hae*III. The reaction mixture contained 3.0 µl of 1X buffer (R-buffer for *Bsu*RI (*Hae*III) and unique-buffer for *Taq*I), 15.0 µl PCR products (approximately 1.0 µg), 1µl of specific endonuclease and 11µl of deionized water with total volume of 30µl. The reaction mixtures were incubated at their specific temperatures as recommended by manufacturer's instructions (Fermentas) The restriction fragments were separated along with a DNA 100bp ladder on 1.5% w/v agarose gel and photographed after visualization under UV light.

DNA SEQUENCING: Out of total amplified PCR products, eleven products (SZ2, SZ7, SZ8, SZ12, SZ13, SZ17, SZ21, SZ24, SZ26, SZ28, and SZ23) were selected based on the restriction pattern of both restriction endonuclease enzymes and sent to Center for Advance Molecular Biology (CAMB) Lahore, Pakistan for sequencing by automated sequencer. Sequenced data obtained was blasted on NCBI and submitted to Bankit for accession numbers.

Results

Isolation and purification of yeast strains from different sources: Purified yeast strains on YEPD were initially morphologically identified as budding yeast of different shape and sizes (Fig. 2) and then subjected to RFLP. Single different colored colonies were selected from specific medium containing triphyltetrazolium. Among 110 colonies, 49 were of pink color and remaining were metallic green in color (Fig. 1b).

Fig. 1. Purified colonies of yeast strains on YEPD (a) and EMBD(b) agar plates.

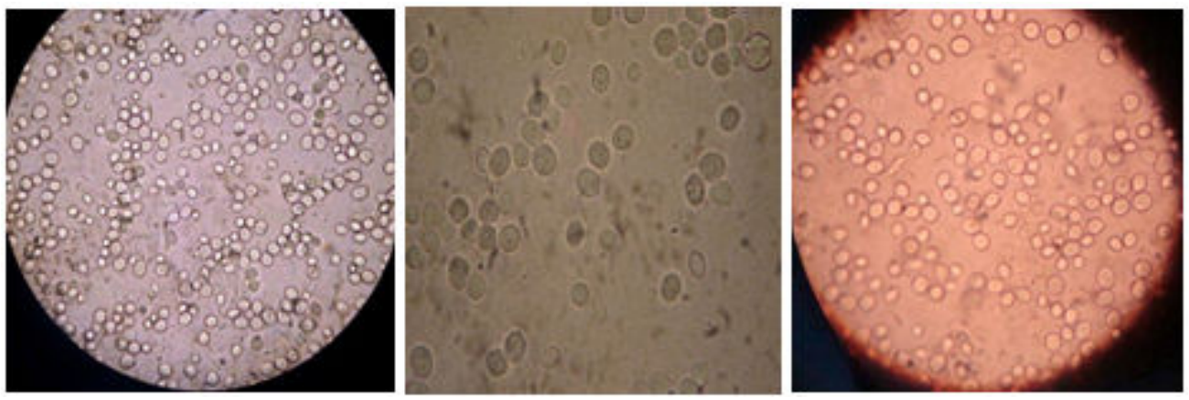

Fig. 2. Budding yeast of different sizes and shapes under microscopic examination.

Amplification of 5.8S-ITS ribosomal gene by PCR: PCR amplified products were resolved on 1% Agarose gel. Different sizes of amplified products of yeast strains ranges from 400bp to 800bp were observed by comparing with 100bp plus DNA ladder (Fig. 3).

Restriction analysis of 5.8S-ITS region by restriction fragment length polymorphism (RFLP): Amplified 5.8S-ITS region of ribosomal RNA gene of yeast strains (Fig. 3) was digested with two restriction endonucleases, *Taq*I and *Hae*III as in methodology. The number and size of different restriction fragments obtained from amplification products with *Taq*I (Fig. 4) and *Hae*III (Fig. 5) are summarized in Tables 1 and 2 respectively. The number and size of bands varied in restriction digestion analysis with both enzymes.

Molecular characterization and phylogenetic analysis of yeast strains on the basis of 5.8S-ITS rRNA Sequencing: Selected strains were restricted by *Taq*I and *Hae*III restriction enzymes and categorized into 11 classes with each class giving specific banding pattern. For molecular characterization, eleven yeast strains from each class were selected and partially sequenced. The nucleotide sequences obtained were blasted by NCBI database. The blast query revealed that yeast strains SZ-02(KF472163), SZ-07(KF472164) and SZ-08(KF472165) were homologous to *Candida etchellsii*, SZ-11(KF472166) was homologous to *Pichia kudriavzevii*, SZ-12(KF472167) was homologous to *Candida tropicalis*, SZ-13 (KF472168) and SZ-17 (KF472169) were homologous to *Candida intermedia* and *Clavispora lusitaniae* respectively (Fig. 6). Similarly SZ-21 (KF472170) was homologous to *Kluyveromyces marxianus*, SZ-24 (KF472171) to *Hansenia sporaovarum*, SZ-26 (KF472172) to *Meyerozyma guilliermondii* and SZ-28 (KF472173) *to Hanseniaspora guilliermondii* (Fig. 6). Other close matches revealed that *Candida etchellsii* (KF472163) has 100% similarity with *C. etchellsii* (JQ653271) whereas C. *etchellsii* (KF472164 and KF472165) showed 99% homology with already reported yeast strains. *Clavispora lusitaniae* (KF472169) and *Candida intermedia* (KF472168) showed 100% similarity between themselves whereas 100% and 79% similarities with already reported *C. lusitaniae* (EU568925) and *C. lusitaniae* (AY321470) respectively. *Candida tropicalis* (KF472167) have 100% homology with *C. tropicalis* (JF922863) and *C. tropicalis* (JN162678) (100% similarity). *Pichia kudriavzevii* (KF472166) was 100% homologous to *P. kudriavzevii* (JQ083432) and *P. kudriavzevii* (KF277144). Likewise, *Kluyveromyces marxianus* (KF472170) and *Meyerozyma guilliermondii* (KF472173) showed 87% homology with each other and 100% similarity with *K. marxianus* (HQ396523) and *Meyerozyma caribbica* (KC544483). Finally phylogenetic analysis also revealed the 100% similarity between *Hanseniaspora ovarum* (KF472171) and *Hanseniaspora guilliermondii* (KF472172) and 97% homology with already reported strains of same species (Fig. 6).

Discussion

Current yeast classification is based on phylogenetic relationships inferred by gene sequencing (Lachance, 2011). DNA analysis has been applied for direct identification of yeasts. In recent studies, molecular techniques such as DNA-DNA hybridization (Libkind & Hittinger & Valério & Gonçalves & Dover & Johnston & Gonçalves & Sampaio, 2011;), electrophoretic karyotyping (Nadal & Colomer & Piña, 1996; Vaughan-Martini & Martini, 1995), allozyme patterns, (Naumov *et al.*, 1997), microsatellite (ssr) analysis (Baleiras Couto & van der Vossen & Hofstra, 1994; Rabbani *et al*., 2010), polymerase chain reaction-based procedures ranging from species-specific PCR, nested-PCR (Ibeas *et al.*, 1997; Josepa & Guillamon & Cano, 2000; Shinwari, 2002), RAPD analysis (Oliveira *et al.*, 2008; Jan *et al*. 2011), amplification of interdelta regions (Legras & Karst, 2003), restriction fragment length polymorphism of chromosomal DNA or restriction pattern of mitochondria1 DNA (Araujo & Gomes & Moreira & Cisalpino & Rosa, 2007; Ibeas & Lozano & Perdigones & Jimenez, 1997; Masood *et al*., 2005), are relied on both similar or dissimilar changes of DNA, RNA or protein. They have certain advantages over phenotypic identification. Yeast ribosomal RNAs (18S, 25S, 5.8S and 5.0S rRNA) are encoded by the genes organized in the rRNA unit that is repeated 100-200 times on the chromosome XII (Montrocher *et al.*, 1998). In this study, primers ITS4 and ITS1 were utilized to amplify ribosomal DNA consisting of 5.8S ribosomal RNA gene and the two non-coding regions (ITS1 and ITS2) described as the internal transcribed spacers referred to as 5.8S-ITS region of yeast strains. Tables 1 and 2 demonstrate the size of amplified products and the restricted fragments achieved after treatment with two restriction endonuclease enzymes *Taq*I and *Hae*III. Fragment smaller than 50bp were not inserted in the Tables because they could not be reproducibly visualized under UV light. The amplified products showed variation in length with range, 400bp for *Candida* and *Clavispora* spp. to 800bp for *Hanseniaspora* spp.

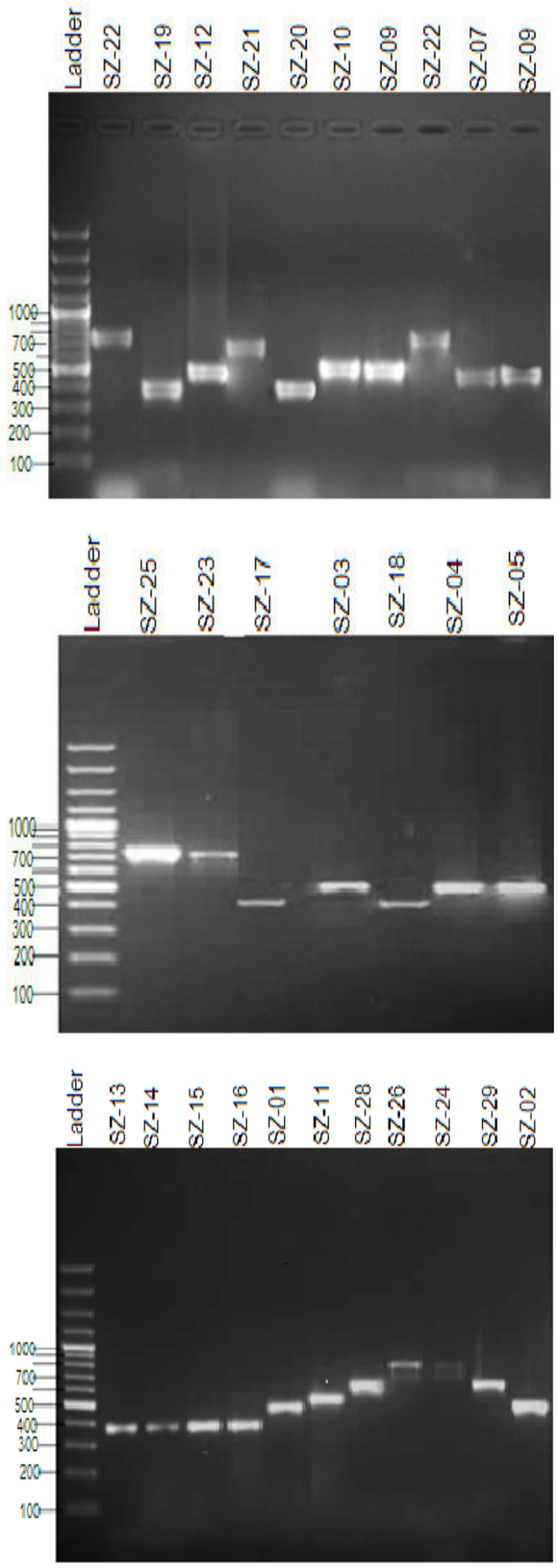

Fig. 3. Agarose gel electrophoresis of amplified PCR products of yeast stains by using ITS1 and ITS4 primers (DNA ladder 100 bp plus).

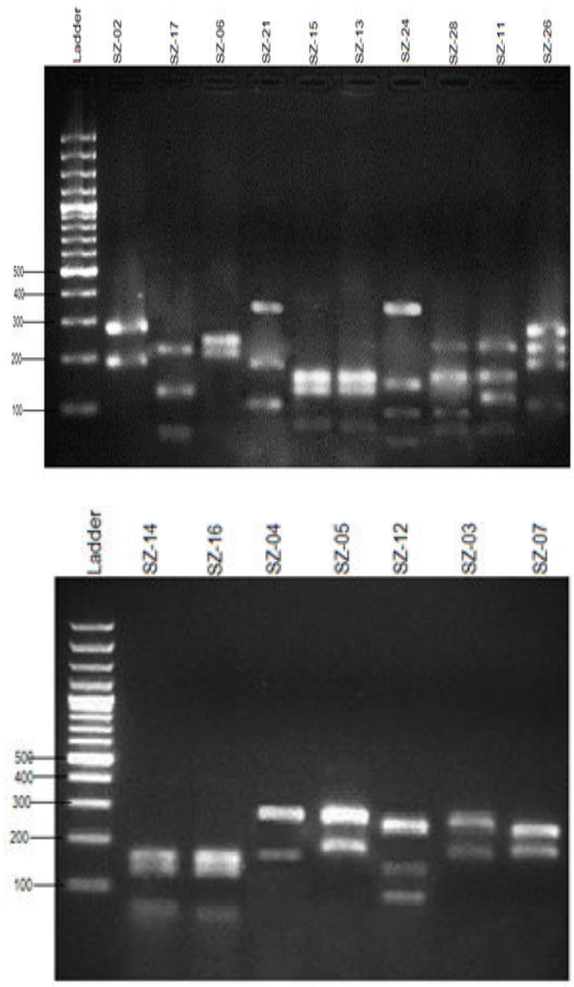

Fig. 4. Restriction fragment length polymorphism (RFLP) analysis of yeast strains after digestion of amplified PCR products (5.8S-ITS region) with restriction endonuclease enzyme *Taq*I.

All yeast species that cannot be classified in other asexual *ascomycetes* yeast genera are included in genus *Candida* is a very heterogeneous genus so those yeast species which do not fit in any other asexual *ascomycete* can be included in this genus. Due to this reason, the perfect state of most *Candida* species are still unknown (Krejer-van Rij, 1984; Kurtzman *et al.*, 2011). In present study, 30 yeast strains belonging to *Candida* spp., *Pichia* spp., *Kluyveromyces* spp., *Clavispora* spp. and *Hanseniaspora* spp., were characterized by RFLP analysis of the 5.8S-ITS region. Each species of selected yeast genera yielded a specific restriction pattern with *Taq*I and *Hae*III restriction enzymes. The variable PCR amplified fragments have range from 400bp for *Candida intermedia* to 500bp for *Candida etchellsii* and C*andida tropicalis,* which shows the heterogeneity and diversity of the genus (Tables 1 and 2). Due to the diverse and complex nature of genus *Candida*, it is recommended that restriction pattern obtained with two endonucleases like *Taq*I and *Hae*III can be uded to get reliable identification of the *Candida* species.

Table 1. RFLP analysis of 5.8S ITS region after digestion with *Taq*I.

Categories	Sr. No	PCR products	Size (bp)	Digestion with *Taq*I& No of fragments	Stains identified on the basis of sequencing and RFLP
	1	SZ-1	500	2(260,210)	
	2	SZ-2	500	2(280,190,)	
	3	SZ-3	500	2(250,190)	
1st	4	SZ-4	500	2(290,140,)	*Candida etchellsii*
	5	SZ-5	500	2(270,190)	
	6	SZ-6	500	2(260,210)	
	7	SZ-7	500	2(280,190)	
	8	SZ-8	500	3(250,150,100)	
2nd	9	SZ-9	500	3(250,150,100)	*Pichia kudriavzevii*
	11	SZ-10	500	3(250,150,100)	
3rd	12	SZ-11	550	4(210,170,100,70)	*Pichia kudriavzevii*
4th	13	SZ-12	500	4(260,170,70)	*Candida tropicalis*
	14	SZ-13	400	3(175,150,75)	
5th	15	SZ-14	400	3(170,160,70)	*Candida intermedia*
	16	SZ-15	400	3(175,150,75)	
	17	SZ-16	400	3(170,160,70)	
	18	SZ-17	400	3(220,130,50)	
6th	19	SZ-18	400	3(200,150,50)	*Clavispora lusitaniae*
	20	SZ-19	400	3(200,150,50)	
	21	SZ-20	400	3(230,110,60)	
7th	22	SZ-21	700	3(400,200,100)	*Kluyveromyces marxianus*
8th	23	SZ-22	750	3(450,200,100)	*Kluyveromyces marxianus*
	24	SZ-23	750	3(400,200,150)	
10th	25	SZ-24	750	4(400,170,130,50)	*Hanseniaspora uvarum*
	26	SZ-25	750	4(400,200,100,50)	
11th	27	SZ-26	800	4(280,240,180,100)	*Hanseniaspora guilliermondii*
12th	28	SZ-27	750	4(280,200,160,60)	*Pichia guilliermondii*
13th	29	SZ-28	600	4(250,170,110,70)	*Meyerozyma guilliermondii*
	30	SZ-29	600	4(230,170,150,50)	*(Pichia guilliermondii)*

Table 2. RFLP analysis of 5.8S - ITS region after digestion with *Hae*III.

Categories	Sr. No.	Isolates	Size (bp)	No. of fragments after digestion with *Hae*III	Strains identified on the basis of sequencing and RFLP
1st	1	SZ-2	500	(500)	*Candida etchellsii*
	2	SZ -7	500	(500)	
	3	SZ -1	500	2(410,90)	
	4	SZ -5	500	2(420,80)	
2nd	5	SZ -4	500	2(400,100)	*Candida etchellsii*
	6	SZ -3	500	2(400,100)	
	7	SZ -6	500	2(400,100)	
3rd	8	SZ -8	500	2(390,110)	*Pichia kudriavzevii*
	9	SZ -9	500	2(410,90)	
4th	10	SZ -12	500	1(500)	*Candida tropicalis*
	11	SZ -11	550	3(400,120)	*Pichia kudriavzevii*
	12	SZ -18	400	1(400)	
5th	13	SZ -17	400	1(400)	*Clavispora lusitaniae*
	14	SZ -19	400	1(400)	
	16	SZ -20	400	1(400)	
	17	SZ -16	400	1(400)	
6th	18	sz-13	400	1(400)	*Candida intermedia*
	19	SZ -14	400	1(400)	
	20	SZ -22	750	2(690,60)	*Kluyveromyces marxianus*
7th	21	SZ -23	750	2(680,70)	
	22	SZ -24	750	1(750)	*Hanseniaspora uvarum*
	23	SZ -25	750	1(750)	
8th	24	SZ -21	700	2(600,100)	*Kluyveromyces marxianus*
9th	25	SZ -26	800	800	*Hanseniaspora guilliermondii*
10th	26	SZ -27	750	3(490,150,130)	*Pichia guilliermondii*
11th	27	SZ -28	600	3(400,130,70)	*Pichia guilliermondii*
	28	SZ -29	600	3(400,180,60)	

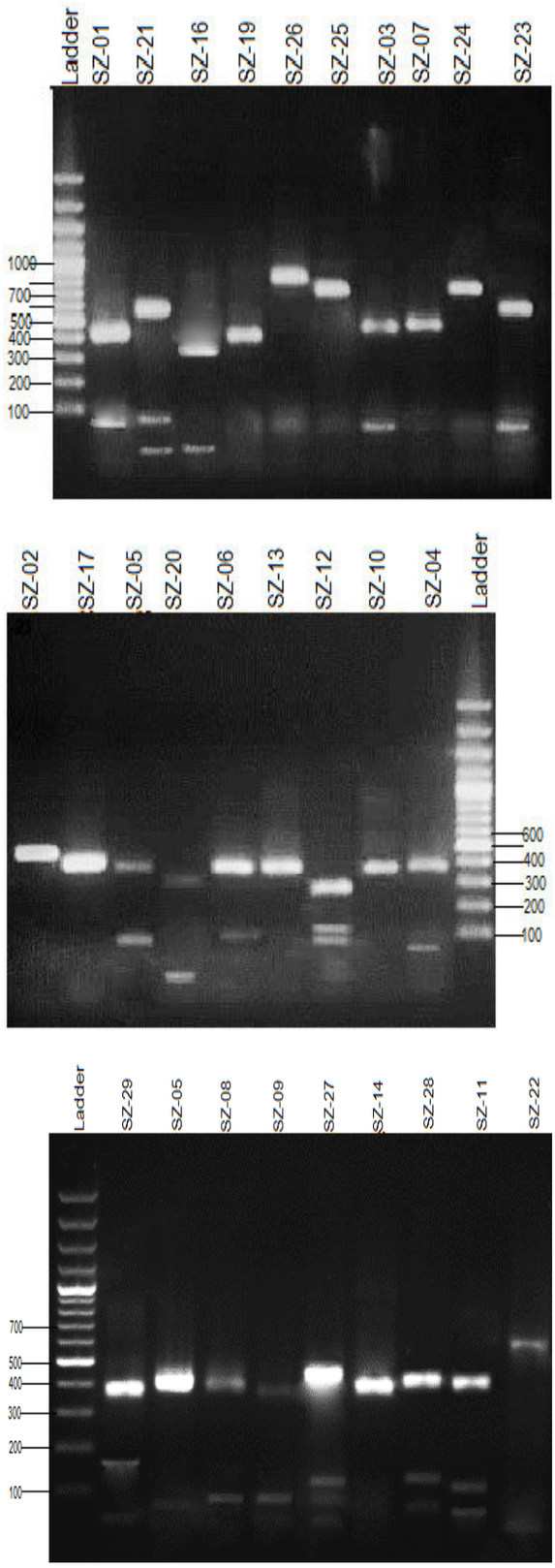

Fig. 5. Restriction fragment length polymorphism (RFLP) analysis of yeast strains after digestion of amplified PCR products (5.8S-ITS region) with restriction endonuclease enzyme *Hae*III.

In the genus *Kluyveromyces*, almost all the species were characterized due to large size of PCR products. All strains belonging to the same species exhibited the similar pattern with the different endonucleases like *Taq*I and *Hae*III (*Bsu*RI) as shown in the Figs. 4 and 5 respectively. *Candida* spp. and as well as *Kluyveromyces* spp. are mostly found in plain yogurt. The capability of these yeasts to grow at very low temperatures to ferment lactose and sucrose and yield lipolytic and proteolytic enzymes which make yogurt by hydrolysis, if milk fat and protein, is very high (Hamad, 2012).

The genus *Hanseniaspora* is an emerging genus of yeast belonging to the *Hemiascomycetes* class, *Endomycetales* order, *Saccharomycetaceae* family.(Suzzi *et al.*, 1996). Three of the isolates (SZ-24,SZ-5 having PCR-product of 750bp and SZ-26 having 800bp) were identified as species *Hanseniaspora ovarum* and *Hanseniaspora guilliermondii* having products sizes of 750bp and 800bp respectively showed a specific restriction pattern with the different endonucleases like *Taq*I and *Hae*III (BsuRI) as shown in the Figs. 4 and 5. Similarly, the isolates belonging to *species*, *Clavispora usitaniae* were also identified having a constant PCR-product size of 400bp and specific banding pattern with endonucleases *Taq*I and *Hae*III (*Bsu*RI) as shown in Figs. 4 and 5.

The genus *Pichia* is the largest in ascomycetous yeast genus. In this study, three *Pichia* species isolated from the different sources were analyzed. The variability in the sizes of PCR products ranges from 500bp in *Pichia kudriavzevii* to 600bp in *Pichia guilliermondii* reflects the complexity of this genus. With the recent advances in automation of nucleic acid sequencing and accumulation of the knowledge of species specific PCR primers development, it seems likely that direct sequencing of PCR amplified DNA fragments could replace the currently used techniques. In perspective, this molecular technique suggested as a rapid, cheap and easy methodology can replace the currently use techniques as a uniform method for the identification of yeasts in general.

The isolates belonging to *Pichia kudriavzevii* produced specifically pink colonies on Eosin-Methylene Blue Differential (EMBD) medium that is not reported still in the literature while this medium already utilized to differentiate common food-borne yeast species on the basis of colored colonies. *Zygomyces balli* produces black to violet colonies whereas *S. cerevisiae* produce metallic green colonies on this medium (Deák, 2007).

Databases already formed on DNA sequence analysis of partial or complete 18S and/or 26S rRNA/DNA present an indispensable source of information for the development of specific PCR primers and also for yeast genera including industrially important yeast species (James *et al.*, 1996; Kurtzman, 2006). However, a development of species or strain-specific PCR primers is still required for most of the industrial yeast strains. Considering the intensity of research in this field of applied science it is realistic to expect one-day procedures for identification of the most important industrial yeasts directly from food in the very near future (Sonja *et al.*, 1997). On the basis of up-to-date technology, direct transfer of this technique from research to routine industrial laboratories remains very limited due to technical pretentiousness, limited education of employees and the price for such type of routine analysis.

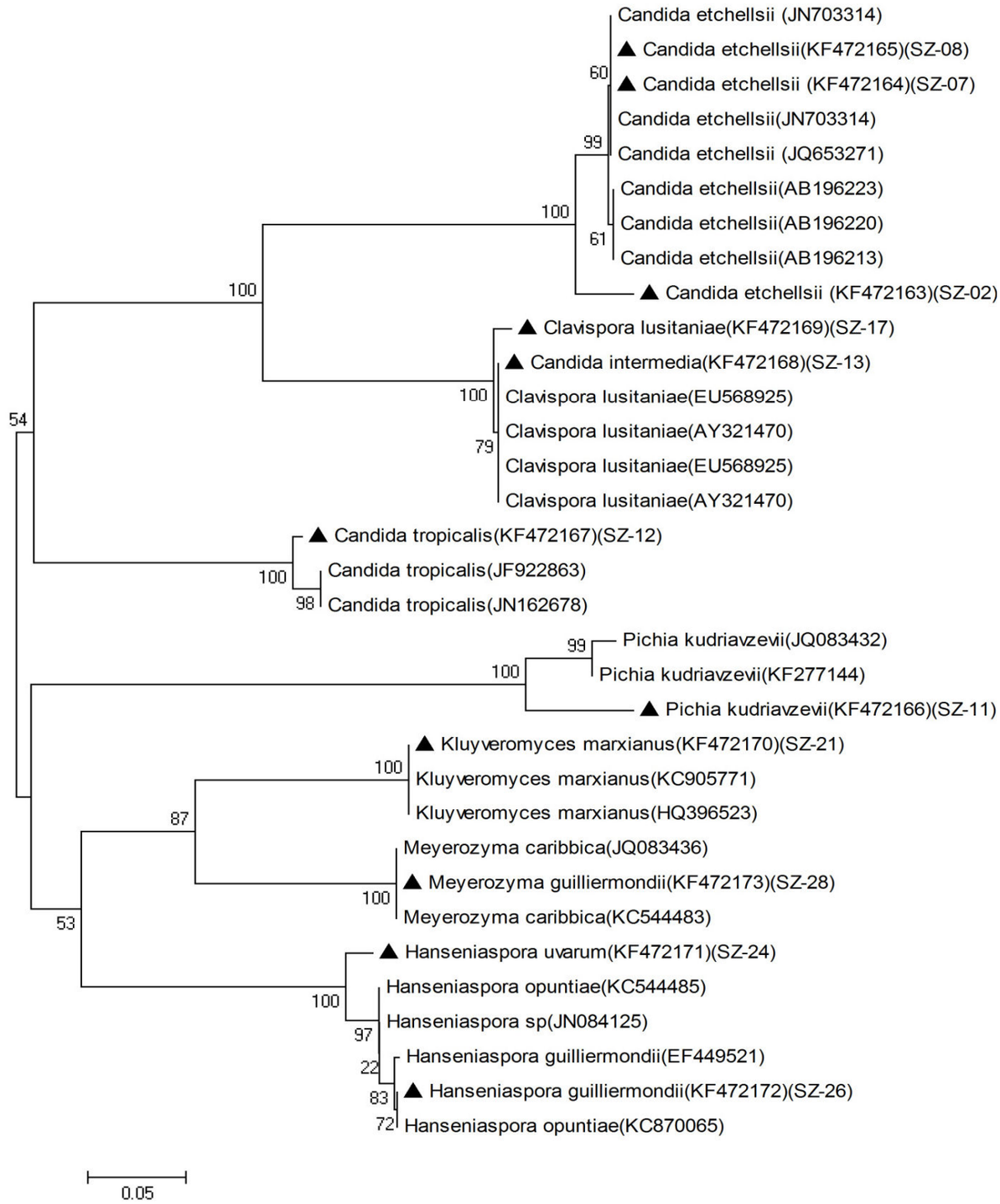

Fig. 6. Neighbor-joining tree showing the phylogeny of selected yeast sp. with already reported and closely related yeast species. Scale bar specifies 0.05 changes per nucleotide position.

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