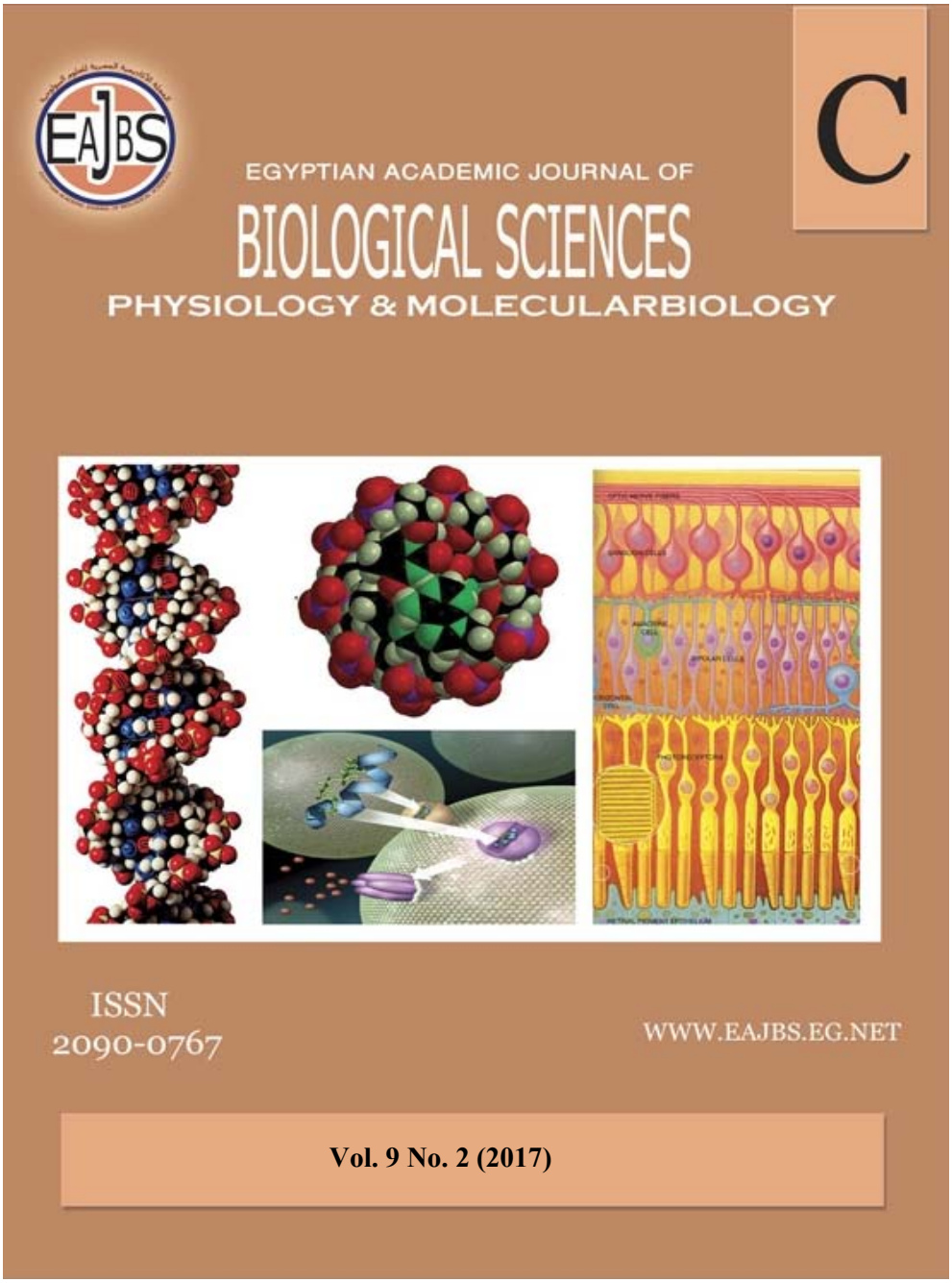

Egyptian Academic Journal of Biological Sciences is the official English language journal of the Egyptian Society for Biological Sciences, Department of Entomology, Faculty of Sciences Ain Shams University.

Physiology & molecular biology journal is one of the series issued twice by the Egyptian Academic Journal of Biological Sciences, and is devoted to publication of original papers that elucidate important biological, chemical, or physical mechanisms of broad physiological significance.

www.eajbs.eg.net

Citation :*Egypt. Acad. J. Biolog. Sci. (C. physiology and Molecular biology) Vol.9(2)pp21-38 (2017)*

Egypt. Acad. J. Biolog. Sci., 9(2): 21- 38 (2017)

Egyptian Academic Journal of Biological Sciences
C. Physiology & Molecular Biology
ISSN 2090-0767
www.eajbs.eg.net

Identification of *Ganoderma* Isolates from Egypt Based on Morphological Characters and ITS1-rDNA Genetic Marker

Labiba Ahmed Reda[1], Naglaa M. Ebeed[2], M.G.E.M. EL-Samman[1], M.H. Mostafa[1] and M.A. Ahmed[1]

1- Plant Pathology Dept., Fac. Agri., Ain Shams Unv., Shoubra El- khima, Cairo, Egypt.
2- Genetics Dept., Fac. Agri., Ain Shams Unv., Shoubra El- khima, Cairo, Egypt.

ARTICLE INFO

Article History
Received: 25/6/2017
Accepted: 28/7/2017

***Keywords*:**
Ganoderma resinaceum, Identification Micromorphology rDNA-ITS1 Phylogeny

ABSTRACT

The basidiomycete fungus Ganoderma Karst., a polyporoid genus within the family Ganodermataceae of the order Aphyllophorales, is worldwide in distribution. The accurate identification of the Ganoderma is still controversial particularly for the tropical species due to high variability in the basidiocarp morphology, and complicated speciation which leads to misidentification by traditional taxonomic methods.

Specimen of *Ganoderma* basidiocarps were collected from different hosts (Navel orange, Oil palm, Fan palm, Casuarina and Morus) in Giza and Qalyubia governorates, Egypt and were identified to species level according to its morphological characters as well as PCR and nucleotide sequence analysis of the ribosomal 5.8S r-DNA gene and the flanking internal transcribed spacers (ITS) utilizing specific primers with ITS 1 region as a target. Isolates of *Ganoderma resinaceum* were described for the first time in Egypt, where, morphological and cultural observations and phylogenetic analysis of ITS1 sequences revealed that all isolates collected from infected trees belong to a single species *Ganoderma resinaceum*.

INTRODUCTION

The basidiomycete fungus Ganoderma Karst., a polyporoid genus within the family Ganodermataceae of the order Aphyllophorales, is worldwide in distribution, growing on numerous coniferous, deciduous and palmaceous hosts. The genus encompasses an extensive and various complex of fungi, a significant number of which are wood rot and others are pathogenic to economically crops and trees. *Ganoderma* give rise to root and stem rots disease that has long been recognized to cause large losses of many equatorial crops such as oil palm (*Elaeis guineensis*), coconut (*Cocos nucifera*), grapevines (*Vitis vinfera*), betelnut (*Areca catechu*), tea (*Camellia sinensis*),Citrus (*Citrus* sp.) (Alfieri, 1977; Adaskaveg and Gilbertson, 1987; Pilotti, 2005; Sankaran *et al*., 2005; Nusaibah *et al*, 2011 and Hushiarian, *et al*., 2013). The genus *Ganoderma* was instituted by Karsten in 1881. Accurate citation of the type species is written as *G. lucidum* (Curt.: Fr.) P. Karst. This genus was later separated into two distinguished groups; the laccate(cutex layer on the outer surface of the mushroom that rendered it is waxy/shiny) (*G. lucidum* complex) and the non-laccate (*G. applanatum* complex) species, which refer to the subgenera *Ganoderma* and *Elfvingia* respectively.

Since then, over 290 taxonomic names in the genus of *Ganoderma* have been published, indicating that this genus is morphologically complex (Roberts, 2004).

The Taxonomic classification of *Ganoderma* species is constructed essentially with respect to morphological features of the basidiocarps, geographical origin, physiological and developmental characters, and chemical components such as secondary metabolites and host specificity (Zheng *et al*. 2009). However, the morphological concept for *Ganoderma* identification is still controversial particularly for the tropical species and can be misguiding due to distinct factors, such as crossbreeding and hybridization (Olson & Stenlid, 2002), ambiguous speciation and convergent evolution (Zhou, *et al*., 2015). As a result, the concept of species in this genus is not well established nor universally accepted. Over the last few decades, it has been demonstrated that the morphology and culture characteristics of species from the same genus can be enormously influenced by growth conditions. Identification of *Ganoderma* species depend on morphology data may occur many synonyms because the number of species the form of the basidiocarp has been affected by the environment. The basidiospores by latitude and altitude and in some species, in southern latitudes, the context color more dark than Northern latitudes on the European continent. A remarkable effect has been shown on the color, size, and brightness of the basidiocarp, and the existence, absence or longitude of the stipe by the age and environment (Moncalvo, 2000).

Ganoderma diversity seems not yet totally discovered, at least in tropical Africa where it and the polypore mycobiota have also been insufficiently probed. Through literature scanning and Index Fungorummyco bank (http://www.indexfungorum.org/Names/Names.asp.), around 473420 unique names have been utilized in Ganoderma, compared to less than one-third which are valid names (Ahmed-Reda., 2007; Douanla-Meliand Langer, 2009).

Molecular procedures could be used to characterize and utilized to describe and distinguish the species. With the improvement of PCR-based techniques, the use of molecular data for taxonomic purposes have been utilized broadly to determine clashing information from morphological attributes (Rolim*etal*, 2011 and Zakaria *et al*., 2005). The Internal Transcribed Spacer (ITS) regions are probably the most important regions in fungi for molecular systematics within a genus for being generally conserved DNA regions within one species because the DNA region has a considerably higher sequence variation between different species. The highly conserved ribosomal genes, which flank the ITS regions, are ideal for universal primer targeting and as a result the ITS regions can be amplified by PCR, then the sequences analyzed, compared and evolutionary trees produced. The ITS regions, a gene marker for fungi are highly variable and for this reason are useful in separating related species and strains of *Ganoderma* was sequenced for the intent of a molecular analysis. (Wang *et al*., 2009). The region of the ribosomal internal transcribed spacer (ITS) are likely the most important regions in fungi for molecular systematics within a genus due to the ITS regions generally conserved DNA regions within one species but, in contrast, the DNA region has a considerably higher sequence variation between different species. ITS is also used to measure the genetic distances between fungal different groups (Del-Prado R *et al*., 2010). The highly conserved ribosomal genes, which flank the ITS regions, are ideal for universal

primer targeting and therefore the ITS regions can be amplified by polymerase chain reaction (PCR), the sequences analyzed and compared, and evolutionary trees produced.

In phylogenetic studies based upon the sequences of (ITS) regions of ribosomal DNA (rDNA), it was demonstrated that extensive assemblage of morphological characters has taken place during the development of *Ganoderma* (Hong and Jung, 2004). It additionally was found that monophyletic groups associate with the geographical source of taxa and host relationships. It is evident that traditional classification systems of genus based on morphological traits should be reviewed considering molecular records. Even though phylogenetic research with ITS sequences gave insights in relationships several the species of *Ganoderma*, relationships of the genus with different genera of the Polyporaceae still are unclear in in many respects after in advance research (Zakaria *et al*., 2009). In addition, molecular mycology was performed easily by the accessibility of fungi DNA sequences in GenBank. Furthermore, the ability to be fit to identify some certain fungi with the aid of using DNA sequences only has confirmed the effectiveness of molecular mycology in issues where conventional taxonomic techniques did not produce conclusive stable classification groups (Ekandjo and Chimwamurombe, 2012).

The aim of the present study was to collect samples of *Ganoderma*-basidiocarps from different hosts (Navel orange, Oil palm, Fan palm, Casuarina and Morus) in Giza and Qalyubia governorates in Egypt; making pure cultures of Ganoderma from its basidiocarps and then, identifying themto species level according to morphological characters as well as ITS1-rDNA genetic marker.

MATERIALS AND METHODS

Source of fungal isolates: Samples of basidiocarps (fruiting bodies) were collected from naturally infected navel orange plants (*Citrus sinensis*) in El Qanater El Khayreya, morus plants (*Morus* sp.) in Kafr EL-Hasa and Casuarina plants (*Casuarina equistifolia*) in Sundanhore (Qalyubia Governorate) and date-palm (*Elaeis guineensis*) and Fan palm (*Washingtonia filifera*) (Giza Governorate), Egypt. Fifteen samples were transferred to the laboratory of Plant Pathology Depart., Faculty of Agriculture, Ain Shams University in ice-boxes for further studies.

Isolation, Purification and Maintenance: Small pieces of basidiocarps were soaked for 1 min. in hydrogen peroxide (5%) solution for surface sterilization, rinsed with sterile water and air dried. Internal tissues of fruiting bodies were removed and cultured on Potato Dextrose Agar (PDA) medium. Standard aseptic laboratory procedures were used, and all plates were incubated at 25°C. Emerging colonies of *Ganoderma* fungi were sub-cultured onto PDA medium till pure cultures were got. The purified isolates were preserved on the same medium at 4-5ºC until used (Kandan *et al*., 2010).

Morphological identification:

Macro-morphological features: Morphological characters such as, type of basidiocarp (stipitate/sessile/dimidiate, imbricate, concave, number of concentric zones, etc.), laccate and non-laccate, in addition, margin shape (lobed, fertile/sterile, rounded/ acute) and color (brown, white, reddish, etc.), pores color, tube size and color, context were recorded.

Micro-morphological features: For internal morphology, free-hand sections were taken from the cutis, context and from the tube layer of each sample (3) respectively. a block of tube layer was used to isolate basidiospores. Then, the sectioned material was handle with KOH

(10%), washed with water to loosen the hyphae. Lactoglycerine (50%) was used as mounting media. Spores were scraped from the pore surface into the mounting solution for observation. Styles of the hyphal system i.e. number, color and diameter were examined. the diameter of Hyphae (20) was measured for each with caution averting collapse hyphae. The diameter and shape of basidiospores were also measured as described before. Caution was considered to avoid very young and immature spores. The slides were examined using light 10X eyepiece of microscope and 10X, 40X and oil immersion (i.e.100X), objectives. Photographs were taken using Motic p 410 attached with photomicrography unit. (Foroutan and Vaidya, 2007; Gottlieb and Wright, 1999).

Cultural characteristics: Culture studies were done to ensure that our data and findings are compatible with those of earlier studies.All tested isolates (3 replicates) were grown up on malt extract agar (MEA) mediaat 25°C. The actively growing margin of mycelia was used for inoculation. Formation of chlamydospores was examined using bright-field microscopy (Hong and Jung, 2004).One drop of Melzer's reagent was mixed with a chlamydospores on a clean, grease-free slide and was allowed to stand for 10-20 minutes and reactions were recordedusing40X and oil immersion (i.e.100X) (Leonard,2006).

Scanning electron microscopy: Fungal isolates were grown on MEA medium at 25ºC for two weeks (Adaskaveg and Gilbertson, 1989). Examination and Photographing of Chlamydospores were carried out using a Jeol Scanning Electron Microscope (T.330A) in the Central Laboratory, Faculty of Agriculture, Ain Shams University.

Molecular identification:

DNA extraction: The DNA extractions were performed using the modified CTAB method (Wu *et al*., 2009) as follows: About 0.2 g mycelium samples were homogenized in liquid nitrogen in cooled mortar, transferred to an 2ml ependorafcontain 800 µL extraction buffer (2%CTAB, 100 mM/L Tris-HCl, 20 mM/L EDTA, 1.4 M/LNaCl, 7%ß-mercaptoethanol and pH 8.0) and mixed gently. The mixture was incubated for 30 min at 60°C in water bath, the homogenate was extracted with chloroform/ isoamyl alcohol (24:1) and centrifuged at 12,000 rpm for 15 min. The supernatant was transferred to new tube and incubated with 1.5 volume of precipitation buffer (1%CTAB, 0.05M/L Tris-HCl, 0.01M/L EDTA, pH 8.0) for 30 min at room temperature, and centrifuged at 12,000 rpm to obtain DNA pellet. Next, the sediment was dissolved in TE buffer (10 mM/L Tris-HCl (pH 8.0), 0.1 mM/L EDTA (pH 8.0), 1M/L NaCl)) and 1µL of RNase A (2.5 U/ml) was added to the solution at 37°C for 30 min to digest RNA. Then the DNA was precipitated by 2.5 volume absolute ethanol and 1/10 volume 3 mol/L NaAC for 1 h at -20°C. The ethanol precipitation was then washed with 70% ethanol, dried and suspended in 100µL of TE buffer. DNA concentrations were estimated and standardized against the known concentrations of DNA on 1.6 % (w/v) agarose gels.

Amplification of ITS region by PCR: Two specific primers of ITS-1 18-mers were designed and synthesized based on the conserved sequence of the ribosomal Internal Transcriber Spacer (ITS) region 1 of rDNA of *Ganoderma boninense* (EMBL accession number X78749).´5TTG ACT GGG TTG TAG CTG 3´ (forward primer)´5GCG TTA CAT CGC AAT ACA3´ (reverse primer). PCR amplification reaction was performed using PCR thermocycler programmed as, 5 min preheating at 95˚C followed by 35 cycles (94 ˚C for 40 s, 52 ˚C for 40 s, 72 ˚C for 45 s) followed by final 12 min extension at 72˚C. The expected DNA fragment product size was 167 bp. The PCR products were

analyzed electrophored on a 1.6% agarose gel, followed by visualized under UV light, photographed and analyzed by documentation using Syngiene Ingenius 3; USA, (Karthikeyan *et al*., 2007).

Sequenceand Phylogenetic analysis

All the PCR products were sequenced, The Internal Trans-cribed Spacer (ITS) fragment was extracted and purified from agarose gels and prepared to be sequenced using the Gene JET™ PCR Purifi-cation Kit (Fermentas). Finally sequencing of PCR product was done via GATC German Company by ABI 3730xl DNA sequences. The sequence data analysis of the ITS gene was received and analyzed. The Sequence assembly was carried out with the Sequencer v. 4.8 program. Sequences obtained from this study were trimmed with Bio Edit software and later BLASTn searched for closest matches in NCBI GenBank database. Phylogenetic tree was constructed to illustrate the relationships among the homologous fungiby using the Maximum Likelihood method based on the Kimura 2-parameter model (Kimura, 1980). The best DNA sequence similarities with our ITS region were obtained from NCBI GenBank and aligned using CLUSTAL W. The treewas drawn to scale, with brlengths measured in the number of substitutions per site. The analysis involved 15 nucleotide sequences. Phylogenetic tree was conducted using MEGA5 (Tamura. *et al*., 2011).

RESULTS

Morphological features:

An artificial key was prepared to differentiate the collected species and for the segregation and assignment of correct taxonomy of the studied samples (Foroutan and Vaidya, 2007).

Taxonomical features:

Ganoderma sp. (Isolate 1) was collected from Navel orange plants (*Citrus sinensis*) in El Qanater El Khayreya (Qalyubia Governorate). The basidiocarp of is woody, sessile, dimidiate, with 13×24 ×1.5 cm. Its upper surface is reddish brown, slightly zonate, laccate, often covered with cinnamon powder of deposited basidiospores (Fig. 1A). The basidiocarp pore surface is creamish brown. Pores are angular to circular, about 4 per mm, brown with 163.8–210.3 µm diameter (Fig. 1F). Tube (10 -19) mm is long brown, unstratified and context is pale brown with a darker zone above the tubes. Cutis type is claviform (Fig. 1B). The hyphal system is trimitic, generative hyphae with (4.2–7.3µm) diameter (Fig. 1C). Skeletal hyphae are (5.3–6.5 µm) diameter, brownish yellow, dichotomously branched (Fig. 1D). and Binding hyphae is 1.7–2.5 µm (Fig. 1E). Basidiospore (8.1–9.8×5.3–6.7 µm) are oval, ellipsoid, truncate at the apex, yellowish brown. Spore index is 1.5 (Fig. 1G). Chlamydosporesar every numerous and the walls are slightly thickened, terminal and intercalary, ellipsoid, spherical or ovoid, smooth, dextrinoid with 13.5-18.7 x 8.2-12.1 µm (Fig. 1H, I).

Ganoderma sp. (Isolate 2) was collected from Morus plants (*Morus* sp.) in Kafr EL-Hesa (Qalyubia Governorate). Basidiocarp of isolate 2 is woody, sessile, dimidiate with dimension of 10×13×1 cm. Its upper surface is dark brown, zonate and laccate (Fig. 2A).Pores are angular to circular, about 4 per mm and brown. Pore surface is creamish brown, 3 per mm and 132.8–294.9 µm diameter(Fig. 2F). Tube is 15 mm long, brown, stratified; context is pale brown with a darker zone above the tubes. Cutis type is claviform (Fig. 2B). The hyphal system is Trimitic, generative hyphae with 1.8–6.8 µm diameter (Fig. 2C). Skeletal hyphae are 4–7.3 µm diameter, brownish yellow and dichotomously branched (Fig. 2D)., in addition to., bindinghyphae are 1–3.8 µm(Fig. 2E). Basidiosporesare (7.6–10.9 ×5.6–6.8 µm), oval, ellipsoid, truncate at the apex, yellowish brown. Spore index

is 1.6(Fig. 2G). Chlamydospores are very numerous, walls slightly thickened, terminal and intercalary, ellipsoid, spherical or ovoid, smooth and dextrinoid with 10-18.6 x 7.6-12.1 µm (Fig. 2H,I).

Ganoderma sp. (Isolate 3) was collected from Casuarina plants (*Casuarinae quistifolia*) in Sundanhore (Qalyubia Governorate). The basidiocarp is woody, stipitate, dimidiate, 17×10×3 cm, stipe darkbrown. Itsupper surface is dark reddish brown, laccate, often covered with cinnamon powder of deposited basidiospores, and subplane to vary irregular. Its margin are 2mm in thickness, sterile and white cream (Fig. 3A).Pore surface is cream white. Pores are angular to circular, about 3 per mm, brown and 174.1–267.4 µm diameter (Fig. 3F). Tube is (2-5) mm long, brown, unstratified and layer of tubes decuurrent on the stipe., furthermore, context is pale brown with a darker zone above the tubes. Cutis type isclaviform (Fig. 3B).Hyphal system is Trimitic, generative hyphae with (3–5.9 µm) diameter (Fig. 3C). Skeletal hyphae are (4–7.4 µm) diameter (Fig. 3D), brownish yellow, dichotomously branched and binding hyphae 1.7–3 µm (Fig. 3E). Basidiosporesare (9.6–11.2 ×5.4–6.7) µm, oval, ellipsoid, truncate at the apex and yellowish brown. Spore index is 1.7(Fig. 3G). Chlamydosporesare very numerous, walls slightly thickened, terminal and intercalary, ellipsoid, spherical or ovoid, smooth and dextrinoid, (11.2-17.6 x 10.8-12.2) µm (Fig. 3H,I).

Ganoderma sp. (Isolate 4) was collected from date palm (*Phoenix dactylifera*) in Giza Governorate. The basidiocarp of it is woody, sessile, dimidiate and 7×5.5×3.2 cm. Its upper surface is dark reddish brown and laccate with margin 2 mm in thickness, sterile, and cream white (Fig. 4A). Pore surface is cream white. Pores are angular to circular, about 3 per mm, brown and (191.4–327.6 µm) diameter(Fig. 4F).Tube is 2 mm long, brown and unstratified, context is pale brown with a darker zone above the tubes. Cutis type is claviform (Fig. 1B). Hyphal system exhibit Trimitic, generative hyphae with 2.3–5.3 µm diameter (Fig. 4C). Skeletal hyphae is (4.2–6.2 µm) diameter, brownish yellow, dichotomously branched (Fig. 4D) and binding hyphae (1–3.1) µm (Fig.4E). Basidiospore are 9.8–11.6×5.9–6.7µm, oval, ellipsoid, truncate at the apex, yellowish brown. Spore index is 1.6(Fig. 4G). Chlamydospores are very numerous, walls slightly thickened, terminal and intercalary, ellipsoid, spherical or ovoid, smooth, dextrinoid, 12.8-21.4 x 8.1-11.1 µm (Fig. 4H,I).

Ganoderma sp. (Isolate 5) was collected from Fan palm (*Washingtonia filifera*) in Giza Governorate. The basidiocarpis woody, sessile, dimidiate, 6×2.5×1.5 cm. Its upper surface is dark reddish brown, laccate with margin 2 mm in thickness, sterile and cream white and pore surface cream white (Fig. 6A). Pore surface is cream white at first, later ochraceous to pale greyish with brown tints and pores are angular to circular, bout 3 per mm, 107.4–347.5 µm diameter (Fig. 6F). Tube is 3 mm long brown, unstratified, context is pale brown with a darker zone above the tubes. Cutis type is claviform (Fig. 6B). Hyphal system exhibited Trimitic, generative hyphae with 2-1–4µm diameter (Fig. 6C). Skeletal hyphaeare (4.6–7.4µm) diameter, brownish yellow, dichotomously branched (Fig. 6D) and binding hyphae (1-2–2.1 µm) (Fig. 6E). Basidiosporeare (7.5–10.8×5.2–6.6µm), oval, ellipsoid, truncate at the apex yellowish brown. Spore index is 1.6 (Fig.6G). Chlamydosporesare very numerous, walls slightly thickened, terminal and intercalary, ellipsoid, spherical or ovoid, smooth, dextrinoid and 11.7-16.2 x 6.9-11.5 µm (Fig. 6H, I).

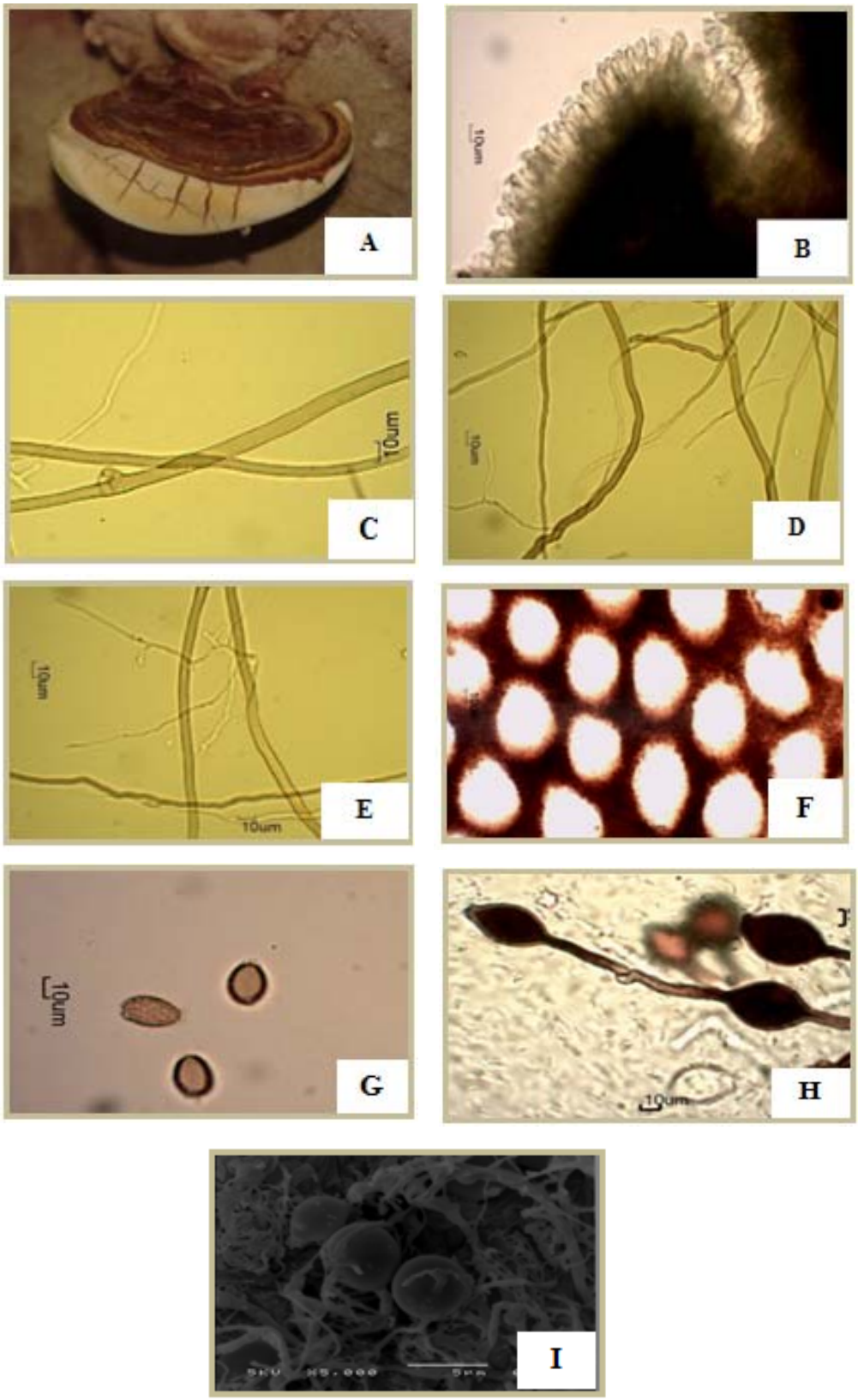

Fig. 1: Isolate 1 of *Ganoderma* sp. was isolated from infected navel orange in El Qanater El Khayreya-Qalyubia. (A) Basidiocarps (holotype); (B) Sections of cutis; (C) Contextual generative hyphae. (D) Contextual skeletal hyphae.; (E) Contextual binding hyphae; (F) pores; (G) Basidiospores showing interwallpillars; (H,I) chlamydospore (H: staining Melzer's reagent; I: SEM photograph).

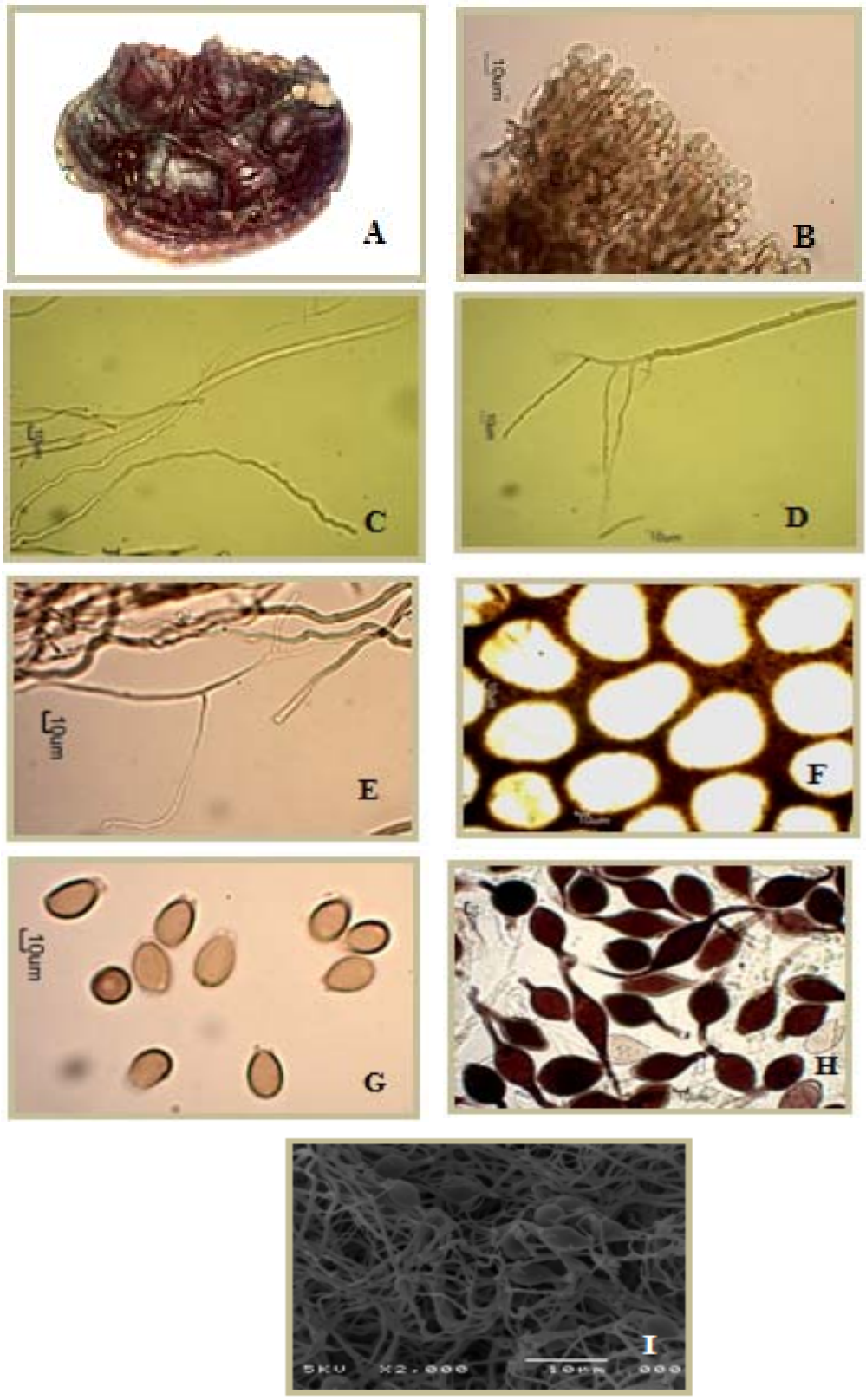

Fig. 2: Isolate 2 of *Ganoderma* sp. was isolated from infected morusin inKafr EL-Hesa, Qalyubia. (A) Basidiocarps (holotype); (B) Sections of cutis; (C) Contextual generative hyphae. (D) Contextual skeletal hyphae. ;(E) Contextual binding hyphae; F) pores;(G) Basidiospores showing interwallpillars; (H,I) chlamydospore (H: staining Melzer's reagent; I: SEM photograph).

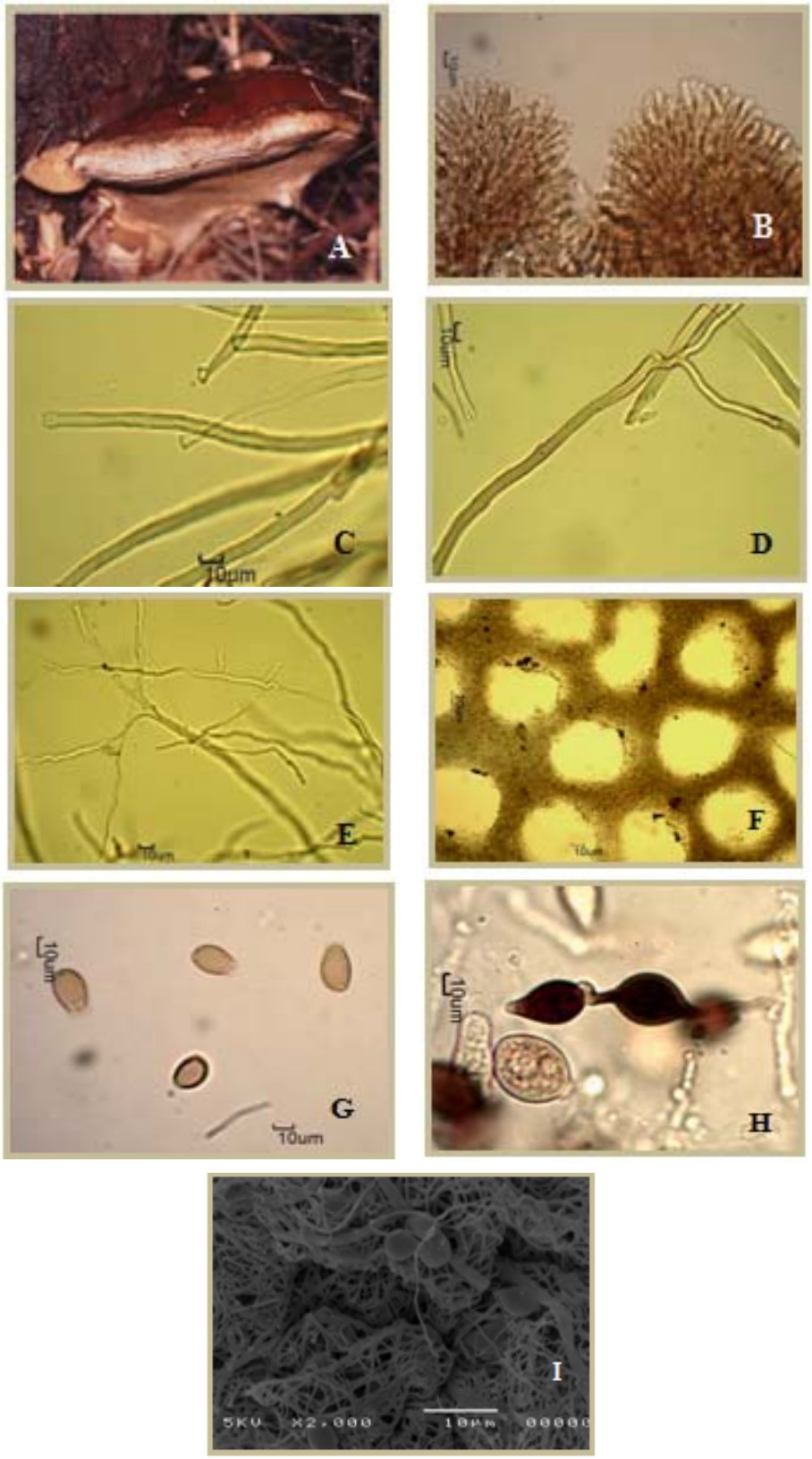

Fig. 3: Isolate 3 of *Ganoderma* sp. was isolated from infected casuarina in, Sundanhor, Qalyubia. (A) Basidiocarps (holotype); (B) Sections of cutis; (C) Contextual generative hyphae. (D) Contextual skeletal hyphae.; (E) Contextual binding hyphae.; (F) pores ;(G) Basidiospores showing interwallpillars; (H,I) chlamydospore (H: staining Melzer's reagent; I: SEM photograph).

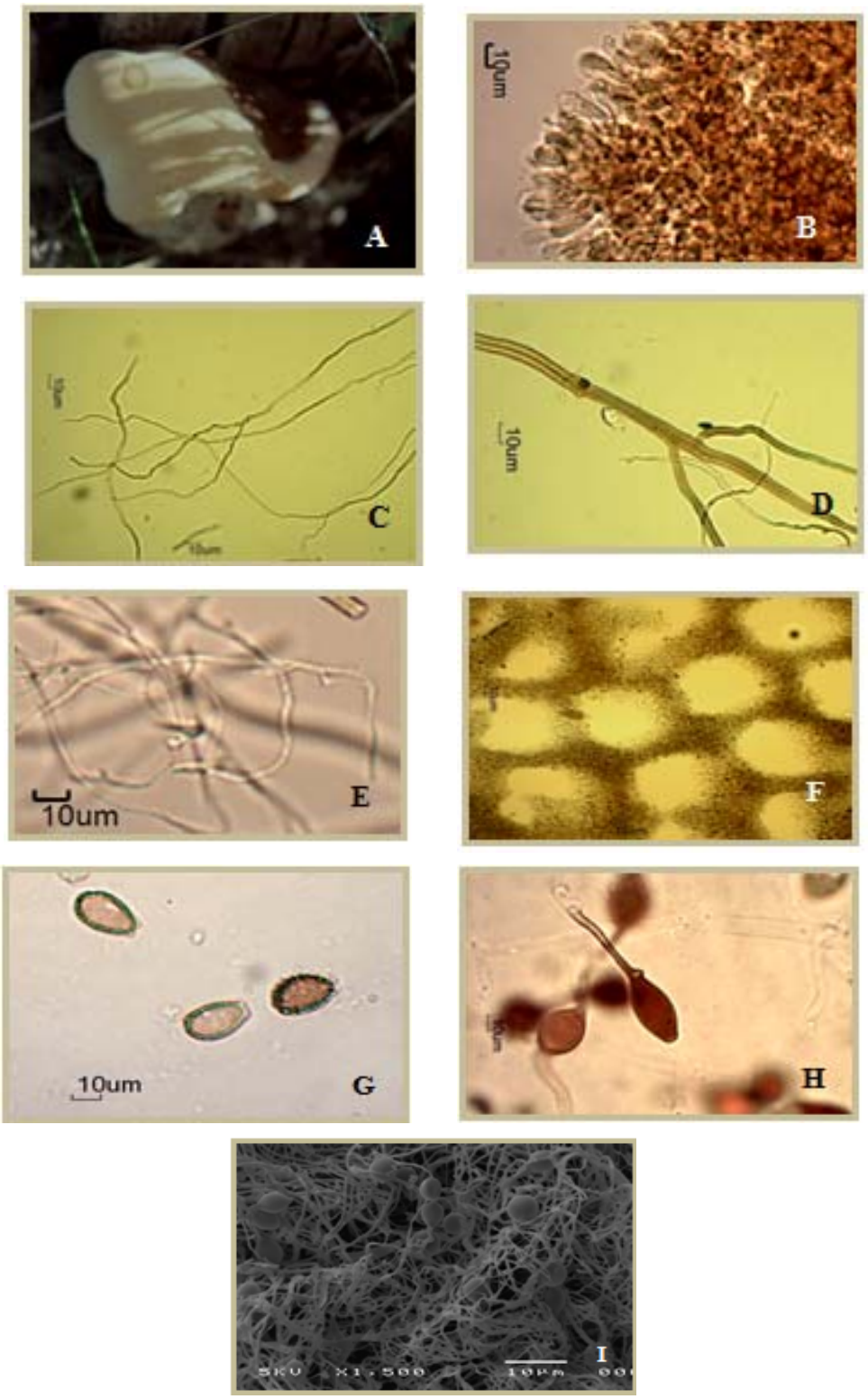

Fig. 4: Isolate 4 of *Ganoderma* sp**.** was isolated from infected date palm in Giza. (A) Basidiocarps (holotype); (B) Sections of cutis; (C) Contextual generative hyphae. (D) Contextual skeletal hyphae.; (E) Contextual binding hyphae.; (F) pores; (G) Basidiospores showing interwallpillars;(H, I) chlamydospore (H: staining Melzer's reagent; I: SEM photograph).

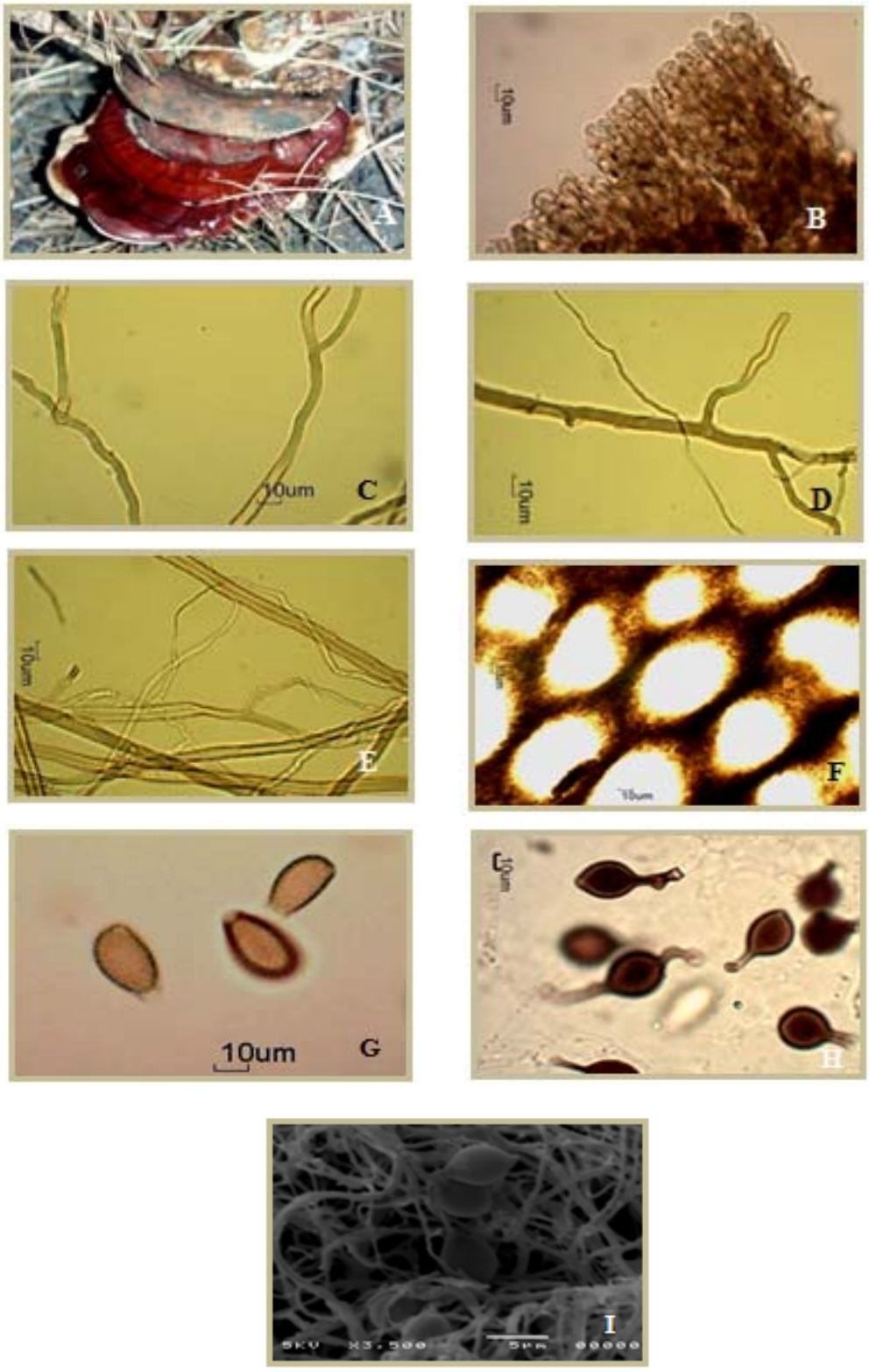

Fig. 5: Isolate 5 of *Ganoderma* sp. was isolated from infected Fan palm in Giza. (A) Basidiocarps (holotype); (B) Sections of cutis; (C) Contextual generative hyphae. (D) Contextual skeletal hyphae.; (E) Contextual binding hyphae.; (F) pores; (G)Basidiospores showing interwallpillars; (H,I) chlamydospore (H: staining Melzer's reagent; I: SEM phot ograph).

Polymerase chain reaction (amplification) Sequence and Phylogenetic analysis

Amplification of the ITS1 region yielded PCR products of approximately 167 bp (Figure 6). BLASns searches revealed a high similarity (91% to100%) between the ITS sequence of isolates in this investigation with those for various *Ganoderma* species in GenBank. The highest similarity was with *G. resinaceum* (98 % to100%), (Figure 7).

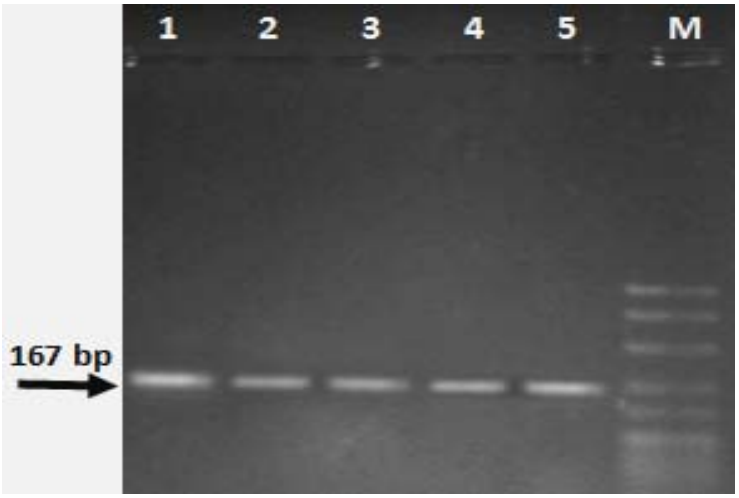

Fig. 6: Amplification of polymerase chain reaction (PCR) product for Ganoderma resina ceumisolates using Gan1 and Gan2 primer. roots); Lane (1) isolate1; Lane (2) isolate2; Lane (3)isolate 3; Lane (4) isolate 4; Lane (5) isolate 5; M, Marker.

Fig. 7: Aligned sequences of the ITS1 regions of rDNA. Ambiguities and polymorphisms are indicated by question marks. Alignment gaps are indicated by dashes, conserved bases by dots and nucleotide base substitution highlighted in yellow. The DNA sequence from left to right reads from 5´ to 3´

A phylogeny generated from sequence data separated the studied isolates into two distinct clades. These two clades are labeled A, B (Figure 8).

Clade A is consisted of five isolated *Ganoderma* from Egypt, with six *G. resinaceum* strains four of which from India (FJ491954, FJ665694, GU451246 and GU451247), and two strains from Korea, and France (JQ 520204 and FJ 805250) respectively. This clade recorded 94% support in the bootstrap analysis.

Clade B represents *G. lucidum* from Russia (GU207321, GU207323 and GU181349), which was robustly supported in the bootstrap analysis (100%).*G. cupreum* was presented as output group.

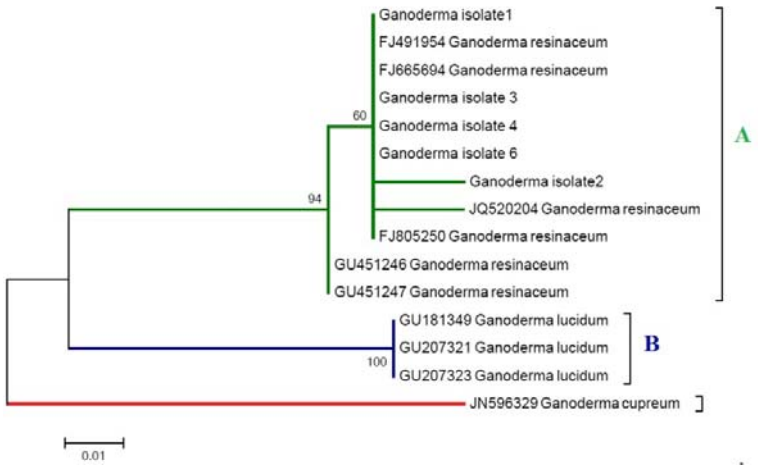

Fig. 8: Rooted phylogenetic tree between isolated Ganoderma (1 – 5); Clad A preresnt *Gandorema resinaceum*, while clad B *Ganoderma lucidum* , *Ganoderma cupreum* use as output group**.**

DISCUSSION

The genus *Ganoderma* includes several wood-decaying fungi on living trees in addition to dead tree trunks and stumps and has been recorded mostly in tropical and temperate areas. Several studies have been carried out on *Ganoderma* diseases focusing on economic damage, the severity of the disease and host range in many regions such as America, Asia, the Middle East and Europe (Fernando, 2008). In Egypt, *Ganoderma* species has been reported as a pathogen on casuarina trees (Ahmed-Reda, 2007; Mahmoud *et al*., 2007). In this study, identification of *Ganoderma* species was commonly found to be associated with stem rot of the navel orange, oil palm, fan palm, casuarina, morus which are selected from two governorates (Giza, Qalyubia) in Egypt.

The morphological similarity of *Ganoderma* species has caused confusion in the identification of these species. Numerous species have been described but many of them were later found to be synonyms or represented species complexes (Muthelo, 2009; Zheng *et al*., 2009).

Identification of *Ganoderma* isolates was carried out depending on morphological features including macroscopic, microscopic, cultural characteristics and chemical reactions as well as DNA based methods.

Two types of basidiocarps can be produced in *Ganoderma* on the particular species. These include species with Lacctebasidiocarps having a shiny upper surface (*G. lucidum*complex), or a non-Laccatebasidiocarps with a dull upper surface (*G. applanatum* complex), (Roberts, 2004). The morphological features of the basidiocarps collected in this study resembled those of the species in the G. *lucidum* complex, which have laccate pilei.

Micromorphological characters of the basidiocarps such as size and morphology of basidiospores, type of hyphal system, as well as the structure of the pileal crust/cuticale surface have been used in the taxonomy of *Ganoderma* (Gottlieb and Wright,1999).

The family Ganodermataceaeis characterized by unique double-walled basidiospores. The differences in basidiospore morphology have been reported for different species within this fungal family. Two kinds of basidiocarps produce this type of basidiospore have been distinguished: one with a shiny

(laccate), yellowish or reddish brown to black pilear surface, and those with a dull (non-laccate), grey-brown to the black pilear surface (Moncalvo, 2000). All the basidiospores examined in this study shared the same characteristics having a yellowish brown to black pilear surface that is well defined.

The structure of the pileal crust/ cuticle cells (Cutis) is a useful character in the taxonomy of the Ganodermataceae. Fruit bodies of *Ganoderma* mostly have an hymenioderm or characoderm and anamixoderm. Characteristics of the cuticle cells have also been valuable in distinguishing species in at least *Ganoderma lucidum* group (Bhosle *et al*., 2010 ; Steyaert, 1980).

The structure of the cutis of all the isolates examined in this study shared the same cutis structure having a hymenioderm (claviform type).The hyphal system in the Ganodermataceae is usually trimitic, occasionally dimitic, the generative hyphae are hyaline, thin walled, branched, septate or not, and clamped. Clamp connections may often be difficult to observe in dried specimens but are easily observed in the youngest parts of the hymenium and context of fresh specimens. Skeletal hyphae are always pigmented, thick-walled, and arboriform or aciculiform. Binding hyphae are usually colorless with terminal branching (Seo and Kirk, 2000). The hyphal system is trimitic in all the isolates was examined in this study.

Morphology of all the isolates was examined in this study as well as appeared to be similar to a number of species in the *G. lucidium* complex. However, a positive identification could not be carried out based on morphology alone. Cultures of *Ganoderma* species produce various hyphal structures, such as generative hyphae with clamp connections, fiber or skeletal hyphae, 'stag-horn' hyphae, cuticular cells and vesicles, and hyphal rosettes as well as chlamdospores. The most useful characters in distinguishing *Ganoderma* cultures are chlamydospore production, growth rate and thermophily (Adaskaveg and Gilbertson, 1989).

High phenotypic plasticity at the macroscopic level, uniformity of microscopic characters, and subjective interpretation of various features such as color or consistency, a lack of handy identification keys and absence of type specimens have resulted in the creation of numerous unnecessary names (synonyms),. The absence of a world monograph has also contributed to problems with species circumscriptions and identifications in *Ganoderma* (Foroutan and Vaidya, 2007).

In this study, all isolate form thick-walled chlamydospores with dextrinoid staining. Ganoderma species based on morphological characteristics were divided into six monophyletic groups (*G. olossums* group, *G. applanatum* group, *G. tsugae* group, Asian *G. lucidum* group, *G. meredithiae* group and *G. resinaceum* group) which included different species. Chlamydospores were observed from the members of the *G. resinaceum* group and *G. oerstedii* of the Asian *G. lucidum* group. Members of the *G. resinaceum* group had negative chlamydospores(*G. subamboinense*, *G. lucidum* ATCC64251 in Taiwan) or dextrinoid staining (*G. resinaceum*, *G. pfeifferi* and *G. lucidum* in North America), but *Ganodermaoerstedii* had amyloid chlamydospores (Hong and Jung, 2004).

Ganoderma pfeifferi is highly characteristic because of the cracked and wrinkled resinous layer on the pileus, the sweet scent in winter and the dark brown context which immediately distinguishes it from old specimens of *G. lucidum* and *G. resinaceum* (Ryvarden et. al., 1993).

Based on morphology alone the *G.lucidum* in North America and *G. resinaceum* in European is considered the same biological species and could not be differentiated from each other (Douanla-

Meli and Langer, 2009). The difficulty of identifying ambiguous *Ganoderma* species based on morphology and cultural property propel the use of DNA sequence data to delineate the isolates Egyptian isolates and distinguish between them easily. Studies using molecular sequencing and phylogenetic relationships revealed that North America *G. lucidum* and European *G. resinaceum* are two species and different (Hong and Jung, 2004) Although, the morphology identification earlier asserted them to the same biological species (Adaskaveg and Gilbertson 1989).

The ITS region, a gene marker was useful in separating related species and strains of *Ganoderma* (Cao and Yuan, 2012; Smith and Sivasithamparam, 2000) was sequenced for a molecular analysis. BLASTn results of the rDNA-ITS sequence data from Egyptian isolates showed that they were similar to various *Ganoderma* species, with the highest similarity being with *Ganoderma resinaceum*.

The phylogram generated from Maximum Likelihood analysis of the ITS sequence data confirmed the results obtained from BLASTn searches. The isolates from Egypt were grouped in one major clade together with sequences labeled as *Ganoderma resinaceum* reported from India (FJ491954, FJ665694, GU451246 and GU451247), and two strains, respectively, from the Korea, and France (JQ 520204 and FJ 805250).

REFERENCE

Adaskaveg, J.E. and Gilbertson, R.L. (1987). Infection and colonization of grapevines by *Ganoderma lucidum*. plant Disease 71: 251-253.

Adaskaveg, J.E. and Gilbertson, R.L. (1989). Cultural studies of four North American species in the *Ganoderma lucidum* complex with comparisons to *G. lucidum*and *G. tsugae*. Mycological Research92: 182–191.

AhmedReda.,labiba (2007).Studies on some Plant Diseases caused by Mushrooms in Egypt. Master of Science Thesis, Ain Shams University, Faculty of Agriculture, Department of Plant Pathology.80p

Alfieri, S. A. J. R. (1977). Heart, Butt, and Root Rot of Redbud, Cercis Cahadensis caused by *Ganoderma curtisii*. Florida. Dept. of Agriculture and Consumer Services. Division of Plant Industry Plant Pathology Circular No.18: pp.5.

Bazzalo, M. E. and Wright, J. E. (1982). Survey of the Argentine species of the *Ganoderma lucidum* complex. Mycotaxon, 16: 293-325 (C.F.CBS).

Bhosle, S., Ranadive, K., Bapat, G., Garad, S., Deshpande, G. and Vaidya, J. (2010). Taxonomy and Diversity of *Ganoderma* from the Western parts of Maharashtra. (india). Mycosphere, 193: 249-262.

Cao. Y. and Yuan, H. S. (2012). *Ganoderma mutabile* sp. nov. from southwestern China based on morphological and molecular data. Mycol. Prog., doi: 10.1007/s11557-012-0819-9.

Del-Prado R, Cubas P, Lumbsch HT, Divakar PK, Blanco O, *et al*. (2010). Genetic distances within and among species in monophyletic lineages of Parmeliaceae (Ascomycota) as a tool for taxon delimitation. Mol Phylogenet Evol 56: 125-133.

Douanla-Meli, C. and Langer, E. (2009). *Ganoderma carocalcareus* sp. nov., with crumbly-friable context parasite to saprobe on *Anthocleista nobilis* and its phylogenetic relationship in G. resinaceum group. Mycological Progress 8:145–155.

Ekandjo LK, Chimwamurombe PM (2012). Traditional Medicinal Uses and Natural Hosts of the Genus *Ganoderma* in North-Eastern Parts of Namibia. J. Pure Appl. Microbiol., 6(3): 1139-1146.

Fernando, K.M.E.P. (2008). The host preference of a *Ganoderma lucidum* strain for three tree species of Fabaceae family; *Cassia nodosa, Cassia fistula* and *Delonix regia* Journal of the National Science Foundation of Sri Lanka. 36 (4): 323-326.

Foroutan, A. and Vaidya, J.G. (2007). Record of new species of *Ganoderma* in Maharashtra India. Asian J. Plant Sci., 6: 913-919.

Gilbertson, R.L. and Ryvarden, L. (1986). North American Polypores. Vol. 1, Fungi flora, Oslo, NorwayPp. 1-433(CBS).

Gottlieb, A.M and Wright, J.E. (1999). Taxonomy of *Ganoderma* from southern South America: subgenus *Elfvingia*. Mycological Research 103 (10): 1289 – 1298..

Hong S. G, and Jung H.S. (2004). Phylogenetic analysis of *Ganoderma* based on nearly complete mitochondrial small-subunit ribosomal DNA sequences. *Mycologia*96 (4): 742–55.

Hushiarian, Roozbeh, Nor AzahYusof, and Sabo Wada Dutse. "Detection and Control of *Ganoderma boninense*: Strategies and Perspectives." SpringerPlus2 (2013). 555. PMC. Web. 22 Oct. 2017.

Kandan A., Ramanathanb A, Raguchanderb, T; Balasubramanianb, Tand Samiyappan R (2010). Early immunodiagnosis of *Ganoderma* infecting coconut seedlings and palms by using monospecific polyclonal antibodies. Arch Phytopath Plant Protect., 43 (17) :1694–1710.

Karthikeyan, M., Radhika, K., Bhaskaran, R., Mathiyazhagan, S., Samiyappan, R. and Velazhahan, R. (2007). Pathogenicity Confirmation of *Ganoderma* Disease of Coconut Using Early Diagnosis Technique .J. Phytopathology 155: 296–304.

Kimura M. (1980). A simple method for estimating evolutionary rate of base substitutions through comparative studies of nucleotide sequences. Journal of Molecular Evolution 16:111-120.

Lempie, K. E. and Percy, M. C. (2012). Genetic Diversity of *Ganoderma* Species in the North Eastern Parts of Namibia using Random Amplified Microsatellites (RAMS). Journal of Pure and Applied Microbiology 6 (3): 1097-1104.

Leonard, L. M. (2006). Melzer's, Lugol's, or iodine for identification of white-spored Agaricales. McIlvainea 16.

Mahmoud,Y. A.G., Eman H. F. A. Mohamed and AbdElzaher, E. H. F. (2007). Response of the Higher Basidiomycetic *Ganoderma resinaceum* to Sodium Chloride Stress.Mycobiology 35(3): 124-128.

Moncalvo, J.M. (2000). Systematics of *Ganoderma*. In: *Ganoderma* diseases of perennial crops, Flood, J. and Bridge, P.D, Holderness, M. (eds). pp. 23-45. CABI Pub., Wallingford.

Muthelo, V.G. (2009). Molecular characterisation of *Ganoderma* species, MSc dissertation, University of Pretoria, Pretoria. 242p.

Niepold,F. B Schöber-Butin (1997). Application of the one tube PCR technique in combination with a fast DNA extraction procedure for detecting Phytophthorainfestans in infected potato tubers .Microbiol.Res. 152:345-351.

Nusaibah, S.A., Latiffah, Z. and Hassaan, A.R. (2011). ITS-PCR-RFLP analysis of *Ganoderma* sp. infecting industrial crops. Pertanika J. Trop. Agric. Sci., 34: 83-91.

Olson A1, Stenlid J. (2002). Pathogenic fungal species hybrids infecting plants.. Microbes Infect. 2002 Nov; 4(13):1353-9.

Pilotti, C.A., (2005). Stem rots of oil palm caused by *Ganoderma boninense:* pathogen biology and epidemiology. Mycopathologia159:129–137.

Roberts, L.M. (2004). Australian *Ganoderma*: Identification, Growth and Antibacterial Properties–PhD thesis. Melbourne: Swinburne University of Technology 271p.

Rolim, L. N.,Cavalcante, M. A. Q, Urben, A. F. and Buso, G. S. C. (2011). Use of RAPD molecular markers on differentiation of brazilian and Chinese *Ganoderma lucidum* strains. *Braz. arch. biol. technol.*, 54, (2): 273-281.

Ryvarden, L. and Gilbertson, R. L. (1993). Polypores Part1: Abortiporus– Lindtneria. Synopsis fungorum.6, pp.272.

Sankaran, K.V., Bridge, P.D., Gokulapalan, C. (2005). *Ganoderma* disease of perennial crops in India-an overview. Mycopathologia159: 143–152.

Seo, G.S. and Kirk, P.M. (2000). *Ganoderma taceae*: Nomenclature and Classification, In: *Ganoderma* Disease of Perennial Crops, Flood, J., Bridge, P.D. and Holderness, P. (Eds.). CABI Publishing, Wallingford, UK., pp. 3-22.

Smith, B. J. and Sivasithamparam, K. (2000). Internal transcribed spacer ribosomal DNA sequence of five species of *Ganoderma* from Australia. Mycol Res.,104: 943-951.

Steyaert, R.L. (1980). Study of some *Ganoderma* species. Bulletin du Jardin Botanique National de Belgique. 50; 135–186.

Tamura K., Peterson D., Peterson N., Stecher G., Nei M., and Kumar S. (2011). MEGA5: Molecular Evolutionary Genetics Analysis using Maximum Likelihood, Evolutionary Distance, and Maximum Parsimony Methods. Mol Biol Evol. (10):2731-9.

Utomo, C . and Niepold, F. (2000). Development of Diagnostic Methods for Detecting *Ganoderma* infected Oil Palms. J. Phytopathology 148:507-514

Wang, D.M., Wu, S.H., Su, C.H., Peng, J.T., Shih, Y.H. and Chen, L.C. (2009). *Ganoderma multipileum*, the correct name for *G. lucidum* in tropical Asia Botanical Studies 50 (4): 451-58.

Wu ,S., Guo, X., Zhou ,X., Li, X., Chen, Y., Lin, J. (2009). AFLP analysis of genetic diversity in main cultivated strains of *Ganoderma* spp. Afr. J. Biotechnol., 8 (15):3448–3454.

Zakaria, Latiffah, Harikrishna, K., Tan, S.G. , Faridah, Abdalha. and Ho, Y.W. (2005). Random Amplified Polymorphic DNA (RAPD) and Random Amplified Microsatellite (RAMS) of *Ganoderma* from infected oil palm and coconut stumps in Malaysia. Asia Pacific J. Mol. Biol. Biotechnol., 13: 23-34.

Zakaria, Latiffah., Ali, N.S., Salleh, B. and Zakaria, M. (2009). Molecular analysis of *Ganoderma* species from different hosts in Peninsula Malaysia. J. Biol. Sci., 9: 12-20.

Zheng, L.Y., Jia, D.H., Fei, X., Luo, X.and Yang, Z.R. (2009). An assessment of the genetic diversity within *Ganoderma* strains with A FLP and ITS PCR-RFLP. Microbiological Research.164: 312-321.

Zhou, L.W., Cao, Y., Wu, S.H., Vlasák, J., Li, D.W., Li, M.J., Dai, Y.C.

(2015). Global diversity of the *Ganoderma lucidum* complex (*Ganoderma taceae*, Polyporales) inferred from morphology and multilocus phylogeny. Phytochemistry 114:7–15.

ARABIC SUMMARY

تعريف عزلات فطر الجانودرما من مصر على أساس الصفات المورفولوجية والعلامة الوراثية -ITS1 rDNA

لبيبة أحمد رضا[١]- نجلاء محمد عبيد[٢] – مجدى جاد الرب السمان[١] - مصطفى حلمى مصطفى[١] - محمد على أحمد[١]

١ - قسم امراض النبات – كلية الزراعة- جامعة عين شمس- القاهرة- مصر

٢- قسم الوراثة – كلية الزراعة- جامعة عين شمس- القاهرة- مصر

يعتبر الفطر *Ganoderma* أحد فطريات عيش الغراب الثقبية الهامة أقتصاديا وهو منتشر فى جميع أنحاء العالم . ومع ذلك لا يزال التعريف الدقيق لفطر *Ganoderma* مثير للجدل خاصة بالنسبة للأنواع الاستوائية بسبب التباين العالي في شكل الثمار البازيدية والخصائص المعقدة التي تؤدي إلى صعوبة التعريف عن طريق أساليب التصنيف التقليدية. وتهدف الدراسة للتعريف الدقيق للعزلات المصرية للجانودرما باستخدام الطرق المورفولوجية وطرق الوراثة الحديثة .وجمعت الأجسام الثمرية البازيدية للفطر من عوائل نباتية مختلفة هى البرتقال ابو سرة والتوت و الكازورينا و نخيل البلح ونخيل المروحة من مناطق مختلفة في بعض محافظات مصر وهى القليوبية و الجيزة. وقد تم الحصول على خمسة عشر عزلة وانماءها وتنقيتها على بيئة آجار مستخلص المولت ، وأوضح الفحص بالمجهر الضوئى العادي أن جميع العزلاتلهاميسليوم ذو هيفات متفرعة ومقسمة كما شوهد تكوين جراثيم كلاميدية.

تم تعريف العزلات الفطرية السابقة (خمسة عشر عزلة) بالاعتماد على الصفات الظاهرية والتشريحية للجسم الثمرى والصفات المزرعية. وقد اتضح ان الاعتماد على الصفات الظاهرية والتشريحية للجسم الثمرى والصفات المزرعية غير كافي لتحديد النوع، لذلك تم أختيارخمسعزلات فطرية وتعريفها الى مستوى النوع بالاعتماد على التقنيات الجزيئية عن طريق التحليل الجزيئي لتتابع لمنطقة internal transcribed spacer region1 of rDNA (ITS1) للعزلات ورسم شجرة القرابة الوراثية وقد أتضح أن جميع العزلات الفطرية تتبع نوع واحد هو *Ganoderma resinaseum*.