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Isolation and Identification of Fungal Species Contaminating the Refrigerators

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Keywords: Fungal Contamination, Refrigerator, Fungi, Food Spoilage, PCR.

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Abstract:

Refrigeration is one of the most widely used methods to control the growth of microorganisms in food products. A number of isolates was 433, including 363 isolates from refrigeration and 70 isolates from freezers. The fungus *Cladosporium* sp. is the large number in both refrigeration and freezer, 187 and 28, respectively, and the lowest numbers of fungi such as *Rhizopus stolonifer*, *Rhizoctonia solani*, and *Fusarium* sp., many fungi were isolated from refrigeration, while fungal isolates from freezing were less numerous and less diverse. Molecular identification of *Cladosporium* sp. because it is the most frequent among the fungal isolated from refrigeration and freezing by using polymerase chain reaction, it has been shown that *Cladosporium sphaerospermum* strain HKA in the gene bank. The aim of the study is to recognize the fungi that contaminate the refrigerator both domestically and commercially.

Keywords: Fungal Contamination, Refrigerator, Fungi, Food Spoilage, PCR.

عزل وتشخيص أنواع الفطريات الملوثة للثلاجات

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الخلاصة:

يُعد التبريد أحد اكثر الطرق المستخدمة على نطاق واسع للتحكم في نمو الكائنات الحية الدقيقة في الأغذية . تضمنت الدراسة الحالية جمع ٢٠ عينة من الثلاجات وعزل العديد من الفطريات وكان عدد العزلات ٤٣٣ عزلة منها ٣٦٣ عزلة من التبريد و٧٠ عزلة من التجميد وظهر الفطر *Cladosporium* sp. بأعداد كبيرة في كلا التبريد والتجميد ١٨٧ و٢٨ على التوالي واقل أعداد كانت للفطريات *Rhizopus stolonifer* ، *Rhizoctonia solani* و *Fusarium* sp. وان العديد من العزلات الفطرية عزلت من التبريد بينما العزلات من التجميد اقل عدد واقل تنوعا ، كما شخصت عينة فطر *Cladosporium* جزيئيا لأنه الفطر الاكثر ترددا من بين العزلات الفطرية المعزولة من التبريد والتجميد باستخدام تفاعل البلمرة المتسلسل وتبيّن أنها *Cladosporium sphaerospermum* strain HKA والمسجلة في بنك الجينات العالمي.

الهدف من الدراسة : التعرف على الأنواع الفطرية الملوثة للثلاجات المنزلية والتجارية .

الكلمات المفتاحية: الفطريات الملوثة، الثلاجات، الفطريات، التعفن الفطري، تقنية تفاعل البلمرة المتسلسل.

1. Introduction:

People in the world are suffering from health problems related to consumption of the contaminated food every year this is one of the problems of the health of our day, many species of microorganisms can be present in food such as bacteria, fungi" yeasts, and molds" the fungi were considered important source causes of the contamination with spoilage of food, fungi damage food by different of food appearance and texture. As well as secreting mycotoxin and consequently the health of consumers for endangering [1]. Refrigeration is one of the most widely used methods to control the growth of microorganisms in food products [2]. Refrigeration is used to the control of rate some enzymes and chemical reactions and also the rate of microorganisms, growth in the food [3]. Studies have shown that food is damaged even in the temperature of the refrigerator because the microorganisms secrete enzymes and oxidation reactions, the type of container or packaging material in which the food is stored and the time of storage also the type of microbial, resulting in food poisoning and damage it while refrigerated [4]. Also, the freezing doesn't prevent the

reproduction of microorganisms [5]. In the presence of nutritious materials, humidity, and appropriate temperatures, increase the growth, leading to food-borne diseases. Microorganisms come from unclean and raw nutrients with unclean surfaces this container directly causes pollution to the other food storage and contamination of inter surface of the refrigerator, which causes indirect food pollution through preparation later [6]. Refrigerated foods can become vectors of disease through contamination with pathogens and microorganisms in markets, through storage, or in consumers' homes [7]. Different foods require different places and temperatures for storage, for example, fresh meat, fish, and poultry are stored in cold storage places, such as freezers, and in containers with ice, as they do with fish sold in markets, but fruits and vegetables are stored in cool places or m, refrigeration [8]. Fungi causes spoilage in foods more than bacteria in refrigerator when humidity and acidity are low and packaging conditions are suitable for their growth, also fungi isolated from fresh refrigerated animal products, fruits, vegetables, and prepared foods to eat [9]. Filamentous fungi produce spores that spread in refrigerators when conditions are favorable, damage food because yeast breaks sugar into Co_2 with alcohol and this causes low quality of the nutrient product [10], [11] fungal spores spread by "air, container, improper packaging, hands, and contaminated food", studies on the isolated filamentous fungi from nutrient materials such as "*Alternaria, Aspergillus, Botrytistis, Cladosporium, Fusarium, Geotrichum, Aureobasidium, Trichothecium, Mortierella, Mucor, Neurospora, Penicillium, Rhizopus, Thamnidium, Manoscus* and among the yeast genera involved *Candida, Cryptococcus, Rhodotorula, Schizosaccharomyces, Trichosporon*" ; however, this fungi is often present in (meat and poultry) but it is also found in many other nutrient products [12]. The harmful effects of fungi are not limited to the spoilage of food only, but they are also harmful to human health and animals, as they secrete toxic metabolites for example, some species of the *Aspergillus* spp. secrete aflatoxin, fungi such as *Cladosporium* cause problems for humans, as it may cause skin and toenail infections, the lung diseases including "nasal congestion, sneezing, coughing, and itchy eyes" *Cladosporium* spores cause airborne allergy and another disease of the respiratory tract, moreover, the mycotoxins which are produced by *Cladosporium* may also make volatile organic compounds (VOCs) related to smells [13].

2. Materials and methods:

Collecting the samples: 20 samples were collected from domestic and commercial refrigerators (refrigeration 4°C and freezing 0°C) from Mosul city from October to December and directly cultured on media.

Prepare medium: Potato Dextrose Agar (PDA) medium was prepared according to the supplying company, United Kingdom-Lab M. Limited, and its pH was adjusted, and then sterilized according to the optimal conditions by placing it in the autoclave (121˚C with pressure 1 atmosphere, for 20 minutes), while the antibiotic was added to the sterile medium cooled to (45-50˚C) after sterilizing it using fine membrane filters (0.22) millimicrons.

Isolation of fungi: Fungi were isolated by using sterile swabs moistened with sterile distilled water, and the PDA medium was inoculated in Petri dishes, with the antibiotic Amoxillin at 100 micrograms/ml, by streaking on the surface of the medium, and then the dishes were incubated at 25˚C for 7 days until fungal colonies appeared, [1]. The percentage of fungal frequency was calculated by the following formula:

$$\text{Frequency percentage} = \frac{\text{Number of colonies}}{\text{Total number of colonies}} \times 100$$

Diagnosis of fungi: The fungi were isolated and diagnosed by taking a part of the fungal colonies after purifying them from the edges of the colony using a method Hyphal tip technique and placing it on a slide with a drop of water in it, then putting the cover slide and it was examined with an optical microscope at 10X and 40X power, according to the shape of the mycelium and conidia, the isolated fungi were diagnosed based on the taxonomic keys of the genus [14]. To the species based on [15], [16], [17]. As well as diagnosing *Aspergillus* to different species according to the taxonomic key by [18], as the following:

1- Growth on Malt Extract Agar medium (MEA) incubated at 25 and 37°C for seven days.
2- Growth on Czapek Yeast Extract Agar medium (CYA) incubated at 25 and 37 °C for seven days.
3- Growth on Glycerol Nitrate Agar medium (G25%N at 25 and 37 °C for seven days.

Transferred discs from fungal colony with 6 mm were using a cork borer under sterile conditions, 3 replicates for each fungus, fungi were also diagnosed *Fusarium* sp. by growing it on a PDA medium according to the taxonomic key Pitt and Hocking (2009).

Molecular diagnosis (DNA extraction): The extraction equipment was used, DNA Geneaid™ according to company instructions in the United States of America. DNA bands are detected by using red safe dye, which is a safe and very sensitive dye for DNA, as an alternative to ethidium bromide dye, which is considered a strong mutagenic agent, as it gives green fluorescence when it binds to DNA [19].

Polymerase chain reaction (PCR): This method was used to detect the gene (*ITS*) Internal Transcribed Spacer, the *ITS* area is the DNA spacer between the small subunit genes of rRNA and genes rRNA with large subunits in the chromosome, it has been used to diagnose fungi and study their molecular evolution for more than twenty years **[20]**.

The Primers used in the study: Two types of specialized primers are *ITS1* the reaction primer and *ITS4* the reverse reaction primer as shown in **Table 1.**

Table 1: Gene-Specific Primers were Chosen *ITS* from (Korean Macro Gene) Company

Name and type primer	Sequence of nitrogenous bases
ITS1(Forward)	5' TCCGTAGGTGAACCTGCGG 3'
ITS4(Reverse)	5' TCCTCCGCTTATTGATATGC 3

Preparation of agarose gel: Prepare agarose gel by dissolving 2 g from agarose in 100 ml of (Tris-Borate-EDTA solution) previously prepared and warmed until boiling then left cool at 45-50°C and gel was poured gently so as to leave no air bubbles and left to cool for thirty minutes. A Comb was then gently isolated from agarose after it had been solid. Plate was fixed on the holder in the horizontal of electrophoresis and then filled the tank with TBE insulating material that covers the surface of the gel **[21]**.

Electrophoresis on agarose gel: Electrophoresis was performed to determine the size of the DNA bands, and to confirm its purity and concentration after extraction. Mix 3 µicroleter from loading solution (Intron /China) with 5 µicroleter from extracted DNA, which is bound with the loading dye, and load the mixture directly into the holes of gel and expose it to electric current at 7v/cm^2 with 1-2 hours until the DNA sample reach to the other side from gel, then gel was exposed to an UltraViolet Transilluminater source at a wavelength of 336 nanometers after placing gel in a water bath with the presence of 30 µicroleter from red safe dye and 500 ml from D.W. then the gel was photographed by using a digital camera to show the bands.

Optimal conditions for the PCR technique: The polymerase chain reaction technique according to the conditions is shown in **Table 2**.

Table 2: Optimal Conditions for Polymerase Chain Reaction Technique [22]

T	Steps	Temperature (°C)	*Duration*	Number of courses
1	Primary denaturation of DNA	95	5 minutes	One cycle
2	Secondary denaturation of DNA	95	30 seconds	35 cycles
3	Annealing process	55	1 minute	
4	First elongation process	72	1 minute	
5	Final elongation process	72	6 minutes	One cycle

Polymerase chain reaction products are sent to the Psomagene sequencing company (USA) to obtain the genetic sequence.

3. Results:

Isolation and diagnosis of fungi: Many fungi were isolated, and the number of isolates was 433, including 363 isolates from refrigeration and 70 isolates from freezers, as shown in **Table 3**.

4. Discussion

Fungi were diagnosed based on phenotypic characteristics as in **Figure 1**, and microscopic characteristics and compared with taxonomic keys, fungi within the genus *Aspergillus* it was diagnosed based on the diameter and color of the colony after growing it on three types of media, also diagnosing fungi within the genus *Fusarium* by comparing them with their taxonomic key, based on the shape of the large and small conidia and the color of the colony after growing it on medium PDA as shown in **Figure 2.**

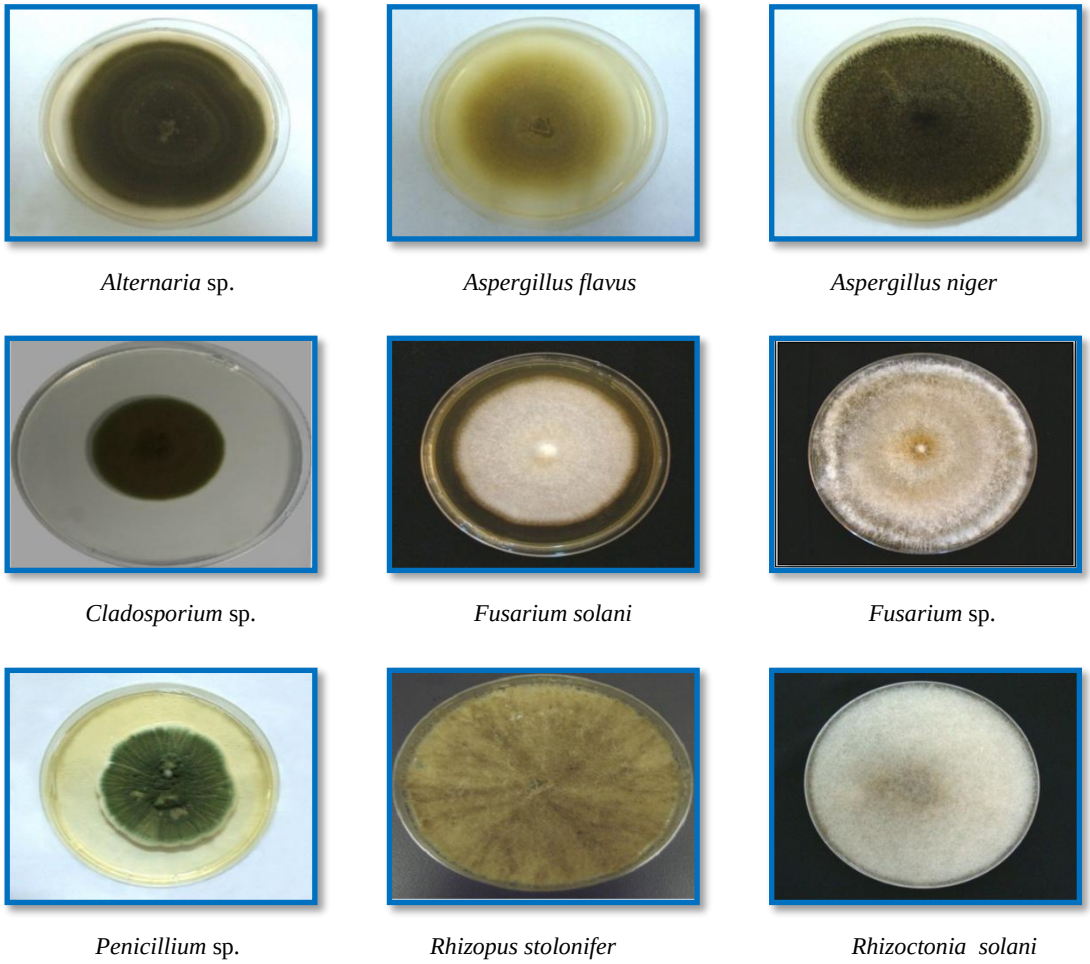
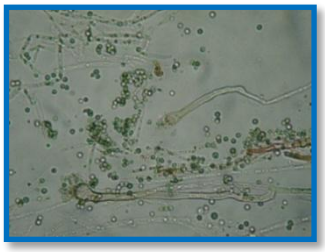


Figure 1: Phenotypic Characteristics of Fungi Isolated on PDA Medium

Alternaria sp.

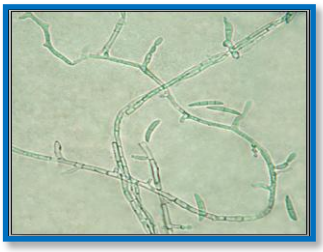


Aspergillus flavus

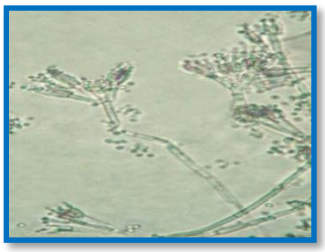
Aspergillus niger

Cladosporium sp.

Fusarium solani



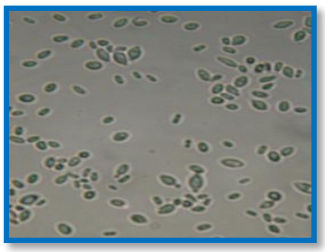
Fusarium sp.



Penicillium sp.

Rhizoctonia solani

Rhizopus sp.



Yeast

Figure (2) Microscopic diagnosis of fungi using an optical microscope at 40X

Cladosporium sp. appeared In large numbers in both refrigeration and freezers 187 and 28 respectively, followed by *Penicillium* sp. with numbers of 70 and 22, respectively, this is consistent with previous studies such as [23], [24]. The lowest numbers of fungi were *Rhizopus stolonifer*, *Rhizoctonia solani*, and *Fusarium sp*. Many fungi were isolated from refrigeration, while isolates from freezing were fewer in number and less diverse. There were fungal genera that were common in isolation from refrigeration and freezing, while there were fungal genera that appeared in

refrigeration only, such as *Aspergillus flavus*, *Alternaria* sp., *Rhizopus stolonifer*, *Rhizoctonia solani*, and *Fusarium* sp. as shown in **Table 3**.

Table 3: Number of fungi isolated from refrigeration and frozen stored food

Isolated fungi	Refrigeration	Freezing
Alternaria sp.	12	----
Aspergillus flavus	2	----
Aspergillus niger	42	14
Cladosporium sp.	187	28
Fusarium solani	4	2
Fusarium sp.	1	----
Penicillium sp.	70	22
Rhizoctonia solani	1	----
Rhizopus stolonifer	1	----
Yeast	43	4
The Total	363	70

The percentage frequency of fungi was calculated, and the highest percentage was the *Cladosporium* refrigeration, it reached 43.2% and after frozen it was about 6.5%, followed by *Penicillium* sp. which reached 16.2% and 5.1%, respectively, and the lowest percentage was for *Rhizopus stolonifer*, *Rhizoctonia* sp., and *Fusarium sp*. which reached 0.2% which was isolated from refrigeration only, as shown in **Table 4**.

Table 4: Percentage Frequency of Isolated Fungi

Isolated fungi	Refrigeration %	Freezing %
Alternaria sp.	2.8	----
Aspergillus flavus	0.5	----
Aspergillus niger	9.7	3.2
Cladosporium sp.	43.2	6.5
Fusarium solani	0.9	0.5
Fusarium sp.	0.2	----
Penicillium sp.	16.2	5.1
Rhizoctonia solani	0.2	----
Rhizopus sp.	0.2	----
Yeast	9.9	0.9
The Total	100	

The percentage frequency of all fungi in refrigeration appeared to be higher than the percentage in freezing, also there is an increase in fungal diversity in refrigeration compared with freezing, which was fewer in number and types of fungi, and thus is consistent with the study [1]. This might be due to high temperatures leading to an increase in the growth and number of microbes [25]. Food-borne diseases are caused by improper storage and unregularly cleaning the refrigerator might increase the risk of infection [26]. Fungi spread in the refrigerator from many sources such as unclean hands, unclean surfaces of the refrigerator, less cleaning of uncooked materials and some material leaks with not good packing such as meat, eggs, and milk, as well as leaving the refrigerator door open and alternative temperature, not washed vegetables and fruits. The refrigerator can contaminate other food items [6]. The current study indicates that the highest percentage of *Cladosporium* followed by *Penicillium* sp. Thus, it matches with many studies, as reports indicate that these two fungi are always found on the sides of the refrigerator and that they can live and reproduce in low temperatures [27]. These fungi produce black spots on the surface of (meat and fatty tissue) in the refrigerator, also producing mycotoxins for several reasons, such as not good packaging of food items [28]. These toxins may cause food toxicity and cancer to humans, such as aflatoxin secreted by *Aspergillus* sp., there are other toxins, such as ochratoxin and zearalenone, which have various harmful effects on the consumer, such as poor digestion, mutagenic effects, or neurological or immune damage [1]. *Cladosporium* also causes spoilage to fatty and butter and causes spoilage of many fruits with production of toxins and causes black spots on the meat [29]. One of the most important causes of contamination may be a continuous opening of the refrigerator door, consequently increasing internal temperature and allowing the entry of contaminated fungal spores, pathogens present in the refrigerator can contaminate food directly or indirectly this dangerous to the health of the consumer in terms of food poisoning even at appropriate storage temperatures [6]. This is because fungi grow well in an environment that contains food with moisture [30]. Fungal growth in refrigerated foods depends on several factors, including temperature, storage time, moisture content, and carbohydrate concentration in the food source [31].

Molecular identification of *Cladosporium* with PCR technique: After examination bands under ultraviolet source it was found that each isolate contained single band with undispersed, and this is evidence of the purity of the DNA and its high concentration. The amplification results after electrophoresis on a 2% agarose gel that the resulting bands were 600 base pairs in size, as shown in **Figure 3**.

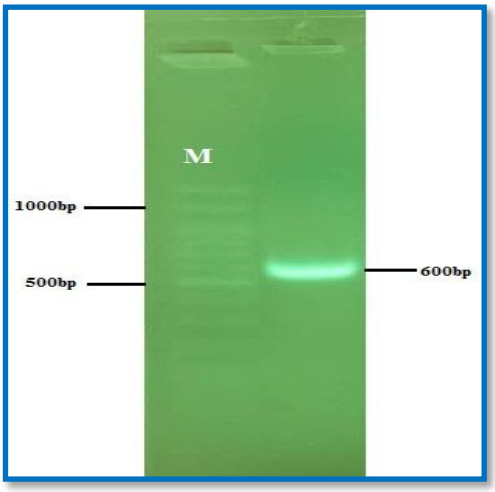


Figure 3: Electrophoresis of amplification products PCR for *Cladosporium* using 2% agarose gel at 5 V/cm^2, at an hour and using 1xTBE, the band size is 600 base pairs. **M:** DNA Ladder 1kb

Results of a study of the sequence nitrogenous bases: Polymerase chain reaction products are sent to the Psomagene sequencing company (USA) to determine and know the sequence of nitrogenous bases and compare with the gene sequence *ITS* storage within the World Gene Bank NCBI. The results showed a match rate of 99.22%, and the presence of site variation in the gene sequence in four locations, as shown in **Figure 4**. Perhaps this variation is due to point mutations that occur spontaneously in nature and include a series of changes in the sequence of nitrogenous bases in the DNA **[32]**.

Cladosporium sphaerospermum isolate 152 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence

Sequence ID: KP794112.1 Length: 509 Number of Matches: 2

Range 1: 4 to 509 GenBank Graphics ▼ Next Match ▲ Previous Match

Score	Expect	Identities	Gaps	Strand
917 bits(496)	0.0	502/506(99%)	0/506(0%)	Plus/Plus

```
Query  1    TCGGGCCGGGATGTTCACAACCCTTTGTTGTCCGACTCTGTTGCCTCCGGGGGCGACCCTG  60
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  4    TCGGGCCGGGATGTTCACAACCCTTTGTTGTCCGACTCTGTTGCCTCCGGGGGCGACCCTG  63

Query  61   CCTCCGGGCGGGGGCCCCGGGTGGACATTTCAAACTCTTGCGTAACTTTGCAGTCTGAGT  120
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  64   CCTCCGGGCGGGGGCCCCGGGTGGACATTTCAAACTCTTGCGTAACTTTGCAGTCTGAGT  123

Query  121  AAATTTAATTAATAAATTAAAACTTTCAACAACGGATCTCTTGGTTCTGGCATCGATGAA  180
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  124  AAATTTAATTAATAAATTAAAACTTTCAACAACGGATCTCTTGGTTCTGGCATCGATGAA  183

Query  181  GAACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTT  240
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  184  GAACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTT  243

Query  241  TGAACGCACATTGCGCCCCCTGGTATTCCGGGGGGCATGCCTGTTCGAGCGTCATTTCAC  300
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  244  TGAACGCACATTGCGCCCCCTGGTATTCCGGGGGGCATGCCTGTTCGAGCGTCATTTCAC  303

Query  301  CACTCAAGCCTCGCTTGGTATTGGGCGACGCGGTCCGCCGCGCGCCTCAAATCGACCGGC  360
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  304  CACTCAAGCCTCGCTTGGTATTGGGCGACGCGGTCCGCCGCGCGCCTCAAATCGACCGGC  363

Query  361  TGGGTCTTTCGTCCCCTCAGCGTTGTGGAAACTATTCGCTAAAGGGTGCCGCGGGAGGCC  420
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  364  TGGGTCTTTCGTCCCCTCAGCGTTGTGGAAACTATTCGCTAAAGGGTGCCGCGGGAGGCC  423

Query  421  ACGCCGTAAAACAACCCCATTTCTAAGGTTGACCTCGGATCAGGTAGGGATACCCGCTGA  480
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  424  ACGCCGTAAAACAACCCCATTTCTAAGGTTGACCTCGGATCAGGTAGGGATACCCGCTGA  483

Query  481  ACTTAAGCATATANANAAGGGGGGAA  506
            |||||||||||  | ||| ||||||
Sbjct  484  ACTTAAGCATATCAATAAGCGGGGAA  509
```

Figure 4: Sequence of Nitrogenous Bases of Cladosporium Sphaerospermum Strain HKA for Genes ITS1, ITS4

Figure 5: Recording the Cladosporium Sphaerospermum Strainhka in The Gene Bank

Cladosporium sp. is diagnosed because it is the most frequent among the fungi isolated from refrigeration and freezing by using polymerase chain reaction, it has been shown that it is *Cladosporium sphaerospermum* the isolate was reported in GenBank by nameHKA as shown in **Figure 5**. After examination of bands under an ultraviolet source, it was found that each isolate contained a single band undispersed, and this is evidence of the purity of the DNA and its high concentration

5. Conclusion

We conclude from the current study that the fungus *Cladosporium sphaerospermum* appeared in large numbers in both refrigeration and freezing and that fungal isolates were more numerous

and diverse from refrigeration compared with freezing, as they were less in number and diversity. and the lowest numbers of fungi such as Rhizopus stolonifer, Rhizoctonia solani and Fusarium sp. Molecular identification of Cladosporium sp. by using PCR, it has been shown that Cladosporium sphaerospermum strain HKA in the gene bank.

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