ANNALI DI BOTANICA
Ann. Bot. (Roma), 2019, 9: 95–106
Journal homepage: http://annalidibotanica.uniroma1.it

THE FIRST IDENTIFICATION OF THE WOOD-INHABITING FUNGUS PLEUROTUS TUBER-REGIUM FROM MADAGASCAR WITH A COMBINED APPROACH BASED ON MORPHOLOGICAL AND MOLECULAR ANALYSES

Vizzini A.[1,2], Voyron S.[1], Tinti F.[3], Cavagna S.[4], Dela Pierre F.[4], Mello A.[2,*]

[1]*Dipartimento di Scienze della Vita e Biologia dei Sistemi, Università di Torino, Viale P.A. Mattioli 25, 10125, Torino, Italy*
[2]*Istituto per la Protezione Sostenibile delle Piante, SS di Torino, Viale P. Mattioli 25, 10125 Torino, Italy*
[3]*Athénée Saint Joseph Antsirabe, Antsohihy, Madagascar*
[4]*Dipartimento di Scienza della Terra, Università di Torino, via Valperga Caluso 35, Torino, Italy*
**Corresponding author: Telephone: +390116502927; email:* antonietta.mello@ipsp.cnr.it

(Received 23 August 2018; received in revised form 09 January 2019; accepted 15 January 2019)

Abstract – Purpose: Madagascar is worldwide recognized as one of the top biodiversity hotspots. Our purpose was to investigate a fungal species found in Madagascar as a sclerotial mass. Methods: A fungal sample consisting of a sclerotium was collected on a *Raphia farinifera* log in Antsohihy (North Madagascar) and sent to our lab for characterization. Morphological and molecular analyses were carried out and small pieces of sclerotium tissue were inoculated in agar in order to isolate mycelium. Results: light and scanning electron microscope observations of the sclerotium revealed a carpet of thick-walled hyphae whereas light microscope observations of mycelium showed nematode-trapping structures. The nrITS sequence obtained from the sclerotium was compared with the GeneBank database showing high similarity with *Pleurotus tuber-regium*. A phylogenetic analysis clearly revealed two clades in this species: one of mainly African origin, the other one of Asian and Australasian origin. Whether these two clades should be considered two subspecies of the same species or two distinct species, is questioned, discussed. Conclusion: This study reports the first molecular identification of *Pleurotus tuber-regium* from Madagascar.

Keywords: Agaricomycetes, Pleurotaceae, *Lentinus*, Phylogeny, Resistance Structures, ITS1-5.8S-ITS2, Madagascar

Introduction

Madagascar is an island between Africa and Asia characterized by enormous climatic and ecological variation. Its rich endemic flora and fauna places Madagascar as one of the top hotspots worldwide for biodiversity conservation (Myers et al., 2000; Wilmé et al., 2006; Ganzhorn et al., 2008; Vences et al., 2009). But, while its plant and animal species richness is relatively well-studied (Schatz et al., 1996; Goodman & Beanstead, 2003; Glaw et al., 2012; Radespiel et al., 2012), fungal species diversity is still far from being fully revealed and understood (Buyck, 2008). The first studies on mushrooms from Madagascar date back to the late nineteenth and early twentieth centuries (Cooke & Massee, 1890; Hennings, 1893, 1908). Later, mycological investigations were carried out in Madagascar by French mycologists, among which the most important studies were those by Patouillard (1924, 1928) on new basidiomycete species, Romagnesi (1941) on the genus *Entoloma*, Métrod (1949) on mycenoid fungi, and Heim who initiated a series of monographs and papers mainly based on his own six–month collecting expedition to Madagascar in 1934–1935 (e.g. Heim 1936, 1938a, b, 1945). After the Second World War, Malagasy mushrooms biodiversity was practically neglected, until the Buyck' s paper of the late

doi: 10.13133/2239-3129/14403

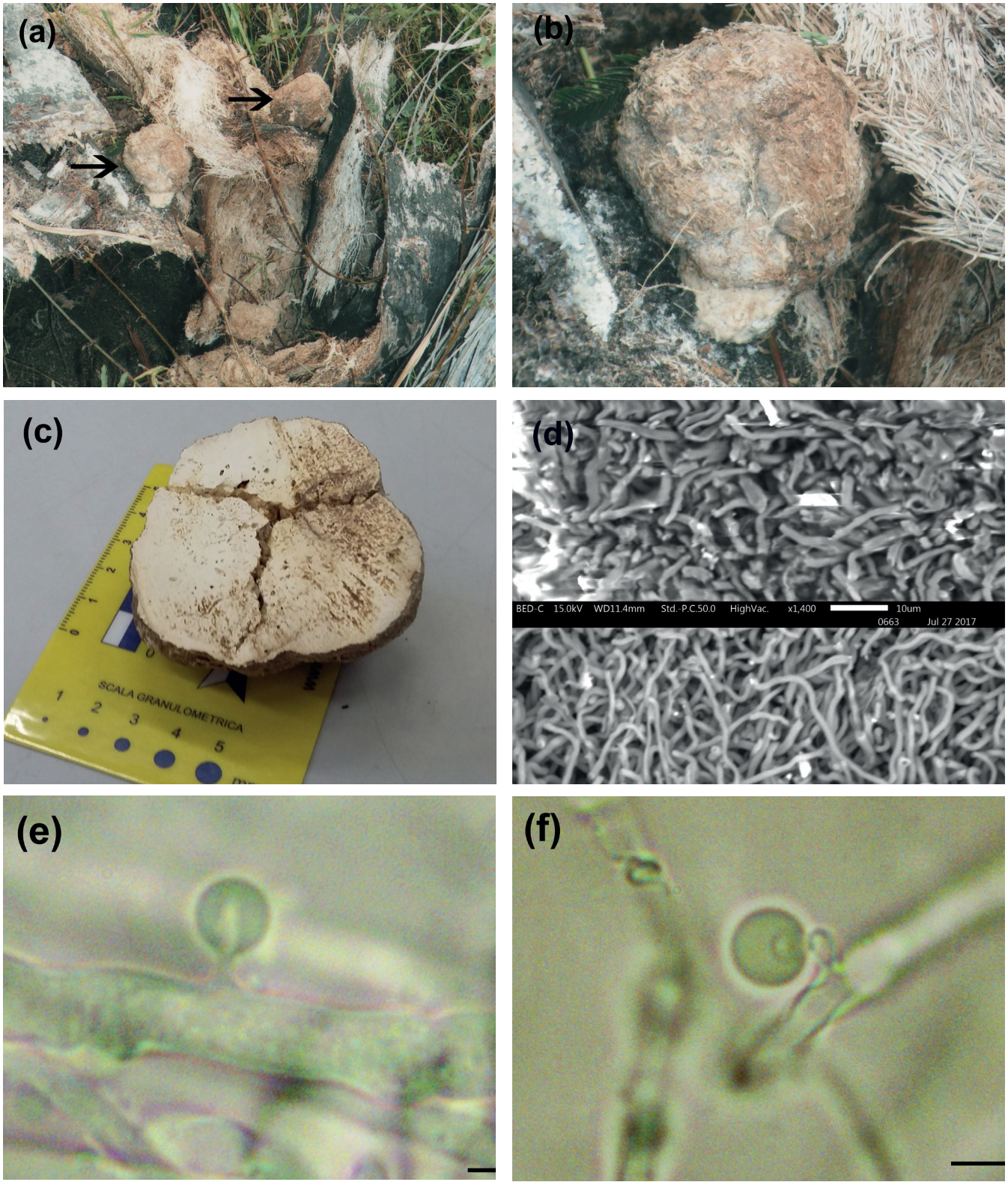

Figure 1. Morphological characters of the *Pleurotus tuber-regium* sclerotium. (a) Two sclerotia, indicated by the two arrows, in habitat (*Raphia farinifera* log). (b) Magnification of the left studied sclerotium. (c) Section of this sclerotium. (d) Hyphae of external layer of the cortex and of the medulla (respectively in the upper and in the lower part of the picture) observed under SEM microscopy. (e, f) Nematode-trapping structures in the mycelium isolated from the sclerotium observed under light microscope. Bars: e, f=10 µm.

twentieth century on the puzzling *Podoserpula pusio* (Buyck 1997). Then Buyck et al. (1998, 2007), Eyssartier & Buyck (1999a, b), Eyssartier et al. (2001) and Antonín & Buyck (2006) provided important contributions to the knowledge of Madagascar mycobiota. While Buyck (2002) provided an overview of the various habitats and hosts for Madagascar's ectomycorrhizal genus *Russula*, Antonín et al. (2005), Duhem & Buyck (2007) and Buyck (2008) reported the most common edible mushrooms from Madagascar. Recently, Shay et al. (2017) studied the biodiversity and phylogeny of the mushroom genus *Marasmius* from Madagascar, documenting 35 species, five of which are new species and 11 are new records for Madagascar. Lichenized fungi were studied and listed by Aptroot (2016).

The aim of the present contribution was to characterize by means of a combined morphological and molecular approach a fungal sclerotium collected in Madagascar and sent to our lab for identification.

Material and Methods

Two sclerotia were collected on the 26 of July 2015 buried in a dead trunk of *Raphia farinifera* (Arecaceae) in a lowland moist forest in Antsohihy (Mahajanga, Sofia) (Fig. 1a); one (Fig. 1b) was sent to our lab where was: i) characterized by light and scanning electron microscope (SEM), ii) used for molecular analysis, iii) mycelium isolation and its morphological observations. A sample of the sclerotium was deposited in the Herbarium Universitatis Taurinensis as TO270715.

Microscopic preparation

For the light microscopic observations, thin hand made sections of the outer and inner parts of the sclerotium were prepared with a razorblade and mounted in 10% KOH. Size range of the microscopic elements is based on 20 measurements.

For the SEM analysis, the sclerotium was cut by a precision cut-off machine generally used for rock samples, in this way coarse slices of the sample have been obtained; thick slices have been successively reduced in 1 mm thick slices suitable for SEM observations through a razorblade. Selected slices from the inner part to the cortex have been later fixed on pin stubs using a Carbon Conductive Cement glue. Carbon sputtering has been then performed on fixed samples. SEM observations have been obtained using a JEOL JSM IT300LV (High Vacuum – Low Vacuum 10/650 Pa - 0.3-30kV) Scanning Electron Microscope operating at 10 kV.

DNA extraction, PCR amplification, and DNA sequencing

Genomic DNA was extracted from 100 mg of a sclerotium by means of the DNeasy Plant Mini Kit (Qiagen, Milan Italy) according to the manufacturer's instructions. Universal primers ITS1F/ITS4 were used for the nrITS region amplification (White et al., 1990; Gardes & Bruns, 1993). Amplification reactions were carried out in PE9700 thermal cycler (Perkin-Elmer, Applied Biosystems) in a 50 µl reaction mixture using the following final concentrations or total amounts: 5 ng DNA, 1×PCR buffer (20 mM Tris/HCl pH 8.4, 50 mM KCl), 1 µM of each primer, 2.5 mM MgCl2, 0.25 mM of each dNTP, 1 unit of Taq polymerase (Promega). The PCR program was as follows: 3 min at 95 °C for 1 cycle; 30 s at 94 °C, 45 s at 50 °C, 2 min at 72 °C for 35 cycles, 10 min at 72 °C for 1 cycle.

PCR products were resolved on a 1.0 % agarose gel and visualized by staining with ethidium bromide. PCR products were purified and sequenced forward and reverse by MACROGEN Inc. (Seoul, Republic of Korea). Sequence assembly and editing were carried out using SeqTrace v 0.9.0 (Stucky 2012). In order to check the identity of the mycelium isolate in pure culture from the sclerotium its genomic DNA was extracted and the nrITS region was amplified as described above.

The sequence was submitted to http://www.ebi.ac.uk/ under the accession number LT992866.

Sequence alignment and phylogenetic analysis

The sequence obtained in this study was compared to those available in the GenBank database (https://www.ncbi.nlm.nih.gov/genbank/) by using the Blastn algorithm (Altschul et al., 1990). Sequences for the phylogenetic analysis dataset were selected according with the Blastn results and the outcomes of recent phylogenetic studies on *Pleurotus tuber-regium* (Isikhuemhen et al., 2000a; Karunarathna et al., 2016). Alignment was originated using MUSCLE (Edgar 2014) with default conditions for gap openings and gap extension penalties.

The alignment was then imported into MEGA v6.0 (Tamura et al. 2013) for manual adjustment, and phylogenetic analysis was performed using the Maximum likelihood (ML) approach, through 1000 bootstrap replicates (Felsenstein, 1985). ML was carried out based on the General Time Reversible (GTR) model (Nei & Kumar, 2000) to perform a tree inference and search for a good topology. Support values from bootstrapping runs (MLB) were mapped on the globally best tree and only MLB values over 50% are reported in the resulting tree (Fig. 2). *Hohenbuehelia petaloides* (KT388021) was used as outgroup taxon according with Thorn et al. (2000).

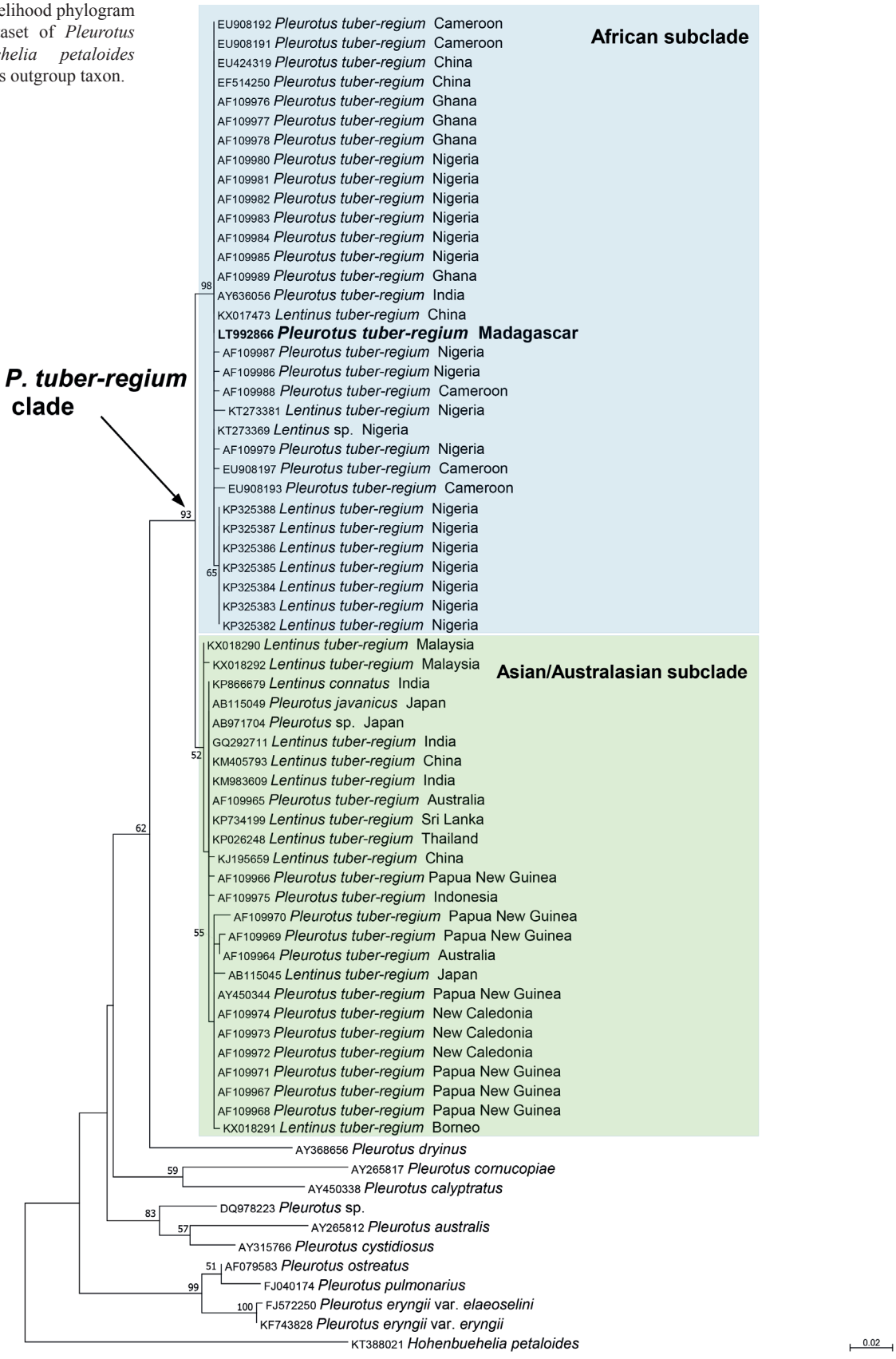

Figure 2. Maximum likelihood phylogram based on a nrITS dataset of *Pleurotus* sequences. *Hohenbuehelia petaloides* (KT388021) was used as outgroup taxon.

Mycelium isolation and morphological characterization

Mycelium was isolated, under sterile condition, by inoculating small pieces of sclerotium tissue (approx. 5 mm of diameter) in 90 mm plates containing Malt extract agar (MEA, malt extract 20 g, peptone from casein, acid digest 5 g, deionized water 1L) added by the antibiotics gentamycin 40 mg/L and chloramphenicol 50 mg/L and incubated at room temperature. DNA sequencing was performed as for the sclerotium to confirm that its sequence was fully congruent to that from the mycelium. Small portions of the mycelium were put on a slide and observed under a light microscope.

Results

The sclerotium collected in Madagascar was first characterized by macro- and micro morphological observations and then used for molecular identification of the species. In parallel it was successfully used for the isolation of the mycelium which was morphologically characterized.

Morphology of the sclerotium

At a macroscopic observation, the sclerotium appears subglobose, 6.5 cm diam., surface ochre to light brown, velutinous-felted, while the interior (medulla) is whitish, hard and homogeneous (Fig. 1b,c). At light microscope the cortex is about 120 μm thick and two-layered. The outer layer measures 40–50 μm and is made of very thick-walled, brown, 2–4 μm wide hyphae, with simple septa and narrow lumen, cemented together by a resinous matter that incorporates plant debris. The inner layer is paler, 70–80 μm thick, without resinous matter, and consisting of interwoven and sinuous hyphae. The medulla is formed by thinner hyphae, 2–3 μm wide coralloid, 10–15 μm wide hyphae and some irregularly formed storage cells (data not shown). Difference between hyphae in the cortex and medulla are shown in Fig. 1d.

Molecular outcomes

The species identification was done on the sequence obtained from the sclerotium that was fully congruent to the sequence obtained later from the mycelium. Comparison of the nrITS sequence with the Genebank database showed high similarity with *Pleurotus tuber-regium* (Blastn result = 99% with *Pleurotus tuber-regium,* query cover = 100%, E-value = 0.0). The phylogenetic analysis performed based on the Blastn results clearly revealed that all the ITS sequences of *P. tuber-regium* present in the GenBank database cluster in a strongly supported major clade (bootstrap value = 93%); two subclades are further recognizable within the major clade (Fig. 2): one of mainly African origin (bootstrap value = 98%), the other one of Asian and Australasian origin (bootstrap value = 52%). Our collection from Madagascar falls in the African subclade.

Characterization of the mycelium

The aerial hyphae of the mycelium isolated from the sclerotium, and observed after 14 days from inoculation show evident and numerous clamp-connections and above all the presence of nematode-trapping structures as blastoconidia-like spherical to ovoid knobs (Fig. 1e,f). Their shape fully fits that of similar structures found in *P. tuber-regium* (Hibbett & Thorn, 1994) and in other *Pleurotus* species (Thorn & Tsuneda, 1993; Thorn et al., 2000; Truong et al., 2007).

Discussion

A combination of morphological and molecular analysis has allowed us to characterize a sclerotium collected in the north of Madagascar, and to isolate and characterize its mycelium. The molecular analysis has assigned the sclerotium to the species *Pleurotus tuber-regium* on the basis of sequences coming from African and Australasian-Pacific isolates, except sequences coming from Madagascar. The absence in Genbank of sequences from this island clearly indicates that this is the first molecular identification of *Pleurotus tuber-regium* from Madagascar. The morphological observations of the mycelium isolated from the sclerotium have revealed nematode-trapping structures typical of the genus *Pleurotus*. They consist of very small cells on a short stalk produced laterally on hyphae and surrounded by a toxin-containing liquid droplet capable of killing or irreversibly paralyzing nematodes (Saikawa & Wada, 1986; Thorn & Tsuneda, 1993; Satou et al., 2008). Clémençon (2004) proposed the term "toxocysts" for these nematocidal structures of *Pleurotus* and the nematotoxic compound of *P. ostreatus* was identified as trans-2-decenedioic acid (Kwok et al. 1992).

Taxonomy, morphology, ethnomycology, and distribution of *P. tuber-regium*

The species was originally described as *Agaricus tuber-regium* by Fries (1821), who quoted a figure by Rumpf based on a collection from Moluccas Islands (Indonesia) (Rumpf 1750), while the sclerotium state was named as *Pachyma tuber-regium* by Fries (1822). Later, the taxon was named *Lentinus pachymae* Fr., (Fries 1825) and then sanctioned as *Lentinus tuber-regium* (Fr.) Fr. (Fries 1836).

The genus attribution of *P. tuber-regium* has been long debated. Because it is characterized by leathery basidiomes with a dimitic hyphal system (with numerous intercalary thick-walled skeletal hyphae that are especially well-developed in the stipe), leading authorities in lentinoid fungi placed it in either *Panus* (Corner, 1981) or *Lentinus* (Pegler, 1972, 1975, 1983). But when *P. tuber-regium* was demonstrated to produce nematode trapping droplets in aerial hyphae in agar cultures (Hibbett & Thorne, 1994), structures that are unique to the genus *Pleurotus* (Thorn & Barron, 1984; Barron & Thorn, 1987), it was evident its classification in *Pleurotus* as previously proposed by Singer (1951, 1986). The presence of these structures is also confirmed in our study (Fig. 1e,f). This taxonomic placement was later supported by molecular phylogenetic analyses, where *P. tuber-regium* is nested in the genus *Pleurotus* (e.g. Hitoshi & Takao, 1995; Vilgalys et al., 1996; Moncalvo et al., 2002; Thorn et al., 2000; Matheny et al., 2006). Consequently, the updated systematic position of the species is: genus *Pleurotus* (Fr.) P. Kumm. (Singer 1951), family Pleurotaceae Kühner (Thorn et al. 2000), suborder Pleurotineae Aime, Dentinger & Gaya (Dentinger et al. 2016) of the order Agaricales Underw. (Matheny et al., 2006).

Within *Pleurotus*, *P. tuber-regium* is aberrant in that its basidiomes arise not from wood directly (Hilber, 1982) or from roots and lower stem residues of Apiaceae (umbellifers) plants (Zervakis et al 2014), but from true sclerotia (Cohn & Schröter, 1891 as *L. woermanni*, Isikhuemhen et al., 2000b). The structure of the sclerotia of *P. tuber-regium* was studied in detail by Bommer (1894, under the name *Lentinus woermannii* Cohn & J. Schröt.). Our observations of the sclerotium revealed that it is characterized by hyphal carpet from medulla to cortex, in agreement with his observations. In fact sclerotia may grow to considerable size (up to 25 cm diam.), are very dense and quite resistant to long periods of desiccation, and have been shown to support successive basidiomes fruiting over consecutive seasons (Isikhuemhen et al., 2000b; Wong & Cheung, 2008). Corner (1981), Pegler (1983) and Karunarathna et al. (2016) stated that the sclerotia of *P. tuber-regium* develop above ground in logs, within rotten wood (where its mycelium produces a white-rot), then might fall to the ground and become covered with soil. Observations by Hibbett & Thorne (1994) do not support this view and indicate that the sclerotia were probably formed directly underground (buried approximately 10 cm underground). Our collection, carried out at a certain height in a dead trunk of *Raphia farinifera,* would make us lean towards the first hypothesis.

Both sclerotia and basidiomes (when young and not of leathery consistency) of *P. tuber-regium* are used for food and medicinal purposes in tropical Africa and Asia (Heim, 1935; Thoen et al., 1973; Zoberi, 1973; Oso, 1975, 1977; Corner, 1981; Pegler, 1983; Nwokolo, 1987; Okhuoya & Ajerio, 1988; Buyck, 1994, 2008; Isikhuemhen & Okhuoya, 1996; Okhuoya et al., 1998; Stametz, 2000; Wong & Cheung, 2008; Dai et al., 2009). In Papua New Guinea, the Gogodala people use its carved sclerotia for making magic club-heads which are attached to wooden clubs (Price et al., 1978). Given its beneficial properties it is widely cultivated on different agricultural wastes (Karunarathna et al. 2016).

The geographic distribution of *P. tuber-regium* includes most of equatorial Africa, India, Sri Lanka, southeast Asia and north Australia, as well as the southern Pacific (Kobayasi et al., 1973; Pegler, 1983; Isikhuemhen et al., 2000 a,b; Karunarathna et al., 2016).

The presence of *P. tuber-regium* in Madagascar, mainly based on old (spanning from 1849 to 1938) and only morphology-based reports on basidiome presence (Heim, 1935; Decary, 1938; Pegler, 1983) is here confirmed by molecular approach (Fig. 2).

Subclades and phylogeny

The phylogenetic analysis with poor taxon-sampling by Isikhuemhen et al., (2000a) strongly associated geographic isolation with patterns of genetic divergence in *P. tuber-regium*; in particular, it indicated that African and Australasian-Pacific isolates of *P. tuber-regium* form at least two distinct evolutionary lineages (clades) even though mating compatibility studies (studies of cross fertility) highlighted that the geographically distinct populations were compatible and suggesting that *P. tuber-regium* (characterized by a tetrapolar mating system) represents a unique intersterility group in *Pleurotus*. Consequently, Isikhuemhen et al. (2000a) recognized the two lineages as a unique species, *P. tuber-regium*, structured into two populations of different geographical distribution.

In our analysis (Fig. 2), we recovered the same two distinct subclades mainly consisting of the collections named as "*P. tuber-regium*" or "*L. tuber-regium*" retrieved from GenBank. The African subclade consists of collections from Africa with the exceptions of EU424319, EF514250 and KX017473 from China and AY636056 from India; the Asian/Australasian subclade encompasses all Asian and Australasian collections. The collection from Madagascar clustered in the first clade. Also two collections named as *Lentinus connatus* (India, KP866679) and *Pleurotus javanicus* (Japan AB115049) clustered in the Asian/Australasian subclade of *P. tuber-regium*. *Pleurotus javanicus* (Lév.) Singer (= *Lentinus connatus* Berk. and *L. infundibuliformis* Berk. & Broome, according with Corner, 1981) is a species signaled in south-east Asia and Australasia (Petch, 1915; Corner, 1981; Pegler, 1983; Sharma et al.,

2015) on rotting trunks and dead wood (often buried), and said to differ from the morphologically allied *P. tuber-regium* only by a slender stipe (3–15 mm vs. 7–35 mm), minutely pubescent (vs. tomentose to subsquamulose) and never arising from a sclerotium but sometimes developing from an irregular-shaped woody pseudosclerotium (a structure consisting of mycelium that merely binds together dead wood substratum of the host plant in a large compact mass) and short cylindric, 6–8.5 × 2.5–3.5 µm spores (vs. elongate cylindric, 7.5–10.5 × 3–4.2 µm spores) (Pegler, 1983, Sharma et al., 2015). Corner, in his monographic work on the genera *Lentinus*, *Panus* and *Pleurotus*, stated that "The species is extremely close to *Pa. tuber-regium* and their specific difference, if tenable, needs investigation" (Corner 1981, p. 94). In addition, the same author reported that for some collections of *P. tuber-regium* from the Solomons islands, "I could find no sclerotium, and the fruit-bodies of others grew directly from rotten trunks without any trace of sclerotium or pseudosclerotium" (Corner 1981, p. 101). In this regard, our phylogenetic analysis would indicate (Fig. 2) a conspecificity between *P. javanicus* and *P. tuber-regium*, and therefore there would be a single species that, depending on so far unknown environmental conditions, can form sclerotia in some cases, in other pseudosclerotia or do not form resistance structures at all. Consequently, it is likely that too much importance has been attributed to the presence of sclerotia and sclerotia-like structures and to its stability within the same species. The value of these characters as taxonomic markers should be carefully re-evaluated also because the mechanisms underlying their development are still poorly investigated. Already Petch, about the possibility for a *Lentinus* species to be able to produce different resistance structures, wrote: "Whether these three types are definitely associated with different species of *Lentinus*, or whether they are merely stages which may be assumed by the sclerotium of any one species, must remain an open question on the available evidence" (Petch 1915, p. 17). Finally, to what taxonomic rank should the two subclades within *P. tuber-regium* be considered? Given the lack of distinctive morphological characters between the collections belonging to the two geographical groups and their inter-compatibility state, Isikhuemhen et al. (2000a) concluded to consider them as conspecific. We now know that the ability of the mycelia to interbreed is not a definitive prove of conspecificity. First of all, mating behavior in fungal species depends not only on the compatibility, but also on environmental factors such as habitat/medium, illumination, pH, humidity, and temperature and other factors (Kerényi et al., 2004). Furthermore, that the ability to interbreed implies conspecificity is not necessarily true (Brasier, 1997). There are several reasons why species recognition based on molecular data (GCPSR, Genealogical Concordance Phylogenetic Species Recognition, Taylor et al. 2000) is better in revealing cryptic species than that based on the intersterility criterion (BSC, Biological Species Criterion, Taylor et al., 2000). Indeed it is well known that the divergence of DNA sequences used in species recognition may occur before intersterility has evolved (Petersen & Hughes, 1999; Taylor et al., 2000; Cai et al., 2011; Giraud & Gourbière, 2012; Liu et al., 2016) and thus be more useful and restrictive to distinguish closely related cryptic (sibling) species. Certain mechanisms of reproductive prezygotic isolation may allow intersterility to evolve much later than the divergence of DNA, again rendering the GCPSR more finely discriminating than the BSC (Le Gac & Giraud 2008; Giraud et al., 2008a, b, 2010). In such cases, close species may remain interfertile for some time, making *in vitro* crosses a quite permissive and inadequate tool for recognizing species.

Data from literature give indications that the application of the molecular species identification method has led and will lead to the recognition and circumscription of far more species than have already been recognized using morphological analyses or cross fertility tests (Cai et al., 2011; Liu et al., 2016).

Therefore, the future sequencing of additional molecular markers will allow to ascertaining whether the Asian/Australasian and African collections represent populations of the same species (then articulated into two subspecies on geographical basis) or two distinct cryptic species. In the latter case, the name *P. tuber-regium* should only be restricted and used for the Asian/Australasian collections as Rumpf (1750) originally described the species from the Moluccas islands (Indonesia), while a new species name will have to be established for the collections in the African subclade.

In conclusion this study provides a glimpse on a fungus collected in Madagascar and here characterized, as well as a short contextualization of this species along previous studies.

Acknowledgements

The collection was possible to the Malagasy people who collected the sample and kindly provided its pictures.

References

Altschul S.F., Gish W., Miller W., Myers E.W., Lipman D.J., 1990. Basic local alignment search tool. Journal of Molecular Biology 215, 403 – 410.

Antonín V., Buyck B., 2006. *Marasmius* (Basidiomycota, Marasmiaceae) in Madagascar and the Mascarenes. Fungal Diversity 23, 17 – 50.

Antonín V., Buyck B., Randrianjohany E., Duhem B., 2005. Edible mushrooms from Madagascar (1): notes on *Collybia tamatavae*. Cryptogamie Mycologie 26, 105 – 111.

Aptroot A., 2016. Preliminary checklist of the lichens of Madagascar, with two new thelotremoid Graphidaceae and 131 new records. Willdenowia 46, 349 – 365.

Barron G.L., Thorn R.G., 1987. Destruction of nematodes by species of *Pleurotus*. Canadian Journal of Botany 65, 774 – 778.

Bommer C.H., 1894. Sclérotes et cordons mycéliens. Mémoires couronnés et Mémoires des savants, Académie Royale des Sciences, des Lettres et des Beaux-Arts de Belgique 54, 1 – 116.

Brasier C.M., 1997. Fungal species in practice: Identifying species units in fungi. In: MF Claridge, HA Dawah, MR Wilson (Eds) Species: The Units of Biodiversity, pp 135–170. Chapman & Hall, London, UK.

Buyck B., 1994. Les champignons comestibles de l'Ouest du Burundi. Publications agricoles N° 34. A.G.C.D., Bruxelles, Belgium.

Buyck B., 1997. *Podoserpula pusio*. Vies en Danger. Espèces disparues, espèces menaces (CD–ROM). Muséum National d'Histoire naturelle. Ed. Emme interactive, Paris, France.

Buyck B., 2002. Preliminary observations on the diversity and habitats of *Russula* (Russulales, Basidiomycotina) in Madagascar. Micologia e Vegetatione Mediterranea 162, 133 – 147.

Buyck B., 2008. The edible mushrooms of Madagascar: an evolving enigma. Economic Botany 62, 509 – 520.

Buyck B., Verbeken A., Eberhardt U., 2007. The Genus *Lactarius* in Madagascar. Mycological Research 1117, 787 – 798.

Buyck B., Eyssartier G., Duhem B., 1998. Contribution à un inventaire mycologique de Madagascar. I. Bulletin de la Société mycologique de France 114, 33 – 59.

Cai L., Giraud T., Zhang N., Begerow D., Cai G., Shivas R.G., 2011. The evolution of species concepts and species recognition criteria in plant pathogenic fungi. Fungal Diversity 50, 121.

Clémençon H., 2004. Cytology and plectology of the Hymenomycetes. Bibliotheca Mycologica 199, 1 – 488.

Cohn F., Schröter J., 1891. Untersuchungen über *Pachyma* und *Mylitta*. Abhandlungen des Naturwissenschaftlichen Vereins in Hamburg 11, 1 – 16.

Cooke M.C., Massee G., 1890. Fungi of Madagascar. Grevillea 18, 49 – 51.

Corner E.J.H., 1981. The agaric genera *Lentinus*, *Panus*, and *Pleurotus*. Beih. Nova Hedwigia 69, 1 – 189.

Dai Y.C., Yang Z.L., Cui B.K., Yu C.J., Zhou L.W., 2009. Species diversity and utilization of medicinal mushrooms and fungi in China (Review). International Journal of Medicinal Mushrooms 11, 287 – 302.

Decary R., 1938. L'Olatafa, champignon de Madagascar. Revue de Mycologie 3(2–3), 91 – 93.

Dentinger B.T.M., Gaya E., O'Brien H., Suz L.M., Lachlan R., Díaz-Valderrama J.R., Koch R.A., Aime M.C., 2016. Tales from the crypt: genome mining from fungarium specimens improves resolution of the mushroom tree of life. Biological Journal of the Linnean Society 117, 11 – 32.

Duhem B., Buyck B., 2007. Edible mushrooms from Madagascar (2). *Clavulina albiramea* comb. nov. (Cantharellales), an edible clavarioid fungus shared between African miombo and Malagasy Uapaca woodland. Nova Hedwigia 85 (3–4), 317 – 330.

Edgar R.C., 2004. MUSCLE: a multiple sequence alignment method with reduced time and space complexity. BMC Bioinformatics 5, 113.

Eyssartier G., Buyck B., 1999a. Contribution à un inventaire mycologique de Madagascar. II. Nouveaux taxons dans le genre *Cantharellus*. Mycotaxon 70, 203 – 211.

Eyssartier G., Buyck B., 1999b. Contribution à un inventaire mycologique de Madagascar. III. Cryptogamie Mycologie 20, 11 – 16.

Eyssartier G., Buyck B., Courtecuisse R., 2001. New species and combinations in cuboid-spored *Entoloma* species from Madagascar. Mycological Research 105, 1144 – 1148.

Felsenstein J., 1985. Confidence limits on phylogenies: an approach using the bootstrap. Evolution 39, 783 – 791.

Fries EM., 1821. Systema Mycologicum. 1. Lundae, 520 p.

Fries EM., 1822. Systema Mycologicum. 2. Gryphiswaldiae, 274 p.

Fries EM., 1825. Systema orbis vegetabilis. Primas lineas novae constructionis periclitator. Pars 1, Plantae Homonemeae. Lundae, 374 p.

Fries EM., 1836 Synopsis generis Lentinorum. Upsaliae, 18 p.

Ganzhorn J.U., Lowry II P.P., Schatz G., Sommer S., 2008. The biodiversity of Madagascar: one of the world's hottest hotspots on its way out. Oryx 35, 346 – 348.

Gardes M., Bruns T.D., 1993. ITS primers with enhanced specificity for basidiomycetes – application to the identification of mycorrhizae and rusts. Molecular Ecology 2, 113 – 118.

Giraud T., Gourbière S., 2012. The tempo and modes of evolution of reproductive isolation in fungi. Heredity 109, 204 – 214.
Giraud T., Refrégier G., de Vienne D.M., Le Gac M., Hood ME., 2008a. Speciation in fungi. Fungal Genetics and Biology 45, 791 – 802.

Giraud T., Yockteng R., López-Villavicencio M., Refrégier G., Hood ME., 2008b. Mating system of the anther smut fungus, *Microbotryum violaceum*. Eukaryotic Cell 7, 765 – 775.

Giraud T., Gladieux P., Gavrilets S., 2010. Linking the emergence of fungal plant diseases with ecological speciation. Trends in Ecology Evolution 25, 387 – 395.

Glaw F., Köhler J., Townsend T.M., Vences M., 2012. Rivaling the microendemic new species of leaf chameleons (*Brookesia*) from northern Madagascar. PLoS ONE 7.

Goodman S.M., Beanstead J.P. (Eds), 2003. The Natural History of Madagascar. University of Chicago Press, Chicago, USA.

Heim R., 1935. L'Olatafa. Archives du Muséum d'Histoire Naturelle, Paris 6 série, 12, 549 – 553.

Heim R., 1936. Observations sur la flore mycologique malgache. III. Trois bolets gigantesques d'Afrique et de Madagascar. Revue de Mycologie (new series) 1, 2 – 18, planches I–IV.

Heim R., 1938a. Les Lactario–Russulés du domaine oriental de Madagascar. Prodromes à une flore mycologique de Madagascar et Dépendances I. Muséum National d'Histoire Naturelle (MNHN), Paris, France.

Heim R., 1938b. Observations sur la flore mycologique malgache. VI. Les champignons des termitières. Première note: Basidiomycètes. Boletim da Sociedade Broteriana 13 (second series), 45 – 63.

Heim R., 1945. Les agarics tropicaux à hyménium tubulé (Madagascar, Côte d'Ivoire, Guinée, Antilles, Insulinde). Revue de Mycologie 10, 3 – 61.

Hennings P., 1893. Fungi Africani II. Engler's Botanische Jahrbuecher 17, 1 – 42.

Hennings P., 1908. Fungi von Madagaskar, den Comoren und Ostafrika. In: Sonderuck aus Voeltzkow. Reise Ostrafrika in den Jahren 1903–1905, Band III. pp. 16 – 33.

Hibbett D.S., Thorn R.G., 1994. Nematode trapping in *Pleurotus tuberregium*. Mycologia 86, 696 – 699.
Hilber O., 1982. Die Gattung *Pleurotus*. J. Cramer, Vaduz, Liechtenstein.

Hitoshi N., Takao N., 1995. Phylogenetic analysis of *Pleurotus* based on data from partial sequences of 18rDNA and ITS-1 regions. Mushroom Science 14, 161 – 168.

Isikhuemhen O.S., Okhuoya J.A., 1996. Cultivation of edible sclerotia of *Pleurotus tuberregium* (Fr.) Singer on agricultural wastes. In Proceedings of the 2nd International Conference on Mushroom Biology and Mushroom Products, 9 – 12 June 1996 (D. J. Royse, ed.): 429–436. Pennsylvania State University, University Park, PA, USA.

Isikhuemhen O.S., Moncalvo J., Nerud F., Vilgalys R., 2000a. Mating compatibility and phylogeography in *Pleurotus tuberregium*. Mycological Research 104, 732 – 737.

Isikhuemhen O.S., Nerud F., Vilgalys R., 2000b. Cultivation studies on wild and hybrid strains of *Pleurotus tuberregium* (Fr.) Sing. on wheat straw substrate. World Journal of Microbiology and Biotechnology 16, 431 – 435.

Karunarathna S.C., Mortimer P.E., Chen J., Li G.J., He M.Q., Xu J.C., Seelan J.S.S., Hassan B.A., Hyde K.D., Zhao R.L., 2016. Correct names of two cultivated mushrooms from the genus *Pleurotus* in China. Phytotaxa 260, 36 – 46.

Kerényi Z., Moretti A., Waalwijk C., Oláh B., Hornok L., 2004. Mating type sequences in asexually reproducing

Fusarium species. Applied and Environmental Microbiology 70, 4419 – 4423.

Kobayasi Y., Otani Y., Hongo T., 1973. Some higher fungi found in New Guinea. Mycological reports from New Guinea and the Solomon Islands. - 14. Reports of the Tottori Mycological Institute (Japan) 10, 341 – 356.

Kwok O.C.H., Plattner R., Weisleder D., Wicklow D.T. 1992. A nematicidal toxin from *Pleurotus ostreatus* NRRL 3526. Journal of Chemical Ecology 18, 127 – 136.

Le Gac M., Giraud T., 2008. Existence of a pattern of reproductive character displacement in Basidiomycota but not in Ascomycota. Journal of Evolutionary Biology 21, 761 – 772.

Liu F., Wang M., Damm U., Crous PW., Cai L., 2016. Species boundaries in plant pathogenic fungi: a *Colletotrichum* case study. BMC Evolutionary Biology 16, 81.

Matheny P.B., Curtis J.M., Hofstetter V., Aime M.C., Moncalvo J.M., Ge Z.W., Yang Z.L., Slot C., Ammirati J.F., Baroni T.J., Bougher N.L., Hughes K.W., Lodge D.J., Kerrigan R.W., Seidl M.T., Aanen D.K., DeNitis M., Daniele G.M., Desjardin D.E., Kropp B.R., Norvell L.L., Parker A., Vellinga E.C., Vilgalys R., Hibbett D.S., 2006. Major clades of Agaricales: a multilocus phylogenetic overview. Mycologia 98, 982 – 995.

Métrod G., 1949. Les Mycènes de Madagascar (*Mycena*, *Corrugaria*, *Pterospora*). Laboratoire de Cryptogamie du Museum National d'Histoire Naturelle, Paris, France.

Moncalvo J.M., Vilgalys R., Redhead S.A., Johnson J.E., Jame T.Y., Aime M.C., Hofstetter V., Verduin S.J.W., Larsson E., Baroni T.J., Thorn R.G., Jacobsson S., Clemencon H., Miller Jr. O.K., 2002. One hundred seventeen clades of euagarics. Molecular Phylogenetics and Evolution 23, 357 – 400.

Myers N., Mittermeier R.A., Mittermeier C.G., da Fonseca G.A.B., Kent J., 2000. Biodiversity hotspots for conservation priorities. Nature 403, 853 – 858.

Nei M., Kumar S., 2000. Molecular evolution and phylogenetics. Oxford University Press, Oxford, UK.

Nwokolo E., 1987. Composition of nutrients in the sclerotium of the mushroom *Pleurotus tuberregium*. Plant Foods for Human Nutrition 37, 133 – 139.

Okhuoya JA, Ajerio C., 1988. Analysis of sclerotia and sporophores of *Pleurotus truber-regium* Fr. an edible mushroom in Nigeria. The Korean Journal of Mycology 6(4), 204 – 206.

Okhuoya J.A., Isikhuemhen O.S., Evue G.A., 1998. *Pleurotus tuberregium* (Fr.) Sing.: Sclerotia and sporophore yield during cultivation on sawdust of different woody plants. International Journal of Mushroom Science 2, 41 – 46.

Oso B.A., 1975. Mushrooms and the Yoruba people of Nigeria. Mycologia 67, 311 – 319.

Oso B.A., 1977. *Pleurotus tuberregium* from Nigeria. Mycologia 69, 271 – 279.

Patouillard N., 1924. Basidiomycetes nouveaux de Madagascar. Bulletin du Muséum National D'Histoire Nature 13, 526 – 532.

Patouillard N., 1928. Contribution a l' étude des champignons de Madagascar. Mémoires de l'Académie Malagache 6, 7 – 49.

Pegler D.N., 1972. Lentineae (Polyporaceae), Schizophyllaceae et especes lentinoides et pleurotoides des Tricholomataceae. Flore illustrée des champignons d›Afrique centrale 1, 1 – 26.

Pegler D.N., 1975. The classification of the genus *Lentinus* Fr. (Basidiomycota). Kavaka 3, 11 – 20.

Pegler D.N., 1983. The genus *Lentinus*: a world monograph. HMSO, London, UK.

Petch T., 1915. The pseudo-sclerotia of *Lentinus similis* and *Lentinus nfundibuliformis*. Annals of the Royal Botanic Gardens, Peradeniya 6, 1 – 18.

Petersen R.H., Hughes K.W., 1999. Species and speciation in mushrooms. Bioscience 49, 440 – 452.

Price T.V., Baldwin J.A., Simpson J.A., 1978. Fungal club-heads in Papua New Guinea. Nature 273, 374 – 375.

Radespiel U., Ratsimbazafy J.H., Rasoloharijaona S., Raveloson H., Andriaholinirana N., Rakotondravony R., Randrianarison R.M., Randranabinina B., 2012. First indications of a highland specialist among mouse lemurs (*Microcebus* spp.) and evidence for a new mouse lemur species from eastern Madagascar. Primates 53,157 –170.

Romagnesi H., 1941. Les Rhodophylles de Madagascar. Laboratoire de cryptogamie du Muséum national d'histoire naturelle, Paris, France.

Rumpf G.E. (Rumphius), 1750. Herbarium Amboinense. Vol. 6. Ed. J. Burmann, Amsterdam, The Netherlands, 256 p.
Saikawa M., Wada N., 1986. Adhesive knobs in *Pleurotus ostreatus* (the oyster mushroom), as trapping organs for

nematodes. Transactions of the Mycological Society of Japan 27, 113 – 118.

Satou T., Kaneko K., Li W., Koike K., 2008. The toxin produced by *Pleurotus ostreatus* reduces the head size of nematodes. Biological & Pharmaceutical Bulletin 31, 574 – 576.

Schatz G.E., Lowry PP II, Lescot M., Wolf A.-E., Andriambololonera S., Raharimalala V., Raharimampionona J., 1996. Conspectus of the vascular plants of Madagascar: a taxonomic and conservation electronic database. In: LJG van der Maesen, XM van der Burgt, JM van Medenbach de Rooy (Eds) The Biodiversity of African Plants, pp 10–17. Kluwer, Wageningen, The Netherlands.

Sharma V.P., Upadhyay R.C., Kamal S., Kumar S., Mohapatra K.B., Sharma M., 2015. Characterization, cultivation, nutritional and antioxidant properties of the culinary edible mushroom *Lentinus connatus*. Sydowia 67, 167 – 174.

Shay J.E., Desjardin D.E., Perry B.A., Grace C.L., Newman D.S., 2017. Biodiversity and phylogeny of *Marasmius* (Agaricales, Basidiomycota) from Madagascar. Phytotaxa 292, 101 – 149.

Singer R., 1951. The Agaricales (mushrooms) in modern taxonomy. Lilloa 22, 5-832.

Singer R., 1986. The Agaricales in modern taxonomy. 4th ed., Koeltz Scientific Books, Koenigstein, Germany, 981 p.

Stamets P., 2000. Growing gourmet and medicinal mushrooms. Ten Speed Press, Berkeley CA, USA, 574 p.

Stucky B.J., 2012. SeqTrace: A graphical tool for rapidly processing DNA sequencing chromatograms. Journal of Biomolecular Techniques 23, 90 – 93.

Tamura K., Stecher G., Peterson D., Filipski A., Kumar S., 2013. MEGA 6: molecular evolutionary genetics analysis version 6.0. Molecular Biology and Evolution 30, 2725 – 2729.

Taylor J.W., Jacobson D.J., Kroken S., Kasuga T., Geiser D.M., Hibbett D.S., Fisher M.C., 2000. Phylogenetic species recognition and species concepts in Fungi. Fungal Genetics and Biology 31, 21 – 32.

Thoen D., Parent G., Lukengu T., 1973. L'usage des champignons dans le Haut-Shaba (République du Zaïre). Bulletin trimestriel du CEPSE "Problèmes Sociaux Zaïrois" 100/101, 69 – 85.
Thorn R.G., Barron G.L., 1984. Carnivorous mushrooms. Science 224, 76 – 78.

Thorn R.G., Tsuneda A., 1993. Interactions between *Pleurotus* species, nematodes, and bacteria on agar and in wood. Transactions of the Mycological Society of Japan 34, 449 – 464.

Thorn R.G., Moncalvo J.M., Reddy C.A., Vilgalys R., 2000. Phylogenetic analyses and the distribution of nematophagy support a monophyletic Pleurotaceae within the polyphyletic pleurotoid-lentinoid fungi. Mycologia 92(2), 241 – 252.

Truong B.N., Okazaki K., Fukiharu T., Takeuchi Y., Futai K., Le X.T., Suzuki A., 2007. Characterization of the nematocidal toxocyst in *Pleurotus* subgen. *Coremiopleurotus*. Mycoscience 48, 222 – 230.

Vences M., Wollenberg K.C., Vieites D.R., Lees D.C., 2009. Madagascar as a model region of species diversification. Trends in Ecology and Evolution 24, 456 – 465.

Vilgalys R., Moncalvo J.M., Liou S.R., Volovsek M., 1996. Recent advances in molecular systematics of the genus *Pleurotus*. In: D.J. Royse (Ed) Mushroom biology and mushroom products (), pp. 91–101. Pennsylvania State University, University Park, PA, USA.

White T.J., Bruns T., Lee S., Taylor J.W., 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: M.A. Innis, D.H. Gelfand, J.J. Sninsky, T.J. White, (Eds) PCR protocols: a guide to methods and applications, pp. 315–322. Academic Press Inc., New York, USA.

Wilmé L., Goodman S.M., Ganzhorn J.U., 2006. Biogeographic evolution of Madagascar's microendemic Biota. Science 312, 1063 – 1065.

Wong K.H., Cheung P.C.K., 2008. Sclerotium of culinary-medicinal King Tuber Oyster mushroom *Pleurotus tuber-regium* (Fr.) Singer (Agaricomycetidea): cultivation, biochemical composition, and biopharmaceutical effects (review). International Journal of Medicinal Mushrooms 10, 303 – 313.

Zervakis G.I., Ntougias S., Gargano M.L., Besi M.I., Polemis E., Typas M.A., Venturella G., 2014. A reappraisal of the *Pleurotus eryngii* complex–New species and taxonomic combinations based on the application of a polyphasic approach, and an identification key to *Pleurotus* taxa associated with Apiaceae plants. Fungal Biology 118, 814 – 834.

Zoberi M.H., 1973. Some edible mushrooms from Nigeria. Nigerian Field 38, 81 – 90.