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DNA-based species identification of Greek macromycetes

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Abstract

Fungi comprise one of the largest and diverse groups of eukaryotes. Macromycetes, which are commonly known as mushrooms, include species in Basidiomycota and Ascomycota. Macromycetes are essential for ecosystem functioning and have high commercial value owing to their nutritional and medicinal properties. Despite the importance of macrofungi for the ecosystem and human welfare, macromycete diversity and phylogeny are poorly characterized, owing to the lack of molecular-based biodiversity descriptors supporting phenotypic classifications, especially for biodiversity rich countries such as Greece. In this study, we implemented a multi-marker DNA barcoding approach, utilizing the Internal Transcribed Spacer 1 (ITS1) and part of the 28S nuclear ribosomal Large Subunit (nrLSU) rDNA regions, for the molecular identification of representative Greek macromycetes. Our analysis involved 103 Greek macromycetes covering seven genera of Basidiomycota (*Agaricus*, *Amanita*, *Boletus*, *Cantharellus*, *Lactarius*, *Pleurotus*, and *Russula*) and one genus of Ascomycota (*Morchella*). Phylogenetic inference based on the generated rDNA sequences, revealed high DNA divergence among most of the examined macromycete genera, which formed discrete monophyletic groups. Our phylogenetic analysis, in accordance with previous studies in the field, further supports the early divergence of the *Cantharellus* clade, followed by the subsequent split of the Russulaceae from a sister clade formed by the *Agaricus*, *Amanita*, *Boletus* and *Pleurotus* genera.

Key words – Ascomycota – Basidiomycota – DNA barcoding – ITS1 – nrLSU – phylogenetic relationships

Introduction

Fungi are one of the largest groups of eukaryotes and represent one of the richest and most diverse groups of organisms in the world (Zotti et al. 2013). According to the Species Fungorum database (http://www.speciesfungorum.org/), more than 150,229 fungal species have been described to date, but the total number is estimated at 2.3-3.8 million species (Hawksworth & Lücking 2017). Macromycete fungi, which are commonly known as mushrooms, include species that are classified under the phyla Basidiomycota and Ascomycota (Singh et al. 2018, Kinge et al. 2020). Basidiomycota and Ascomycota are considered monophyletic sister groups, which likely share a hypothetical common ancestor (Sugiyama 1998, Van de Peer et al. 2000, Berbee & Taylor

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2001, Barseghyan et al. 2012).

Basidiomycota is one of the largest and most diverse phylum of higher fungi, with more than 41,000 species corresponding to approximately 30% of the total characterized fungal diversity (He et al. 2019). The phylum is comprised of the clades Agaricomycotina, Ustilaginomycotina, and Pucciniomycotina (Hibbett et al. 2007, Matheny et al. 2007, Padamsee et al. 2012). Within the subphylum Agaricomycotina, the basidioma-forming Agaricomycetes constitute more than 90% of the total clade diversity, including the gilled mushrooms, puffballs, bracket fungi, crust fungi, coral fungi, jelly fungi, and chanterelles (Hibbett 2006). Given the role that fruiting bodies play in sexual reproduction in Agaricomycetes, it has been postulated as one of the driving mechanisms shaping macromycete evolutionary radiations (Hibbett et al. 2007, Varga et al. 2019). Ascomycota, the sister group to Basidiomycota, constitutes the largest phylum of fungi and includes approximately 110,000 species (Wijayawardene et al. 2021). According to the current classifications, Ascomycota include the subphyla Saccharomycotina, Pezizomycotina, and Taphrinomycotina (Spatafora et al. 2006, Jaklitsch et al. 2016). Saccharomycotina includes the majority of the ascomycetous yeast species such as *Candida albicans*. Pezizomycotina is considered the largest subphylum of Ascomycota; it includes more than 82,000 described filamentous and ascoma-producing species with important roles in ecosystem functioning and symbioses (Spatafora et al. 2006, James et al. 2020).

Macrofungi play vital roles in ecosystem functions as recyclers, decomposers, bioremediators, parasites and/ or symbiotes, but they are also valuable for their influence on humans and human-related activities (Peláez et al. 1995, Pointing 2001, Pointing et al. 2005, Oberwinkler 2012, Zotti et al. 2013, Kinge et al. 2020). Besides their environmental importance, macromycetes are also an important feed for animals and a nutrient-rich food source for humans, given their high content in vitamins, fibres, proteins, and minerals, as well as their low caloric content (Zhang et al. 2015, de Mattos-Shipley et al. 2016). Furthermore, bioactive compounds in macromycetes have been traditionally used in medicine for their anti-carcinogenic properties and other health-promoting effects (de Silva et al. 2012a, b, 2013), though they have also been reported as the cause of food poisoning (Dai et al. 2009, Chen et al. 2014).

Despite the importance of macromycetes for the ecosystem and human welfare, macromycete diversity has been largely unexplored. Macromycete phylogenies have been traditionally examined by the exclusive utilization of morphological characteristics for species identification and phenotypic classifications. Although phenotypic characterization is important for identifying species correctly, classifications based solely on morphological characteristics can be sometimes misleading, given that hybridization (Olson & Stenlid 2002), convergent evolution (Brun & Silar 2010) and cryptic speciation (Harrington & Rizzo 1999, Kohn 2005) are very common phenomena in fungi. Thus, a more effective approach necessitates the combination of several descriptors, including molecular, morphological, as well as ecological information (Hyde et al. 2013, Kuhnert et al. 2015, Maharachchikumbura et al. 2015, 2016).

Coupling phenotypic taxonomy with DNA-based technologies, such as DNA barcoding, enables a more detailed view in the phylogeny of said cryptic species. DNA barcoding analyses using the ribosomal ITS rDNA region, which is the universal barcode for fungi (Schoch et al. 2012), enabled species identification and phylogenetic resolutions in a variety of fungal lineages, such as macromycetes (Zhang et al. 2004, Frøslev et al. 2007, Zhang et al. 2010), smuts (Vialle et al. 2009), even for pin molds and lichenized fungi (Kelly et al. 2011), and the Mucorales of Mucoromycotina (Schwarz et al. 2006). Several groups even adopted a multi-marker barcoding approach in order to facilitate fungal species identification either for specific clades or for megaphylogenies. For instance, the ITS barcoding region has been used along with the nrLSU to increase identification accuracy in *Lactarius* (Geml et al. 2009), *Russula* (Park et al. 2014), and several species across the kingdom (Taylor & McCormick 2008). Other examples include the combination of multiple barcodes for molecular phylogenetics in *Russula* (Park et al. 2013, Li et al. 2019b) and *Boletus* (Dentinger et al. 2010). Multi-marker analyses have also been implemented in higher-level taxa phylogenies for 392 genera of the phylum Basidiomycota (Zhao et al. 2017), as

well as for understanding the broad patterns of diversification and evolution drivers for mushroom-forming fungi by analysing Agaricomycetes megaphylogenies (Varga et al. 2019). Very recently, complete mitochondrial genomes have been utilized for the phylogenetic analysis of *Lactarius* and *Cantharellus* ectomycorrhizal fungi (Li et al. 2018, 2019a), even eDNA and metabarcoding technologies have also been evaluated as an effective tool for screening macromycete biodiversity (Frøslev et al. 2019).

Despite the progress in the field, DNA-based genetic inventories of the extant Greek macromycetes biodiversity, for which species delimitation is primarily based on phenotypic descriptors, are still severely lacking. Combining precise morphological and molecular data is necessary to achieve a more accurate species delimitation and identify cryptic species. Morphological identification at the species level is often problematic due to the lack of sufficient discriminative characteristics among closely related species, large intraspecific variability of the characters and their dependence on physiological growth parameters (Hibbett et al. 2007, Barseghyan et al. 2012). The problem of misidentification is exacerbated for macromycete species that are usually used for culinary purposes, since misidentification in those cases could cause serious health issues. This is especially apparent for industrialized/ processed mushroom products that impede species authentication by phenotypic means (Raja et al. 2017, Jensen-Vargas et al. 2018).

Given the apparent lack of available barcoding data for Greek macromycetes, herein, we performed DNA barcoding analysis using the ITS1 and partial nrLSU rDNA regions for the molecular characterization and identification of major Greek macromycete species covering seven taxonomic families. The research presented in this work is a pioneer approach in implementing DNA-based methodologies for the genetic identification of Greek mushroom-forming fungi and a step towards resolving Greek macromycete phylogeny.

Materials & Methods

Taxon sampling and DNA extraction

In this study, we used 103 Greek macromycete species; 99 species corresponding to 7 genera of the Basidiomycota division (*Agaricus*, *Amanita*, *Boletus*, *Cantharellus*, *Lactarius*, *Pleurotus*, *Russula*), and four species from the *Morchella* genus of the Ascomycota, which was used as an outgroup (Table 1). Fruit body collections were derived from the herbarium collection of the Forest Research Institute (FRI) of Thessaloniki, Greece. The specimens have been collected from a wide range of Greek ecosystems and landscapes. All taxa used in this work have been morphologically characterized and identified to the species level by professional taxonomists prior to their deposit in the herbarium collection.

Table 1 Macromycetes used in this study. GenBank accession numbers of the ITS1 and nrLSU sequences generated in this study for the corresponding specimens are highlighted in **bold**

Taxon	Herbarium ID	GenBank Accession number	
		ITS1	nrLSU
Agaricus arvensis	247	**MT747198**	-
A. arvensis†	-	AJ887993	-
A. augustus	179	**MT747196**	-
A. campestris	1436-16	**MT747203**	-
A. campestris var. *squamulosus*	562	**MT747199**	-
A. campestris†	-	KJ877749	-
A. cupreobrunneus	1064	**MT747201**	-
A. placomyces	721	**MT747200**	-
A. silvaticus	101	**MT747195**	-
A. silvaticus	1464	**MT747202**	-
A. xanthodermus	246	**MT747197**	-
A. xanthodermus†	-	AJ131127	-

Table 1 Continued.

Taxon	Herbarium ID	GenBank Accession number	
		ITS1	nrLSU
Amanita caesarea	176	**MT747205**	**MT747315**
A. caesarea†	-	MH508283	MK277517
A. ceciliae	897	**MT747212**	-
A. ceciliae†	-	MN490655	-
A. citrina	531	**MT747210**	-
A. citrina	1443	**MT747220**	**MT747318**
A. citrina var. alba	1437	**MT747219**	-
A. citrina†	-	-	MH486456
A. gemmata	843	**MT747211**	-
A. muscaria	468	**MT747208**	-
A. ovoidea	818	**MT747213**	-
A. pantherina	446	**MT747206**	**MT747313**
A. pantherina	1441	**MT747218**	-
A. pantherina†	-	MK402132	MK204473
A. phalloides	140	**MT747204**	-
A. rubescens	499	**MT747209**	-
A. rubescens	1027	**MT747216**	**MT747312**
A. vaginata var. *alba*	455	**MT747207**	**MT747316**
A. velosa	823	**MT747215**	-
A. virosa	988	**MT747214**	**MT747314**
A. vittadinii	1475	**MT747217**	-
Boletus calopus†	-	-	AF456833
B. reticulatus	810	**MT747230**	-
Butyriboletus appendiculatus	120	**MT747223**	-
Cantharellus cibarius	1434	**MT747303**	**MT747323**
C. cibarius†	-	-	KX592708
C. infundibuliformis	959	**MT747300**	-
C. lutescens	1216	**MT747301**	-
C. lutescens	1346	**MT747302**	**MT747324**
C. lutescens†	-	GU373513	JQ976982
Craterellus tubaeformis†	-	HM468496	-
Lactarius acerrimus	1077	**MT747247**	-
L. azonites	1313	**MT747253**	-
L. blennius	704	**MT747240**	-
L. blennius	1256	**MT747251**	-
L. chrysorrheus	141	**MT747233**	-
L. chrysorrheus	484	**MT747236**	-
L. circellatus	705	**MT747239**	-
L. controversus	211	**MT747234**	-
L. controversus	2011-1447	**MT747257**	-
L. controversus†	-	MH930301	-
L. deliciosus	1325	**MT747252**	-
L. deterrimus	874	**MT747241**	-
L. deterrimus†	-	AF140267	-
L. mitissimus	684	**MT747238**	-
L. mitissimus†	-	EF493295	-
L. pallidus	1245	**MT747248**	-
L. pallidus	2010-1447	**MT747254**	-
L. pallidus	1258	**MT747249**	-
L. piperatus	136	**MT747299**	**MT747330**
L. piperatus	397	**MT747235**	-
L. piperatus	493	**MT747237**	-
L. piperatus†	-	-	KF220215
L. pubescens	1255	**MT747250**	-
L. salmonicolor	1448	**MT747256**	-

Table 1 Continued.

Taxon	Herbarium ID	GenBank Accession number	
		ITS1	nrLSU
L. scrobiculatus	1054	**MT747245**	-
L. tesquorum	997	**MT747243**	-
L. torminosus	930	**MT747242**	-
L. vellereus	1451	**MT747311**	**MT747329**
L. vellereus†	-	-	KR364237
L. volemus	1022	**MT747244**	**MT747328**
L. volemus†	-	-	JQ348397
L. zonarius	1444	**MT747255**	**MT747331**
Lactifluus luteolus	1081	**MT747246**	-
Morchella conica	28	**MT747288**	-
M. conica†	-	MG431337	-
M. elata	740	**MT747293**	**MT747332**
M. elata†	-	-	KM485984
M. esculenta	37	**MT747289**	**MT747334**
M. esculenta†	-	EU600240	MH868892
M. vulgaris	64	**MT747290**	-
Neoboletus erythropus	1446	**MT747232**	-
Panellus mitis	528	**MT747298**	**MT747335**
Pleurotus mitis†	-	-	AY014288
P. ostreatus	263	**MT747295**	**MT747339**
P. ostreatus	353	**MT747296**	**MT747338**
P. ostreatus†	-	MT644908	MH874388
Rubroboletus rhodoxanthus	776	**MT747228**	-
R. rhodoxanthus	1070	**MT747310**	**MT747322**
Russula acrifolia	1479	**MT747282**	-
R. albonigra	1478	**MT747286**	-
R. aurata	379	**MT747259**	**MT747340**
R. aurata†	-	-	MN710556
R. azurea	992	**MT747269**	-
R. cf. *amoenoides*	819	**MT747274**	-
R. cf. *graveolens*	1029	**MT747275**	-
R. cyanoxantha	408	**MT747261**	-
R. cyanoxantha	1046	**MT747276**	-
R. cyanoxantha	1314	**MT747278**	-
R. cyanoxantha	1442	**MT747283**	-
R. delica	1453-108	**MT747258**	-
R. foetens	330	**MT747260**	-
R. foetens	1453-187	**MT747308**	-
R. fragilis	473	**MT747262**	-
R. heterophylla	1480	**MT747284**	-
R. illota	1452	**MT747306**	**MT747341**
R. illota†	-	-	DQ422024
R. integra	825	**MT747266**	-
R. laurocerasi	1015	**MT747272**	-
R. lepida	1302	**MT747279**	-
R. livescens†	-	JN129398	-
R. lutea	1495	**MT747287**	-
R. monspeliensis	1000	**MT747271**	-
R. nigricans	798	**MT747305**	-
R. nigricans	983	**MT747268**	-
R. nigricans†	-	KF306040	-
R. ochroleuca	987	**MT747270**	-
R. palidospora	1298	**MT747307**	-
R. rosea	1436-144	**MT747280**	-
R. sanguinea	808	**MT747265**	-

Table 1 Continued.

Taxon	Herbarium ID	GenBank Accession number	
		ITS1	nrLSU
R. sanguinea	879	**MT747264**	-
R. sanguinea†	-	MH930200	-
R. sardonia	534	**MT747263**	-
R. sororia	952	**MT747267**	-
R. virescens	1445	**MT747281**	-
Suillellus comptus	814	**MT747229**	-
S. queletii	324	**MT747292**	-
S. queletii	1068	**MT747231**	-
S. queletii†	-	MH011918	-
S. luridus	1494	**MT747309**	-
Suillus luteus	382	**MT747225**	**MT747321**
S. luteus†	-	KU059580	MH867249
Xerocomellus chrysenteron	100	**MT747221**	-
X. chrysenteron	118	**MT747222**	**MT747320**
X. chrysenteron	262	**MT747226**	-
X. redeuilhii	720	**MT747227**	-

† DNA sequences for the reference species were retrieved from GenBank

Total DNA was extracted from 0.1 g of lyophilized tissue after grinding with liquid nitrogen. DNA extraction was carried out with a modified version of the Cetyl Trimethyl Ammonium Bromide (CTAB) protocol, previously described by Doyle & Doyle (1987). DNA concentration and quality were assessed using the UV-Vis Spectrophotometer Q5000 (Quawell Technology Inc., U.S.A.) and optically with gel electrophoresis in 1% agarose gel. Working DNA stocks were prepared in 20 ng/μL.

PCR amplification and sequencing

PCR amplification was performed on a Rotor-Gene 6000 real-time 5-Plex HRM PCR Thermocycler (Corbett Research, Sydney, Australia), using the Rotor-Gene Q software version 2.0.2 (Corbett Life Science, Cambridge, UK). PCR reaction mixtures were prepared in a total volume of 20 μL consisting of 20 ng genomic DNA, 1× PCR buffer, 0.5 μM forward and reverse primers, 0.2 mM dNTPs, 1.5 mM SYTO™ 9 Green Fluorescent Nucleic Acid Stain (Invitrogen, Eugene, Oregon, USA), and 1 U Kapa Taq DNA polymerase (Kapa Biosystems, USA). The ITS1 region was amplified using the ITS1 [5'-TCCGTAGGTGAACCTGCGG-3'] and ITS2 [5'-GCTGCGTTCTTCATCGATGC-3'] primers (White et al. 1990), with the following protocol: initial denaturation at 94°C for 3 min, followed by 35 cycles of 94°C for 30 sec, 54ºC for 30 sec, and 72°C for 40 sec. Amplification of the partial nrLSU region was performed with the LR0R [5'-GTACCCGCTGAACTTAAG-3'] (Rehner & Samuels 1994) and LR5 [5'-ATCCTGAGGGAAACTTC-3'] (Vilgalys & Hester 1990) primer pair, using the following protocol: initial denaturation at 94°C for 3 min, followed by 35 cycles of 94°C for 30 sec, 55ºC for 30 sec, and 72°C for 90 sec. After successful amplification, PCR products were sequenced in two directions with Big Dye terminator v3.1 Cycle sequencing kit (PE Applied Biosystems, Foster City, CA, USA) in an ABI 3730 sequencer (PE Applied Biosystems). Sequences were manually curated for sequencing ambiguities using the CHROMAS trace viewer software version 2.6.6 (Technelysium Pty Ltd).

Species identification

The generated ITS1 and nrLSU sequences were validated based on their similarity with entries in the GenBank (https://www.ncbi.nlm.nih.gov/genbank/) and UNITE (https://unite.ut.ee/) sequence repositories, using the default search parameters and optimizing for highly similar sequences (megablast). Species identification was deemed succesful when the morphological

classification agreed with the BLAST (Basic Local Alignment Search Tool) analysis for at least one of the two available DNA barcodes. The newly generated DNA barcoding sequences were submitted to GenBank with accesion numbers MT747195-MT747311 for ITS1 and MT747312-MT747341 for nrLSU.

Phylogenetic analysis

Multiple sequence alignments were generated for both the ITS1 and nrLSU sequences along with representative reference sequences from GenBank for all the genera used in this work (Table 1). Alignments were performed with the Molecular Evolutionary Genetics Analysis X (MEGA X) software version 10.05 (Kumar et al. 2018) using the MUSCLE algorithm. For each alignment, Maximum Likelihood fitness assessment was carried out for 24 nucleotide substitution models using MEGA X (Tables 2, 3). The model with the lowest Bayesian Information Criterion (BIC) scores was chosen as the best model to describe the substitution pattern observed in the data. Maximum Likelihood trees were subsequently generated for each alignment using MEGA X, based on the parameters defined by the aforementioned fitness analysis with 1000 bootstrap replicates. The Hasegawa-Kishino-Yano (HKY) model (Hasegawa et al. 1985) with discrete Gamma distribution (+G = 1.0650) was used for the ITS1, and the Kimura 2-parameter (K2-P) model (Kimura 1980) with discrete Gamma distribution (+G = 0.4962) was used for the nrLSU phylogenetic tree reconstructions. The *Morchella* clade of the Ascomycota was used as an outgroup to root the trees. The phylogenetic tree images were designed and annotated using the FigTree software version 1.4.4 (http://tree.bio.ed.ac.uk/software/figtree/) and the iTOL (https://itol.embl.de/) online tool. The multiple sequence alignments and the phylogenetic trees were submitted to TreeBASE (http://treebase.org) with submission ID 27492 (http://purl.org/phylo/treebase/phylows/study/TB2:S27492?x-access-code=84adf247127337d3f745f2cf8e5901fe&format=html).

Results

In accordance with the morphological classification and in scopes of enriching species identification and classification inventories of Greek macromycetes, we performed DNA barcoding analysis on representative species from the Agaricaceae, Amanitacea, Boletaceae, Cantharellaceae, Pleurotaceae, and Russulaceae families of the Bosydiomycota fungi, as well as the closely related sister group of Morchellacheae of the Ascomycota (Tables 1-3).

The ITS1 barcoding region was successfully amplified and sequenced for all 103 macromycete species used in this work, generating sequence reads of 232 bp on average. According to the ITS1 BLAST analysis, the majority of the macromycetes used in this work were confidently identified to the species level with identity values above 90% (Table 2). However for the rest of the species, albeit with lower percent identity values, the corresponding species informed by the morphological classification could be detected within the list of entries and manualy checked in terms of their sequence to ensure their correct clasification.

Table 2 ITS1 BLAST analysis of the Greek macromycetes

Taxon	Herbarium ID	GenBank acession number	GenBank/ UNITE best hit	Score (bits)	e-value	Identity
Agaricus arvensis	247	MT747198	AJ887993	569	3.00E-165	98.7%
A. augustus	179	MT747196	KC797151	217	3.00E-59	80.1%
A. campestris	1436-16	MT747203	KJ877749	564	2.00E-163	98.1%
A. campestris var. *squamulosus*	562	MT747199	KJ877749	564	2.00E-163	98.1%
A. cupreobrunneus	1064	MT747201	AY484680	374	9.00E-108	88.5%
A. placomyces	721	MT747200	MN892622	387	5.00E-111	89.8%
A. silvaticus	101	MT747195	AF438556	377	1.16E-107	89.1%
A. silvaticus	1464	MT747202	AF438556	346	3.00E-98	87.4%

Table 2 Continued.

Taxon	Herbarium ID	GenBank acession number	GenBank/ UNITE best hit	Score (bits)	e-value	Identity
A. xanthodermus	246	MT747197	AJ131127	538	8.00E-156	98.0%
Amanita caesarea	176	MT747205	MH508283	412	4.06E-111	93.3%
A. ceciliae	897	MT747212	MN490655	313	3.16E-81	92.8%
A. citrina	531	MT747210	KY587527	383	5.00E-109	96.1%
A. citrina	1443	MT747220	AJ889920	377	2.00E-107	93.0%
A. citrina var. *alba*	1437	MT747219	UDB011892	438	1.00E-127	97.3%
A. gemmata	843	MT747211	FJ890024	132	6.00E-29	75.0%
A. muscaria	468	MT747208	MT578069	366	3.00E-103	96.8%
A. ovoidea	818	MT747213	KY606969	344	1.36E-90	91.4%
A. pantherina	446	MT747206	MK402132	425	4.19E-115	100%
A. pantherina	1441	MT747218	MK402132	425	4.19E-115	100%
A. phalloides	140	MT747204	MK580738	446	3.00E-127	100%
A. rubescens	499	MT747209	MK580678	102	2.00E-24	95.3%
A. rubescens	1027	MT747216	KJ638284	281	6.00E-78	86.0%
A. vaginata var. *alba*	455	MT747207	KM658296	300	7.00E-82	92.9%
A. velosa	823	MT747215	GQ250409	300	7.00E-82	93.3%
A. virosa	988	MT747214	MT345282	351	1.00E-99	94.4%
A. vittadinii	1475	MT747217	MH603603	483	2.84E-132	100.0%
Boletus reticulatus	810	MT747230	AB821461	97	9.00E-23	100.0%
Butyriboletus appendiculatus	120	MT747223	GU233428	318	7.00E-90	89.6%
Cantharellus cibarius	1434	MT747303	MF589901	68	1.00E-11	88.5%
C. infundibuliformis	959	MT747300	HM468496	377	1.00E-100	100.0%
C. lutescens	1216	MT747301	GU373513	324	1.16E-84	99.4%
C. lutescens	1346	MT747302	GU373513	195	9.00E-46	86.9%
Lactarius acerrimus	1077	MT747247	KX610689	459	4.64E-125	99.2%
L. azonites	1313	MT747253	KR082889	169	6.00E-45	81.4%
L. blennius	704	MT747240	MK028448	444	1.00E-120	98.8%
L. blennius	1256	MT747251	MK028446	346	4.00E-91	92.3%
L. chrysorrheus	141	MT747233	AF096983	313	3.00E-88	91.6%
L. chrysorrheus	484	MT747236	KT165289	438	5.66E-119	99.6%
L. circellatus	705	MT747239	MT006008	329	6.00E-94	92.7%
L. controversus	211	MT747234	MH930301	457	1.62E-124	100.0%
L. controversus	2011-1447	MT747257	MH930301	438	5.58E-119	100.0%
L. deliciosus	1325	MT747252	MH038154	392	3.00E-111	95.2%
L. deterrimus	874	MT747241	AF140267	460	1.26E-125	100.0%
L. mitissimus	684	MT747238	EF493295	436	2.00E-118	100.0%
L. pallidus	1245	MT747248	KT881542	468	7.78E-128	99.6%
L. pallidus	2010-1447	MT747254	KT881542	388	1.00E-111	94.2%
L. pallidus	1258	MT747249	KT881542	460	1.28E-125	99.6%
L. piperatus	136	MT747299	KX267652	235	7.54E-58	90.1%
L. piperatus	397	MT747235	KX267652	414	9.12E-112	99.1%
L. piperatus	493	MT747237	MK269093	121	2.00E-29	97.2%
L. pubescens	1255	MT747250	JQ888184	460	1.29E-125	99.2%
L. salmonicolor	1448	MT747256	MK028450	385	5.00E-110	97.4%
L. scrobiculatus	1054	MT747245	KX441098	372	4.00E-106	95.0%
L. tesquorum	997	MT747243	DQ116913	429	3.69E-116	97.2%
L. torminosus	930	MT747242	MK167422	398	1.00E-106	95.2%
L. vellereus	1451	MT747311	MH125239	449	2.65E-122	100.0%
L. volemus	1022	MT747244	JQ753946	381	8.36E-102	99.0%
L. zonarius	1444	MT747255	JF908280	442	4.35E-120	100.0%
Lactifluus luteolus	1081	MT747246	KU885434	191	2.00E-44	98.2%
Morchella conica	28	MT747288	MG431337	427	1.00E-122	99.6%
M. elata	740	MT747293	GU373499	302	2.00E-84	90.2%

Table 2 Continued.

Taxon	Herbarium ID	GenBank acession number	GenBank/ UNITE best hit	Score (bits)	e-value	Identity
M. esculenta	37	MT747289	EU600240	401	4.00E-114	97.5%
M. vulgaris	64	MT747290	JQ691469	370	3.00E-105	96.0%
Neoboletus erythropus	1446	MT747232	AJ496595	110	5.41E-20	83.3%
Panellus mitis	528	MT747298	UDB023600	379	7.00E-104	100.0%
Pleurotus ostreatus	263	MT747295	MT644908	464	9.97E-127	99.6%
P. ostreatus	353	MT747296	MT644908	464	9.97E-127	99.6%
Rubroboletus rhodoxanthus	776	MT747228	MH011924	464	1.05E-126	98.1%
R. rhodoxanthus	1070	MT747310	MH011924	213	4.08E-51	82.1%
Russula acrifolia	1479	MT747282	DQ421998	324	8.00E-92	91.9%
R. albonigra	1478	MT747286	MH930188	420	1.95E-113	99.6%
R. aurata	379	MT747259	AY061659	241	2.00E-59	87.2%
R. azurea	992	MT747269	JN944002	248	3.00E-69	89.6%
R. cf. amoenoides	819	MT747274	KU205321	388	5.02E-104	99.5%
R. cf. graveolens	1029	MT747275	KX905044	398	8.00E-107	100.0%
R. cyanoxantha	408	MT747261	AY606960	412	3.00E-111	99.6%
R. cyanoxantha	1046	MT747276	AY606960	252	7.00E-63	88.8%
R. cyanoxantha	1314	MT747278	JF908699	252	7.00E-63	88.9%
R. cyanoxantha	1442	MT747283	KR364093	315	1.00E-88	92.3%
R. delica	1453-108	MT747258	MG687336	407	2.00E-109	96.7%
R. foetens	330	MT747260	MG687323	337	1.00E-94	91.7%
R. foetens	1453-187	MT747308	AY061677	411	1.00E-110	99.6%
R. fragilis	473	MT747262	DQ367914	294	7.00E-83	91.1%
R. heterophylla	1480	MT747284	MG680180	398	8.44E-107	100.0%
R. illota	1452	MT747306	MG687367	431	1.00E-116	98.4%
R. integra	825	MT747266	MN959792	425	4.19E-115	100.0%
R. laurocerasi	1015	MT747272	JF908694	442	2.00E-127	99.6%
R. lepida	1302	MT747279	MG383648	383	2.00E-102	99.5%
R. lutea	1495	MT747287	LC192788	261	1.00E-65	88.5%
R. monspeliensis	1000	MT747271	KX537645	403	1.93E-108	98.7%
R. nigricans	798	MT747305	KF306040	418	6.98E-113	99.6%
R. nigricans	983	MT747268	KF306040	383	2.26E-102	100.0%
R. ochroleuca	987	MT747270	MT644930	219	1.00E-59	86.4%
R. palidospora	1298	MT747307	DQ422032	370	2.00E-98	97.3%
R. rosea	1436-144	MT747280	AY061715	394	1.00E-105	99.%
R. sanguinea	808	MT747265	MH930200	401	6.60E-108	100.0%
R. sanguinea	879	MT747264	MH930200	401	6.60E-108	100.0%
R. sardonia	534	MT747263	JF908641	350	7.00E-100	95.9%
R. sororia	952	MT747267	JN129398	442	4.00E-120	99.6%
R. virescens	1445	MT747281	MG687338	433	2.63E-117	99.2%
Suillellus comptus	814	MT747229	MH011923	472	6.00E-129	99.6%
S. queletii	324	MT747292	MH011918	436	2.00E-118	98.4%
S. queletii	1068	MT747231	MH011918	431	1.00E-116	98.0%
S. luridus	1494	MT747309	MH011915	191	2.00E-44	81.8%
Suillus luteus	382	MT747225	KU059580	418	6.87E-113	100.0%
Xerocomellus chrysenteron	100	MT747221	KU355473	420	3.00E-120	100.0%
X. chrysenteron	118	MT747222	KX449432	420	1.92E-113	100.0%
X. chrysenteron	262	MT747226	DQ822793	230	6.00E-63	83.8%
X. redeuilhii	720	MT747227	KX905051	435	8.00E-118	99.2%

To further support the morphological and DNA-based species identification, as well as tentatively test phylogenetic relationships amongst the studied taxa, multiple sequence alignments were assembled for all the 103 macromycete ITS1 sequences with reference sequences from GenBank (Table 1). The resulting alignment generated a matrix of 362 sites in total, with 343

variable and 304 parsimony-informative sites. Based on the ITS1 phylogenetic tree, the various macromycete species were largely clustered in distinct clades for the majority of the taxonomic groups analysed (Fig. 1). This was especially characteristic for the *Agaricus*, *Cantharellus* and *Morchella* groups, which formed monophyletic clades, whilst for several individual species and clades the topology was not so clearly defined (Fig. 1). Taxon misplacements were remarkably pronounced for the *Pleurotus* clade, and several individual species from the *Russula*, *Lactarius*, *Boletus*, and *Amanita* clades (Fig. 1), though broad phylogenetic patterns can be clearly observed. Nevertheless, it is noteworthy that the *Pleurotus ostreatus* species are positioned much earlier than expected in the topology of the ITS1 tree, whilst the entirety of the *Cantharellus* clade showed a more recent divergence (Fig. 1).

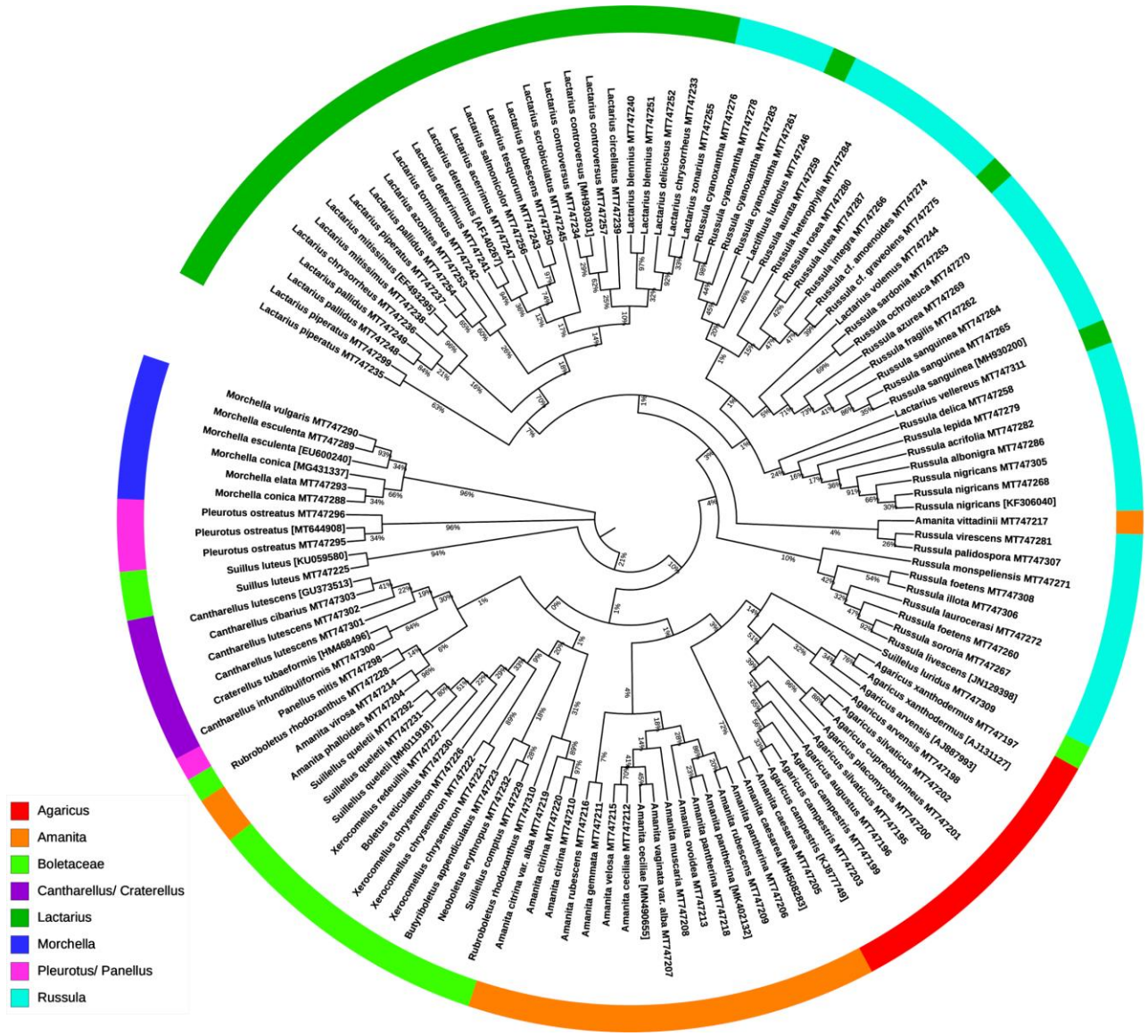

Fig. 1 – ITS1 phylogenetic tree of major Greek macromycetes. The bootstrap consensus phylogenetic tree shown here (1000 replicates) was generated based on the Maximum Likelihood method and the Hasegawa-Kishino-Yano model with +G = 1.0650. Branch labeling represents bootstrap values (%). The GenBank reference sequences are indicated by accession numbers in brackets. Colour-strip annotations were used for visualization purposes.

To further clarify broad phylogenetic topologies and taxon misplacements observed in the ITS1 tree, especially for the *Pleurotus* and *Cantharellus* clades, DNA barcoding analysis and phylogenetic tree inference were subsequently pereformed for selected misplaced species and other

representative lineages using the nrLSU barcoding region. The BLAST analysis involved 22 species in total; 13 misplaced species and 9 representative species with correct topology along the ITS1 tree (Fig. 1, Table 1). The nrLSU amplification and sequencing was successful for all 22 samples (Table 1), generating reads of approximately 790 bp in length. All 22 species were correctly identified to the species level with more than 94% identity using the more conserved nrLSU barcode (Table 3).

Table 3 nrLSU BLAST analysis of the Greek macromycete species

Taxon	Herbarium ID	GenBank accession number	GenBank/ UNITE best hit	Score (bits)	e-value	Identity
Amanita caesarea	176	MT747315	MK277517	3241	0	99.9%
A. citrina	1443	MT747318	MH486456	1541	0	99.0%
A. pantherina	446	MT747313	MK204473	1635	0	99.8%
A. rubescens	1027	MT747312	MT644892	1528	0	99.5%
A. vaginata var. *alba*	455	MT747316	KM658311	1587	0	98.2%
A. virosa	988	MT747314	HQ539756	1580	0	98.6%
Cantharellus cibarius	1434	MT747323	KX592708	1604	0	100.0%
C. lutescens	1346	MT747324	JQ976982	1075	0	94.1%
Lactarius piperatus	136	MT747330	KF220215	1570	0	99.9%
L. vellereus	1451	MT747329	KR364237	1626	0	99.8%
L. volemus	1022	MT747328	JQ348397	1509	0	97.6%
L. zonarius	1444	MT747331	MG712343	1454	0	96.0%
Morchella elata	740	MT747332	KM485984	1480	0	98.7%
M. esculenta	37	MT747334	MH868892	1384	0	96.2%
Panellus mitis	528	MT747335	AY014288	1472	0	99.7%
Pleurotus ostreatus	263	MT747339	MH874388	1587	0	99.8%
P. ostreatus	353	MT747338	MH877725	1559	0	99.5%
Rubroboletus rhodoxanthus	1070	MT747322	DQ534647	1592	0	99.7%
Russula aurata	379	MT747340	MN710556	1362	0	97.8%
R. illota	1452	MT747341	DQ422024	1640	0	99.8%
Suillus luteus	382	MT747321	MH867249	1639	0	99.9%
Xerocomellus chrysenteron	118	MT747320	KC215211	1587	0	100.0%

Alignment of the generated nrLSU sequences with reference species sequences from GenBank resulted in a matrix of 951 total sites, with 520 variable and 457 parsimony-informative sites. In contrast to the ITS1–based phylogeny, the misplaced species were correctly clustered in monophyletic groups in the nrLSU phylogenetic tree (Fig. 2). Furthermore, the nrLSU tree retains the general topology observed also in ITS1, while clarifying the topology of the *Cantharellus* and *Pleurotus* clades (Fig. 2). Overall our analysis further supports the expected early divergence of the *Cantharellus* clade within the Basidiomycota and the subsequent divergence of the Boletaceae with two sister clades; one culmintating to the radiation of the Russulaceae (*Russula*, *Lactarius*), whilst the other giving rise to the rest of the genera in this work.

Discussion

Despite the fact that comparative genomics have been widely applied in systematics and phylogenetic analyses of fungi, fungal diversity and broad-scale phylogenies are still poorly characterized for many taxonomic clades, especially for the sister macromycete clades of Basidiomycetes and Ascomycetes (Liu et al. 2017). This is especially apparent in fungal communities for which species identification is primarily limited to phenotypic characteristics. To date, identification of Greek macromycete communities is mainly facilitated by morphological descriptors, which can lead to many issues of misidentification and misclassification considering the “cryptic” nature of many fungal taxa.

In an attempt to moleculary characterize and identify extant Greek macromycete species we performed DNA barcoding analysis for 109 Greek macromycete species of the Basiodiomycota and Ascomycota fungi. For our purposes, in accordance with the Fungal Barcoding Consortium, the ITS1 barcoding region was selected as the primary DNA barcoding region, given its universality in fungi identification (Schoch et al. 2012). The ITS1-based phylogenetic analysis presented herein could effectively discriminate almost all of the macromycete genera as distinct monophyletic groups, even though some taxon misplacements have been observed, especially for the *Pleurotus*, *Cantharellus* and Russulaceae clades (Fig. 1). This indicates that the ITS1 region is likely not ideal for resolving the phylogeny in these groups, which might be the result of incorrect phenotypic characterisation or high similarity in terms of sequences. Especially for the Cantharellacea, the ITS1 region was previously reported as being rather variable (Feibelman et al.1994), which might explain the observed phylogenetic discrepancies in that tree (Fig. 1). As such, for selected taxa the nrLSU region was also used to further resolve their phylogenies. Such multi-marker barcoding approaches have been proven quite effective in species identification and phylogenetic analyses, and specifically for macromycete species, combination of the ITS and nrLSU have been previously used to increase phylogenetic accuracies in several clades, incuding *Lactarius* (Geml et al. 2009) and *Russula* (Park et al. 2014). Although the nrLSU barcode was previously reported to have low interspecies variation within the *Russula* genus (Li et al. 2019b), herein, we could effectively resolve broad-scale phylogenies and clarify topological misplacements observed among several macromycetes, especially for the *Amanita*, *Boletus*, *Cantharellus* and *Pleurotus* genera (Fig. 2), which could not be resolved with ITS1 alone (Fig. 1).

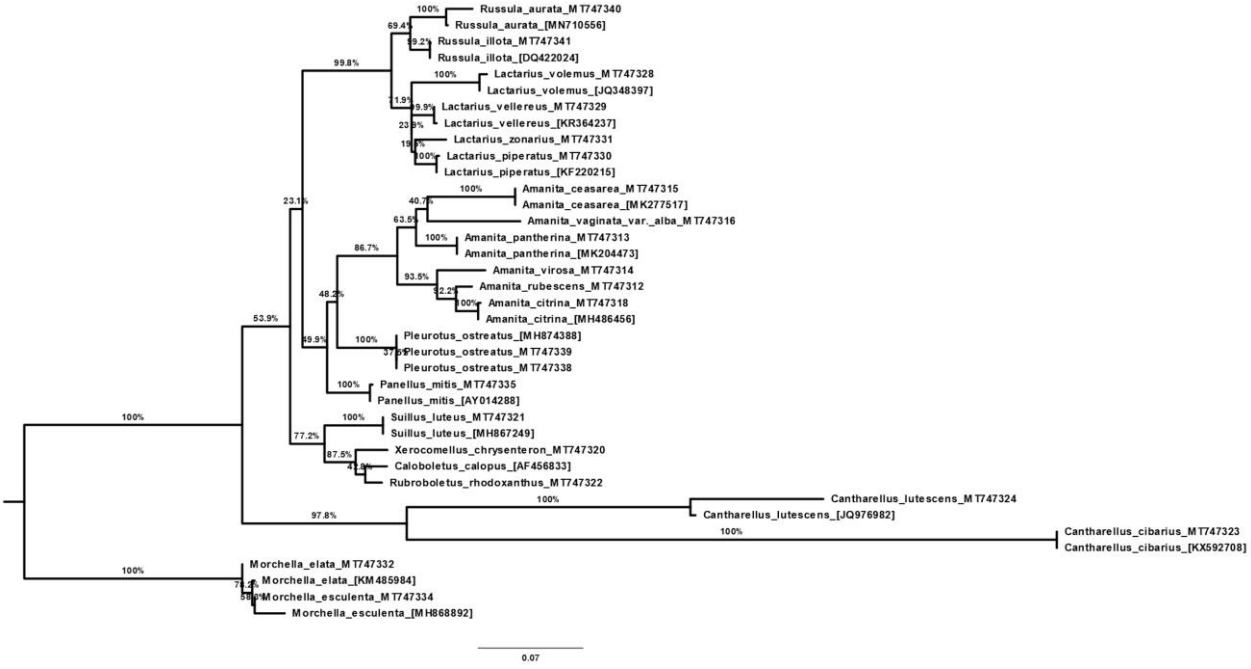

Fig. 2 – nrLSU phylogenetic tree of major Greek macromycete species. The phylogenetic tree was generated based on the Maximum Likelihood method and the Kimura 2-parameter model with +G = 0.4962. The tree with the highest log likelihood (-4805.92) is shown here. Branch labeling represents the percentage of replicate trees in which the corresponding taxa are clustered together (1000 bootstrap replicates). The GenBank reference sequences are indicated by accession numbers in brackets. Scale bar = number of substitutions per site.

Phylogenetic discrepancies are not uncommon for Russulaceae fungi, given their phylogeny is still inconclusive, owing to their morphological similarities. Besides, in taxonomically rich families, such as the family of Russulaceae, phyllogenetic distance among species is expected to be higher due to small scale patterns caused by enviromental or regional clines, which often cause

phyllogenetic constraints, even in well analyzed clades (Gómez-Hernández et al. 2016). Although *Russula* species can be reliably distinguished morphologically from species of other clades, high intraspecies variation, the large species number within the genus, and inaccurate species descriptions could cause several inaccuracies in *Russula* phylogeny (Li et al. 2019b). Similarly, classification of *Lactarius* has been problematic since mycologists often use different morphological characterstics for infrageneric classifications (Lee et al. 2019), even though multi-marker approaches have been succesfully used in identifying new *Lactarius* species and enhancing the accuracy of their phylogeny (Verbeken 2001, Verbeken et al. 2014, Wisitrassameewong et al. 2016).

Regardless of some taxon misplacements observed in the ITS1 phylogenetic tree, overall tree topology in both the ITS1 and nrLSU trees showed very clear patterns of macromycetes phylogeny (Figs 1, 2). In our analysis, all Basidiomycetes clustered together and the only examined group of Ascomycetes (*Morchella*) clustered in a different terminal clade (Figs 1, 2). Ascomycota and Basidiomycota are well characterized monophyletic sister groups that share a hypothetical common ancestor (Barseghyan et al. 2012). The genus *Morchella* is considered as one of the most complex and taxonomically problematic genera, mainly due to the high polymprhism exhibited by the species. Many mycologists suggest that the high degree of polymorphism is possibly induced by enviromental and climatic factors. Although classification above the species level is largely clear, species identification still remains intriguing for taxonomists (Barseghyan et al. 2012).

To date, the majority of the analyses that have been performed on the Cantharellaceae, have focused primarily in differentiating species and very few studies attempted to determine their evolutionary relationships to other species or families (Hibbett et al. 2007, Varga et al. 2019, He et al. 2019). However, in order to construct a comprehensive phylogeny of the genus many more species need to be examined (Dunham et al. 2003a, b). According to the preliminary phylogenetic analyses presented herein, especially for nrLSU, the *Cantharellus* clade showed an early divergence in Basidiomycota evolution, followed by the divergence of the Boletaceae and a split that resulted in the radiation of the clade containing the Russulaceae along with a sister clade containing the rest of the studied genera. This early divergence of the *Cantharellus* clade, as well as the overall topology of the ITS1 and nrLSU phylogenetic trees, show high similarity with macromyctete phylogenies in relevant studies (Hibbett 2006, Varga et al. 2019, He et al. 2019), which further supports the accuracy of our phylogenetic reconstructions.

Conclusion

The high diversity of vascular plants and the variaty of ecosystems serve Greece as a country with an extremely rich fungal diversity. Systematic studies in mycology are necessary in order to support ecological and conservation work of fungi. Yet, measures which bring to light the phylogenetic structure of communities may reveal the relative importance of the different ecological processes that organize assemblages (Kembel & Hubbell 2006). This study presents the first attempt in implementing molecular-based identification in Greek macromycetes classifications, as well as getting an insight into their phylogeny. Our study suggests high DNA divergence among major macromycete genera, although taxon delimitations were not so clear for the highly radiating and phylogenetically similar groups within the Russulaceae family. Our phylogenetic analysis further supports previous work in the field that report an early divergence of the *Cantharellus* clade within the Basidiomycota, followed by the subsequent radiation of the the *Boletus*, *Agaricus*, *Amanita*, *Pleurotus* and the Russulaceae clades. We expect the work presented here to not only enrich macromycete species identification inventories, but also to form the basis for future mycological work in the country for the comparative analysis of molecular, morphological and ecological data. Finally, any data that is submitted at the global fungal genetic base will contribute significantly in resolving and understanding fungal diversity and phylogeny among countries and continents.

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