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Research Article

Molecular and morphological identification of wild mushrooms isolated in Basrah Province with the global recording of new strains

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Abstract

The present work aimed to collect various types of mushrooms from different regions in Basrah province and their identification based on morphological and molecular data using ITS1-ITS4 primers. During the present study, eight taxa were identified viz. wild *Agaricus bisporus, A. bitorquis, Coprinopsis picaceus, Panaeolus campanulatus, P. papilionaceus, Psathyrella candolleanl, Psathyrella* sp.1 and *Psathyrella* sp.2. Based on sequencing results, five new strains were recorded in the gene bank: two strains of *A. bisporus* (*A. bisporus* IQ Inaam1 and *A. bisporus* IQ Nassar), two strains of *P. papilionaceus* (*P. papilionaceus* INNASA2 and *P. papilionaceus* IQ Zeena), and one strain of *P. candolleana* as a new globally (*P. candolleana* IQ M. Jawad).

Keywords: Mushrooms, Molecular, Morphological identification, New strains.

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Introduction

In the past, Linnaeus classified mushrooms as part of the Thallophyta division, which included the so-called lesser plants. This was partly because the structural features were quite simple and anatomically uncomplicated (lack of true roots, stems, leaves, flowers, and seeds). They were more closely linked to plants than animals since they had cell walls. Later, researchers have determined that mushroom biota, like other fungi, have sufficiently and significantly different characteristics to place them in a separate fungal kingdom, the kingdom fungi. The term mushroom is generally defined as: "is a macrofungus with a characteristic fruiting body which can be either epigeous (above ground) or hypogenous (underground) and big abundant to be appreciated by the eye". The structure we refer to as a mushroom is the fungus' fruiting body, while the mycelium indicates a vegetative portion of the fungus (Chang & Miles 2004). Mushrooms are a great and differentiated group of macrofungi belonging to basidiomycetes and ascomycetes. The important parts of mushrooms for classifying groups and species of fungi consist of the cap, gills, stipe, ring, and valva. Main features include shape, size, color, topology, ornamentation, and texture; these characteristics vary depending on the species group (Rajesh et al. 2014). Molecular genetic markers have been used to quickly identify distinct types of mushrooms due to advancements in biotechnology (Froslev et al. 2007; Urbanelli et al. 2007). The internal transcribed spacer (ITS) region has long been a good target for species-level molecular identification of fungi (Sanchez-Ballesteros et al. 2000).

Generally, studies that were interesting with mushrooms in Iraq are limited to the northern and western regions (Aziz & Toma 2012). Aziz & Toma (2012) collected mushrooms from mountain areas in Sulaymania, Soran, Joman, Sedakan districts Qandil and Zalm village Amad Hawa, and identified several

genera and species of basidiomycetes, such as *Agaricus, Macrolep, Cystoderma amianthinum, Crepidotus variabilis, Inocybe godeyi, Hypholoma, Entoloma, Clitocybe, Tricholoma, Micromphale, Mycena, Pleurotus, Panaeolus, Stereum, Lactarius, Phellinus, Chondrostereum, Fomes, Trametes, Fomitopsis* and *Paxilus corrugatus*. Moreover, Al-Khesraji et al. (2017) surveyed mushrooms was performed in Al-Alam and Tikrit provinces from Tikrit district in Salahadin Governorate, north-central Iraq, with the identification of mushrooms represented *Coprinellus disseminates*, *Ganoderma lucidum*, *Lichenomphalia umbellifera, Montagnea arenaria, Phellinus pomaceus, Podaxis pistillaris* and *Trametes trogii*. In addition, Suliaman et al. (2017) & Toma et al. (2018) studied macrofungi from various areas in northern Iraq.

Furthermore, investigations of macrofungi in southern Iraq have been restricted to Khalaf (2015), who isolated *Coprinus* sp.. Muhsin et al. (2012) reported *Podaxis pistillaris* from Basrah Province. It is worth mentioning, that all previous studies in Iraq are constricted to morphological identification only. Therefore, the present study has involved isolating and identifying mushrooms using morphological features and molecular techniques.

Material and methods

Collection and preserving of samples: Basidiocarps of different species of native mushrooms were collected from different regions in Basrah from December 2017 to April 2018. The collecting, preservation, and identification protocols were followed according to standard methods. Photographs of samples were taken and stored for laboratory identification.

Isolation of mushrooms on culture media: Fruiting bodies were cleaned with tap water to eliminate dust and dirt before being chopped into small pieces under aseptic circumstances with a sterile blade. After sterilizing each sample with 70% ethanol for 3 minutes, the parts were submerged in a sodium hypo chloride (NaOCl) solution for 30 seconds to 1min (Tiwari et al. 2017). Representative pieces of fruiting body samples were washed for 1 minute in sterile distilled water before drying on filter paper. Four pieces of fruit body were inoculated in GPA, PDA, and MDA media plates after adequate drying and incubated at 25-30°C (7-10 days). Pure colonies were placed onto PDA slants and kept at 4°C in the fridge.

Identification of collected macrofungi

Morphological identification: All collected macrofungi were identified based on their macroscopic features including cap, scale, gills, stalk and cup of mushrooms, and microscopic characteristics (spore and hyphal morphologies). Tissues sections were prepared for non-fleshy mushrooms to measure the dimensions of spores. Macrofungi identification was carried out depending on their taxonomy using taxonomic references (Hall et al. 2003; Chang & Miles 2007; Hibbett et al. 2007).

Spore fingerprint: The spore print color is the foremost imperative character for delimiting the families. The fruiting body was sliced at the location of the stipe combine with the cap at that point found on a white paper whereas covered at that point cleared out for 23 h (Bazzle et al. 2015).

Molecular identification

DNA extraction of mushrooms from culture: The DNA extraction was done based on Manjunathan et al. (2011), using a sterile toothpick method. The DNA precipitates were diluted in 50µl of TE buffer and preserved at -20°C until used. DNA bands were detected and examined under UV transilluminator.

Polymerase Chain Reaction (PCR): The amplification processes of the internal transcribed spacer (ITS 1-5.8S-ITS 2) region for isolates were carried out according to Mirhendi et al. (2006) and two universal primers were used (ITS1 F-5-TCCGTAGGTGAACCTGCGG-3 and ITS4 R-5-TCCTCCGCTTATTGATATGC-3) for amplification. While, the extension of desired DNA was achieved by a mixture of master mix (Promega) 25µl, DNA template 4µl, forward and reverse primers 2µl for each one, and the volume was completed to

50µl with nuclease-free water. PCR reactions were performed in a thermocycler and the process took two hours according to the following setups: 5min at 94°C as the first step of denaturation, followed by 35 rhythms involving a denaturation step at 94°C for 30sec, an annealing step at 56°C for 45sec, and extension stage at 72°C for 1min, and final extension step at 72°C for 7min. After the end of the amplification period, 5.5µl of each PCR product for all isolates were electrophoresed in 2% agarose gel that was performed in TBE buffer and stained with ethidium bromide, 3.5µl of molecular marker (100-2000bp) was added to 1st well, then the product was detected and examined by illumination with UV light.

Results and Discussion

During the study periods, the various wild mushroom was reported belonging to four genera of *Agaricus*, *Coprinopsis*, *Panaeolus*, and *Psathyrella.* Temperatures and rainy seasons are the most common factors that limit mushroom growth. When the year is characterized by abundant rain and frequent humidity, many types of wild mushrooms, particularly those belonging to the genus *Agaricus*, thrive. *Panaeulous* and *Psathyrella* species prefer warm, moderate humidity conditions of 30°C for growth and development. As a result, *Agaricus* spp. accounted for the highest percentage of species collected during the winter. *Panaeulous* spp. and *Psathyrella* spp. are among the species collected during non-rainy seasons. Obviously, the winter of 2018 in Iraq was characterized by a lack of rain, resulting in a paucity of mushroom samples obtained throughout the study period. This explains the reduction in the annual product of *Agaricus* spp. in comparison with foregoing heavy rainfall years. Furthermore, samples of wild mushrooms were collected in different habitats in Basrah, some of them were brought from general and home gardens, in addition to their occurrence in agriculture fields, while others were associated with trees as a mycorrhizal relationship.

Classification and identification of mushrooms are established on morphological characteristics of the fruiting body. Different species of wild mushrooms were subjected to molecular identification by utilizing amplification of ITS1-5.8S-ITS2 rDNA region by universal primers ITS1 and ITS4 for discrimination and identification of DNA extraction. The DNA yields were shown high purity and quantities to be ready for amplification and suitable for sequencing. The ITS region is rather useful for molecular characterization in fungi at the species level and within the species; this portion has become an important molecular target for taxonomy and identification because it has been a greater variety of nucleotides succession (Raja et al. 2017b).

Figure 1 shows DNA bands of mushroom samples to confirm the size of the gene was approximate700-1000bp. Mushrooms species were compared to determine similarity with other reliable sequences deposited in the gene bank, a part of National Center of Biotechnology Information (NCBI) and European Nucleotide Archive (ENA). The sequencing of ITS1 - 5.8S - ITS2 rDNA with the program BLAST revealed five species dissimilar from their reference strains in several positions of nucleotide sequences so that it was recorded as new isolates for the first time. The size of these non-coding regions evolves more rapidly and their lengths rely on the species, while the lengths of coding genes are identical in all fungal species with slow development. Therefore, the present study was dependent on this part to identify mushrooms as in many foregoing studies (Lee et al. 2006; Leon et al. 2013; Appiah et al. 2017; Raja et al. 2017b).

The novel strains reported and published in NCBI included two strains of *A. bisporus* (*A. bisporus* IQ Inaam1, which had 99% similarity with the sequences of its reference strains and *A. bisporus* IQ Nasir. In addition, two strains of *Panaeolus papilionaceus* were identified, represented *P. papilionaceus* INNASA1. This strain was associated with its reference strain by 97% and

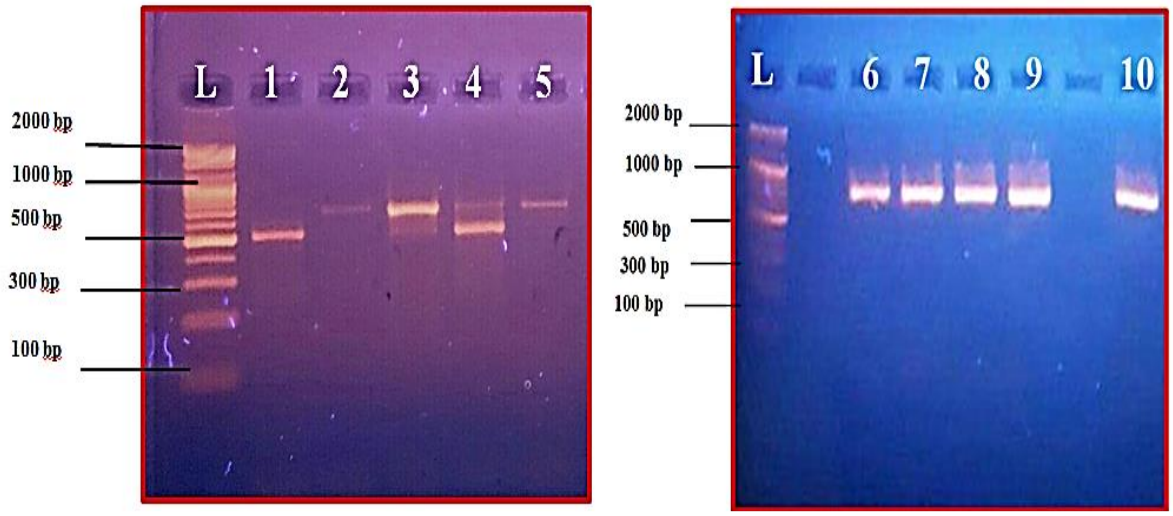

Fig. 1. 2% agarose gel electrophoresis analysis for PCR assay with two primers ITS1, ITS4 L= (100 bp) DNA marker. Lanes: 1-10 bands of amplification gene of mushrooms species, the size of gene was approximately 500-700bp.

Fig. 2. Wild *Agaricus bisporus*: Fruiting bodies, and B: spores (100x).

P. papilionaceus IQ Zeena with 99% similarity, and one isolate of *Psathyrella candolleana* was recorded as a new globally (*P. candolleana* IQ M. Jawad) with a similarity of 99% with their reference strains.

The present study was in agreement with Rajaratnam & Thiagarajan (2012) who obtained genomic DNA from fruit bodies of wild mushrooms using ITS1 and ITS4 with the aligned sequence (559bp) had 88% similarities with *Perenniporia* sp. (GQ982890.1). Whereas, Dung et al. (2012) described 6 Oyster mushroom samples using morphological and molecular data. The present study revealed that 97-99% similarities of mushroom isolates with their related type strains deposited in GenBank. They were recorded as new glob isolates due to their difference with reference isolate as a result of mutations in nucleotide sequence.

Classification and Description of collected samples

Genus: *Agaricus* (2 species)

Agaricus bisporus strain IQ Inaam1

The sample was collected as dry in July-2018 from agricultural areas in Basrah Province.

Macroscopic features: The cap is dry, smooth, and yellowish, 4-15cm in diameter, and convex to flat. The stipe is 3-11cm long, cylindrical to clavate, enlarged at the base, brown, with a membranous veil

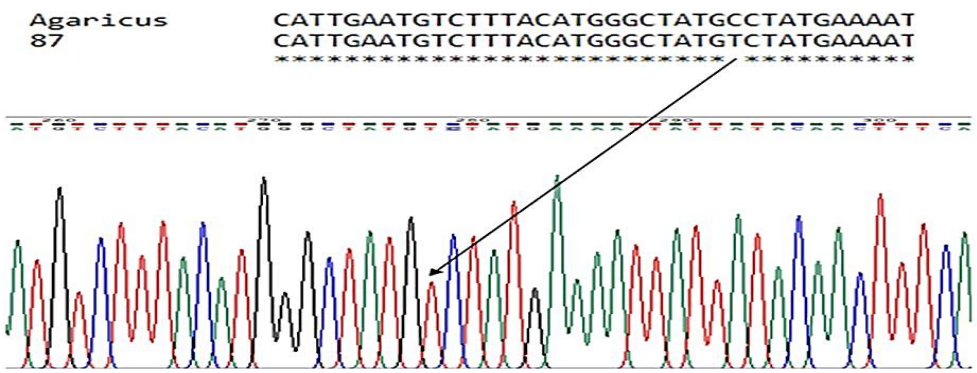

Fig. 3. Comparison between the new strain *Agaricus bisporus* IQ Inaam1 (with peaks) and its reference isolate CBS 505.73. Gene or point mutation type transversion (T instead of C) at the position 279bp.

Fig. 4. Fruiting bodies of wild *Agaricus bisporus.*

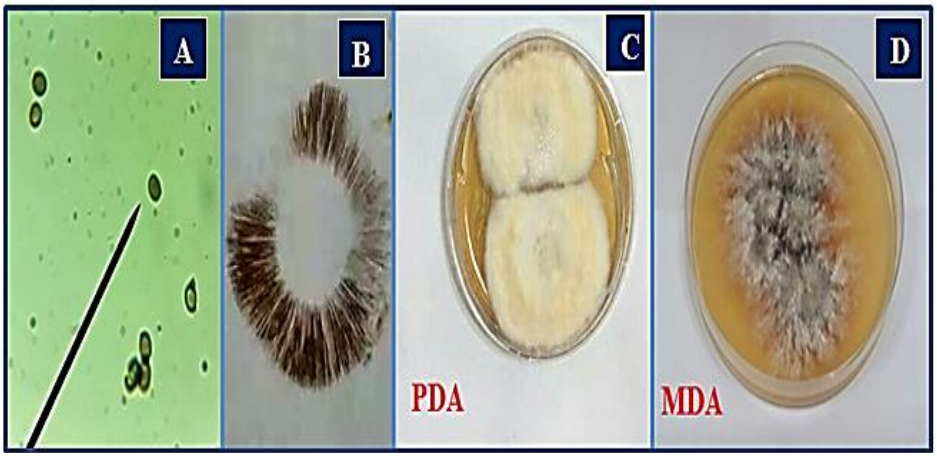

Fig. 5. Wild *Agaricus bisporus*, A: Spores (100x), B: Spore print, and C, D: colony growth on PDA and MDA.

and thick white mycelial sheathing near the base. The flesh is solid and firm, the gills are free, very narrow, close, dark reddish-brown. While, the cap appeared smooth, white in the young fruiting body, but staining yellowish in age, often with dirt on the cap (Fig. 2).

Microscopic features: Size of spores are about 3-5 x 2-4µm ellipsoid, smooth, thick-walled, brownish (Fig. 2B). Spores color are light green when young, becoming dark reddish-brown when reaching maturation. Wild *A. bisporus* IQ Inaam1 revealed morphological similarities to other strains of

Fig.6. Comparison between the new isolate *Agaricus bisporus* IQ Nasir (with peaks) and its reference isolate CBS 505.73. Gene or point mutation type transversion (C instead of T) at the position 278bp.

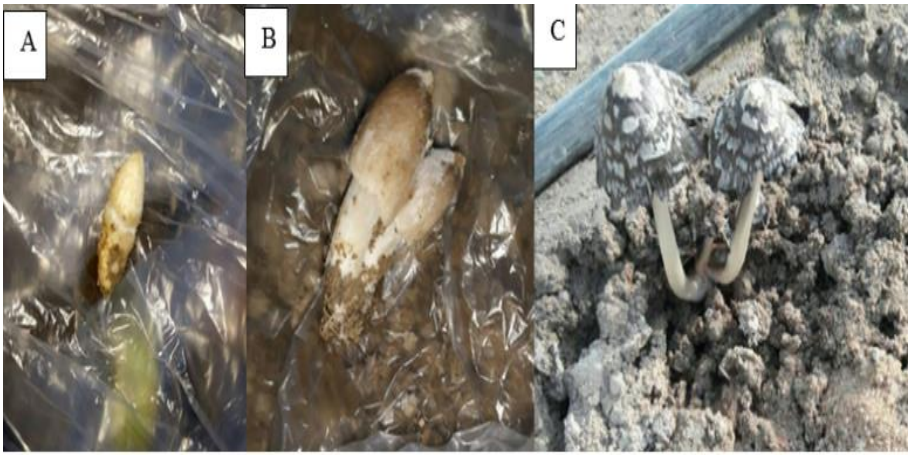

Fig. 7. Fruiting bodies of wild *Agaricus bitorquis*, gills (A), two ring (B) and cup (C).

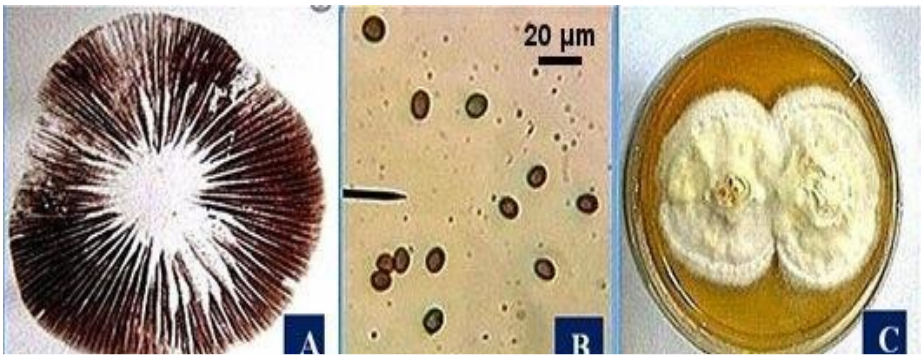

Fig. 8. Wild *Agaricus bitorquis.* A: Spore print, B: Spores (100x), C: colonies of *Agaricus bitorquis* on PD.

A. bisporus CBS 505.73. Whereas the molecular identification by sequencing ITS1-5.8S-ITS2 region and compared that by BLAST with sequences of *A. bisporus* isolate CBS 505.73 which available in NCBI 99% similarity and difference in one nucleotide at the position 279bp as a result of point mutation type transversion (Fig. 3).

Agaricus bisporus strain IQ Nasir

Macroscopic features: The cap is 3-14cm, white,

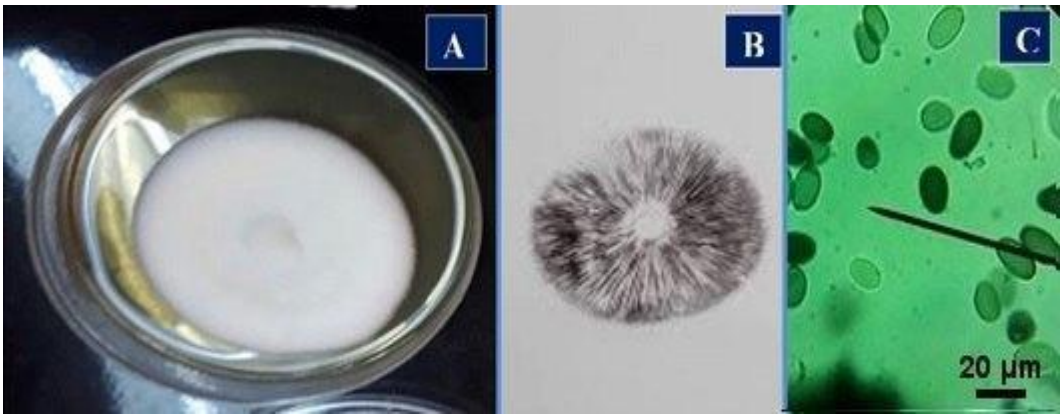

Fig.9. *Coprinopsis picaceus*. A: Colonies on PDA, B: Spore print and C: Spores (100x).

Fig.10. Fruiting bodies of *Panaeolus campanulatus*.

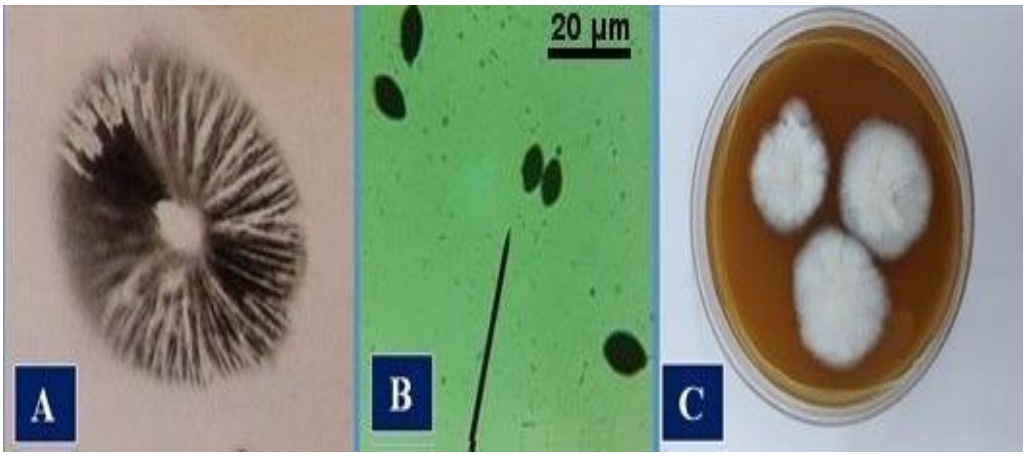

Fig.11. *Panaeolus campanulatus*. A: Fruiting bodies, B: spores (100x) and C: Colonies on MDA.

spherical with rounded shape at first, expanding to broadly convex or nearly flat, dry bald or with pressed-down fibers or small scales, brown. Gills are free, narrow, brown, while stipe is long, cylindrical, white, and has no ring. Scales and cup not found (Fig. 4).

Microscopic features: Spores 2–3 x 4–6µm, ellipsoid, smooth, thick-walled, and brownish (Fig. 5A).

Growth on culture media: The growth pattern of wild *A. bisporus* on PDA and MDA appeared as white colonies with a diameter of 6-9 cm after 7 days

Fig. 12. *Panaeolus papilionaceus* in nature.

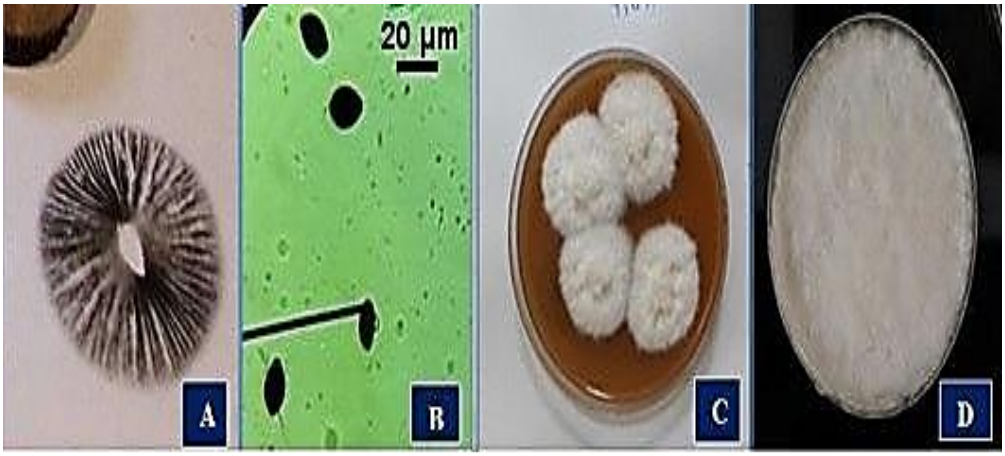

Fig.13. *Panaeolus papilionaceus.* A: Spore print, B: Spores, C and D: Colonies on MDA and PDA respectively.

of incubation at 25-28˚C. The slide culture of these structures revealed filamentous organization without any distinguished structure (Fig. 5C, D).

Wiled *A. bisporus* IQ Nasir was revealed morphological similarity with other isolates of **A.** *bisporus* CBS 505.73, but the molecular identification by sequencing the ITS1-5.8S-ITS2 region and compared that by BLAST with sequences of *A. bisporus* isolate CBS 505.73which available in NCBI was showed similarity with 99% and difference in one nucleotide at the position 278bp. as a result of point mutation type transversion (Fig. 6).

***Agaricus bitorquis* (Quel) Sacc.**

Macroscopic features: The cap is dry, smooth, white, and convex to flat; the gills are free, narrow, dark reddish-brown, stipe with 3-11cm long, cylindrical to clavate, white, without ring. Cup is adnate while scales are absent (Fig. 7, 8A).

Microscopic features: Spores 5-11 x 2-4µm, ellipsoid, smooth, thick-walled, and brownish (Fig. 8B).

Growth on culture media: The growth pattern of *A. bitorquis* on PDA and MDA showed white colonies with a diameter of 4-6 cm after 10 days of incubation at 25-28˚C. The slide culture of these structures has appearance of filamentous organization without any distinguished structure (Fig. 8C).

Genus: *Coprinopsis*

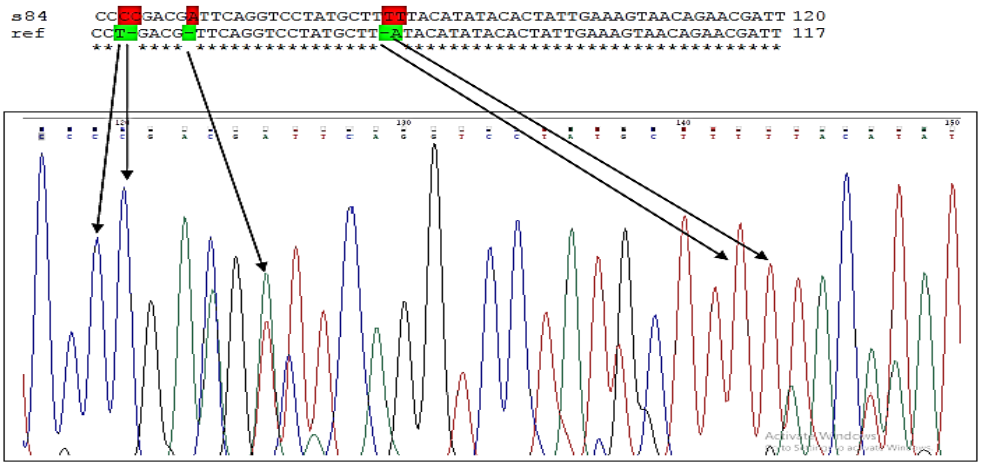

Fig.14. Comparison between the new strain *Panaeolus papilionaceus* INNASA1 (peaks) and its reference strain NAMA 2017-161. Gene or point mutations type transversion (C and T instead of T and A) at the position 67 and 89. Frame shift mutation (Insertion C, A and T) at the position 68, 72 and 88bp.

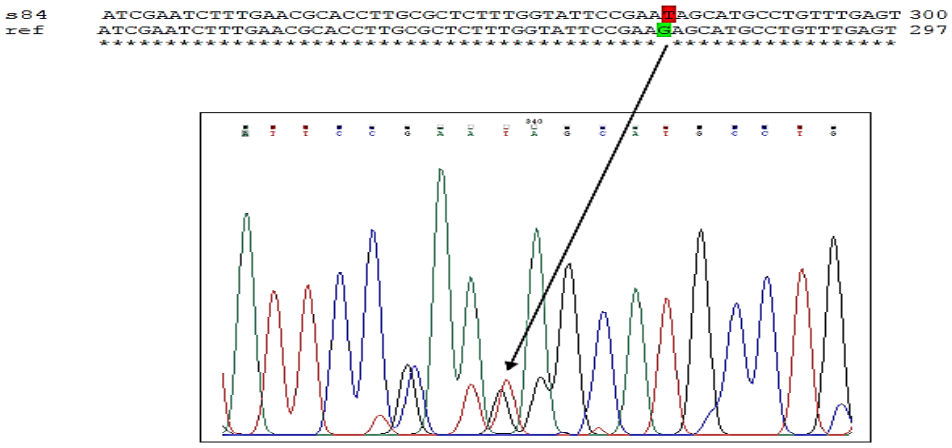

Fig.15. Comparison between the new strain *Panaeolus papilionaceus* INNASA1 (peaks) and its reference strain NAMA 2017-161. Gene or point mutations type transversion (T instead of G) at the position 283bp.

***Coprinopsis picaceus* (Bull.) Redhead, Vilgalys &Moncaluo**

Macroscopic features: The cap is dry, conical and bright brown; gills are free, narrow, and black. The stipe is cylindrical to clavate, pale brown, and has no ring. Scales are smooth with unavailability of cups (Fig. 9, 10).

Microscopic features: Spores 14.0-18.8 x 9.6-13.0µm, ellipsoid or ovoid, rounded at apex, very dark brown, almost black smooth, and thick-walled (Fig. 9C).

Growth on culture media: White appearance colonies on PDA and MDA with a diameter of 7-4 cm following 10 days of cultivation. The optimum growth of mycelia is shown in PDA higher than MDA at 28˚C for one week (Fig. 9A).

Genus: *Panaeolus*

Panaeolus campanulatus

Macroscopic features: The cap is dry, spherical and brown. The gills are adnate, narrow, and black. The stipe appeared without ring, cylindrical and brown. Scales and cup are absent (Figs.11, 12A).

Microscopic features: Spores 7-11x 4-6µ, more or less elliptical, smooth, and black (Fig. 12B).

Growth on culture media: The growth pattern revealed white colonies with a diameter of 3-5cm

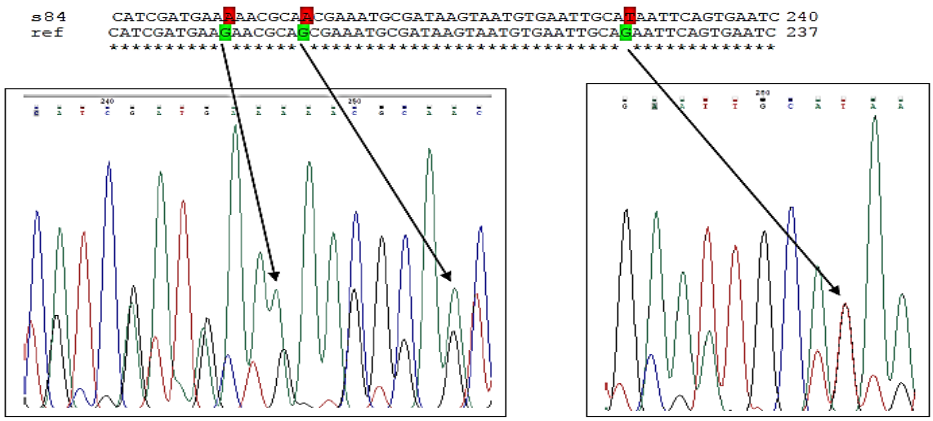

Fig.16. Comparison between the new strain *Panaeolus papilionaceus* INNASA1 (peaks) and its reference strain NAMA 2017-161. Gene or point mutations type transversion (A, A and T instead of G, G and G) at positions 179, 185 and 227bp.

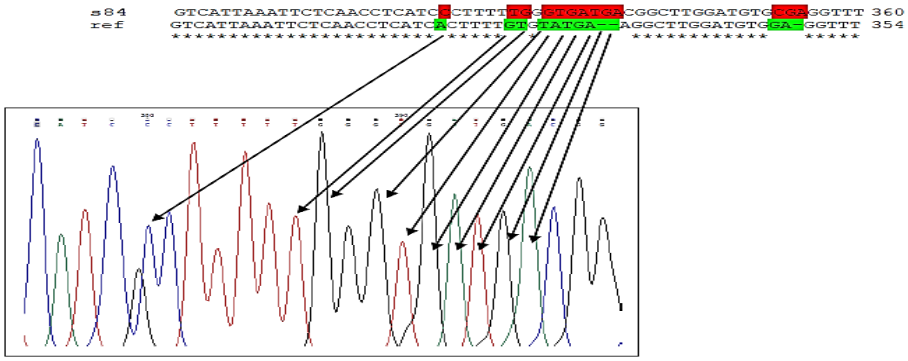

Fig.17. Comparison between the new strain *Panaeolus papilionaceus* INNASA1 (peaks) and its reference strain NAMA 2017-161. Gene or point mutations type transversion (C, T, G, G, T, G, A, T, C and G instead of A, G, T, T, A, T, G, A, G and A) at the position 329, 334, 335, 336, 337, 338, 353 and 354bp. Frame shift mutation (Insertion G, A and A) at positions 339, 340 and 355bp.

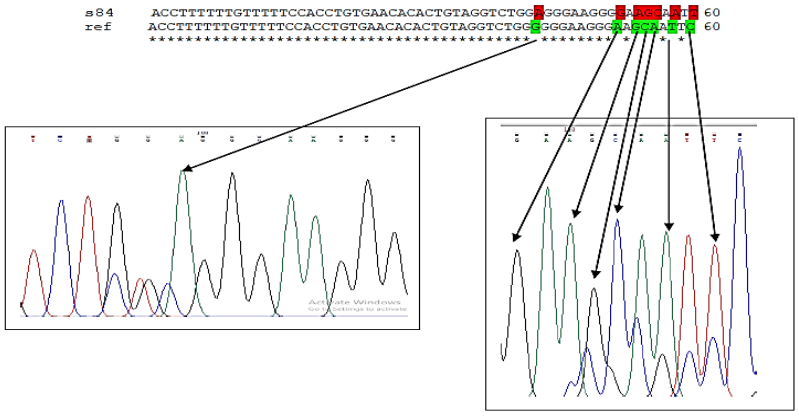

Fig.18. Comparison between the new strain *Panaeolus papilionaceus* INNASA1 (peaks) and its reference strain NAMA 2017-161. Gene or point mutations type transversion (A, G, A, G, C, A and T instead of G, A, G, C, A, T and C) at positions 41, 52, 54, 55, 56, 58 and 68bp.

after 10 days of incubation on PDA and MDA at 25-28˚C (Fig. 12C).

Panaeolus papilionaceus (Bulliard) Quelet

Panaeolus papilionaceus strain INNASA1

Macroscopic features: The cap is dry, bell-shaped, and bright grey. The gills are adnate, narrow, and

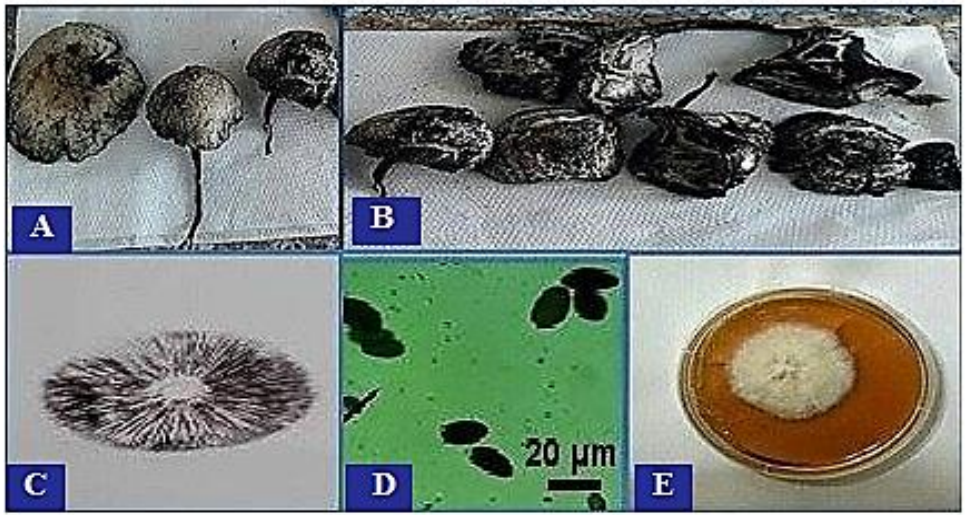

Fig.19. *Panaeolus papilionaceus*, A, B: Fruiting body, C: Spore print, D: Spores 100x and E: Colony on MDA.

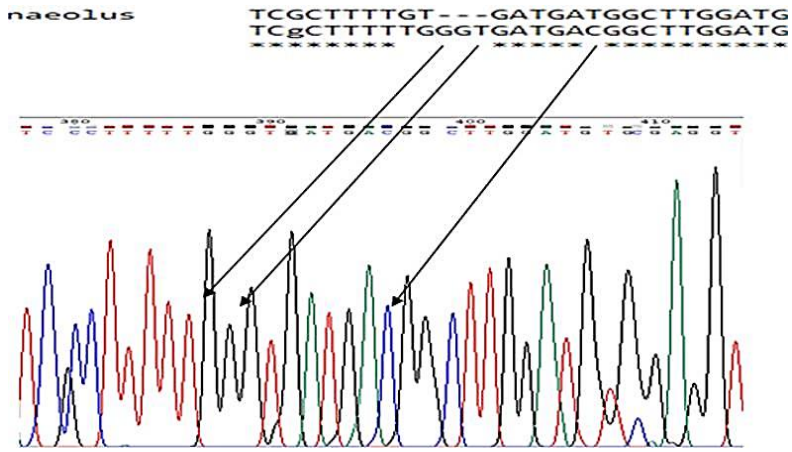

Fig.20. Comparison between the new strain *Panaeolus papilionaceus* IQ Zeena (peaks) and its reference strain RA400. Gene or point mutations type transversion (C instead of T) at the position 123. Frame shift mutation (Insertion G, G and T) at the position 115, 116 and 117bp.

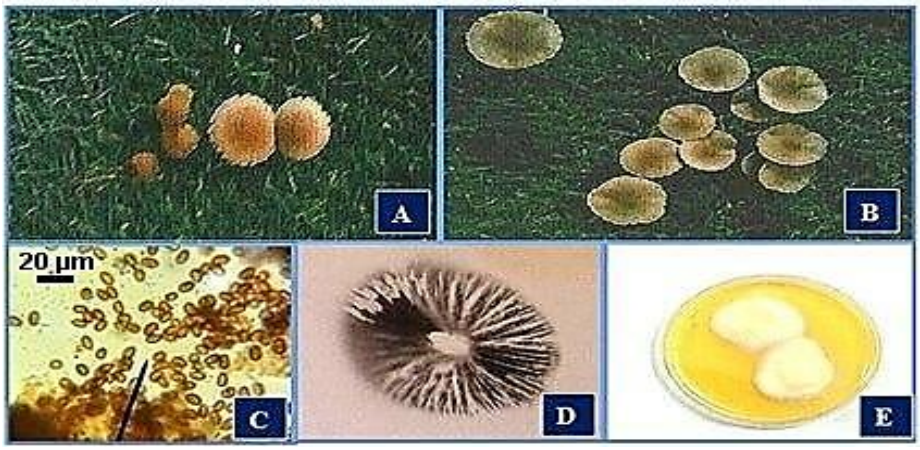

Fig.21. *Psathyrella candolleana.* A, B: Fruiting bodies, C: spores (100x) D: spore print, E: Colonies on PDA.

black. The stipe is 3-15cm long, cylindrical to clavate, pale brown, no ring. Scales and cup disappeared (Fig. 13).

Microscopic features: Spores 11-18.5 x 7.5-12µ, more or less elliptical, smooth, and black (Fig. 14B). **Growth on culture media:** White colonies on PDA

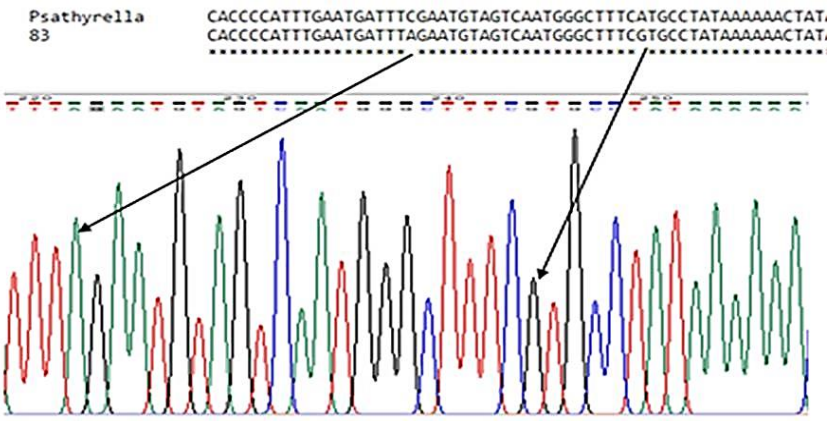

Fig.22. Comparison between the new strain *Psathyrella candolleana* IQ M. Jawad (with peaks) and its reference isolate WB5140, point mutation type (A and G instead of C and A) at the position 122 and 144bp.

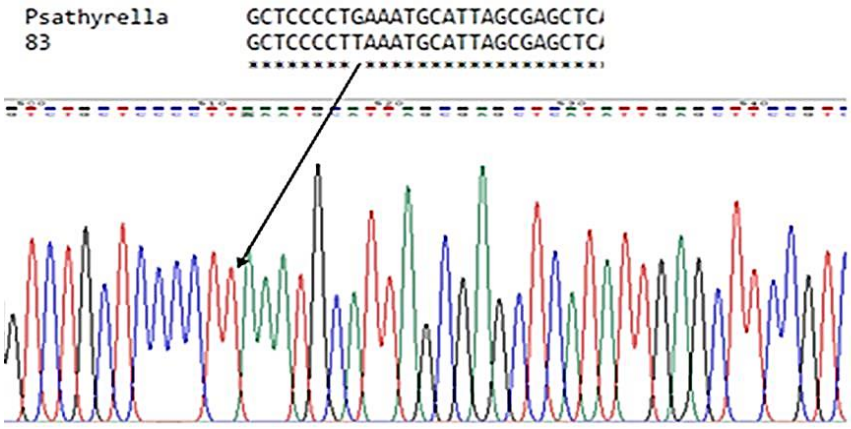

Fig.23. Comparison between the new strain *Psathyrella candolleana* IQ M. Jawad (with peaks) and its reference isolate WB5140, point mutation type transveration (T instead of G) at the position 511bp.

and MDA with a diameter of 4-9cm following 7 day incubation time at 25-28˚C (Fig.14C, D).

Sequencing of ITS1-5.8S-ITS2 for *P. papilionaceus* INNASA1 revealed similarity with their reference strain NAMA 2017-161 as 97% with a difference in some nucleotide due to gene or point mutations type transversion (C and T instead of T and A) at the position of 67 and 89bp. Frameshift mutation (Insertion C, A and T) at position 68, 72 and 88bp (Fig. 15).

Furthermore, at position 283bp, gene or point mutations type transversion (T instead of G) has occurred (Fig. 16), in addition at positions of 179, 185, and 227bp. There is point mutations type transversion (A, A, and T instead of G, G, and G) (Fig. 17). Comparison between the new strain *P. papilionaceus* (peaks) and its reference strain NAMA 2017-161 was showed gene or point mutations type transversion (C, T, G, G, T, G, A, T, C and G instead of A, G, T, T, A, T, G, A, G, and A) at the position 329, 334, 335, 336, 337, 338, 353, 354 and 360bp. Frameshift mutation (Insertion G, A, and A) at positions 339, 340, and 355bp. (Fig. 18). The new strain *P. papilionaceus* differs from its reference strain NAMA 2017-161 in six nucleotides due to gene or point mutations type transversion (A, G, A, G, C, A and T instead of G, A, G, C, A, T and C) at the position 41, 52, 54, 55, 56, 58 and 68bp. (Fig. 19).

Panaeolus papilionaceus strain IQ Zeena

Macroscopic features: The cap is dry, spherical, and white; gills are adnate, narrow, and black. The stipe

with long 3-11cm, cylindrical, pale, while, and ring; scales and cup are lacking (Fig. 20A, B, C).

Microscopic features: Spores appeared smooth, black, and ellipsoidal and pierced with dimensions 11-18.5 x 7.5-12µm (Fig. 20D).

Growth on culture media: The growth pattern on PDA and MDA after incubation of 10 days showed white colonies with a diameter of 3-6cm (Fig. 20E). Sequencing of ITS1-5.8S-ITS2 for *P. papilionaceus* strain IQ Zeena revealed similarity with their reference isolate RA400 by 99% with the difference in some nucleotide due to gene or point mutations type transversion (C instead of T) at the position 123. Frameshift mutation (Insertion G, G, and T) at positions 115, 116, and 117bp. (Fig. 21).

Genus: *Psathyrella*

Psathyrella candolleana strain IQ M. Jawad

Macroscopic features: The cap is dry, spherical and white; with crenate margins. The gills are adnate, narrow, and black. The stipe is cylindrical to clavate, pale, and without a ring. Scales and cups are unavailable (Fig. 22 A, B, D).

Microscopic features: Spores appeared ellipsoidal, smooth, thick-walled, and yellow to brownish with size 6-9 x 2-4µm (Fig. 22C).

Growth on culture media: Colonies of *P. candolleana* displayed a white appearance on PDA and MDA with a diameter of 3 - 5 followed by 7 days of cultivation (Fig. 22E). Sequencing of ITS1-5.8S-ITS2 for *P. candolleana* IQ M. Jawad revealed similarity with their reference strain WB5140 by 99% with the difference in three nucleotides as a result of a point mutation at the position 122, 244, and 511bp. (Figs. 23, 24).

Conclusions

The current study reveals that the isolation of new strains of mushrooms that were recorded for the first time in Iraq due to changes in nucleotides bases may lead to a variety of environmental factors.

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