Diversity of Algerian basidiomycetes, morphological, macrochemical and genotypic characterization

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Abstract

A collection of 16 basidiomycetes was carried out in northwestern Algeria. During the harvest, the average temperature of the sites was between 11.2 to 19.3 °C with an average humidity of 67 to 90%. The dominant plants observed at the sampling sites are pine, oak and false pepper. The collected mushrooms were identified by their macroscopic, microscopic (light microscope and scanning electron microscope) and macrochemical characteristics using chemical reagents to distinguish among the different species. Of the 16 fungi, a genotypic identification was made using the tools of molecular biology and bioinformatics analysis. The results of the phenotypic analysis showed that the fungi in question were: *Lactarius zonarioides, Amanita proxima, A. virosa, Agaricus bisporus*, *Suillus mediterraneensis, Boletus* sp., *Xerocomus* sp., *Coprinus atramentarius*, *Pleurotus ostreatus, P. pulmonarius, P. eryngii, P. dryinus, Lepiota* sp., *Cortinarius orellanus, Armillaria mellea* and *Trametes quercina.* The comparison of the ITS1 sequences (143 bp) with those of the databases was carried out using the BLAST algorithm, which confirmed the identification of *Agaricus bisporus*.

Keywords: *Agaricus bisporus, Basidiomyces,* Bioinformatics, Macrochemical characterization, SEM.

Introduction

Poor agricultural practices in developing countries are reported to be a major source of pesticide poisoning through dermal and oral ingestion in humans (Kumari and John, 2018; Mohamed *et al.,* 2018). In response to this situation, the world has adopted alternatives, including organic agriculture, which excludes the use of chemical pesticides, using natural phytosanitary products known as: biopesticides (COLEACP, 2011). Research suggests that biopesticides made from basidiomycetes could be a safer and more planet-friendly alternative (Barseghyan *et al.,* 2016).

Basidiomycetes are macromycetes that grow prolifically. These constitute one of the most species-rich phyla of richest phylum of fungi with nearly 32,000 described species in almost all terrestrial species and some aquatic habitats, both freshwater and marine (Lin *et al*., 2019; Mao and Wang, 2019). They from a more important group than the Ascomycetes (Blandeau, 2012). They are cosmopolitan heterotrophic organisms, which play an important role in nutrient cycling and maintaining a healthy state of forests in addition to the medicinal and nutritional importance (Kumar *et al*., 2015; Singh *et al*., 2019). They present a great morphological diversity with non-flagellated spores and compartmentalized mycelium (Blandeau, 2012). There are a large number of reports that have demonstrated the role of basidiomycetes and their potential use as biocontrol agents against phyto-fungi (Ishihara *et al.,* 2018; Sidorova and Voronina, 2019; Ait-Hamadouche *et al.,* 2021). Therefore, the objective of this study was to show the diversity of basidiomycetes from western Algeria, their morphological and macro-chemical characterization and finally a molecular and bioinformatics analysis of the species *Agaricus bisporus*.

Materials and Methods

Collection of basidiomycetes

Basidiomycetes fungi (E1, E2, E3.... E16) were collected in northwestern Algeria at different sites during the year 2018-2019 (Fig. 1). The collected mushrooms were photographed *in situ* in their natural habitats (Fig. 2), identified by their macroscopic, microscopic and macrochemical characteristics (Benazza-Bouregba, 2017) and then sun-dried for preservation (Moglad and Saadabi, 2012).

Phytoecological characteristics of harvesting sites

During the harvest, we noted the geographical and bioclimatic characteristics of the sampling sites, which were provided to us by the Office of Meteorology of Oran. The average temperature of the sites ranged from 11.2 and 19.3 °C. The average humidity was between 67 and 90%. Table 1 shows the collection sites of the 16 samples (E1 to E16), their habitat and the coordinates of the geographical position. The table also showed that the average temperature of the sites ranges from 11.2 to 19.3 °C.

Macroscopic characterization

Macroscopic characterization was carried out with the naked eye and with the Leica EZ4HD stereomicroscope. This description was based on: the cap (size in cm, shape, margin, color and texture), the foot (height and diameter in cm, color, presence of ring, volva or basal bulb), the flesh (color and texture) and the hymenium (lamellae/tubes, color, shape, mode of insertion on the stipe) (Champon, 2009; Eyindong *et al.,* 2011; Adrien, 2013). Organoleptic characteristics and latex flow as well as the place of harvesting are to be taken into consideration (Eyindong*et al*., 2011; Krishna *et al.,* 2015).

Macrochemical characterization

Macrochemical characteristics are also necessary for the description of the basidiomycete. The technique consists of placing a fragment of the cap of the fungus in a watch glass, which will serve as a support for the reactions, and then adding a few drops of the reagent prepared beforehand. The reagents used were: Ammonia 34%, formalin 35-40%, phenol 3%, iron sulphate 10%, potash 10%, sulphovanillin and aniline). Wait a few minutes to 1 hour to note the change in colour of the reagent (Courtecuisse and Duhem, 2011).

Light microscopy

Before microscopy, the caps of basidiomycetesare cut out using the freehand technique. This technique consists of using two sharp razor blades, Gilettes brand, directed in the direction of the cap lamellae (Ebika, 2013). The cut tissue was then placed in a drop of Congo Red with 10% SDS between the slide and the lamella. The dimensions, shape, ornamentation, and the presence of apical pore (germinative pore) of the spores are recorded. The dimensions of the basidia were noted and the number of basidiospores from sterigmata (monosporic, bisporic, trisporic and tetrasporic) is counted. The shape and type of apex of the cystidia were also noted. Dimensions were measured using the micrometer on the Olympus CX22 light microscope.

Scanning electron microscopy SEM

The observation of the samples was carried out by the JEOL Scanning Electron Microscope (JSM-6610LA) at the Laboratory of Electron Microscopy & Material Sciences "LMESM" at the USTO University. This equipment benefits from an integrated EDS analyser (energy dispersive spectroscopy) as well as the possibility of working under partial vacuum, at an acceleration voltage of 500V and above, for taking images with a resolution of 5120×3840. The secondary electron detector was of the Everhart Thornley type and used at magnifications from x5 to x300,000 (polaroid format). The camera was connected to a computer to record the results of the observations.

Molecular approach

We used the products of the Wiragen kit for the extraction of the genomic DNA of the basidiomycete fungus. The resulting DNA was then amplified by PCR and visualized by electrophoresis. The amplicons as well as the ITS1 and ITS4 primers were sent to GENEWIZ in Germany for Sanger sequencing (https://www.genewiz.com/fr-FR/Public/Services).

Bioinformatic analysis

After receiving our sequences from GENEWIZ, the sequence identity of the basidiomycete fungus was obtained after a homology search in the "Genbank" database of the National Center for Biotechnological Information NCBI (https://www.ncbi.nlm.nih.gov).

The comparison of the sequences with those of the databases was carried out via the BLAST algorithm (Altschul *et al*., 1990), providing the list of probable results corresponding to our sequence, together with the sequence similarity values (Altschul *et al.,* 1997). Nucleotide sequence alignments were performed using the "Clustal W" software (Thompson *et al*., 1994). The phylogenetic analysis was carried out using the "Neighbor-Joining" method (Saitou and Nei, 1987) using the Mega 6 version 6.06 software (Tamura *et al*., 2013).

Results and Discussion

According to the criteria for determining the main families of basidiomycetes (Champon, 2009; Eyindong*et al.,* 2011; Adrien, 2013), the fungi collected from the forests and sites are grouped together in the mapping in Fig. 3. These fungi are identified by macroscopic and microscopic characteristics (Eyindong *et al*., 2011; Krishna *et al.,* 2015).

After a systematic identification, the collected basidiomycetes belong to 8 families. Referring to Gerhardt (2008), Losange (2015) and Redeuilh *et al*. (2015), we classified samples into the following families: Russulaceae, Amanitaceae, Agaricaceae, Suillaceae, Psathyrellaceae, Pleurotaceae, Cortinariaceae, Boletaceae, Physalacriaceae and Fomitopsidaceae.

The macroscopic and microscopic description of some basidiomycete samples are shown in figures (4 to 10). We have *Agaricus bisporus* (Fig. 4), *Coprinus atramentarius* (Fig. 5), *Suillus mediterraneensis* (Fig. 6), *Lactarius zonarioides* (Fig. 7), *Amanita proxima* (Fig. 8), *P. dryinus* (Fig. 9) and *Amanita virosa* (Fig. 10). The identified basidiomycetes enrich the list of fungi already reported by other works in Algeria like those of Benazza-Bouregba (2016); Yakhlef *et al.* (2020) and Mesfek *et al.* (2022).

For macrochemical identification, the results

obtained are recorded in Table 2. We speak of a positive reaction if there is a change in color of the tested part (cap, foot, cuticle, flesh or pores) which can be slow, thus taking 10 min to 1 h, or an immediate reaction, thus an instantaneous change on the tested tissue; or by negative reactions (no reaction) if there is no change in color. For the reagents tested, aniline reacted positively only on the cap and lamellae of the small lepiota (E2 *Lepiota* sp.) with a yellow coloration. It remained unchanged on the rest of the samples.

The subjectivity of the manipulator is important because everyone sees the colours with a different hue and intensity (Courtecuisse and Duhem, 2011). Take the example of iron sulphate, used in mycology (green in colour) which, once applied, turns orange (Courtecuisse and Duhem, 2011). This was confirmed in oyster mushrooms and the two amanitas, *T. quercina* and *L. zonarioides* which gave a pale or intense orange reaction depending on the basidiomycete tissue tested. For *Boletus* sp., an intense red reaction was revealed, and according to Theirs (1975) several species of *Boletus* give different colors when the reagent comes into contact with the skin or flesh or pores of the fungus (pale grey, orange-yellow, orange); other species such as *B. smithii*; *B. spadiceus*; *B. submentosus* with black skin (Theirs, 1975). In *S. mediterraneensis*, the reagent gave a black colour on the pores and flesh, and this has been seen in other species of this fungus with colurs varying between grey, grey-black and grey-blue (Theirs, 1975). Ammonia is the only reagent used without direct contact between the substance and the fungus (fumes are sufficient) (Courtecuisse and Duhem, 2011). In this study, 34% ammonia was used in direct contact with the fungal tissue and was positive on only 4 samples including: *C. atramentarius* (dark black on the tested tissues) whereas according to the bibliography, other species such as *C. balteatus* (yellowish green), *C. largus* (yellow), *C. variecolor* (bright yellow) (Bon *et al.,* 1987). For *S. mediterraneensis*, the cuticle and pore tissue turn dark black on contact with ammonia, while different reactions vary from species to species (grey, pink, pale and dark red, blue to green) which is consistent with the results of Lange and Hora (1963) and Theirs (1975). Ammonia is also used for the identification of *Xerocomus*, whose chemism changes according to the species (blue reaction, blue-green, negative to pale pink, pale green, negative to black, negative or pale pink, greenish) (Theirs, 1975; Bon *et al.,* 1987). The reaction is absent for the rest of the samples, including *L. zonarioides*, while a purple reaction is seen in two species (*L. turpis* and *L. plumbeus*) (Lange and Hora, 1963; Bon *et al.,* 1987) ; and for *Boletus* species that react with the reagent (greenish, blue skin, black, yellow, dark red, dark green, red to deep pink, purplish) (Theirs, 1975).

Potash or KOH at 10% lightened the cuticle of *A. bisporus*, but Bresinsky (1990) reported a potash reaction (intense yellow) on *A. placomyces*. Comparing the two amanites, potash reacted only on the cuticle of *A. virosa* after 15min giving a chromium yellow color change which was confirmed by Miller (1978). Potash at 10% revealed an unpleasant smell on *L. zonarioides* and gave an orange-yellow reaction on the lamellae and cuticle, this was also found in other species like *L. pyrogalus* (Lange and Hora, 1963). It can be concluded that macrochemical characterization allowed us to differentiate between different species of the same genus, and the chemism changes according to the species.

Finally, the comparison of the ITS1 sequences (143 bp) with those of the databases was performed using the BLAST algorithm (Altschul *et al*., 1990). Blast identified the basidiomycete E1 as *Agaricus bisporus*, which confirms the morphological identification. The molecular phylogeny was based on the use of nucleotide sequences. It made possible to establish the links of relationship between different living beings. Over the last 20 years, molecular techniques such as PCR and sequencing have been developed and have led to the production of a large number of DNA sequences. The use of these sequences has led to profound changes in systematic classification (Adrien, 2013). Figure 11 showed the phylogenetic position of the basidiomycete E1 (*Agaricus bisporus*) in relation to the other basidiomycetes closest to it according to the Blast and to an ascomycete fungus (outgroup: *Morchella esculenta*). The percentage of replicate trees in which E1 (*Agaricus bisporus*) clustered in the bootstrap test (500 replicates) is shown next to the branches. Only the identity of *Agaricus bisporus* was molecularly characterized because of its culinary and economic interest.

Conclusion

This research work is part of the development of Algeria's natural and especially mycological heritage, and in the light of recent work on the use of wild basidiomycetes in agriculture. These 16 fungi have been the subject of research work demonstrating their antifungal phytosanitary power on phytopathogenic strains because there is an urgent need for alternative, environmentally friendly products to control plant diseases in Algeria. Among them, we mention *Amanita proxima, A. virosa, Armillaria mellea, Trametes quercina, Pleurotus pulmonarius, P. ostreatus, Lepiota* sp. and *Xerocomus* sp.

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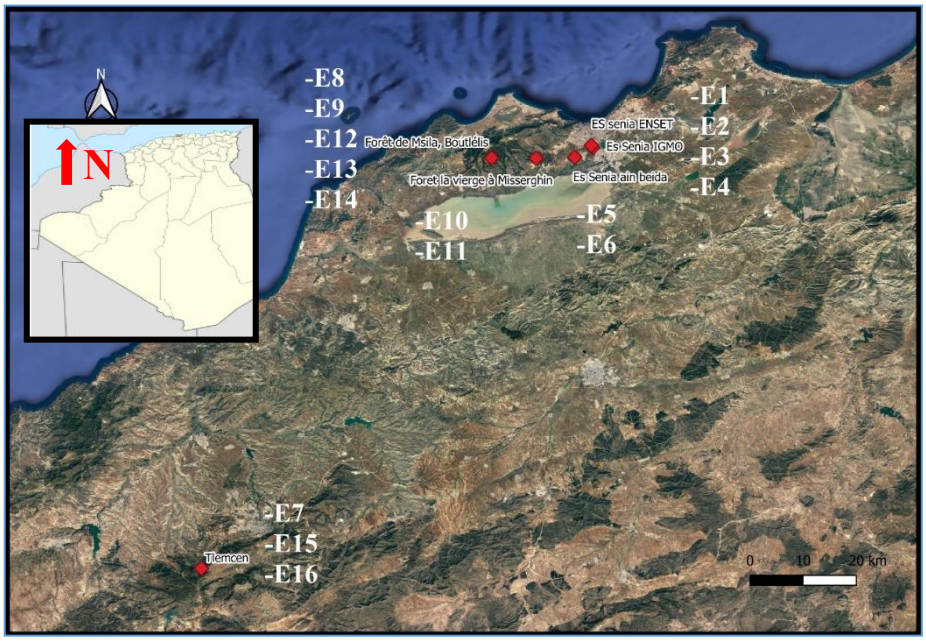

Fig. 1: Cartography of basidiomycete samples E1 to E16

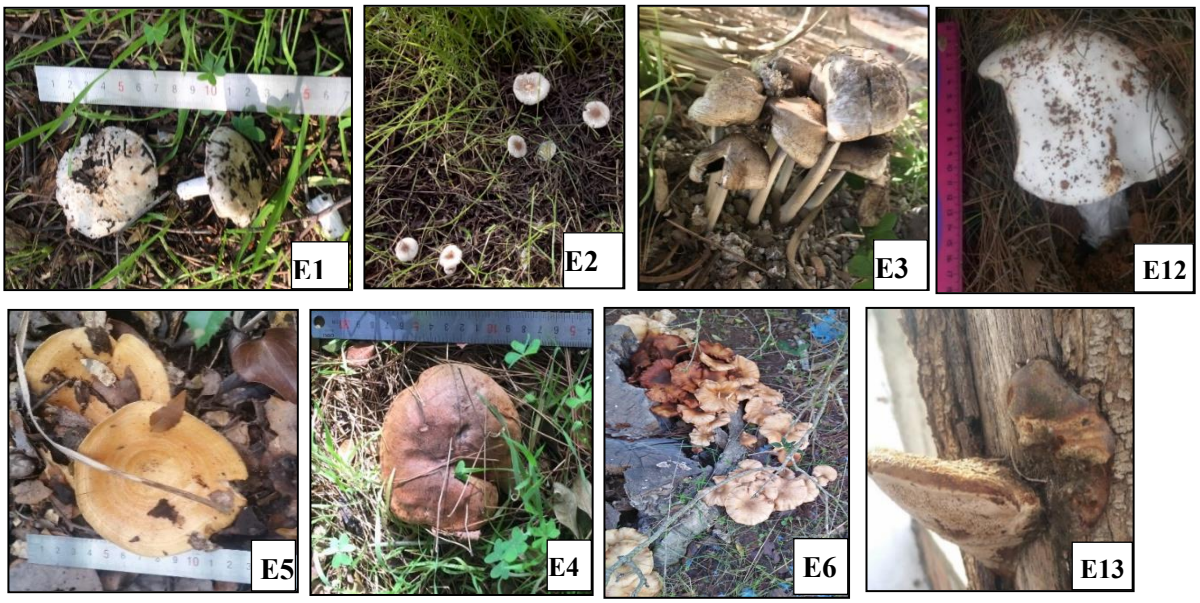

Fig. 2: Collection of some basidiomycete samples *in situ:* E1, E2, E3, E12, E4, E5, E6 and E13.

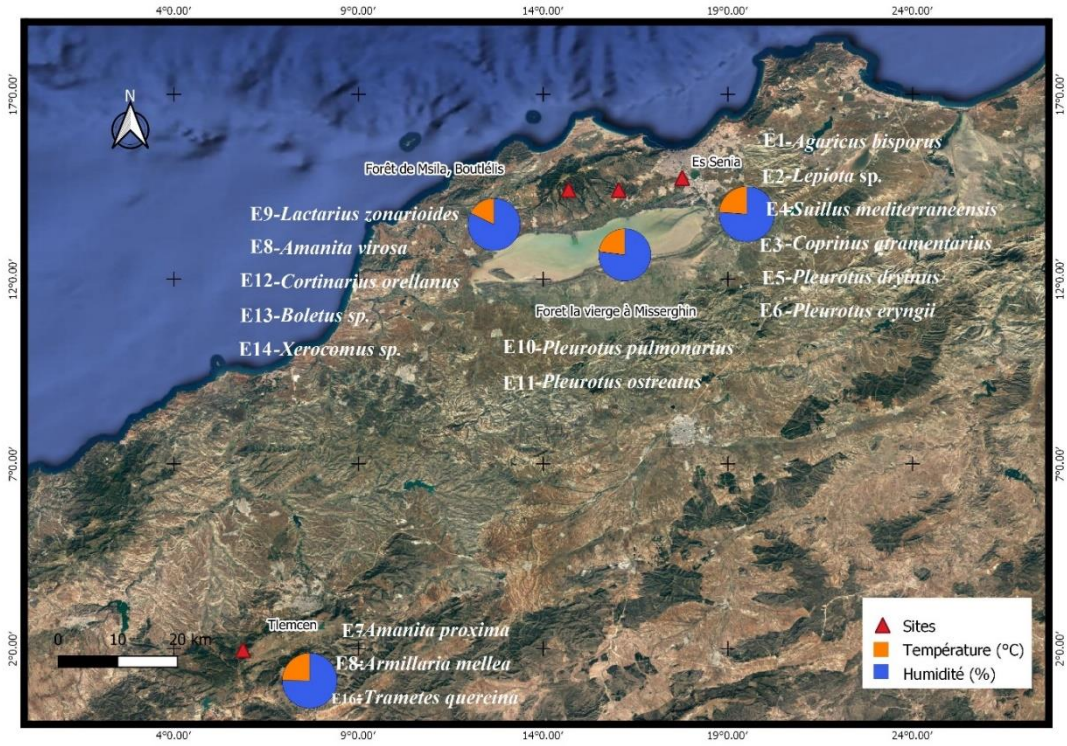

Fig. 3: Basidiomycete sites (QGIS software). **E1:** *Agaricus bisporus;* **E2:** *Lepiota* sp. *;* **E3:** *Coprinus atramentarius* **;** **E4:** *Suillus mediterraneensis ;* **E5:** *Pleurotus dryinus* **;** **E6 :** *Pleurotus eryngii* **;** **E7 :** *Amanita proxima* **;** **E8:** *Amanita virosa* **;** **E9:** *Lactarius zonarioides* **;** **E10:** *Pleurotus pulmonarius* **;** **E11:** *Pleurotus ostreatus* **;** **E12:** *Cortinarius orellanus* **;** **E13 :** *Boletus* sp. *;* **E14:** *Xerocomus* sp**.** **;** **E15:** *Armillaria mellea;* **E16:** *Trametes quercina.*

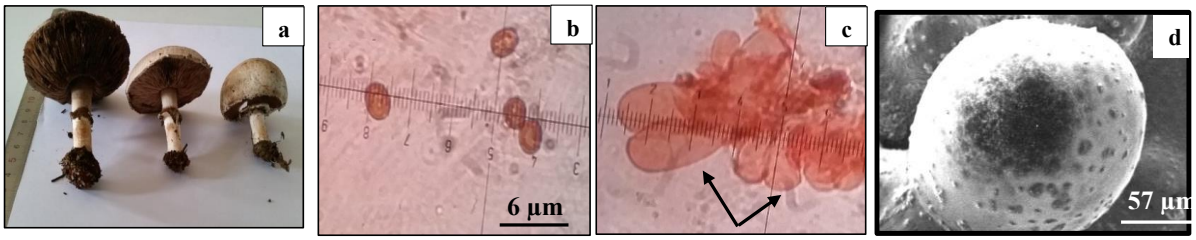

Fig. 4: Macroscopic and microscopic characterization of *Agaricus bisporus.* **a):** Sporophore of *A. bisporus* or two-spore agaricus; **b):** Basidiospores 4-8 × 5-8 µm, brown, rounded to elliptical, smooth without germinal pore(G×1000); **c):** Subhymenium with 8×15 µm ellipsoidal cystids with rounded apex (black arrow) (stained with Congo Red plus SDS, G×1000) under light microscope. **d):** Spore at 57 µm, rounded to elliptical, smooth without germinal pore, observed under SEM, G×270.

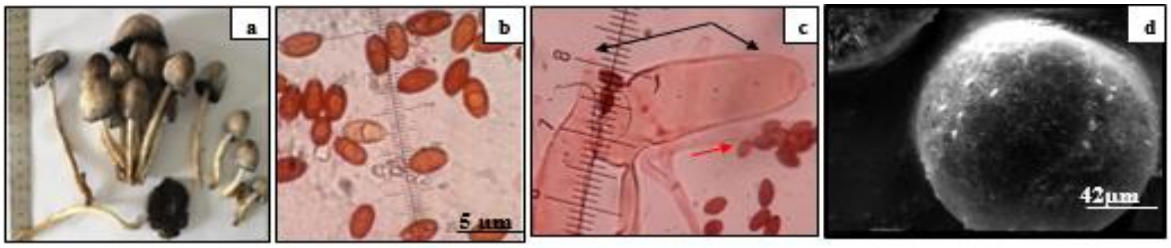

Fig. 5: Macroscopic and microscopic characterization of *Coprinus atramentarius.* **a):** Sporophore of *C. atramentarius* or Ink black Coprin, **b):** Basidiospores 4-6 × 6-12 µm ellipsoid, brown with clear germinative pore (G×1000); **c):** Subhymenium with trabecular cystidia (black arrow) and septate hyphae with attached spores (red arrow) (Stained with Congo Red plus SDS, G×1000) under light microscope, **d):** Spore at 42 µm, ellipsoid, observed under SEM, G×370.

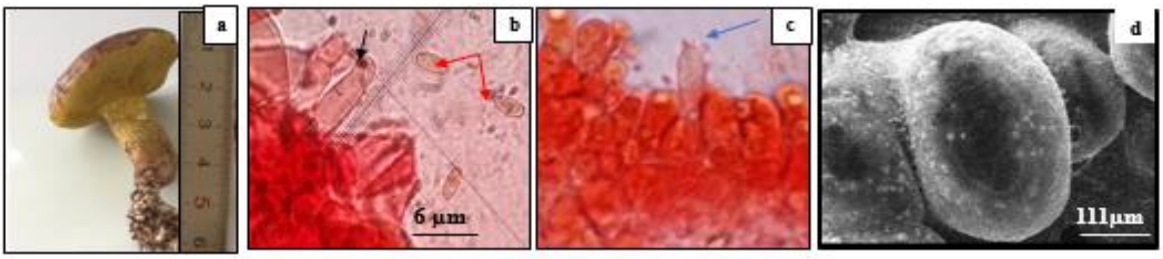

Fig. 6: Macroscopic and microscopic characterization of *Suillus mediterraneensis*. **a):** Sporophore of *S. mediterraneensis*, **b):** Basidiospores : 3-4 × 9–10 µm fusiform, smooth without germinative pore (red arrow), Subhymenium with cystidia 6-8 × 12-22 µm claviform with captive apex (black arrow) (G×1000); **c):** Subhymenium with tetraphonicbasidia 5 × 15 µm (blue arrow) (Stained with Rougeongo plus SDS, G×1000) by light microscopy, **d):** Spore at 111 µm, fusiform, smooth without germinative pore, observed by SEM G×370.

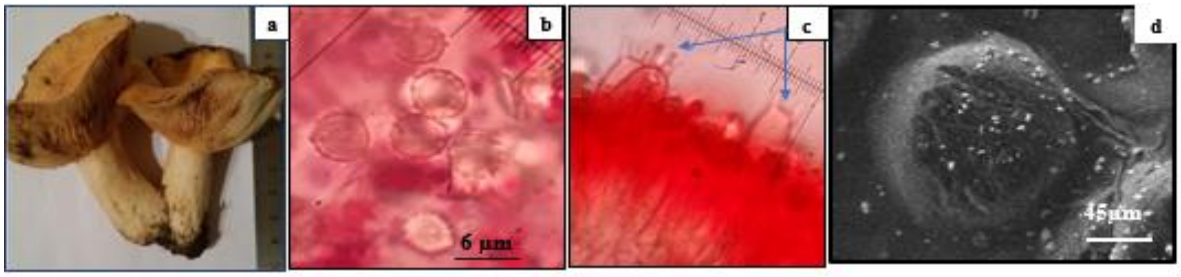

Fig. 7: Macroscopic and microscopic characterization of *Lactarius zonarioides.* **a):** Sporophore of *L. zonarioides*, **b):** Basidiospores: 4–6 × 5–12 µm, ovoid-elliptical, verrucose, reticulate or bristly, without germinative pore (G×1000), **c):** Subhymenium with basidiospores 9 × 12 µm (blue arrow) (stained with Rougeongo plus SDS, G×1000) under light microscope, **d):** Spore at 45 µm, ovoid, elliptical, verrucose, reticulate without germinative pore, observed under SEM, G×330.

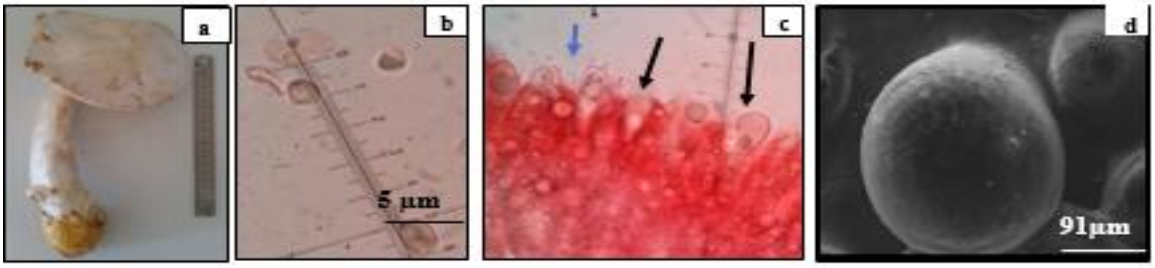

Fig. 8: Macroscopic and microscopic characterization of *Amanita proxima.* **a):** Sporophore of *A. proxima* or red volva *Amanita*, **b):** Basidiospores: 7–10 × 4–7µm, smooth with apicle (G×1000), **c):** Subhymenium with tetraphonicbasidia (blue arrow) and piriformcystidia with rounded apex (black arrow) (Stained with Congo Red plus SDS, G×1000) by light microscopy, **d):** Spore at 91 µm, smooth with apiculus, observed by SEM, G×250.

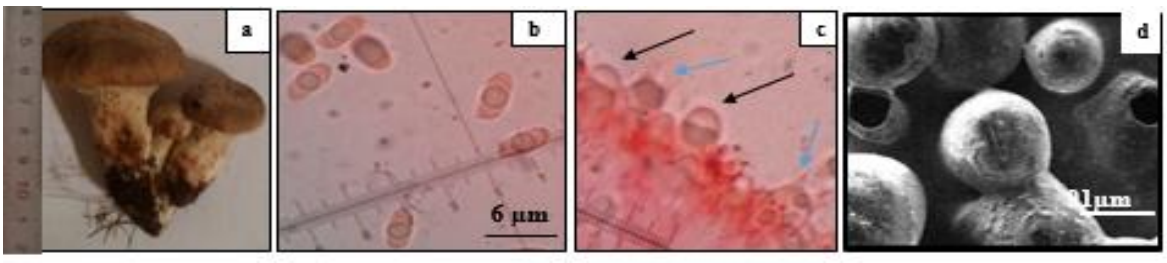

Fig. 9: Macroscopic and microscopic characterization of *Pleurotus eryngii.* **a):** Sporophore of *P. eryngii* or panicum oyster mushroom, **b):** Basidiospores: 5-6 x 11-13µm smooth with apical (G×1000); **c):** obovoid cystids with rounded apex (black arrow), bisporicbasidia (blue arrow) (stained with Congo Red plus SDS, G×1000) under the light microscope, **d):** Spore at 91 µm, smooth with apical, observed under the SEM, G×170.

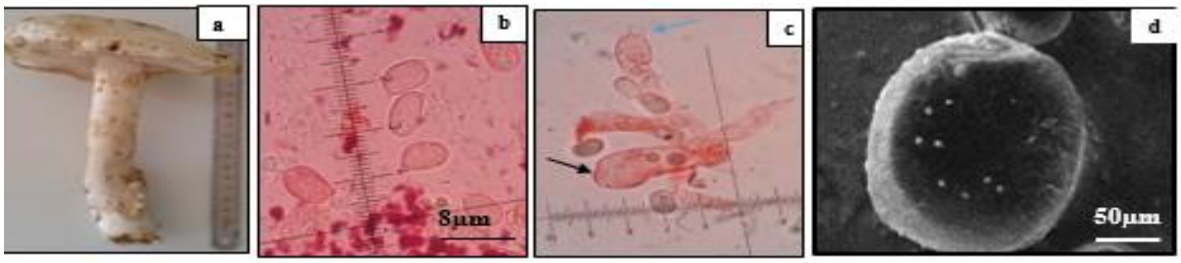

Fig. 10: Macroscopic and microscopic characterization of *Amanita virosa.* **a):** Sporophore of *A. virosa* or Worm-eaten Amanite, **b):** Basidiospores: 5–7 × 9–10 µm, smooth with apicle (G×1000), **c):** Subhymenium with tetraphonicbasidia (blue arrow) and attached spores; obovoidecystids with caped apex (black arrow) and attached spores, (stained with Congo Red plus SDS, G×1000) by light microscopy, **d):** Spore at 50µm, smooth with apicle, observed by SEM, G×300.

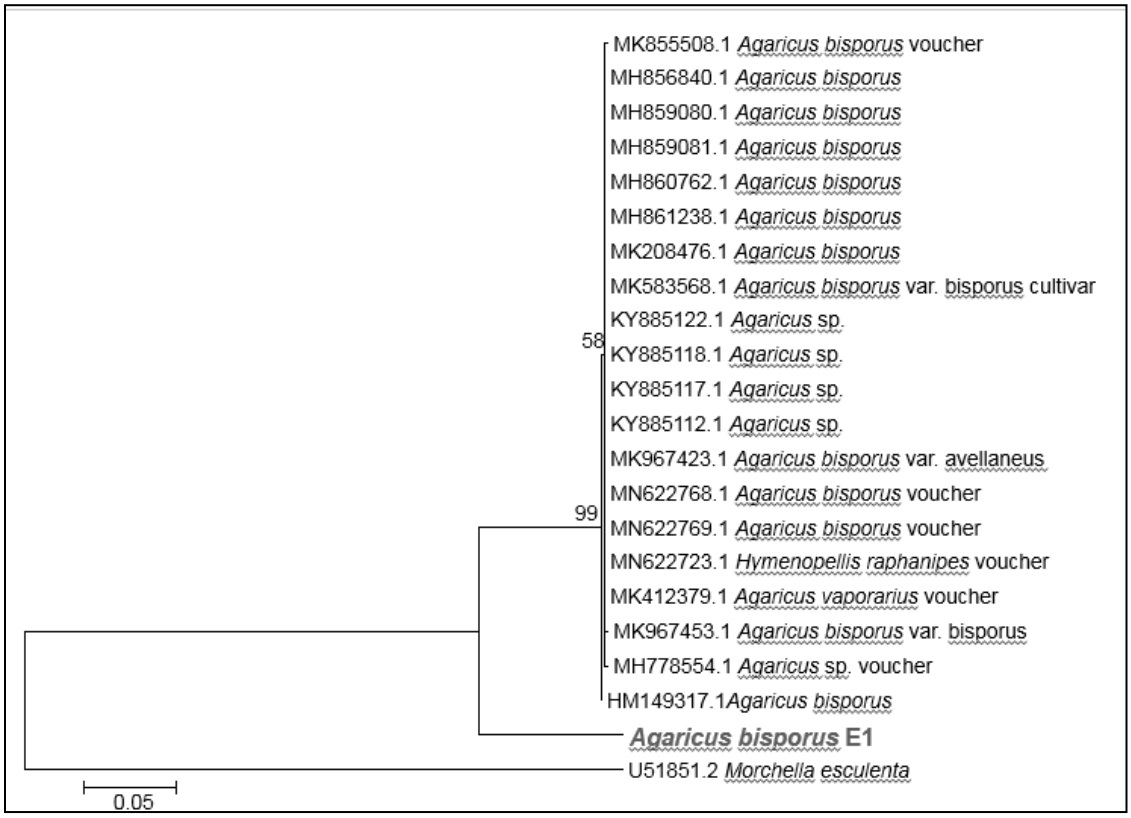

Fig. 11: Molecular phylogenetic analysis using the maximum likelihood method. The evolutionary history was inferred using the Neighbor-Joining method (Saitou and Nei, 1987). The optimal tree with the sum of the branch lengths = 0.76816331 is presented. The percentage of replicate trees in which the associated taxa clustered in the bootstrap test (500 replicates) is shown next to the branches (Felsenstein, 1985). The tree is drawn to scale, with branch lengths in the same units as the evolutionary distances used to derive the phylogenetic tree. The evolutionary distances were calculated using the 2-parameter method of Kimura (Kimura, 1980) and are expressed in units of the number of base substitutions per site. Twenty-two nucleotide sequences were analysed. All positions containing gaps and missing data were eliminated. There were a total of 570 positions in the final dataset. Evolutionary analyses were performed in MEGA6 (Tamura *et al*., 2013).

Table 1: Geographical and climatic characteristics of basidiomycete collection sites.

Samples	Harvesting location	Ecology (habitat)	Temperature	GPS coordinates	
				Longitude	Latitude
E1	Es-Sénia university	False pepper tree (*Schinus* molle)	21°C	0°37′26″ ouest	35°38′52″ Nord
E2					
E3					
E4		Pine tree (*Pinus*)			
E5	Farm of AinBeida (Es-Sénia)	Nd	20 °C	7°23′34″ ouest	35°47′47″ Nord
E6		Nd			
E7	Forest of Tlemcen	Pine tree (*Pinus*)	12 °C	1°18′53″ ou est	34°52′41″ Nord
E8		Treetrunk of oak			
E9		Cork oak (*Quercus suber*)			
E10	Virgin forest at Misserghin	Nd	13 °C	0° 44' Ouest	35° 37' Nord
E11		Nd			
E12	Forest of Msila, Boutlélis	spruce		-0°52'24.56" Ouest	35° 37' Nord
E13		Pine tree (*Pinus*)			
E14		Hardwood undergrowth			
E15		Pedunculate oak (*Quercus robur*)			
E16					

Nd: not determined

Table 2: Macrochemical characteristics of basidiomycetes.

Samples	NH_3 34%	Pure form	Phenol 3%	Ferroussulphate (10%)	KOH (10%)	Sulfovanillin
E1	NR	NR	NR	NR	Cuti.clears up	Red, purple (after 1 hour)
E2	NR	NR	NR	NR	Cap and lam. Intense yellow, cuti. (15min) yellow ochre	Red, purple (after 1 hour)
E3	Black Dark	NR	Black Dark	Black Dark	Black Dark	Red, purple (after 1 hour)
E4	Pore and cap Black Dark	NR	NR	Black	Pore, flesh andcuti. Black Dark	Purpleflesh (IR)
E5	NR	Cap and lam. dark yellow	Cap and lam. transparent	Flesh, cap and lam. orange	yellow	Lam., cap and flesh rouge à mauve (15min)
E6	NR	NR	NR	flesh orange (10min) the rest NR	NR	Lam.,cap and flesh Red, purple (after 15min)
E7	NR	NR	Cap orange, lam. (NR)	Cap, flesh and lam. orange (IR)	NR	Cap and lam. mauve pink (SL)
E8	Orange side	Cap and lam. orange dark	Orangy	light orange	Golden yellowcuticle (15min)	Lam. mauve (IR) ; cap (NR)
E9	NR	NR	Mauve or brown (SL)	light orange	Unpleasant odour, lam.	Cap, flesh and lam. mauve to blue

					and cuticule orange-yellow	anchor (SL)
E10	Transparent	NR	flesh and lam. And cap yellow dark	Orange (15min)	Lam. yellowochre	Cap and flesh mauve
E11	NR	Flesh, lam. and cap yellow dark	After 15 minutes it clears up	Flesh and lam. orange (RI)	Lam. yellow ochre	Cap and flesh mauve
E13	NR	NR	NR	Cap, flesh and pore (red dark)	Black dark	Cap, flesh and pore (deep pink then mauve)
E14	NR	NR	NR	Blackish flesh and pore	Black dark	Same as E 13
E15	NR	Black side	Clears up	Brow dark	NR	Deep pink foot
E16	NR	Lam., cuti. Orange(10 min)	light orange	Cuti. orange Lam. **(**NR**)**	Light green then brown (SL)	Lam., cuti. purple

IR: Immediate reaction; **NR:** no reaction; **SL:** slow reaction; **lam:** Lamella; **cuti:** Cuticule; **Tran:** Transparent ; **E1:** *Agaricus bisporus;* **E2:** *Lepiota* sp.*;* **E3:** *Coprinus atramentarius ;* **E4:** *Suillus mediterraneensis;* **E5:** *Pleurotus dryinus;* **E6:** *Pleurotus eryngii;* **E7:** *Amanita proxima;* **E8:** *Amanita virosa*; **E9:** *Lactarius zonarioides;* **E10:** *Pleurotus pulmonarius;* **E11:** *Pleurotus ostreatus;* **E12:** *Cortinarius orellanus*; **E13:** *Boletus* sp. *;* **E14:** *Xerocomus* sp.*;* **E15:** *Armillaria mellea;* **E16:** *Trametes quercina.*

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