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PHYLOGENETIC BIODIVERSITY OF YEASTS IN THE REPUBLIC OF NORTH OSSETIA-ALANIA *(first report)*

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Abstract:

The authors identified yeast strains from the surface of grape cultivars Interes (strain 3K (14) and Dekabrskiy (strain 5e), as well as from the wild hops cones (strain BUG1) and performed their identification. The primary identification of the selected strains was performed by studying cultural, tinctorial, morphological, physiological-biochemical, and technological properties in the Research Institute of Biotechnology of Gorsky SAU, using traditional research methods. For the final identification based on the analysis of ribosomal genes sequence, 3K (14), 5e, BUG1 strains were sent to the Bioresource Centre – All-Russian Collection of Industrial Microorganisms (BRC VKPM) National Research Centre "Kurchatov Institute" – State Research Institute of Genetics. According to the results of the performed in BRC VKPM NRC "Kurchatov Institute" – State Research Institute of Genetics analysis of the nucleotide sequence, coding part of genes rRNA it was found that 3K (14) strain is the closest to the species ***Metschnikowia pulcherrima****; BUG1 strain is closest to the species* ***Saccharomyces;*** *5e strain is closest to the species* ***Rhodotorula mucilaginosa.***

Keywords: *yeasts, strain, gene encoding 18S rRNA, DNA, PCR mode.*

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INTRODUCTION:

Relevance. Despite the difference in human relationships with their microscopic satellites, researchers have always been interested in the details of morphological, physiological, biochemical, tinctorial, genetic and other characteristics of each species. This knowledge makes it possible to avoid undesirable consequences of contact with some microbes or, conversely, to meet different needs of people in agriculture, medicine, food industry or to solve environmental problems using the potential of microorganisms.

Such representatives of the microbial world as yeast fungi are of particular importance for the quality of human life.

Currently, as a result of increasing anthropogenic pressure on the biosphere, the preservation of the microbiota diversity and study of species characteristics for each representative of the microworld is a very important area of research in the field of biotechnology.

Besides its economic importance, yeast is also known as the main source of products spoilage. [4]. Therefore, it is necessary to know which strain is used and all its properties.

In the work of Ling L. (2019), various isolates leading to spoilage of traditionally cultivated crops were investigated, for example, cultures of microorganisms isolated by Lanzhou Lily from fallen fruits in Gansu province, China during 2016-2017. A total of four isolates and basing on their DNA genes sequences of 16S rDNA and 26S rDNA in combination with morphological characteristics of cultures and sporulations were obtained and identified. Of four isolates, one was identified as *Bacillus safensis*, one – as *Stenotrophomonas maltophilia*, and two isolates – as *Metschnikowia pulcherrima.* [9]

Gan Kh.M. in his article states the unconditional benefit of yeast. In industry, the yeast *Rhodotorula mucilaginosa* is usually used for carotenoids production. Carotenoids are important because they are used as natural dyes in food production, and some carotenoids are precursors of retinol (vitamin A). However, the identification and molecular characterization of the carotenoids pathway in species belonging to the genus *Rhodotorula* are scarce due to the lack of genomic information, which prevents the effective metabolic engineering of these yeast strains to improve carotenoids production [7].

Caudy A.A. (2019) believes that mass spectral measurement of cellular metabolites shows complexes in cells, which are not explained by current maps of metabolic reactions, and existing computational models are unable to make all the actions observed in cells. Mass spectrometry in full metabolism survey of polar small molecules to explore the enzymes deletion mutants in the model yeast *Saccharomyces cerevisiae* was used in this study [6].

New molecular approaches have shown that in *Aspergillus Nigri* cut there is high biodiversity, but species are sometimes difficult to identify based solely on their phenotypic characteristics. Using this type of approach, 19 species of Aspergillus – Nigri cut were adopted [12]. Varga and co-authors (2011) reported that in section Aspergillus Nigri there were 26 taxa [13].

Vesth T.S. (2018) in his paper approve that the Aspergillus section *Nigri* includes mold fungi related to biomedicine, bioenergy, health and biotechnology. To find out what genetically separates these species, as well as their possible application in biotechnology and biomedicine, 23 genomes de novo have been ordered, forming a complete genome summary for the section (26 species) [14].

The grapevine is one of the most cultivated and productive plants species in the world. The vineyards are distributed over five continents and has a surface area of 7,5 million hectares (International Organization of Vine and Wine (OIVV) (2016)).

In the work of Mayssa Arfaui et al. (2019) strains were selected from the soil in which the grapes grew. To study the bacterial microflora, sampling was carried out in two vineyards [5].

Wines produced using yeasts *H. vineae* and *Saccharomyces cerevisiae*, fermented sequentially, show more intense fruit flavor and complexity than wines produced using only S. cerevisiae yeast. In the work of F. Giorello (2019), ordering the genome, assembly and phylogenetic analysis of two *H. vineae* stresses showed that they are members of a large complex *Saccharomycetes*. [8].

MATERIAL AND RESEARCH METHODS:

We have identified 3K (14), 5e, BUG-1 yeast strains from the surface of different grape varieties. The primary identification of the selected strains was performed by studying cultural, tinctorial, morphological, physiological-biochemical, and technological properties in the Research Institute of Biotechnology of Gorsky SAU, using traditional research methods [1], [3].

For the final identification based on the analysis of ribosomal genes sequence, 3K (14), 5e, BUG1 strains were sent to the Bioresource Centre – All-Russian Collection of Industrial Microorganisms (BRC VKPM) National Research Centre "Kurchatov Institute" – State Research Institute of Genetics.

Researches in Bioresource Centre – All-Russian Collection of Industrial Microorganisms (BRC VKPM) NRC "Kurchatov Institute" – State Research Institute of Genetics included:

I. Culture screening to individual colonies and biomass obtaining for RNA 18S analysis.

II. DNA extraction. (Genomic DNA Purification Kit)

III. Strain identification based on 18S rDNA sequence

III.1 Selection of primers and PCR modes.

Conservative primers to develop gene sequence coding 18S rRNA were selected [10]:

NS1-gtagtcatatgcttgtctc;

NS4 – cttccgtcaattcctttaag.

PCR mode:

1. 95ºC – 3 min.
2. 35 cycles: 95º C - 30 sec., 57ºC- 30 sec., 72ºC - 30 sec.
3. 72ºC – 5 min.

Conservative primers to develop gene sequence coding 5,8S rRNA and internal transcribed spacers ITS1 and ITS2:

ITS1 – TCCGTAGGTGAACCTGCG

ITS4 – TCCTCCGCTTATTGATATGC

PCR mode:

1. 95ºC – 3 min.
2. 35 cycles: 95ºC – 30 sec., 57ºC – 30 sec., 72ºC – 30 sec.
3. 72ºC – 5 min.

Consecutive primers to amplify D1/D2 domain of 26S rRNA gene:

NL-1 GCATATCAATAAGCGGAGGAAAG

NL-4 GGTCCGTGTTTCAAGACGG

PCR mode:

1. 95ºC – 3 min.
2. 35 cycles: 95ºC – 30 sec., 52ºC – 30 sec., 72ºC – 30 sec.
3. 72ºC – 5 min.

III.2 18S rRNA and 5,8 rRNA genes sequencing, comparison of sequences and building phylogenetic trees was performed using automatic sequencer AE3000.

To analyze sequences the specialized computer program BLAST was used [15].

Validity of the results. Sequences sufficient to classify strains into a certain taxonomic group of microorganisms were determined.

IV. Identification using species-specific primers

Primers specific to the genus *Fusarium equiseti* [11]:

FEF1 5' - CATACCTATACGTTGCCTCG-3'

FER1 5' - TTACCAGTAACGAGGTGTATG-3'

Primers specific to *Fusarium oxysporum* [11]:

FOF1 5' - ACATACCACTTGTTGCCTCG-3'

FOR1 5' - CGCCAATCAATTTGAGGAACG-3'

PCR mode:

1. 95ºC – 3 min.
2. 35 cycles: 95ºC – 30 sec., 52ºC – 30 sec., 72ºC – 30 sec., 72ºC – 5 min.

V. Conditions for PCR electrophoresis of the samples under study:

1,0% agarose gel, electrophoresis at an electric field strength of 5 V/cm.

RESEARCH RESULTS:

3K (14) strain identification based on ribosomal genes sequence analysis.

Analysis stages:

a) Sequencing of sections of the sequence coding 18S rRNA gene of **3K (14)** strain based on the ribosomal genes sequence analysis.

When sequencing the DNA section coding 18S rDNA gene of the studied strain, the following sequence was obtained:

GTTATCGTTTATTTGATAGTACCTTGCTAATTGGCATACGCCTGGCAATTCTAGAGCTAAT
ACATGCACACAAGCCCAACCTCCGGAAGGGCTGTATTTATTAGATAAAAAATCAACACTCCAGAA
GATTCATAATAACTTGTCGAATCGCAGGGCCTCGGGCCGGCGATGGTTCATTCAAATTTCTGCCCT
ATCAACTTTCGATGGTAGGATAGAGGCCTACCATGGTTTCAACGGGTAACGGGGAATAAGGGTTC
GGTTCCGGAGAGGGAGCCTGAGAAACGGCTACCACATCCAAGGAAGGCAGCAGGCGCGCAAATT
ACCCAATCCCGACACGGGGAGGTAGTGACAATAAATAACGATACAGGGCCCTTTCGGGTCTTGTA
ATTGGAATGAGTACAATGTAAATACCTTAACGAGGAACAATTGGAGGGCAAGTCTGGTGCCAGCA
GCCGCGGTAATTCCAGCTCCAAGAGCGTATATTAAAGTTGTTGCAGTTAAAAAGCTCGTAGTTGAA
TTTTGGGGCGGCCGCGGAGGTCCACTTCTTTGTGAGTACTTTTTGCGGCGGCCCTTCCATGGCCCYT
WAYMGGGCCATAGTTACTTTGAGTAAATGAGAGTGTTCAAAGCAGGCAAGCGCTTGAATCTTTTA
GCATGGAATAATAAAATAGGACGATGATTCTATTTTGTTGGTTTCTAGGACCATCGTAATGATTAA
TAGGGACGGTCGGGGGCATTAGTATTCAGTTGTAAGAGGTGAAATTCTTAGATTTTCTGAGACTAA

CTACTGCGAAAGCATTTGTCAAGGGACGTTTTCATTAATCAGAACGAAAGTTAGGGGATCGAGAT
GATCAGATWCGGTCGTAGTCTTACCATAAACTATGCCGAACTAGGGAATTGG

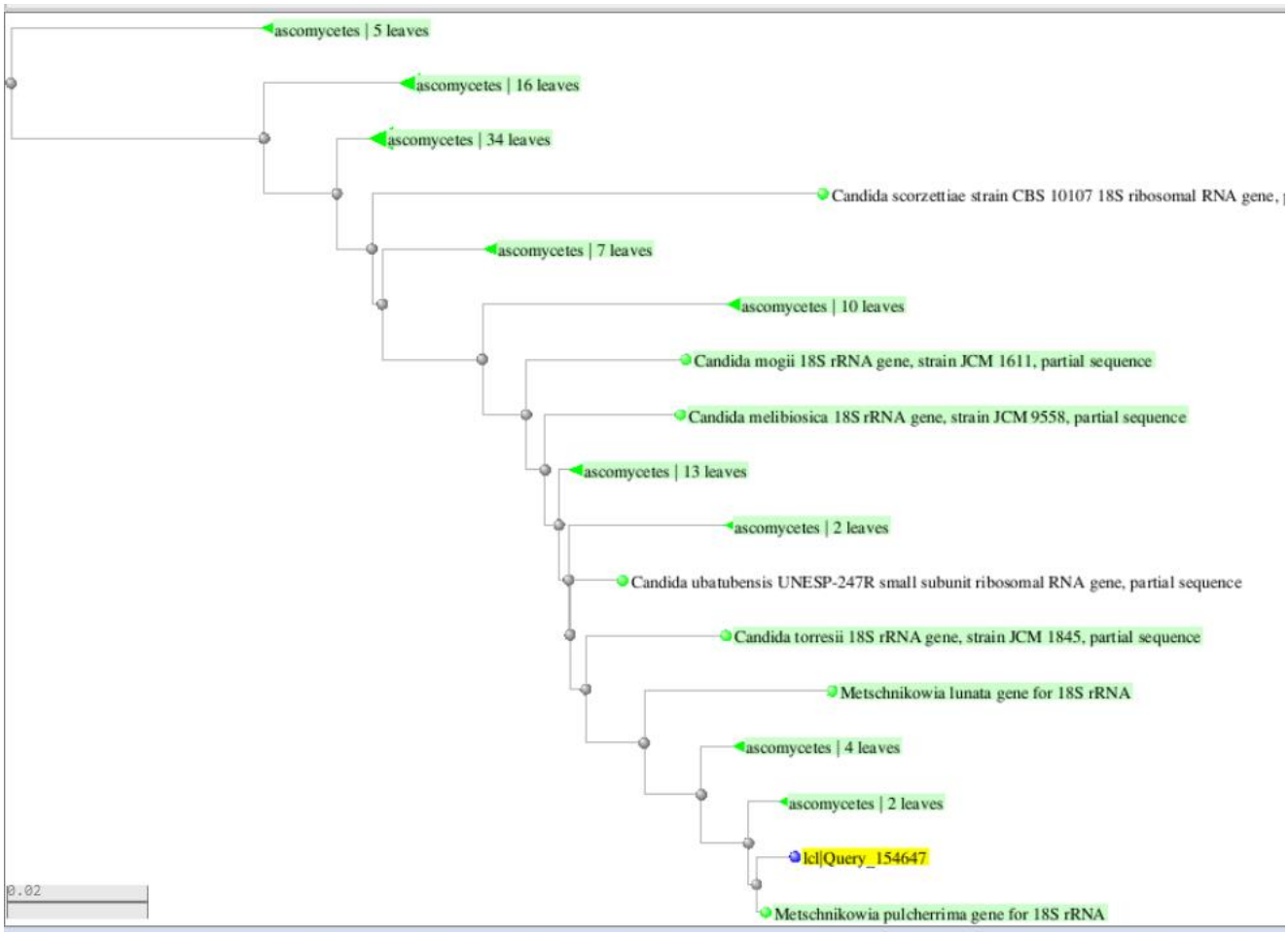
b) Analysis of the gene sequences coding 18S rRNA.

The similarity of the nucleotide sequence of the gene coding 18S rDNA of the studied strain was analyzed using BLAST server [http://www.ncbi.nlm.nih.gov/blast].

RESULTS:

Primary screening on the GenBank database showed that the studied strain belongs to the following systematic group: Eukaryota; Fungi; Dikarya; Ascomycota; Saccharomycotina; Saccharomycetes; Saccharomycetales; *Metschnikowiaceae; Metschnikowia.*

Homology of at least 97% is considered to be the criterion for classifying a microorganism to a particular species. As shown by fig. 1, the analyzed strain can be classified into several species.



The method to compare nucleotide sequences coding D1/D2 domain of 26S rRNA gene was also used to establish phylogenetic affinity of similar species.

When sequencing the DNA section coding D1/D2 domain of the 26S rRNA gene, the following sequence was obtained:

CMAGGGMATTGCCTCAGTAACGGCGAGTGAAGCGGCAAAAGCTCAAATTTGAAATCCCCCGGGA
ATTGTAATTTGAAGAGATTTGGGTCCGGCCGGCRGGGGTTAAGTCCACTGGAAAGTGGCGCCACA
GAGGGTGACAGCCCCGTGAACCCCYTCAACGCCCTCATCCCAGATCTCCAAGAGTCGAGTTGTTTG
GGAATGCAGCTCTAAGTGGGTGGTAAATTCCATCTAAAGCTAAATACCGGCGAGAGACCGATAGC
GAACAAGTACAGTGATGGAAAGATGAAAAGCACTTTGAAAAGAGAGTGAAAAAGTACGTGAAAT
TGTTGAAAGGGAAGGGCTTGCAAGCAGACACTTAACTGGGCCAGCATCGGGGCGGCGGGRARCAA
AACCACCGGGGAATGTACCTTTCGAGGATTATAACCCCGGYCYYWAYTYCCTYRYYGYCCCGAGG
CCTGCAATCTAAGGATGCTGGCGTAATGGTTGCAAGTCGCCCGTCTTGAAACACGGACCYATA.

Phylogenetic analysis, made by using strains of closely related microorganisms, showed that species *Metschnikowia pulcherrima* (99 %) is the closest to the studied strain.

The analysis of the nucleotide sequence coding a part of rRNA genes showed that the studied **3K (14)** strain is the closest to the species ***Metschnikowia pulcherrima.***

5e strain identification based on ribosomal genes sequence analysis.

Analysis stages.
a) Sequencing of sections of the sequence coding 18S rRNA gene.
When sequencing the DNA section coding the 18S rDNA gene of 5e strain the following sequence was obtained:

GGTAATTCTAGAGCTAATACATGCTGAAAAATCCCGACTTCTGGAAGGGATGTATTTATTAGATCC
AAAACCAATGGCCTTCGGGTCCCTATGGTGAATCATGATAACTGCTCGAATCGCATGGCCTTGCGC
CGGCGATGCTTCATTCAAATATCTGCCCTATCAACTTTCGATGGTAGGATAGAGGCCTACCATGGT
GATGACGGGTAACGGGGAATAAGGGTTCGATTCCGGAGAGAGGGCCTGAGAAACGGCCCTCAGG
TCTAAGGACACGCAGCAGGCGCGCAAATTATCCCCTGGCAACACTTTGCCGAGATAGTGACAATA
AATAACAATGCAGGGCTCTTACGGGTCTTGCAATTGGAATGAGTACAATTTAAATCCCTTAACGAG
GATCAATTGGAGGGCAAGTCTGGTGCCAGCAGCCGCGGTAATTCCAGCTCCAATAGCGTATATTA
AAATTGTTGCCGTTAAAAAGCTCGTAGTCGAACTTCGGGCTCTGTCAGCCGGTCCGCCTTCTTGGT
GTGTACTTGTTTGACGGAGCCTTACCTCCTGGTGAACGGCGATGTCCTTTACTGGGTGTCGTCGCA
AACCAGGACTTTTACTTTGAAAAAATTAGAGTGTTCAAAGCAGGCCTTTGCCCGAATACATTAGCA
TGGAATAATAAAATAGGACGCGCGTTCCCATTTTGTTGGTTTCTGAGATCGCCGTAATGATTAATA
GGGATAGTTGGGGGCATTTGTATTCCGTCGTCAGAGGTGAAATTCTTGGATGCCGGGAGACAAACT
ACTGCGAAGCATTGCAGAATGTTTTCATTGATCAAGACGAAAGAAGGGGG

b) Analysis of the gene sequences coding 18S rRNA.
The similarity of the nucleotide sequence of the gene coding 18S rDNA of 5e strain was analyzed using BLAST server [http://www.ncbi.nlm.nih.gov/blast].

Results.
Primary screening on GenBank database showed that 5e strain belongs to the following systematic group: Eukaryota; Fungi; Dikarya; Basidiomycota; Pucciniomycotina; Microbotryomycetes; Sporidiobolales; *Sporidiobolaceae; Rhodotorula.*

The strains involved in the analysis and the sequence similarity level of 18S rDNA of 5e strain are shown in fig. 2.

Homology of not less than 97% is considered to be a criterion for identifying microorganisms to a particular species [2]. As shown by fig. 2, the analyzed strain can be classified into several species.

The method to compare nucleotide sequences coding D1/D2 domain of 26S rRNA gene was also used to establish phylogenetic affinity of similar species.

When sequencing the DNA section coding D1/D2 domain of the 26S rRNA gene, the following sequence was obtained:

GSSGGGGTATTCCCCTAGTAGCGGCGAGCGAAGCGGGAAGAGCTCAAATTTATAATCTGGCACCTT
CGGTGTCCGAGTTGTAATCTCTAGAAATGTTTTCCGCGTTGGACCGCACACAAGTCTGTTGGAATA
CAGCGGCATAGTGGTGAGACCCCCGTATATGGTGCGGACGCCCAGCGCTTTGTGATACATTTTCGA
AGAGTCGAGTTGTTTGGGAATGCAGCTCAAATTGGGTGGTAAATTCCATCTAAAGCTAAATATTGG
CGAGAGACCGATAGCGAACAAGTACCGTGAGGGAAAGATGAAAAGCACTTTGGAAAGAGAGTTA
ACAGTACGTGAAATTGTTGGAAGGGAAACGCTTGAAGTCAGACTTGCTTGCCGAGCAATCGGTTT
GCAGGCCAGCATCAGTTTTCCGGGATGGATAATGGTAGAGAGAAGGTAGCAGTTTCGGCTGTGTT
ATAGCTCTCTGCTGGATACATCTTGGGGGACTGAGGAACGCAGTGTGCCTTTTGGCGGGGGTTTCG
ACCTCTTCACACTTAGGATGCTGGTGGAATGGCTTTAAACGACCCGTCTTGAAACACGGACCA

Phylogenetic analysis, made by using strains of closely related microorganisms, showed that species *Rhodotorula mucilaginosa* (99 %) is the closest to the studied strain.

The analysis of the nucleotide sequence coding a part of rRNA genes showed that the studied **5e** strain is the closest to the species ***Rhodotorula mucilaginosa.***

BUG1 strain identification based on ribosomal genes sequence analysis.

Analysis stages.

a) Sequencing of sections of the sequence coding 18S rRNA gene.

When sequencing the DNA section coding the 18S rDNA gene of **BUG1** strain the following sequence was obtained:

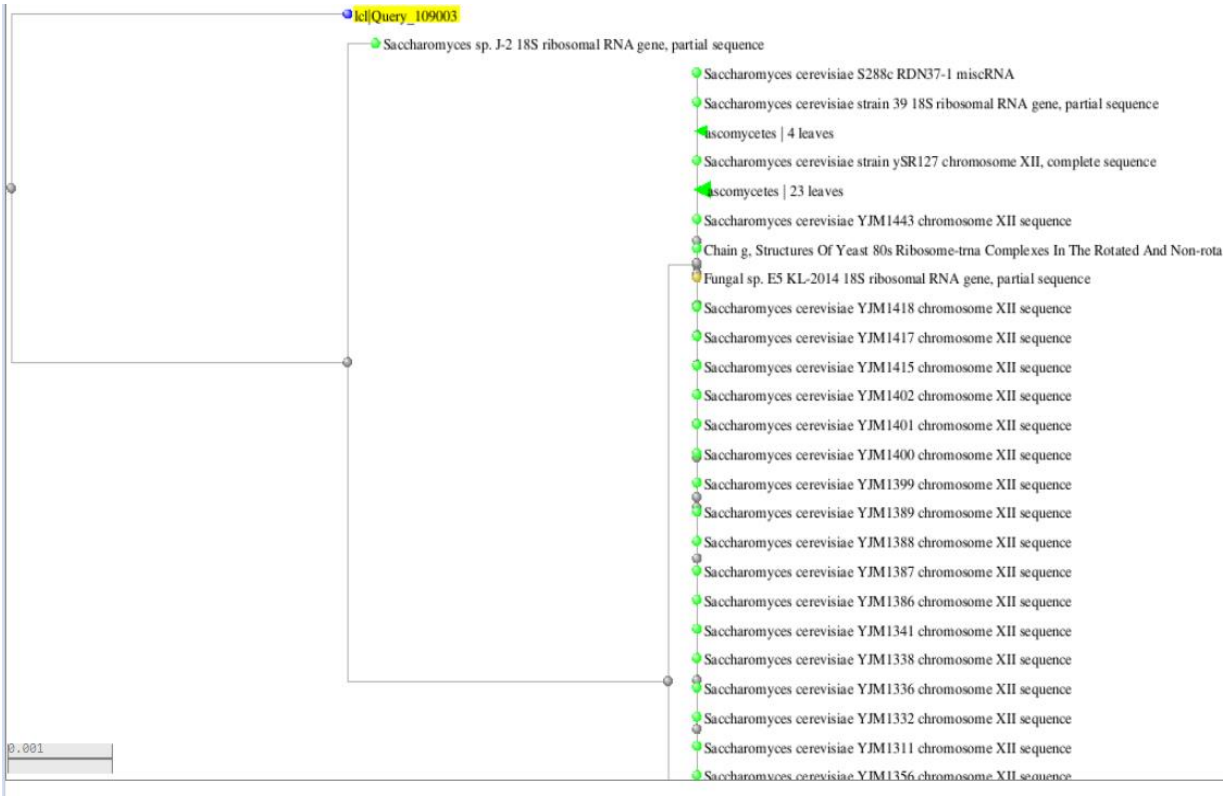
AACTGCGTTGGGCTCAAAAAATCAGTTATCGTTTATTTGATAGTTCCTTTACTACATGGTATAACTG
TGGTAATTCTAGAGCTAATACATGCTTAAAATCTCGACCCTTTGGAAGAGATGTATTTATTAGATA
AAAAATCAATGTCTTCGGACTCTTTGATGATTCATAATAACTTTTCGAATCGCATGGCCTTGTGCTG
GCGATGGTTCATTCAAATTTCTGCCCTATCAACTTTCGATGGTAGGATAGTGGCCTACCATGGTTTC
AACGGGTAACGGGGAATAAGGGTTCGATTCCGGAGAGGGAGCCTGAGAAACGGCTACCACATCC
AAGGAAGGCAGCAGGCGCGCAAATTACCCAATCCTAATTCAGGGAGGTAGTGACAATAAATAACG
ATACAGGGCCCATTCGGGTCTTGTAATTGGAATGAGTACAATGTAAATACCTTAACGAGGAACAA
TTGGAGGGCAAGTCTGGTGCCAGCAGCCGCGGTAATTCCAGCTCCAATAGCGTATATTAAAGTTGT
TGCAGTTAAAAAGCTCGTAGTTGAACTTTGGGCCCGGTTGGCCGGTCCGATTTTTTCGTGTACTGG
ATTTCCAACGGGGCCTTTCCTTCTGGCTAACCTTGAGTCCTTGTGGCTCTTGGCGAACCRGGACTTT
TACTTTGAAAAAATTAGAGTGTTCAAAGCAGGCGTATTGCTCGAATATATTAGCATGGAATAATAG
AATAGGACGTTTGGTTCTATTTTGTTGGTTTCTAGGACCATCGTAATGATTAATAGGGACGGTCGG
GGGCATCAGTATTCAATTGTCAGAGGTGAAATTCTTGGATTTATTGAGACTAACTACTGCGAAGCA
TTTGCCAAGGACGTTTCATATCAAGAACGAAGTAAGGGGAATCGAGAATGATCAGA

b) Analysis of the gene sequences coding 18S rRNA.
The similarity of the nucleotide sequence of the gene coding 18S rDNA of the studied strain was analyzed using BLAST server.

Results.
Primary screening on GenBank database showed that BUG1 strain belongs to the following systematic group: Eukaryota; Fungi; Dikarya; Ascomycota; Saccharomycotina; Saccharomycetes; Saccharomycetales; *Saccharomycetaceae; Saccharomyces*.

The strains involved in the analysis and the sequence similarity level of 18S rDNA of the studied strain are shown in fig. 3.

Homology of not less than 97% is considered to be a criterion for identifying microorganisms to a particular species. As shown by fig. 3, the analyzed strain can be classified into several species.



The method to compare nucleotide sequences coding 5,8 S rRNA gene and internal transcribed spacers ITS1 and ITS2 was also used to establish phylogenetic affinity of similar species.

When sequencing the DNA section coding 5,8 S rRNA gene and internal transcribed spacers ITS1 and ITS2, the following sequence was obtained:

GCARCGGCATCATCCGSWTATGAGGTSMRCWTKWTCAMCATAYGKWCGCCTASACGCTCTCTTCT
TATCGATAACGTTCCAATACGCTCAGTATAAAAAAGATTAGCCGCAGTTGGTAAAACCTAAAACG
ACCGTACTTGCATTATACCTCAAGCACGCAGAGAAACCTCTCTTTGGAAAAAAAAAAMATCCAAK
GAAAAGGCCARCAATTTCAAGTTAACTCCAAARAGTATCMCTCMCTACCAAAMARAAKGKTTGA
AAAGGAAAWGACSCYCAAACAGGCATGCCCCCKGGAAWACCAAGGGGSGCAAKGKGSGTTCAAA
RATTCSATGATTCMCGGAATTCTGCAATTCMCATTACGTATCGCATTTCSCTGCGTTCTTCATCRAT
GCRARAACCAARAAAWCCGTTGTTGAAAGTTTTTAAWATTTTAAAATTTCCMGTTACRAAAATTC
TTGTTTTTGAMAAAAATTTAAKGAAWARAWAAAATTGTTTGKGTTTGTTAACCTCKGGGCCCCGA
TTGCTCRAATGCCCAAARAAAAAGTTGCAAAGATATGAAACYCCACAGTGTGTTGTATTGAACGG
TTTAATGTCCTATACAAAGCACAGAATCTCTCACGTTGAATAGCAGAAGAACTACAGCTAGCAGA
CGCGCACTAGCGCAGCCGCTGACTCTCATCTCTGTCTCTGCCAGTAAGCTCATGCYTGCAACAAAA
CAATCATTCAAGTWATSTCATGTCTCCRTCMCTMGAGGYACGAYTAGAGCTTMCTGGATGATTAT
CTCTGAGCACGCTAGCGCTCACGCTGAGCACTAGTGTATC.

The method to compare nucleotide sequences coding D1/D2 domain of 26S rRNA gene was also used to establish phylogenetic affinity of similar species.

When sequencing the DNA section coding D1/D2 domain of the 26S rRNA gene, the following sequence was obtained:

AMGGGGKCAATGCCTTAGTACGGCGAGTGAAGCGGCAAAAGCTCAAATTTGAAATCTGGTACCTT
CGGTGCCCGAGTTGTAATTTGGAGAGGGCAACTTTGGGGCCGTTCCTTGTCTATGTTCCTTGGAAC
AGGACGTCATAGAGGGTGAGAATCCCGTGTGGCGAGGAGTGCGGTTCTTTGTAAAGTGCCTTCGA
AGAGTCGAGTTGTWTGGGAATGCAGCTCTAAGTGGGTGGTAAATTCCATCTAAAGCTAAATATTG
GCGAGAGACCGATAGCGAACAAGTACAGTGATGGAAAGATGAAAAGAACTTTGAAAAGAGAGTG
AAAAAGTACGTGAAATTGTTGAAAGGGAAGGGCATTTGATCAGACATGGTGTTTTGTGCCCTCTGC
TCCTTGTGGGTAGGGGAATCTCGCATTTCACTGGGCCAGCATCAGTTTTGGTGGCAGGATAAATCC
ATAGGAATGTAGCTTGCCTCGGTAAGTATTATAGCCTGTGGGAATACTGCCAGCTGGGACTGAGG
ACTGCGACGTAAGTCAAGGATGCTGGCATAATGGTTATATGCCGCCCGTCTTGAACMMSGGRAMM
MCAAAAAA

Phylogenetic analysis, made by using strains of closely related microorganisms, showed that species *Saccharomyces cerevisiae* (99 %) is the closest to the studied strain.

CONCLUSIONS:

The analysis of the nucleotide sequence coding a part of the rRNA genes showed that **3K (14)** strain is the closest to the species ***Metschnikowia pulcherrima;*** **BUG1** is the closest to the species ***Saccharomyces cerevisiae;*** **5e** strain is the closest to the species ***Rhodotorula mucilaginosa.***

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