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IDENTIFICATION AND PHYLOGENY OF SOME SPECIES OF THE GENERA *SPORIDIOBOLUS* AND *RHODOTORULA* USING ANALYSIS OF THE 5.8S RDNA GENE AND TWO RIBOSOMAL INTERNAL TRANSCRIBED SPACERS

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Abstract - Due to the problems encountered in routine morphological and physiological procedures that are used in yeast identification, DNA-based methods have recently been developed. In the present study, l66 yeast strains were isolated from several apple and citrus cultivars. After analysis by basic morphological methods, the ITS1 and ITS2 regions of the isolates were amplified separately, and the isolates were grouped based on fragment size polymorphism (FSP) of the amplicons. By comparing the electrophoretic patterns of the PCR products with *Rhodotorula mucilaginosa,* species were identified as *Rhodotorula*. For precise and final identification, the ITS-PCR products were subjected to sequencing followed by Blast analysis. As a result, eight isolates were identified as belonging to the *Rhodotorula* genus, of which five were identified as *R. mucilaginosa* and three as *R. glutinis*, and one as a *Sporidiobolus*. We conclude that the method PCR-FSP, in combination with other approaches, is useful for the identification of yeast species.

Key words: Yeast, identification, ITS, *Rhodotorula*, *Sporidiobolus*

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INTRODUCTION

Rhodotorula Harrison is a member of the basidiomycetous yeasts, existing normally in air and soil from which it can be easily isolated. This yeast can also be obtained from human skin, stool, food and fruits. Although most species of the genus *Rhodotorula* are non-pathogenic, some species are infectious (Hazen et al., 1995). It is necessary to understand that numerous *Rhodotorula* species play a role as biological control agents of fruit diseases following rhodotorulic acid production (Gholamnejad et al., 2009). The *Sporidiobolus* species have mostly been isolated from the soil and wilting leaves of some trees (Drex et al., 1930; Last et al., 1955; Nagahama et al., 2001; Shadd et al., 2001). It was reported that *Sporobolomyces roseus* is a synonym of *Sporobolomyces shibatanus*. The anamorph of *Sporidiobolus pararoseus* has been studied by phylogenetic analysis (Boekhout et al., 1991; Boekhout and Nakase, 1998; Hamamoto and Nakase, 2000). Both of these species (*Rhodotorula* and *Sporidiobolus*) produce some pink, orange and red pigments (Kreger-Van Rij 1984).

Red yeasts are the predominant yeasts recognized in many studies, and are primarily members of the genera *Rhodotorula*, *Rhodosporidium* and *Sporobolomyces* (Hagler et al., 1987). The genus *Rhodotorula* has been mentioned as a polyphyletic genus and it is a phylogenetically mix-group with the genera *Rhodosporidium, Sporidiobolus* and *Sporobolomyces* (Fell et al., 1995, 1998, 2000).

Identifications of anamorphic yeasts based on phenotype were remarkably problematic because of the variable or ambiguous results of these kinds of tests. It has been reported that some of their characteristics are highly influenced by culture conditions (Guillamón et al., 1998; Valente et al., 1999). Recently, molecular identification techniques, particularly those based on the ribosomal RNA gene (rRNA gene), have been developed for the precise identification of these microorganisms, including random amplified polymorphic DNA (RAPD)-PCR, restriction fragment length polymorphism (RFLP) analysis, real-time PCR with species-specific probes, and sequence analysis of amplicons (Esteve-Zarzoso et al., 1999; Kami et al., 2001; Loeffler et al., 2000; Millon et al., 2002; Pryce et al., 2003).

Different parts of rDNA complex, especially ITS1 and ITS2 which are separated by the 5.8S gene, are widely used for species identification of fungi (James et al., 1996; Kurtzman et al., 1998, 2003, 2008). In the present study, we used morphological and molecular methods based on the rDNA region to identify the *Rhodotorula* and *Sporidiobolus* species.

MATERIALS AND METHODS

Isolates

The yeast species were isolated from the surfaces of healthy fruits including apple, sweet lemon, sour lemon, orange, and tangerine. Fruit skins were submerged in about 150 ml double-distilled water and shaken for 30 min at 100 RPM. Then, 0.3 ml of each sample was placed on potato dextrose agar (PDA) and incubated at 25-28 ° C for 48 h. The yeast isolates were stored at -20° C in a 1.5 ml tubes containing 30% glycerol and 70% double distilled water to keep them alive for a long period (Kreger-Van Rij 1984; Yarrow et al., 1998).

A total of 55 yeast strains were isolated, namely M11 – M40 from golden delicious , red delicious and golab apples from the cities of Damavand, Eyvanakey, Shiraz, Urumia, Pakdasht, Tehran, Karaj, Yazd, and M41 – M55 for sweet and sour lemon, tangerine, orange obtained from Tehran and Karaj. We also had a standard strain of *Rhodotorula mucilaginosa* from CBS named M1, M3, and M7.

Phenotypic tests

Morphological properties were determined for each group (Yamamoto et al., 1991). The production of pigments and shape of colonies of yeast was examined. Biochemical tests including the assimilation of sucrose, arabinose, dextrose, mannose, fructose, glucose, rhamnose were tested on PDA for some isolates, and the isolates were cultured on CHROMagar Candida which frequently is used for the differentiation of the medically important *Candida* species (Shadd et al., 2001).

Molecular tests PCR-FSP

rDNA regions are unique and can be used to identify common yeasts at the species level (Iwen et al., 2002; Mirhendi et al., 2008). A preliminary grouping of the isolates was performed on the basis of fragment size polymorphism (FSP) of both the internal transcribed spacer (ITS1) and (ITS2) regions in rDNA using colony-PCR (Mirhendi et al., 2007). The forward ITS1 (5'-TCCGTAGGTGAAC CTGCGG-3') and reverse ITS2 (5'-GCTGCGTTCTTCATCGATGC-3') primers were used to amplify the ITS1 region and the forward (ITS3: 5'-GCATCGATGAAGAACGCAGC-3') and ITS4 (5'-TCCTCCGCTTAT TGATATGC-3') primers were used for amplification of ITS2 region, respectively. The amplicons of each region for each individual yeast isolate were mixed and subjected to 1.5% agarose gel electrophoresis, stained with ethidium bromide and visualized under UV light. The molecular sizes of the DNA fragments were judged in comparison with molecular standards 100bp. The identification of the yeasts was based on the electrophoretic pattern for each species. This means that we amplified the ITS1 region with two primers, ITS1 and ITS2, and the ITS2 region with ITS3 and ITS4 primers. Next, we mixed the two amplified PCR-products for electrophoresis that included both ITS1 and ITS2 regions. The conditions of PCR amplification were:

7 min at 94 °C, followed by 30 cycles to denaturing, 45 s at 94 °C, 1 min at 56 °C to annealing, and 1 min at 72 °C with a final extension of about 7 min at 72 °C.

ITS-sequencing and phylogenetic analysis

A representative isolate for each group of red yeasts was selected for sequencing and phylogenetic analysis. A part of the rDNA region ITS1–5.8S rDNA–ITS2 was amplified using the forward (ITS1) and reverse (ITS4) primer pairs.

PCR was performed in the same program with PCR-FSP. The PCR products were sent to the Kawsar Company (Tehran, Iran) and Macrogene Company (Korea) for sequencing to identify the yeast species accurately. The sequences were analyzed using BLAST program (http://www.ncbi.nlm.nih.gov/

Table 1. Yeast growth on PDA and CHROM agar Candida media

Isolate	PDA	Chrome agar
M34	orange to red	red
M16	orange to red	strong red
M18	orange to red	pink
M23	orange to red	purple
M27	light pink	purple
M60	light pink	purple
M22	light pink	pink to red
m24	shiny pink	pink to red
M35	light pink	pink to red
m42	light pink	purple
M40	orange to red	purple
M65	light pink	Strong pink
M50	pink to red	purple

Table 2: Assimilation of carbohydrates tests on the yeast strains of apple and citrus

ISOLATES		LACTOSE	ARABINOSE	GLUCOSE	DEXTROSE	MANOSE	RAMNOSE	GALACTOSE	FRUCTOSE
M42	*R.glutinis*	_	_	*	*	_	_	*	*
M22	*R.glutinis*	_	*	*	*	_	_	*	*
M24	*R.glutinis*	_	V	*	*	_	_	*	*
M35	xxx	_	V	*	*	_	_	*	*
M40	xxx	_	_	*	*	_	_	*	*
M34	*R.mucilaginosa*	_	*	*	*	_	_	*	*
M16	*R.mucilaginosa*	_	*	*	*	*	_	*	*
M18	*R.mucilaginosa*	_	*	*	*	*	_	*	*
M23	xxx	_	*	*	*	_	_	*	*
M27	xxx	_	*	*	*	*	_	*	*
M60	xxx	_	V	*	*	*	_	*	*
M61	xxx	_	*	*	*	V	_	*	*
M50	*Sporidiobolus pararoseus*	_	_	*	V	V	_	W	V
M65	xxx	_	_	*	*	_	_	*	*

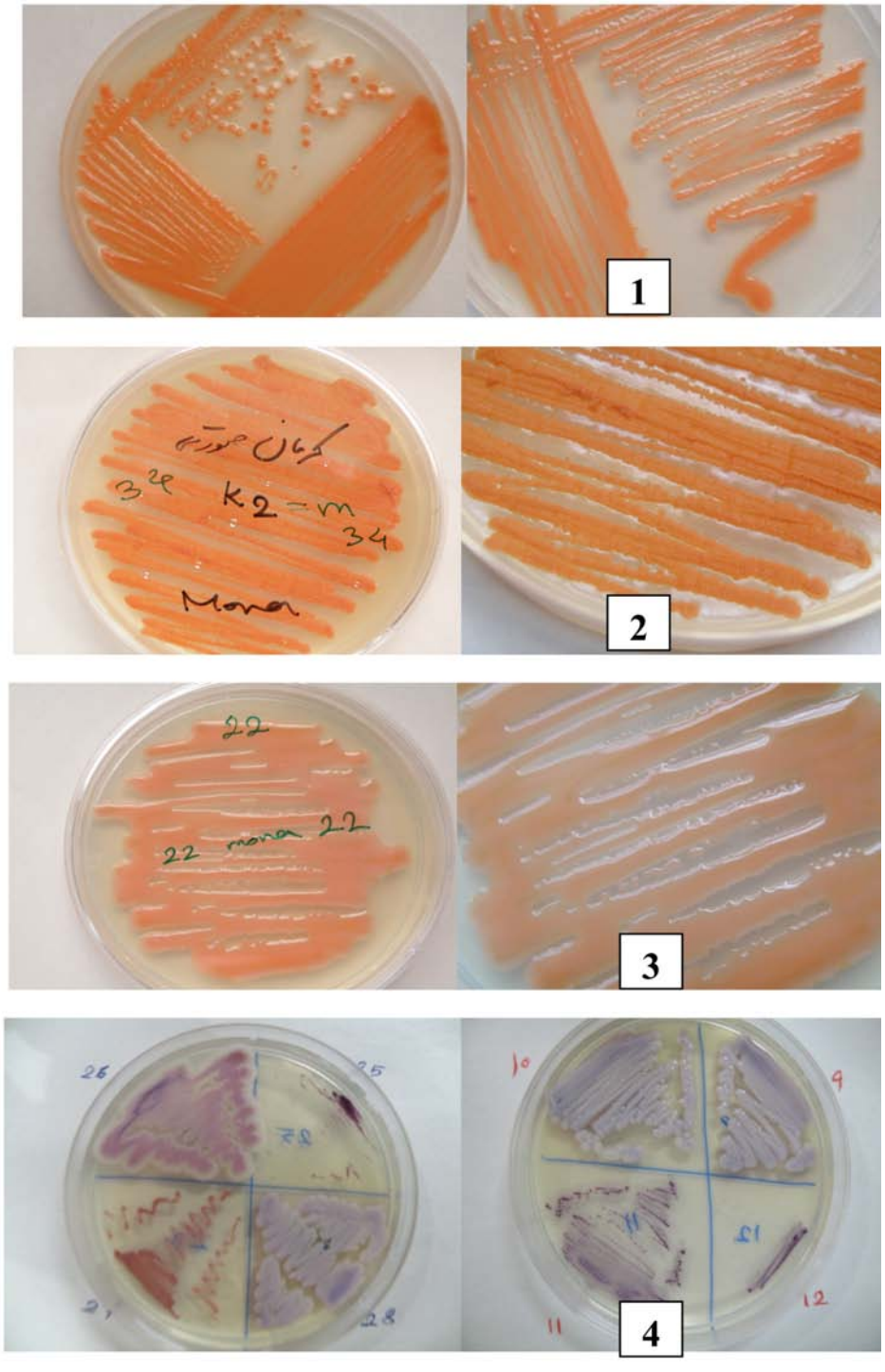

Fig. 1. The main groups of colony color of the yeast isolates on PDA and CHROM agar Candida, 1: isolate M50 (*Sporidiobolus pararoseus*), 2: isolate M34 *(Rhodotorula mucilaginosa*), 3: M22 (*Rhodotorula glutinis*), 4: Examples of the colony of some isolates on CHROM agar Candida.

Table 3. Blast results of isolates after sequencing and their highest homology with GeneBank strains. ITS sizes from PCR-FSP are added in the table

isolate	result	ITS1/ITS2 size(bp)	blast homology
M61	*Rhodotorula mucilaginosa*	230/390	99%
M60	*Rhodotorula mucilaginosa*	230/390	99%
M16	*Rhodotorula mucilaginosa*	230/390	99%
M18	*Rhodotorula mucilaginosa*	230/390	99%
M34	*Rhodotorula mucilaginosa*	230/390	100%
M22	*Rhodotorula glutinis*	230/390	99%
M24	*Rhodotorula glutinis*	230/390	98%
M42	*Rhodotorula glutinis*	230/390	99%
M50	*Sporidiobolus pararoseus*	230/380	99%

BLAST/). Sequences were aligned using CLUSTAL_X1.83 (Thompson et al., 1997). A neighbor-joining phylogenetic tree was constructed using MEGA4 software (Tamura et al., 2007). Bootstrap analysis was carried out with 1000 replications. Reference sequences were retrieved from the GeneBank database and included in the analysis.

RESULTS

Throughout the morphological tests, including the color of colonies on the PDA medium, the yeast isolates were categorized into groups that are shown in Table 1. On the basis of colony colors on the CHROM agar Candida medium they were classified into different groups which are also shown in Table 1. Thirteen isolates that produced pigments were selected for the molecular tests. Thus only 13 selected isolates are shown in Table 1.

Throughout the physiological tests containing eight carbohydrates substrates, 13 selected yeast species were divided into groups that are given in Table 2; some related yeast tests are shown in Fig. 1.

On the basis of the similarity in size of the banding patterns of the PCR-FSP products (ITS1 and ITS2), our 13 isolates were clustered into two groups as follows: group1: (M16, M18, M22, M23, M24, M27, M34, M40, M60) with 410bp for ITS2 size band and 230 bp for ITS1, and group2: (M50, M65) with 380bp size band for ITS2 and 220 bp for ITS1. Some typical electrophoretic bands belonged to each group and standard isolates are illustrated in Table 3.

Some representative isolates were then subjected to sequence and phylogenetic analysis. BLAST analysis of the nucleotide sequence of the ITS region of M16, M18, M27 showed 99% similarity with *Rhodotorula mucilaginosa*. On the basis of the Blast search, M22, M24 and M42 exhibited high similarity with *Rhodotorula glutinis*. Isolate M50 showed the highest homology with *Sporidiobolus pararoseus*. Following phylogenetic analysis, each isolate located in the same clade of the phylogenetic tree (with reference isolates from GeneBank with a high percentage of bootstrap support) are shown in Fig. 2. This can be validation for our sequencing process and indicates it occurred correctly.

Nucleotide sequence accession numbers

The determined ITS1-5.8S-ITS2 of the rDNA gene sequences were deposited in the GeneBank using the

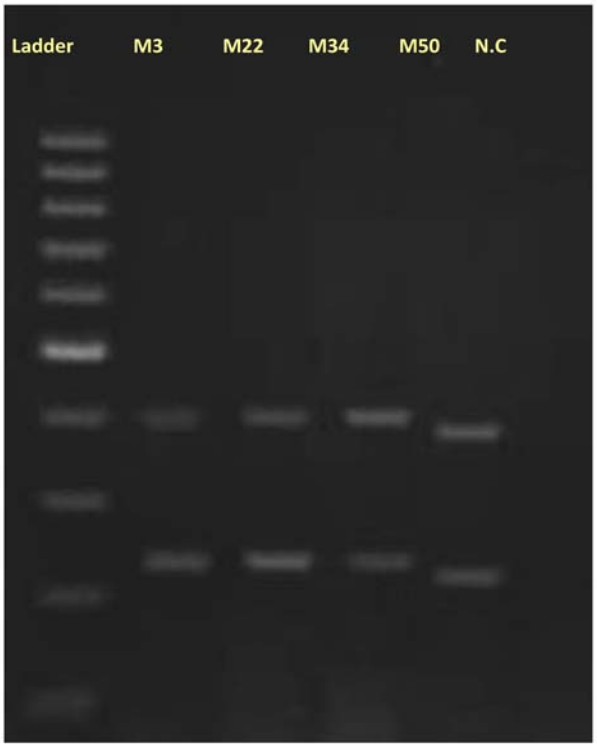

Fig. 2. Phylogenetic tree based on the sequence of the total ITS region. The tree was constructed by using UPGMA analysis. Each number indicates the percentage of bootstrap samplings, derived from 1000 samples, supporting the internal branches

NCBI database under the accession numbers shown below:

HQ379144, HQ379145, HQ379146, HQ379147, HQ379148, HQ379154, HQ379160, HQ379162, HQ379163.

DISCUSSION

Phenotypic tests can lead to unreliable results in the identification of yeast species. Reported misidentifications abound (Arroyo-Lo'peza et al., 2006; Guillamón et al., 1998). We separated our yeast isolates on the basis of their morphological character. We kept the colorful colonies of yeasts producing pigments (orange, pink, red) and left out those isolates with white or cream colony colors. Therefore, we continuously only had 13 *Rhodotorula* and *Sporidiobolus* species. In order to obtain a precise identification, it is necessary to use molecular techniques based on DNA sequence analysis. The use of ribosomal DNA (rDNA) genes for identification of fungal species is based on the detection of conserved sequences in 5.8S rDNA and 28S rDNA that enable the amplification of the ITS2 region between these two genes**.** Chen et al. established an ITS database that provides the precise identification of at least 40 species of medically important yeasts (Chen et al., 2001; Thompson et al., 1997, Hibbet et al., 2007, Enache et al., 2009, 27). Thus, we focused on the total ITS region of yeast species. As a result of PCR-FSP, our red yeast isolates divided into two major groups. We were forced to select some isolates in each group based on their morphologies. Subsequently, 7 isolates from group 1 were chosen for ITS-sequencing and one from group 2. After sequencing, 4 isolates belonged to *R. mucilaginosa* (M16, M18, M34, M60); 3 isolates belonged to *R. glutinis* (M22, M24, M42), and the last isolate M50 belonged to *S. pararoseus*. These results are in agreement with other studies that have reported misidentification of species by morphological methods. We chose different yeasts from our 2 groups morphologically. For example, the isolates M16 and M18 that had similar patterns in the PCR-FSP, exhibited an orange-to-red colony color on the PDA medium. The M16 colonies were bright red on the CHROM agar medium, whereas the M18 colonies were pink. Thus, we selected both of them for sequencing. However, they showed the same result as *R. mucilaginosa*. Another result in the physiological tests was not reliable because they were similar in numerous species and there is no specific biochemical test to identify one species and the results may be affected by culture conditions. Thus, none of our phenotypic methods could help us to identify the yeast accurately.

The isolates M16, M18, M22, M24 and M34 belonged to apple samples and M42, M50, M60 belonged to citrus samples. It seems there is no significant correlation between these two source fruits

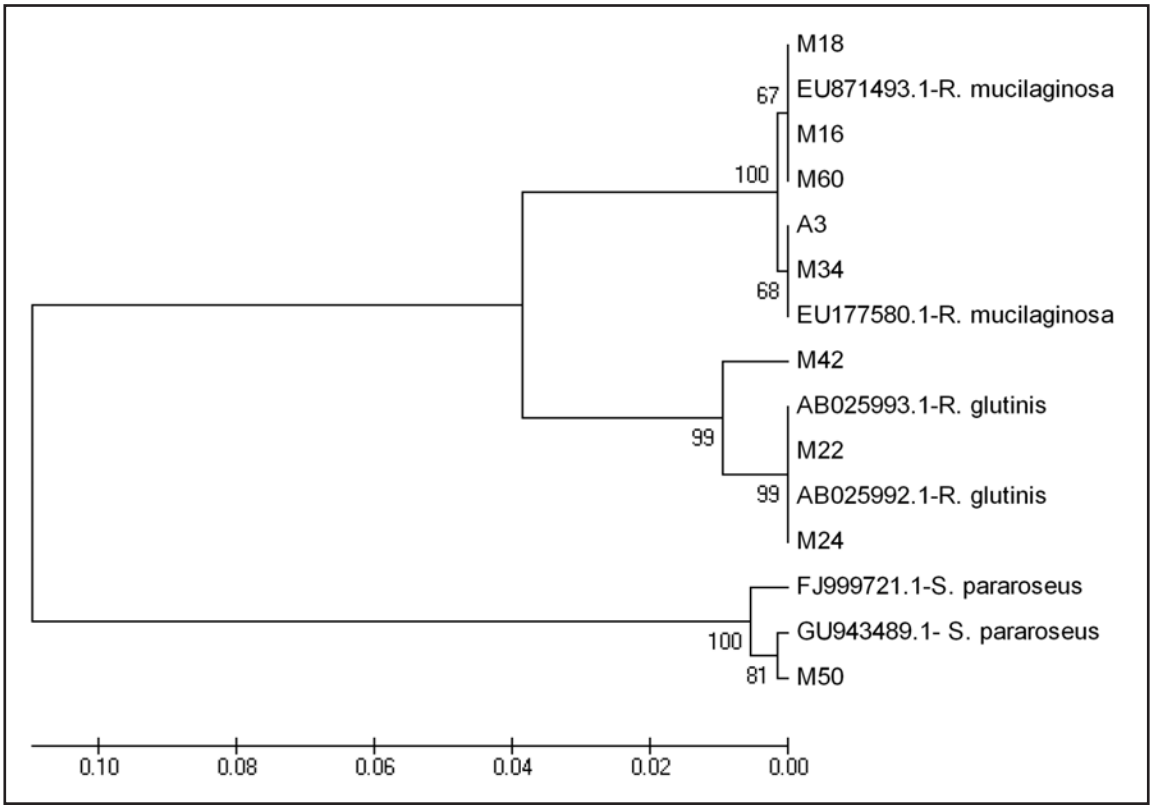

Fig. 3. Phylogenetic tree based on the sequence of the total ITS region. The tree was constructed by using UPGMA analysis. Each number indicates the percentage of bootstrap samplings, derived from 1000 samples, supporting the internal branches.

in their derived yeasts. But isolate M50 belonged to *Sporidiobolus* genus and it is a telomorphic type of *Sporobolomyces*. We had only one of this species and it was obtained from the Pakdasht tangerine. It might refer to a differentiation in the nutrition source of the citrus and apple that can lead to the growth of *S. pararoseus* on the tangerine. But more statistical tests and research are needed to gain an absolute result. The M50 located in the reference isolate of the GeneBank with 100% bootstrapping support.

The isolates M16 and M18 that are in the same phylogenetic clade were isolated from Damavand apples, but M34 was isolated from Kerman apples, which placed it in another clade near the A3 n standard CBS strain. Nonetheless, these two *R. mucilaginosa* clades are located in the same larger clade with 100% bootstrap support which means that all these isolates are closely related based on the total ITS region sequences. The *Rhodotorula* species would be useful in the control of biological activities on fruit wilt (Janisiewicz et al., 2010; Hagler et al., 1987).

Isolates M22 and M24, which were isolated from Eyvanakey apples, clustered in our reference *R. glutinis* of the GeneBank with 99% bootstrap support, which shows these are more similar genetically. Isolate M42 from the Tehran sweet lemon located in another nearby clade. This may be due to their source nutrition or source isolation, and this needs more research. It is necessary to say that both these clades of *R. glutinis* fell into the same further clade with 99% bootstrap support.

The last clade of the phylogenetic tree showed isolate M50 placement in the reference *S. pararo-*

seus clade with 100% bootstrap support. It shows isolate M50 is surely *S. pararoseus* and supports our sequencing process. This is the first report of *S. pararoseus* from tangerine in the world and it would be helpful to it study more for the biological control of fruit diseases. A number of studies have reported *S. pararoseus* activity in the control of certain post-harvest diseases of fruits (Janisiewicz et al., 2010).

One important consequence of the morphological test is the performance of colony color application in our yeast identification. As we saw in the first step, we selected red yeasts with the probability of them being *Rhodotorula* and *Sporobolomyces*. Our final result illustrated this morphological test at this level and could help us considerably in showing that we did our sampling correctly. This characteristic of these kinds of yeasts was mentioned by Kreger-Van Rij in 1984 and Nagahama in 2001. Nagahama et al. studied deep-sea environment red yeasts containing many *Rhodotorula* and *Sporobolomyces* species. Thus, phenotypic methods are useful complimentary methods for identification of yeast (Kreger-Van Rij 1984; Nagahama et al., 2001).

We report that the most frequent red yeasts on apples and citrus are due to *Rhodotorula* specifically, partially *R. mucilaginosa* and then *Sporidiobolus*. In other words, the proportion of *Rhodotorula* species were about 85% and *Sporidiobolus* was about 15%.

It seems that PCR-FSP is a precise molecular method for yeast identification but sometimes it works in on the genus level such as *Rhodotorula* in this approach and sometimes can help in species level such as *Candida* species (Mirhendi et al., 2008). On the basis of all our identification methods in this approach the isolates M27, M40, M23, M35 belonged to the *Rhodotorula* species and M61 belonged to *Sporidiobolus*. Red yeasts obtained from the fruits do not differ genetically at a high level but differ in their phylogenetic location and evolutionary divergence. For unambiguous identification of all red yeast species, that comprise most of the yeasts on fruit surfaces, it is obvious that study on their phylogenetic relationships on the basis of molecular techniques is needed.

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