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Research Article

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Molecular identification and genetic diversity study of the Iraqi truffles

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ABSTRACT

The aim of this study is to investigate the molecular identification of the Iraqi truffles species and a better understanding of genetic diversity in the center of the truffles habitat. Thirty-two samples were collected from the Iraqi desert and local markets. Samples were chosen depending on the morphological diversity of the fruit body and sample collection area. Results of ITS region sequencing for the 32 samples showed two genuses *Tirmania* and *Terfezia* are the main dominant, 4 species of *Tirmania pinoyi* and 28 species of *Terfezia claveryi*. All species sequences were deposited in NCBI GenBank and all had accessions number. The neighbor-Joining method was used to generate a phylogenic tree to study the genetic diversity of the ITS sequences for the 32 Iraqi truffle samples. Results showed a high genetic diversity for the Iraqi truffles samples. The phylogenic study showed Iraqi truffles clustered with different groups as a clade with the reference sequences from other countries represent three continents Asia, Africa, and Europe. Also, we found in this study a unique cluster group for the Iraqi sequences for *T. pinoyi* and *T. claveryi* truffles cluster in one group and do not match with any reference sequences used in this study. This is a piece of strong evidence proofed the Iraqi habitat could be the origin of center diversity for the *T. pinoyi* and *T. claveryi* truffles.

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INTRODUCTION

Historically, the desert truffles have been mentioned in the old ancient manuscripts and Egyptian temples in the Arabian region, also known as a "poor native food as alternative to meat", in Iraq its call kamaa, kima or chima, depending on local dialects [1]. Truffles known as hypogeous ascocarps "grow underground" related Eukaryota; Fungi; Dikarya; Ascomycota; Pezizomycotina; Pezizomycetes; Pezizales; Pezizaceae. Predominantly grow in the desert and live in ectomycorrhizal association with *Helianthemum* species locally name "Jaraida" [2]. The largest portion of truffles is locally consume, it's have a unique medicinal and organoleptic properties as well as their protein content (20-27%), and fiber (7-13%), and fat (3-7.5%), and ascorbic acid (2-5%), and also minerals, and 65-67% carbohydrate [3-5]. It has been reported that *Terfezia claveryi* and *Tirmania nivea* occur in deserts of salty and/or gypseous soils [6].

The first scientific reference reported a truffle in Iraq was in 1892 for both *Terfezia hafizi* the white and *Terfezia metaxasias* bIack truffles, made by Chatin [7]. Malencon [8] reported *T. claveryi* in Iraq. Other study description three species of truffles in Iraq *T. claveryi*, *Tirmania pinoyi* and *T. nivea* [9]. Abdullah et al [10] reported five hypogeous ascomycotina in Iraq *Terefezia boudieri*, *T. claveryi*, *Tirmania nivea*, *T. pinoyi* and *Phaeangium lefebvrei*. In study done by Owaid [11] found two genera of truffles *Terefezia* and *Tirmania* depend on the morphological characteristics. In Sulaymaniyah governorate north of Iraq, a study showed only two truffle species *T. claveryi* and *T. boudieri* identified using morphological classification [12](Al-jaff et al, 2018).

All above studies rely on the morphological characteristics to identify truffles. The truffles known to have a wide morphological diversity in color, size and shapes depend on soil type and environment where its grow in the wild, truffle development with fall–winter rainfalls and start to harvest in early spring. The dessert truffles are well distributed around the world, it have been reported in arid and semiarid zones area in different countries, like Iraq and Kuwait, Iran, the Sahara regions of Saudi Arabia and parts of the Magreb [13], Tunisia [14] Algeria[15]. Also in Europe like Hungary and Yugoslavia [16,17], and China [18], the Kalahari Desert [13,19,20], Australia [21,22], and North America [23,24]. Sbissi et al [14] studied genetic diversity of *T. boudieri* and *T. claveryi* in Tunisia by using PCR–RFLP technique, and found three haplotype (I, II, and III), the haplotype I was founded in a low pH soil association with *Helianthemum kahiricum*. The species from *Tirmania* and *Terfezia* is known as mycorrhizal associated with genus

Helianthemum [25]. A genetic diversity of desert truffles study showed several morphological species by using PCR-RFLP and DNA sequences of (ITS), *Terfezia arenaria*, *T. boudieri*, *T. claveryi*, *T. leptoderma*, *T. terfezioides* (=*Mattirolomyces terfezioides*), *Tirmania nivea* and *T. pinoyi*, the phylogenetic analyses showed that both genus *Tirmania* and *Terfezia* are genetically closely to each other [26].

The proposed of this study aims to describe the molecular identification of truffle species and distribute in the Iraqi habitat, and understanding the genetic diversity among truffle species in the bioclimatic.

MATERIAL AND METHODS

Sample Collection

Samples of truffles were collected from the local markets and desert land known with a natural grow habitat for truffles. Samples collected from these following cities: Alnakhab, Rhatbua, Kahlar, Falluja, Hadeetha, Ana, and Rawa Figure 1. All samples were brought to the plant disease laboratory in the Department of Plant Protection, University of Baghdad. The samples were washed and cleaned with tap water to remove soil. The samples were collected and selected randomly depending on the diversity of color, shapes, and size. All samples were documented with record information of location, collection date, and color. Pictures were taken for the exterior and interior features for the truffle's fruit body. Five grams from each sample were collected with clean knife from the core of the fruit body and kept in -20 °C in plastic tubes for use in the DNA extraction.

DNA Extraction

Five-gram of tissue truffles fruit body samples was mixed in liquid nitrogen in a ceramic mortar and grounded with a pestle to a powder. DNA extraction kit was used, EZ-10 spin column fungal genomic DNA (Bioneer Corporation, South Korea). The company protocol for DNA extraction was followed. The final DNA products were kept at -20 °C to do further test.

PCR Assay

The internal transcribed spacer region of ribosomal DNA (ITS) was amplified by using ITS primer ITS1-F (TCC GTA GGT GAA CCT GCG G) and ITS4-R (TCC TCC GCT TAT TGA TAT GC) [27] (Bioneer Co, Korea). Total 20 µL volume of PCR reaction mix were amplified using a thermo-cycler model (My™ Genie 32 Thermal Block- Bioneer, South Korea). The PCR reaction was performed in 20 total volumes consisting of 5 µL of PCR PreMix (Bioneer Corporation, South Korea), and 5 µL of DNA (50 ng) template, and 3µL of primer ITS1-F, and 3 µL ITS4-R, and 4µL of PCR demonized water. The following program settings were used: initial denaturation (95°C, 2 min), 35 cycles of denaturation 94°C for 30s, annealing (55°C, 1 min) and extension (72°C, 1 min), final extension phase was performed at 72°C during 10 min.

The PCR final products were loaded in a 1.5% agarose gel with Ethidium- bromide dye. DNA marker 100 bp was used (Bioneer Co., South Korea). Samples of 2µL mix with 2µL of 6X loading buffer and loaded in to the gel. Electrophoresis (model Bioneer Co., South Korea) was run at 80 V for 2 h. The DNA bands were visualized and photographed using special UV camera (model AE-9000 E-Graph Atto-Jaban).

DNA Sequencing and Data Analysis

The PCR products were purified using the AccuPrep® PCR Purification Kit (Bioneer Co., South Korea) and sequenced commercially by facility at Bioneer Co., Korea. All sequenced data were blasted on NCBI nucleotide blast GenBank (www.blast.ncbi.nlm.nih.gov) [28] to reviewing the chromatograms, and any similarities above 98% from the data base were accepted to identify the species. All the sequences data were deposited in the NCBI gene bank and get accessions number. For the genetic diversity study, MAGA 6.06 [29] program was used to generate the phylogenic tree figure by using the ITS region sequence samples.

RESULTS AND DISCUSSION

Morphology and Molecular Characterization

All truffle samples collected in this study showed a wide diversity of morphology characteristics as in color gradients of the fruit body, and core texture and pattern (Figure 2). These characteristics are one of main criteria to the morphology classification for truffles species [31]. Sometime of these characteristic especially fruit body color can be changeable depends on temperature, moisture and the soil chemical and physical properties. The diversity on color and texture pattern for the fruit body can be very tricky even to the specialists to classify the species of the truffles. Also we found the truffles ascocarps inseparable with *Helianthemum spp* plants Figure 3. Fortas and Chevalier [25] report that species of *Tirmania* and *Terfezia* is known as mycorrhizal associated with genus *Helianthemum*.

Sequencing results of the ITS region, final alignment were 615 and 650 bp, confirmed the identity of samples to be among the *Tirmania pinoyi* and *Terfezia claveryi* species group (all identities >98% sequence similarity). Samples sequences were blasted in the NCBI GenBank to compare the alignment with the GenBank sequences. The results showed that 4 samples were belong to *T. pinoyi* and 28 samples belong to *T. claveryi* from total 32 samples collected in this study. All the sequences were deposited in the NCBI GenBank and accession numbers were issued for all sequences start with MK478851 to MK910038 (Table 1). Many previous studies used the sequence of ITs region to identify truffles species [5,32].

This results disagree with previous study survey on truffles in Iraq done by Abdullah et al [10] who reported five hypogeous ascomycotina in Iraq *Terefezia boudieri*, *T.claveryi*, *Tirmania nivea*, *T.pinoyi* and *Phaeangium lefebvrei*. Climate change may

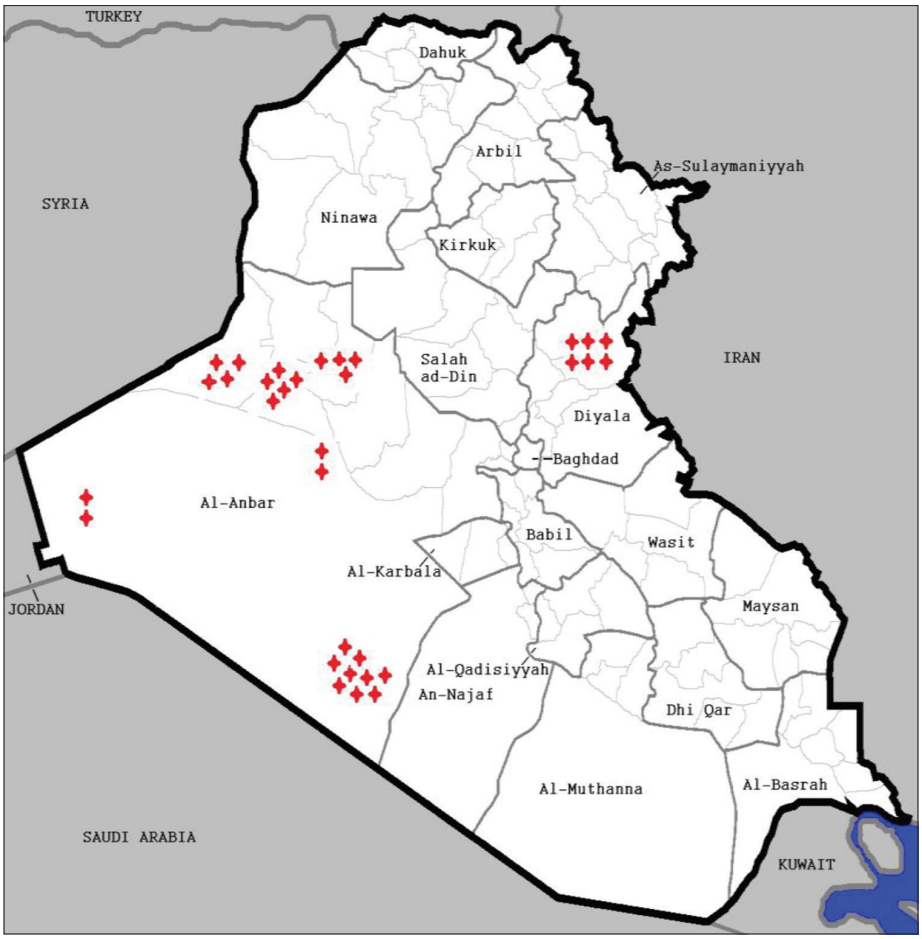

Figure 1: Sites of truffles sampling collected in this study from Iraq truffles habitats, every red star mean sample present

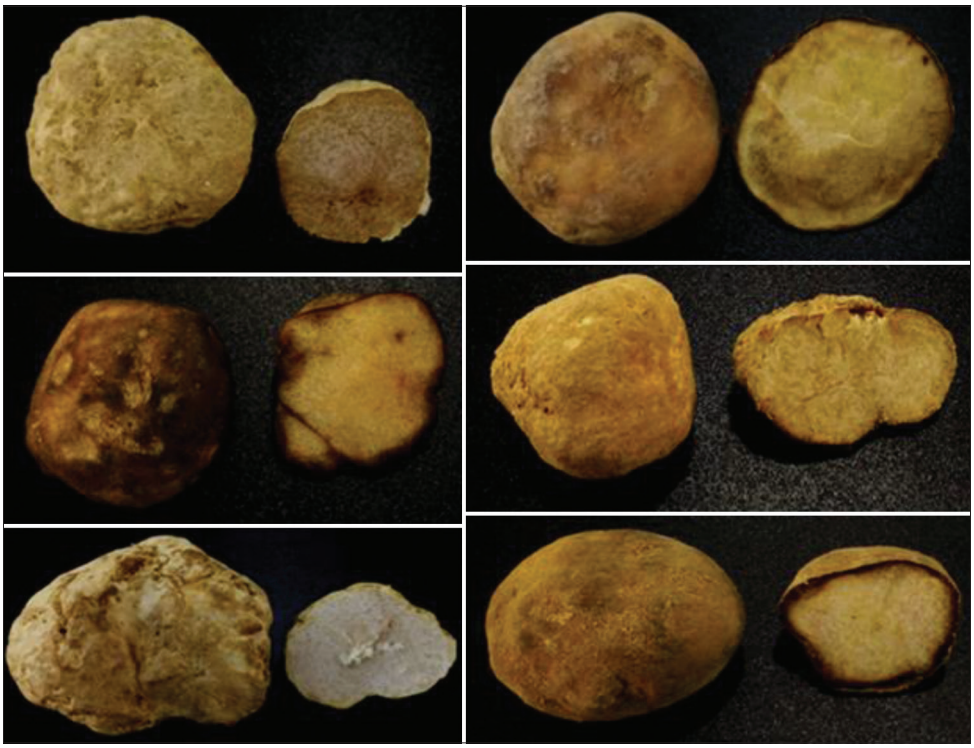

Figure 2: Truffles samples collected in this study show the morphological diversity in shape, core texture, and color

Table 1: Truffles species found in this study and some accession sequences download from NCBI GenBank use to generate phylogenic tree

Sample NO.	Location	Fruit body color	Species	Accession number
1	Iraq-Rawa	White-light brown	*Tirmania pinoyi*	MK478851
2	Iraq-Rawa	White-light brown	*T. pinoyi*	MK478852
3	Iraq-Rawa	Light-brown	*T. claveryi*	MK478853
4	Iraq-Rawa	Dark-brown	*T. claveryi*	MK478854
5	Iraq-Alnakhab	Dark-brown	*T. claveryi*	MK478855
6	Iraq-Alnakhab	Dark-brown	*T. claveryi*	MK478856
7	Iraq-Alnakhab	Dark-brown	*T. claveryi*	MK478857
8	Iraq-Alnakhab	Dark-brown	*T. claveryi*	MK478858
9	Iraq-Alnakhab	Dark-brown	*T. claveryi*	MK478859
10	Iraq-Alnakhab	Dark-brown	*T. claveryi*	MK478860
11	Iraq-Alnakhab	Dark-brown	*T. claveryi*	MK478861
12	Iraq-Alnakhab	Dark-brown	*T. claveryi*	MK478862
13	Iraq-Alnakhab	White-light brown	*T. pinoyi*	MK478863
14	Iraq-Alnakhab	White-light brown	*T. pinoyi*	MK478864
15	Iraq-Ana	White-light brown	*T. claveryi*	MK880339
16	Iraq-Ana	White-light brown	*T. claveryi*	MK880340
17	Iraq-Ana	White-light brown	*T. claveryi*	MK880341
18	Iraq-Ana	White-light brown	*T. claveryi*	MK880342
19	Iraq-Ana	White-light brown	*T. claveryi*	MK880343
20	Iraq-Hadeetha	White-light brown	*T. claveryi*	MK880344
21	Iraq-Hadeetha	White-light brown	*T. claveryi*	MK880345
22	Iraq-Hadeetha	White-light brown	*T. claveryi*	MK880346
23	Iraq-Alrhatbua	White-light brown	*T. claveryi*	MK880347
24	Iraq-Alrhatbua	White-light brown	*T. claveryi*	MK880348
25	Iraq-Fallouja	White-light brown	*T. claveryi*	MK880349
26	Iraq-Fallouja	White-light brown	*T. claveryi*	MK880350
27	Iraq-Kahlar	Dark-brown	*T. claveryi*	MK910033
28	Iraq-Kahlar	Dark-brown	*T. claveryi*	MK910034
29	Iraq-Kahlar	Dark-brown	*T. claveryi*	MK910035
30	Iraq-Kahlar	Dark-brown	*T. claveryi*	MK910036
31	Iraq-Kahlar	Dark-brown	*T. claveryi*	MK910037
32	Iraq-Kahlar	Dark-brown	*T. claveryi*	MK910038
Reference sequences				
33	Iran	_	*T. pinoyi*	MH084953
34	Iran	_	*T. pinoyi*	GQ228094
35	Spain	_	*T. pinoyi*	MG917773
36	Slovenia	_	*T. pinoyi*	FN395012
37	France	_	*T. pinoyi*	AF276669
38	Hungary-	_	*T. claveryi*	HQ698074
39	Spain	_	*T. claveryi*	AF387647
40	Hungary	_	*T. claveryi*	HQ698071
41	Spain	_	*T. claveryi*	MN326673
42	Iran	_	*T. claveryi*	GQ888693
43	Iran	_	*T. claveryi*	EU519461
44	Tunisia	_	*T. claveryi*	GU474801
45	Tunisia-	_	*T. claveryi*	GU474804
46	Southern African	_	*T. claveryi*	AF301421
47	Algeria	_	*T. claveryi*	MF940189
48	Algeria	_	*T. claveryi*	MF940197
49	Hungary	_	*T. claveryi*	HQ698076
50	Hungary	_	*T. claveryi*	HQ698072
51	Spain	_	*T. claveryi*	MN326672
52	Spain	_	*T. claveryi*	MN326671
53	Hungary	_	*T. claveryi*	HQ698086
54	France	_	*T. claveryi*	AF276670
55	France	_	*T. claveryi*	AF387645
56	Algeria	_	*T. claveryi*	MF940182
57	Algeria	_	*T. claveryi*	MF940186
58	Algeria	_	*T. claveryi*	MF940185
59	Iran	_	*T. claveryi*	GQ228093
60	Tunisia	_	*T. claveryi*	GU474805
61	Iran	_	*T. pinoyi*	GQ888695

Figure 3: Right picture represent swelling of the ground for *T. claveryi*, ascocarp. Left picture represent *T. pinoyi* ascocarp over soil surface. White arrow shows *Helianthemum spp* plants

be a big factor for disappearance for some species and change the microflora, or the molecular identification is more accurate compare to the morphological identification that depend on the morphology characteristics.

Genetic diversity study

The Neighbor-Joining method used in this study to show the genetic diversity of the truffles species collected from the different geographic area. The ITS region sequencing of 32 sequence were use in this analyses, also 29 random sequence downloaded from NCBI GenBank website used as reference sequence belong to *T. pinoyi* and *T. claveryi* species selected from different bioclimatic zone countries that considered as habitat for truffles. These sequences were used as a reference to generating the phylogenic tree using MEGA6 software [29,30].

The phylogenetic analysis result revealed that there is a two major groups A and B for the ITS sequences of the species in this study. Two subgroups under (B group) b1 and b2 Figure 4. Two *T. claveryi* isolate MK478854 and MK910038 were identified to be in group A cluster, isolate MK478854 were alimented is same group with sequences references from Algeria and Hungary as one cluster, and sample MK910038 was alimented with the Spain sequence reference. The isolate sequence MK880350, MK880348, MK880339, MK478860, MK478855, MK478857, MK478861, MK880347, MK880349, and MK910034 of *T. claveryi* were clustered as a clade under subgroup b2 and aliment with Spanish and Hungarian sequence references. While sample MK880345, MK910037, MK880340, MK478858, MK910036, MK478853, MK880341and MK880343 were clustered in a group under b2 cluster alimented with the Iranian and Tunisian sequence references. The samples MK880346 and MK910033 were very unique clade under the subgroup b1 were alimented with sequence references from four different countries Hungary, France, Iran, and South Africa belong to three continents Asia, Africa, and Europe. Samples sequences for MK478856, MK880342, MK910035, MK478862, MK880344, and MK478859 belong for *T. claveryi* species were separated in clade under subgroup b1 with no sequence references match to it Figure 2, that keeps these truffles isolates possible to be a unique races belong to Iraqi habitat. A unique cluster of four *T. pinoyi* sequences under subgroup b1 that not matching with

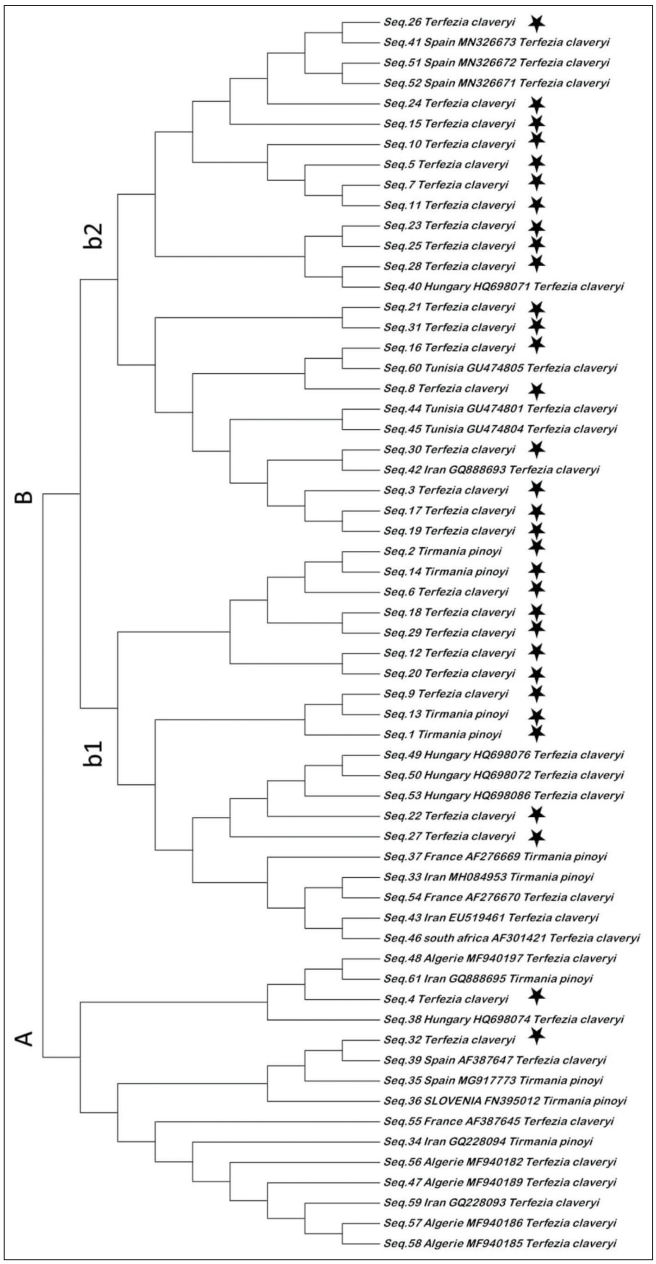

Figure 4: Neighbor-Joining phylogenic tree of 32 of Iraqi truffles samples using the ITS sequencing region and the reference sequences from NCBI GenBank

any reference sequences, which give a strong evidence to be possible unique a races, belong to the Iraqis habitat. Similar phylogenic study has been done by Bouzadi et al [5] in Libya, results showed all samples of Libyan truffle sequences were separated in a unique cluster, genetically far from the reference sequences, this approve a low genetic diversity for the individual separated glades. Also a previous study showed high genetic diversity for *T. claveryi* in Tunisia among the other species [14]. Diez et al [26] found a close genetic relationship between *Tirmania* and *Terfezia* by phylogenetic analyses method, a single evolutionary lineage maybe arose from pezizalean fungi that developed in the hypogeous habit that adapted to heat and drought in Mediterranean ecosystems.

CONCLUSIONS

The conclusion results from this study showed that Iraqi truffles have a wide genetic diversity compared to the global reference sequences obtained from the NCBI GenBank for this study. This study give an idea and strong evidence that Iraq truffles have a high genetic diversity for both species *T. claveryi* and *T. pinoyi*, also this study found a high intraspecific variation of both morphological characters compared to the rDNA ITS sequences. From a genetic perspective, this is the first study focus on molecular classification and genetic diversity for Iraqi truffles. This study opens the horizons for the scientist to do more deep research studies to understand the fitness of the center diversity of Iraqi truffles.

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