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Morphological And Molecular Identification Of *Leucoagaricus Leucothites* (Agaricales) From Salahadin Governorate, North Central Iraq

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Abstract

Leucoagaricus leucothites (Agaricaceae/ Agaricales) is identified based on morphological and molecular data from Tikrit University campus –Salahadin Governorate,north central Iraq.

Key words: *Leucoagaricus leucothites*, macromycota, molecular analysis

Introduction:

The genus *Leucuagaricus* (Locq. ex) Singer (Agaricales /Agaricaceae) includes 150-277species(1, 2.,3). These species are saprotrophic occurring on diverse habitats such as grassy areas, soil, wood chips, leaf litter, pasture, and gardens (4).And are widely distributed in the Northern and Southern hemispheres (5,2,6). Species of Leucoagaricus have white and fleshy basidiomata, free gills, central and bulbous stipe, presence of membranous annulus, basidiospores dextrinoid and mostly lack germ pores, white spore print, absence of clamp connections and pleurcystidia are rarely present (4, 5). Identification of Leucoagaricus specimens is difficult due to its close relationships with other lepioid genera like *leucocoprinus* (2). Previous molecular phylogenetic studies implied that both Leucoagaricus and *Leucocoprinus* form a monophyletic clade (7). Vellinga and Davis (2006)8 indicated the feasibility of treating the resolved monophyletic *Leucoagaricus*/ *Leucocoprinus* clade either as a single large genus or splitting it into smaller genera. These two genera were treated as separate genera by some researchers (9, 1). Due to limited molecular data, taxonomic and phylogenetic relationships of Leucoagaricus and *Leucocoprinus* are still unresolved. (10, 2). However, No information is available on the genus *Leucoagaricus* from Iraq. In this study, *Leucoagaricus leucothites* was identified as a new record to Iraq based on morphological and molecular data.

Materials and methods:

Fungal samples were collected from the gardens of Tikrit University during September-November 2020. Macroscopic and microscopic features of samples were reported. Samples were photographed in natural habitats. Distilled water and 5 %KOH were used for microscopy. Fungal samples were identified according to (11, 12, 6, 13 ,14). Identified samples were deposited in the Department of Biology, College of Education for Pure Sciences, Tikrit University, Iraq.

DNA extraction, PCR and sequencing:

Genomic DNA was isolated using a kit (ZR Plant/Seed DNA MiniPrep™), Purity and concentration were measured by nanodrop (2000c) and adjusted to 25 ng/µl. PCR achieved

by using a fragment of ITS as a target to amplify with a forward primer (ITS1 F:5'-TCCGTAGGTGAACCTGCGG-3') and a reverse primer (ITS4 R:5'- TCCTCCGCTTATTGATATGC-3') (Primers set supplied by IDT (Integrated DNA Technologies company, Canada.) ([15]). The PCR amplification was performed in a total volume of 25µl containing 1.5µl DNA (25 ng/µl), 5 µl Taq PCR PreMix (Intron, Korea), 1µl of each primer (10 pmol) then distilled water was added to a total volume of 25µl. The thermal cycling conditions were done as follows: Denaturation at 94 °C for 3 min, followed by 35 cycles of 94 °C for 45s, 52°C for 1 min and 72 °C for 1min with final incubation at 72 °C for 7 min using a thermal Cycler (Gene Amp, PCR system 9700; Applied Biosystem). The PCR product was run in 1.5% agarose gel electrophoresis, and positive reactions were sequenced with forward and reverse PCR primers sent to Macrogen Inc., South Korea .Sequence similarity tool BLAST was employed to find the similarity of the sequences with known 5.8S rDNA sequences in the GenBank database and the sequences were deposited in Gen Bank of NCBI to obtain the accession numbers. A phylogenetic tree was constructed using the ITS-rDNA sequences of the macrofungal strains along with other similar or related macro fungal ITS-rDNA sequences retrieved from the NCBI GenBank. The sequences were aligned using the Maximum Likelihood (ML) method based on the Tamura Nei model (16)..

Results and discussion:

Macro and microscopic features

Cap 3- 4cm in diameter, plano-convex, white, smooth, and light brown disc, margin involute, white, lamellae, white, crowded, not attached to the stipe. Stipe 4.5- 6.0 x0.7 – 1.0cm, white, smooth, cylindrical, expanding at the base, solid, hollow when removed from the substrate, ring present, context white 1-2 mm thick. Basidia 25-35×8-10µm, clavate, 4-spored, spores 7.5-10×5-7.5µm, elliptical, hyaline, with one large oil droplet and one tiny pore. Habit and habitat: Saprotrophic, solitary on soil, grassy areas in gardens.Tikrit University campus, Salahadin Governorate, Iraq. September – November. This species occurs in Europe, North America, and in many other parts of the world, like Turkey (2), Iran (17), and India (18).

Molecular identification of *Leucoagaricus leucothites***:**

The DNA was extracted with high purity and concentration , Also, a perfect PCR was achieved that was confirmed by obtaining the target band, which is 550 bp , *Leucoagaricus leucothites* Identified sample was deposited in NCBI Gen Bank with accession number (MZ149999.1) and in Biology Department, College of Education for Pure Sciences, Tikrit University, Iraq,(Fig 1).

This confirms that the selection of the rDNA site through ITS1 and ITS4 was effective in identifying the sites of variation between the local sample and the global samples, and was adopted as a basis for giving it the accession number in the database in the NCBI, and despite the few sites, it contributed to determining its location on the phylogenetic tree that includes The earliest recorded samples from different environments of the world.

Although the ITS region is not uniformly variable among all fungus, it has been used to identify the majority of fungal species, and as a result, most accessible sequences belong to this widely used marker. This suggests that, despite a number of drawbacks, the ITS region will most likely remain the primary marker for fungal identification in the near future. For these reasons It was chosen as a marker in this investigation (19).

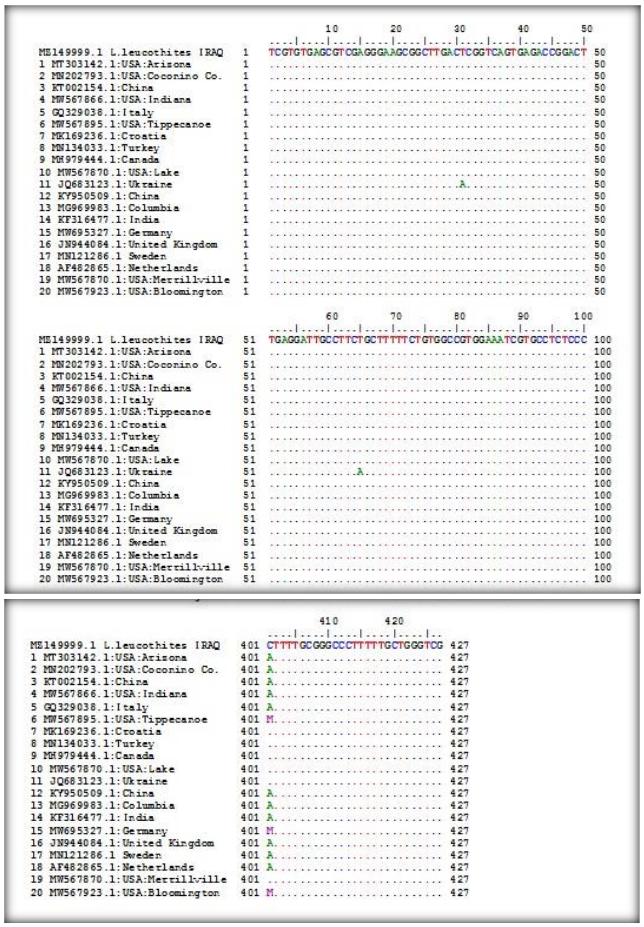


Fig.1: Part of DNA sequencing results of the study sample, showing the sites of nucleotides variation comparison with GenBank samples.

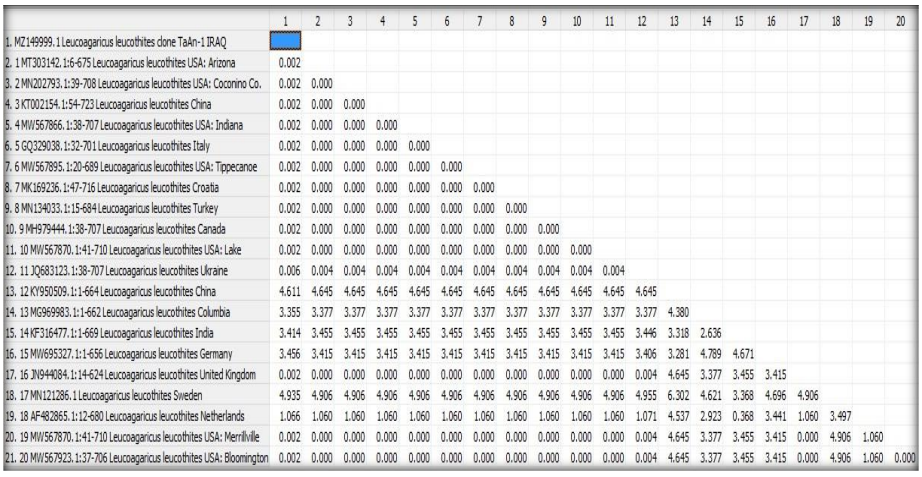
Sequences of *Leucoagaricus leucothites* generated from the current study and additional 20 sequences of taxa retrieved from GenBank were combined and analyzed together to identify our samples correctly and see phylogenetic relationships among species in the phylogenetic tree. (Table1) DNA fragment of the region was approximately 550 bp length encompassing complete ITS1 and ITS4 subregions. The aligned data included all fragments positions of which 427 were conserved, and three positions were variable (Fig1). Also this result was evident in the genetic distance table (Table 2).

Table1: Percentage of match between local sample MN221244.1 and NCBI samples.

	Accession	Country	Source	Compatibility
	ID: MT303142.1	USA: Arizona	Leucoagaricus leucothites	99%
	ID: MN202793.1	USA: Coconino	Leucoagaricus leucothites	99%

		Co.		
	ID: KT002154.1	China	Leucoagaricus leucothites	99%
	ID: MW567866.1	USA: Indiana	Leucoagaricus leucothites	99%
	ID: GQ329038.1	Italy	Leucoagaricus leucothites	99%
	ID: MW567895.1	USA: Tippecanoe	Leucoagaricus leucothites	99%
	ID: MK169236.1	Croatia	Leucoagaricus leucothites	99%
	ID: MN134033.1	Turkey	Leucoagaricus leucothites	99%
	ID: MH979444.1	Canada	Leucoagaricus leucothites	99%
	ID: MW567870.1	USA: Lake	Leucoagaricus leucothites	99%
	ID: JQ683123.1	Ukraine	Leucoagaricus leucothites	99%
	ID: KY950509.1	China	Leucoagaricus leucothites	99%
	ID: MG969983.1	Columbia	Leucoagaricus leucothites	99%
	ID: KF316477.1	India	Leucoagaricus leucothites	99%
	ID: MW695327.1	Germany	Leucoagaricus leucothites	99%
	ID: JN944084.1	United Kingdom	Leucoagaricus leucothites	99%
	ID: MN121286.1	Sweden	Leucoagaricus leucothites	99%
	ID: AF482865.1	Netherlands	Leucoagaricus leucothites	99%
	ID: MW567870.1	USA: Merrillville	Leucoagaricus leucothites	99%
	ID: MW567923.1	USA: Bloomington	Leucoagaricus leucothites	99%

Table2: genetic distance values between local sample MN221244.1 and NCBI samples.



	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
1. MZ149999.1 Leucoagaricus leucothites clone TaAn-1 IRAQ																				
2. 1 MT303142.1:6-675 Leucoagaricus leucothites USA: Arizona	0.002																			
3. 2 MN202793.1:39-708 Leucoagaricus leucothites USA: Coconino Co.	0.002	0.000																		
4. 3 KT002154.1:54-723 Leucoagaricus leucothites China	0.002	0.000	0.000																	
5. 4 MW567866.1:38-707 Leucoagaricus leucothites USA: Indiana	0.002	0.000	0.000	0.000																
6. 5 GQ329038.1:32-701 Leucoagaricus leucothites Italy	0.002	0.000	0.000	0.000	0.000															
7. 6 MW567895.1:20-689 Leucoagaricus leucothites USA: Tippecanoe	0.002	0.000	0.000	0.000	0.000	0.000														
8. 7 MK169236.1:47-716 Leucoagaricus leucothites Croatia	0.002	0.000	0.000	0.000	0.000	0.000	0.000													
9. 8 MN134033.1:15-684 Leucoagaricus leucothites Turkey	0.002	0.000	0.000	0.000	0.000	0.000	0.000	0.000												
10. 9 MH979444.1:38-707 Leucoagaricus leucothites Canada	0.002	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000											
11. 10 MW567870.1:41-710 Leucoagaricus leucothites USA: Lake	0.002	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000										
12. 11 JQ683123.1:38-707 Leucoagaricus leucothites Ukraine	0.006	0.004	0.004	0.004	0.004	0.004	0.004	0.004	0.004	0.004	0.004									
13. 12 KY950509.1:1-664 Leucoagaricus leucothites China	4.611	4.645	4.645	4.645	4.645	4.645	4.645	4.645	4.645	4.645	4.645	4.645								
14. 13 MG969983.1:1-662 Leucoagaricus leucothites Columbia	3.355	3.377	3.377	3.377	3.377	3.377	3.377	3.377	3.377	3.377	3.377	3.377	4.380							
15. 14 KF316477.1:1-669 Leucoagaricus leucothites India	3.414	3.455	3.455	3.455	3.455	3.455	3.455	3.455	3.455	3.455	3.455	3.446	3.318	2.636						
16. 15 MW695327.1:1-656 Leucoagaricus leucothites Germany	3.456	3.415	3.415	3.415	3.415	3.415	3.415	3.415	3.415	3.415	3.415	3.406	3.281	4.789	4.671					
17. 16 JN944084.1:14-624 Leucoagaricus leucothites United Kingdom	0.002	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.004	4.645	3.377	3.455	3.415				
18. 17 MN121286.1 Leucoagaricus leucothites Sweden	4.935	4.906	4.906	4.906	4.906	4.906	4.906	4.906	4.906	4.906	4.906	4.955	6.302	4.621	3.368	4.696	4.906			
19. 18 AF482865.1:12-680 Leucoagaricus leucothites Netherlands	1.066	1.060	1.060	1.060	1.060	1.060	1.060	1.060	1.060	1.060	1.060	1.071	4.537	2.923	0.368	3.441	1.060	3.497		
20. 19 MW567870.1:41-710 Leucoagaricus leucothites USA: Merrillville	0.002	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.004	4.645	3.377	3.455	3.415	0.000	4.906	1.060	
21. 20 MW567923.1:37-706 Leucoagaricus leucothites USA: Bloomington	0.002	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.004	4.645	3.377	3.455	3.415	0.000	4.906	1.060	0.000

This resulted in a congruence of 99% with the comparison samples, the percentage of differences was very low due to the number of different bases between the local samples recorded by serial number MN221244.1 with the NCBI samples shown in Table 1,

Phylogenetic analysis:

The sequences obtained in this study were checked, assembled and compared to those available in the GenBank database by using the BLASTn algorithm. the results (sequences

were selected based on the greatest similarity) and outcomes of recent phylogenetic studies focused on section. (Fig 2)

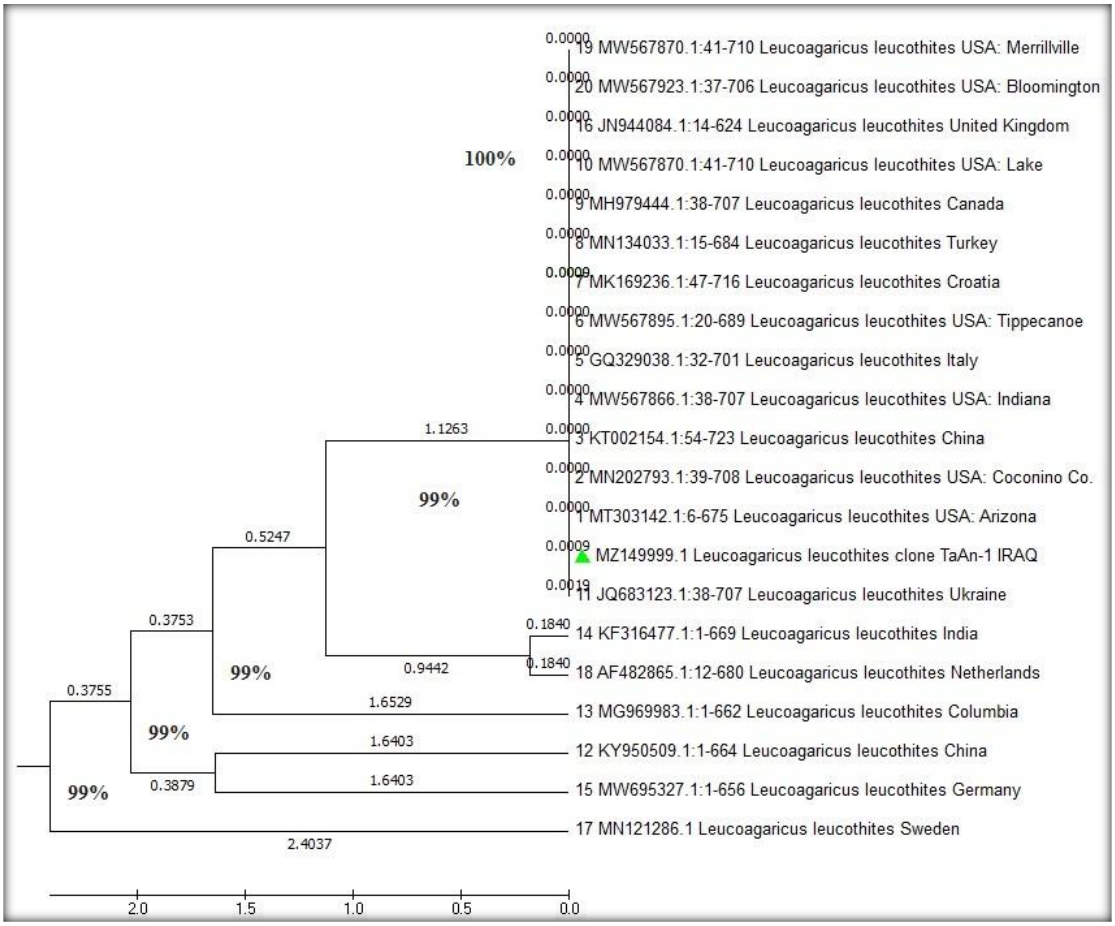


Fig2. Phylogenetic tree of *Leucoagaricus leucothites* species based on ML analysis of the ITS region. Green triangular indicates studied specimen.

Although there was low discrepancy between the studied sample sequences and other species of *Leucoagaricus leucothites* of GenBank derived from ITS sequences, it showed a well-defined Phylogenetic tree in terms of branches and ancestry. The ITS phylogeny showed that the location of the studied sample clearly between its closest species MT303142.1 Arizona and JQ683123.1 Ukraine, and it was formed one group with the species (MW567870.1 Merrillville, MW567923.1 Bloomington, JN944084.1 United Kingdom, MW5678700.1 Lake, MH979444.1 Canada, MN134033.1 Turkey, MK169236.1 Croatia, MW567895.1 Tippecanoe, GQ329038.1 Italy, MW567866.1 Indiana, KT002154.1 China and MN202793.1 Coconino Co.), a perfect Matching ranges from 99% to 100%. As for the rest of the species, they formed other groups and were located on other branches of the Phylogenetic tree.

Molecular information has enhanced the morphological data in identifying the studied sample and supported it as a new record of this species in Iraq. The lack of variance in the

sequence made the morphological data the ruling and deciding factor in the recording of the studied sample.

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