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The molecular diagnosis of some yeast species isolated locally from olive tree fruits, leaves, and soil

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Abstract
The current study involved the isolation of 60 local yeast isolates from the fruits, leaves, and soil of olive trees. The isolates were identified after being cultured on Yeast Extract Malt Extract Medium (YM Agar) based on their molecular characteristics, using the Internal Transcribed Spacer (ITS) region with Polymerase Chain Reaction (PCR) technology. The results showed that they belonged to four different yeast species: *Candida tropicalis*, *Rhodotorula mucilaginosa*, *Nakaseomyces glabratus*, and *Saccharomyces cerevisiae*. The isolates were registered in the National Center for Biotechnology Information (NCBI) under the names Alias 2, Alias 3, Alias 4, Alias 5, and Alias 6, and were assigned the accession numbers PQ810339, PQ810340, PQ810341, PQ881603, and PQ881604, respectively.

Keywords: Yeasts isolation, olive tree fruits, PCR, NCBI

Introduction

Yeasts are a group of unicellular fungi belonging to three divisions: Ascomycota, Basidiomycota, and Deuteromycota (Kai *et al*., 2024). In recent years, interest in yeasts has significantly increased due to their benefits and importance to human health (Badia & Nadeem, 2020). They serve as a source for ethanol production (Wani *et al*., 2023) [24], biofuels, and medically valuable compounds such as vaccines (Zhao *et al*., 2023) [26], carotenoids—precursors of vitamin A (Malla Obaida *et al*., 2018), various fatty acids (Zhang *et al*., 2022) [25], and enzymes such as pectinase (Ametefe *et al*., 2021) [7]. Yeasts are found in various environments, including food products (Malla Obaida, 2020a) [17]. Plants (fruit surfaces, flowers, leaves, and grains) are among the most common environments for yeast growth. They grow on plant leaf surfaces and obtain nutrients from leaf secretions (Pollock, 1992) [20]. Several studies have reported the isolation of yeasts from fruits, leaves, and soil (Suryaningsih *et al*., 2018) [23]. Researchers such as Hernandez *et al*., (2007) successfully isolated *Pichia anomala*, *Kluyveromyces marxianus*, and *S. cerevisiae* from green olives. Sánchez *et al*. (2020) identified 14 different yeast species belonging to various genera (*Zygosaccharomyces, Pichia, Lachancea, Kluyveromyces, Saccharomyces, Candida*, and *Torulaspora*) in olives from Castilla, Spain. Mean while, Obaida (2020b) isolated *Debaryomyces hansenii* from home garden soil. Additionally, Malla Obaida (2020c) observed the presence of *C. cerevisiae* and *D. hansenii* in the leaves of different citrus tree species. Furthermore, Al-Taei and Malla Obaida (2023) isolated 17 yeast strains from fruits and leaves of various plant species belonging to different genera (*Debaryomyces, Rhodotorula, Pichia, Candida, Kluyveromyces, Geotrichum, Kloeckera, Saccharomyces, Cryptococcus, Zygosaccharomyces*, and *Trichosporon*). The aim of this study is to isolate and identify yeasts present in the local environment, which could serve as valuable strains for various bioremediation applications.

Materials and Methods

Samples Collection

The samples were collected starting from September 1, 2024, for a duration of three months from various sources, including fruits, leaves, and soil of olive trees obtained from different areas such as Bashiqa district, the village of Fazliya, as well as home gardens and local farms in Mosul city. The samples were placed in sterile, tightly sealed glass bottles inside clean, sterilized bags and then transported to the Postgraduate Research Laboratory, Department of Biology, College of Science, University of Mosul.

Yeast extract malt extract medium (YM agar)

The medium was prepared by dissolving 5 g of peptone, 10 g of glucose, 3 g of yeast extract, 3 g of malt extract, and 20 g of agar in 1000 mL of distilled water. After heating the medium to boiling on a magnetic stirrer with heating, it was left to cool, and the pH was adjusted to 7.0 using a pH meter. The medium was sterilized in an autoclave at 121°C for 15–20 minutes. After cooling to 45–50°C, the antibiotic Ampicillin was added at a concentration of 100 µg/mL (Kusmiyati *et al*., 2022; Kreger-Van, 1984) [11].

Molecular Diagnosis of Yeast Isolates

With the emergence of modern diagnostic techniques, such as molecular diagnosis, which relies on DNA-based characteristics due to their stability against environmental changes, researchers have recently adopted this method for identifying various yeast species that are difficult to diagnose accurately based on their morphological or microscopic traits. To ensure the accuracy of morphological, microscopic, and biochemical diagnoses, molecular diagnosis was conducted following these steps:

Cultivation and Activation of the Isolate for DNA Extraction

For the molecular diagnosis of selected yeast isolates, the isolates were cultivated in liquid Malt Extract Agar (MEA) medium for 48 hours at 28°C.

DNA Extraction from Yeast Samples Under Study

The Genomic DNA Mini Kit from Geneaid was used for DNA extraction as follows:

1. The yeast pellet obtained from the liquid medium, equivalent to 1×10^9 cells, was transferred to a 1.5 mL Eppendorf tube.
2. 400 µL of GP1 buffer and 5 µL of RNase were added, and the mixture was thoroughly vortexed.
3. The tubes were incubated at 60°C for 10 minutes, with mixing every 3 minutes.
4. 100 µL of GP2 buffer was added, mixed, and incubated on ice for 3 minutes.
5. The mixture was transferred to a Filter Column and centrifuged at 10,000 rpm for 1 minute, and the filtrate was collected in a 1.5 mL Eppendorf tube.
6. 1.5 volumes of GP3 buffer were added and mixed immediately for 5 seconds.
7. 700 µL of the solution was transferred to a GD Column and centrifuged at 16,000 rpm for 2 minutes.
8. The filtrate was discarded, and the GD Column was reattached to a new collection tube.
9. 400 µL of W1 buffer was added and centrifuged at 16,000 rpm for 30 seconds.
10. The filtrate was discarded, and the GD Column was placed into a fresh collection tube.
11. 600 µL of Wash Buffer was added and centrifuged at 16,000 rpm for 30 seconds. The filtrate was discarded, followed by an additional centrifugation at 16,000 rpm for 3 minutes.
12. The GD Column was transferred to a fresh 1.5 mL Eppendorf tube, and 100 µL of Elution Buffer was added. The tube was incubated at room temperature for 3 minutes.
13. The final centrifugation was carried out at 16,000 rpm for 30 seconds. The GD Column was discarded, and the extracted DNA was stored at -20°C until use.

The Quality Control of DNA Extraction Test

This test includes three assessments to determine the suitability of the extracted DNA for molecular experiments during the current study, which involved the following:

First: DNA Integrity Detection This test was carried out through a series of sequential steps as follows:

1. After extracting the genomic DNA (gDNA), electrophoresis was performed using a 2% agarose gel prepared by dissolving 2 grams of agarose powder in 100 mL of 10% TBE solution. The mixture was heated and continuously stirred using a magnetic stirrer until the turbidity disappeared, and it became clear while boiling. It was then left to cool until it reached 60˚C.
2. The mixture was poured into the tray of the electrophoresis apparatus after placing the comb at one end of the gel to form wells. The pouring was done gently to avoid the formation of bubbles, which were removed with a pipette if they appeared. The mixture was left to solidify, then the comb was carefully removed to prevent any scratching of the gel at the well formations, and the gel was placed in the electrophoresis tank containing 200 mL of 10% TBE solution to cover the surface of the gel.
3. 7-10 microliters of the sample DNA were injected into the wells formed by the comb.
4. The electrophoresis tank was connected to the power supply and operated by applying an electrical current with a voltage difference of 80 volts/cm, ensuring that the migration direction was from the negative electrode to the positive electrode. The electrophoresis process took 180 minutes.
5. After the electrophoresis process was completed, the gel was transferred to a tray containing a mixture of TBE (X10) and 5 microliters of Midori Green Advance DNA stain for every 40 mL of gel. The gel was gently stirred, then lifted and transferred to another tray containing distilled water to remove any remaining dye.
6. The gel was examined by exposure to ultraviolet (UV) light using a UV light generator at a wavelength of 240 nm to visualize the DNA bands.
7. The Gel Documentation System was used to capture and document the electrophoresis results in the agarose gel. This system is equipped with a software program to measure the sizes of the bands.

Second: DNA Concentration Evaluation

The concentration of genomic DNA extracted from the selected yeast isolates for this field was measured using the Spectronanometer, a light spectrophotometer. The test procedure was as follows:

1. The device was powered on, and we waited for several minutes until the device's program loaded.
2. The device was calibrated by adding 3-5 microliters of Elution Buffer solution to the lower lens using a micropipette. The upper lens was then lowered to place the drop between the two lenses.
3. After calibrating the device, the drop was removed using a micropipette, and a new drop of the DNA solution dissolved in the same Elution Buffer was added. The reading appeared on the device's screen thereafter. The ideal DNA concentration for the extracted DNA ranged between 27-52 micrograms/ml, while the DNA concentrations of the isolates under study varied (Table 5).

Third: DNA Purification Evaluation

The purity of DNA extracted from yeast isolates was determined using the same technique mentioned in the second test section. The ideal purity ratio for DNA is between 1.9 and 1.7. If it falls below this range, it indicates contamination with compounds detectable by the device, such as proteins, phenols, or other substances. If the ratio exceeds 1.9, it suggests the presence of RNA. The results for DNA purity evaluation were within the ideal standards and ratios mentioned above, and the steps followed were the same as those for measuring DNA concentration.

Primers Used

The specialized primers, both forward and reverse types, were prepared for the molecular diagnosis of yeast isolates obtained from the English company Biolab in a lyophilized form. They were prepared according to the specifications of the supplying company by analyzing the primers. 10 microliters of each primer were taken and added to 90 microliters of deionized water (DDH2O) in a 1.5 ml Eppendorf tube (Abdul-Hadi *et al*., 2022) [1], as shown in Table (1).

Table 1: Nucleotide sequences of the primers used

Type of Primer	The Nucleotide Sequence Order of The Primer (5´-3´)	Used Gene	Output Size (BP)
Forward (ITS1)	TCCGTAGGTGAACCTGCGG	ITS	***700***
Revers (ITS4)	TCCTCCGCTTATTGATATGC		

The Polymerase Chain Reaction (PCR) Technique

After extracting and electrophoresing the DNA, the PCR reaction was performed based on the method described by Al-Dabbagh (2022) [2]. The primers were prepared separately, each with a volume of 1 microliter and a concentration of 10 picomoles/microliter from the stock primers solution. The reaction mixture, with a total volume of 50 microliters, was placed in special 0.5 ml Eppendorf tubes and thoroughly mixed using a vortex for 3–5 seconds, following the instructions provided with the Master Mix supplied by the English company Biolaps, as shown in Table (2). The reaction mixture was distributed into five Eppendorf tubes, with 10 microliters per tube. The tubes were then placed in the thermal cycler (Bridge et al., 1998), and the device was programmed as indicated in Table (3).

Table 2: contains the components of the tubes for the polymerase chain reaction

Components	Quantity (µL)	Concentration
DNA Template	15	-
Free nuclease water	13	-
Master Mix	20	1 X
Forwared primer	1	10 pica moles/microliter
Reverse primer	1	10 pica moles/microliter

Table 3: steps and stages of the implemented program for Polymerase Chain Reaction (PCR) interaction

Polymerization reaction stages (steps)	Time duration	Temperature °C	number of courses
1. Denaturation	6 min	95	1
2. Denaturation	45 Sec	95	35
Annealing	1 min	58	
Extention	1 min	72	
3. Final Extention	5 min	72	1

Investigation of DNA Nucleotide Sequences

To investigate the nitrogenous base sequences, the sample was sent to the Korean company Macrogene. The bands resulting from the PCR reaction were extracted from the gel for purification and sent for nucleotide sequence testing, relying on the kit provided by Geneaid and following its protocol as follows:

1. The bands were cut from the agarose gel using a sterile scalpel, removing as much surrounding gel as possible.
2. Approximately 300 mg of each gel piece was transferred to a 1.5 ml Eppendorf tube, and 500 µl of Buffer DF was added, then mixed using a vortex mixer.
3. The tubes were incubated at 55-60°C for 15 minutes, with inversion every 3 minutes during incubation, then left at room temperature.
4. 800 µl of the sample mixture was transferred to a DF column placed in a collection tube, followed by centrifugation at 16,000 rpm for 30 seconds, then the filtrate was discarded.
5. The DF column was reattached to a new collection tube, and 600 µl of wash buffer was added, left for 1 minute, then centrifuged at 16,000 rpm for 30 seconds, and the filtrate was discarded. The washing step was repeated.
6. An additional centrifugation step was performed for 3 minutes at 16,000 rpm to ensure the DF column was completely dry.
7. The DF column was transferred to a new 1.5 ml Eppendorf tube, then 20-50 µl of Elution Buffer was added to the center of the column and left for 2 minutes to ensure absorption of the buffer. Finally, centrifugation was performed at 16,000 rpm for 2 minutes to obtain the dissolved DNA.

After receiving the results within approximately one month, the National Center for Biotechnology Information (NCBI) database was used to compare the nucleotide sequences of the sample with reference sequences available on the platform. The BLAST program was employed to align the nucleotide sequences of the studied isolates to achieve an accurate identification of yeast isolates at the species level.

Results and Discussion

Isolation of Yeasts

The growth of colonies on the isolation media indicated the presence of 60 yeast isolates. The highest number of isolates (25) was found in soil samples taken from beneath olive trees, whereas the lowest number (15) was obtained from fruit samples. Meanwhile, the isolation from olive tree leaves revealed 20 isolates, as shown in Table (4). Several studies have indicated the possibility of isolating yeasts from fruits, leaves, and soil (Suryaningsih *et al*., 2018) [23]. Sánchez *et al*., (2020) successfully isolated 14 different yeast species belonging to various genera (Zygosaccharomyces, Pichia, Lachancea, Kluyveromyces, Saccharomyces, Candida, and Torulaspora) from olive fruits in Castilla, Spain. Additionally, Hernandez *et al*., (2007) observed the presence of yeasts such as *Pichia anomala*, *Kluyveromyces marxianus*, and *Saccharomyces cerevisiae* in green olive fruits. The widespread presence of yeasts in soil, fruit, and plant leaves (Al-Taie and Malla Obaeda, 2022) [6] is attributed to their ability to tolerate a wide range of temperatures and sugar concentrations. It is worth mentioning that food contamination with yeasts varies depending on its moisture content. A decrease in moisture content leads to the disappearance of yeasts (Al-Khafaji *et al*., 1992).

Table 4: Sources of yeast isolation and isolation preparation.

Solation Sources	Solation Code	Total Isolations
Fruits	H1, H2, H3, H4, H5, H6, H7, H8, H9, H10, H11, H12, H13, H14, H15	15
Leaves Olives	H16, H17, H18, H19, H20, H21, H22, H23, H24, H25, H26, H27, H28, H29, H30, H31, H32, H33, H34, H35	20
Soil	H36, H37, H38, H39, H40, H41, H42, H43, H44, H45, H46, H47, H48, H49, H50, H51, H52, H53, H54, H55, H56, H57, H58, H59, H60	25
Total		60

Molecular Diagnosis of Selected Yeast Isolates Using Specific-PCR Technique

Evaluation of DNA Concentration and Purity

The results of measuring DNA concentration and purity using the Spectronanometer (a nano-drop spectrophotometer) showed variations in the DNA concentrations of the isolates under study, as presented in Table (5).

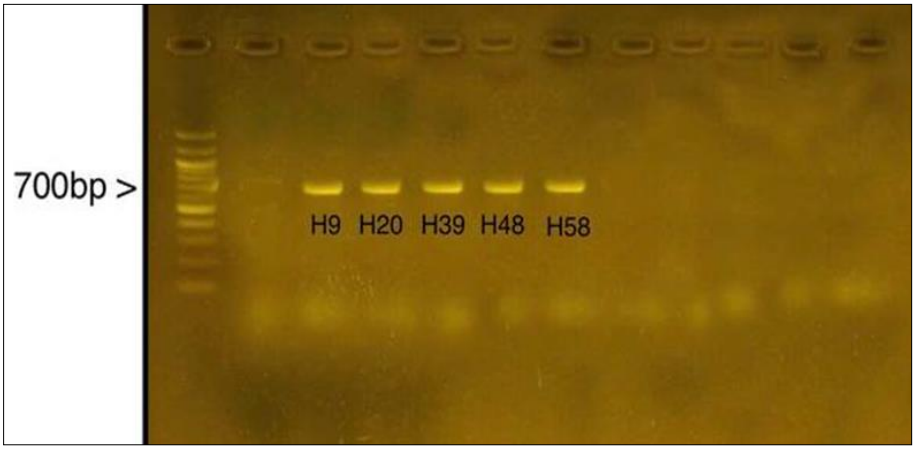
Table 5: DNA Concentrations and Purity of the Extracted DNA from the Studied Isolates

Yeast Strains	DNA concentration (micrograms/mL)	Purity
S. cerevisiae H9	42.7	1.78
N. glabratu H20	44.5	1.7
C. tropicali H39	38.3	1.8
C. tropicalis H48	40	1.79
R. mucilaginosa H58	33.1	1.8

DNA Amplification

The results of genomic DNA (gDNA) amplification of the yeasts *S. cerevisiae* H9, *N. glabrata* H20, *C. tropicalis* H39, *C. tropicalis* H48, and *R. mucilaginosa* H58, using a combination of the specialized primers ITS1 and ITS4 to amplify the 18S rRNA sequences for detecting the ITS region via thermal polymerase, revealed a single distinct band for each yeast isolate at a molecular size of 700 base pairs during the electrophoresis of the amplification products on agarose gel (Figure 1). This is consistent with the information related to the design of the aforementioned primers available on the website of the National Center for Biotechnology Information (NCBI).

When the specific bands of each yeast isolate were extracted from the agarose gel and sent to the Macrogen company in Korea for sequencing of the nitrogenous bases, they were registered under the names Alias 2, Alias 3, Alias 4, Alias 5, and Alias 6, as shown in Figures (2,3,4,5,6). Our results were similar to those obtained by Alkhuwailidy and Alrufae (2022) [4], who conducted a study on the identification of *Candida* sp. yeasts and observed single bands with molecular weights reaching 500 base pairs. Another study by Nouraei *et al*. (2024) [18], using ITS1 and ITS4 primers for the amplification of DNA isolated from different yeast species (*R. sloofia*, *R. mucilagenosa*, *C. guilliermondii*, *Aureobasidium pullulans*, and *Trichosporon asahii*), showed molecular weights of up to 100 base pairs. Additionally, another study used the specialized primers ITS1 and ITS4 to register five isolates of the yeast *Meyerozyma caribbica* (Al-Sumaidaie, 2023) [5] and three isolates of *C. albicans*, along with one isolate each of *C. tropicalis* and *C. glabrata*, in the National Center for Biotechnology Information (Hadeed, 2023).



H9 **:***S. cerevisiae* H9*:* Alias 2
H20 **:***N. glabratu* H20*:* Alias 3
H39*:* H39 *C. tropicali* **:**Alias 4
H48*:* H48 *C. tropicalis* **:**Alias 5
H58 **:**H58 *R. mucilaginosa:* Alias 6

Fig 1: PCR reaction product of the ITS region with a 700 bp amplicon for yeast and analyzed on a 2% agarose gel.

```
  1 caaacacaaa caattttatc tattcattaa atttttgtca aaaacaagaa ttttcgtaac
 61 tggaaatttt aaaaatatta aaaactttca acaacggatc tcttggttct cgcatcgatg
121 aagaacgcag cgaaatgcga tacgtaatgt gaattgcaga attccgtgaa tcatcgaatc
181 tttgaacgca cattgccccc ttggtattcc agggggcatg cctgtttgag cgtcatttcc
241 ttctcaaaca ttctgtttgg tagtgagtga tactctttgg agttaacttg aaattgctgg
301 ccttttcatt ggatgttttt tttccaaaga gaggtttctc tgcgtgcttg aggtataatg
361 caagtacggt cgttttaggt tttaccaact gcggctaatc ttttttatac tgagcgtatt
421 ggaacgttat cgataagaag agagcgtcta ggcaacaatg ttcttaaagt
```

Fig 2: Sequence of nitrogenous bases for the yeast *S. cerevisiae* H9

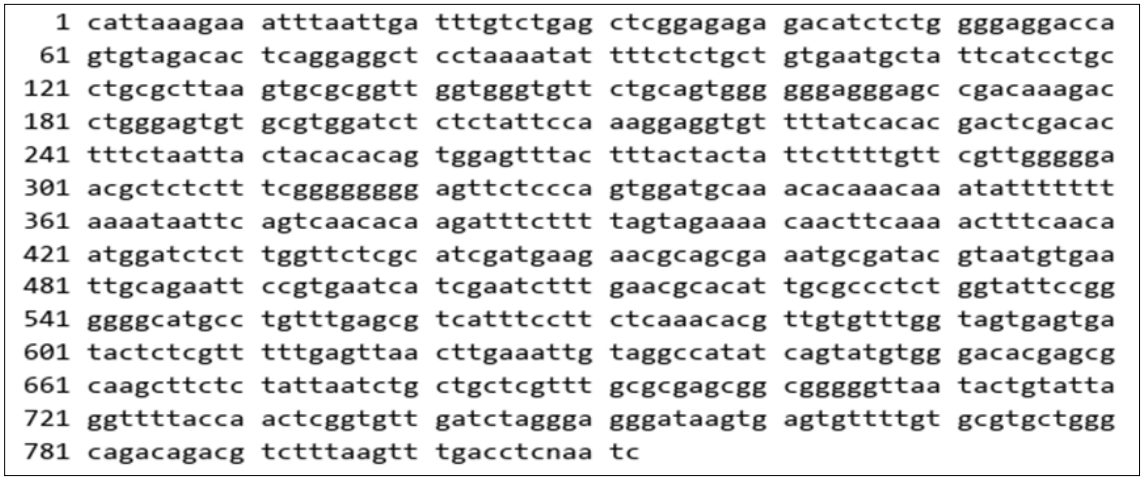


Fig 3: Sequence of nitrogenous bases for the yeast *N. glabrata* H20

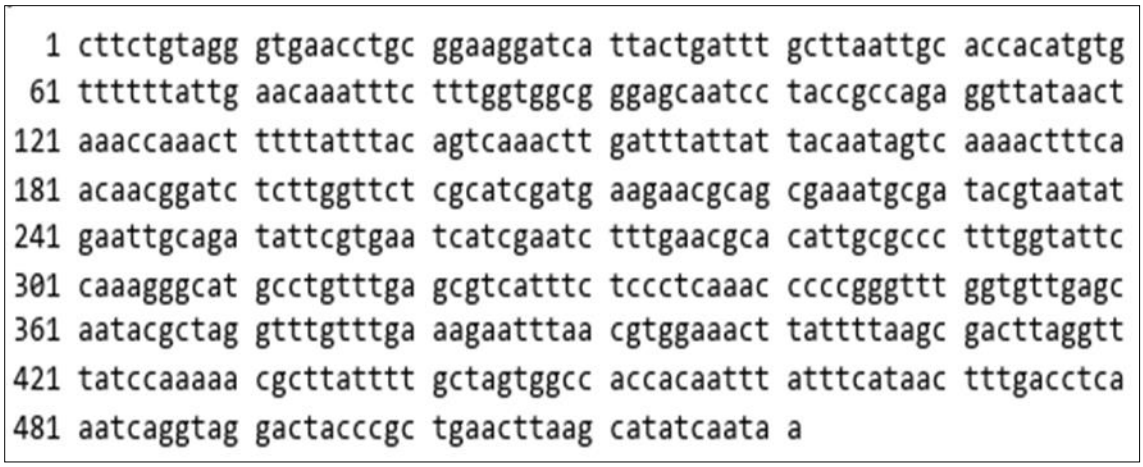


Fig 4: Sequence of nitrogenous bases for the yeast C. *tropicalis* H39

Fig 5: Sequence of nitrogenous bases for the yeast *C. tropicalis* H48

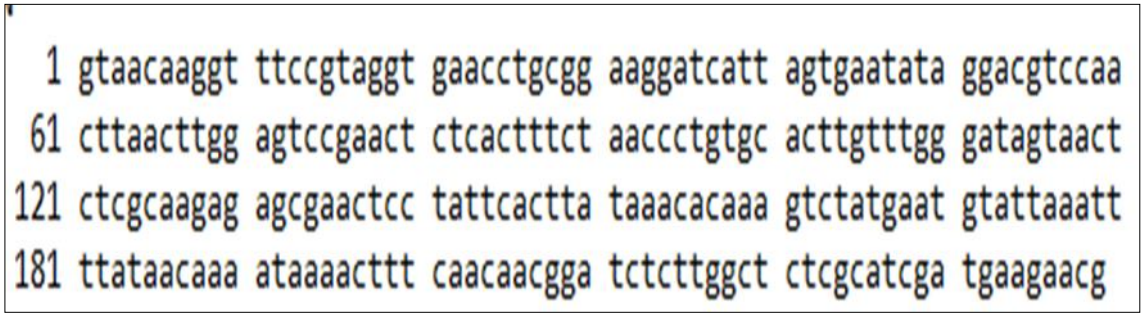


Fig 6: The sequence of nitrogenous bases of the yeast R. *mucilaginosa* H58.

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