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I.C. Saré, M.S. Ndir, A. Manga, Mamadou Ndiaye, Gérard Barroso, T.A. Diop

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Research Article

MOLECULAR CHARACTERIZATION OF WILD SENEGALESE VOUCHER BELONGING TO THE *AGARICUS*, *GANODERMA* AND *TERMITOMYCES* GENERA: EVIDENCES OF NEW SPECIES PHYLOGENETICALLY DISTANT FROM DESCRIBED ONES

Saré IC[1]*, Ndir MS[1], Manga A[1,2], Ndiaye M[1], Barroso G[3], Diop TA[1]

[1]Laboratoire de Biotechnologies des Champignons/FST/UCAD. Sénégal

[2]Université Gaston Berger de Saint Louis. Sénégal

[3]Université de Bordeaux, INRA unité UR1264, Bordeaux, France

***Correspondence**

Saré IC

Laboratoire de Biotechnologies des

Champignons/FST/UCAD. Sénégal

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ABSTRACT

Mushrooms collected from different areas in Senegal were molecularly characterized in order to establish phylogenetic relationships with previously described species. For this, PCR products were analyzed by electrophoresis and sequenced. Orthologous Sequences were deduced by Blastn analyses from those available in GenBank and belonging to clearly identified taxonomic units and species. Phylogenetic analyses were performed by the Maximum likelihood method. These analyses led to determine species belonging to three important genera: *Agaricus*, *Ganoderma* and *Termitomyces*. Moreover, phylogenetic analyses have allowed the location of these Senegalese fungal strains in relation with reference species. However, all these Senegalese strains appeared phylognetically distant from the others species and, consequently can be considered as new and still un-described species.

Keywords: *Agaricus, Ganoderma*, *Termitomyces*, molecular methods, phylogeny

INTRODUCTION

Among the 1.5 million species identified in the fungal kingdom, only less than 10 % have been formally described, to date[1]. Among them, only 2327 useful wild species were identified including 2166 edible and alimentary species and 470 medicinal species from a compilation of 200 different sources from 110 countries[2]. Hence, Fungi are little known and also used, despite their important potential. Indeed, the nutritional and medicinal values of fungi have already been recognized[3]. Likewise, economic value of wild mushrooms is very considerable for human species[4]. Despite their importance on food, medicinal and economic plans, the knowledge and use of fungi remain weak in Africa, particularly in Senegal. Only ten edible species (*Afroboletus costatisporus, Amanita crassiconus, Amanita hemibapha, Cantharellus congolensis, Amanita rubescens, Cantharellus pseudofriesii, Lactarius gymnocarpus, Russula foetens, Russula pectinata, Tubosaeta brunneosetosa*), three consumed species (*Gyrodon intermedius, Phlebopus sudanicus, Pisolithus sp*) and one species for medicinal use (*Polyporus sp.*) were reported in this country[2,5,6]. The objective of the present study was to evaluate Senegalese's mushroom following molecular and phylogenetic approaches.

MATERIALS AND METHODS

Biological materials

Mushrooms samples were collected in two distinct regions of Senegal (Dakar and Saint Louis) between July and September. Once localized in the field, each mushroom was photographed and the morphology features of the fruiting body (color, shape….), the gills, the stipe, the pileus and the annulus (when present) were described. Figure 1 shows some morphological aspects of collected Senegalese's mushrooms. Then, the collected samples were dried and placed in bags in herbarium; one part (0.2 g) was used for DNA extraction and molecular characterization.

Methods

DNA extraction

Biological material was crushed to fine powder in liquid nitrogen in a mortar. Grinding allowed to destroy the walls and to weaken the membranes. The resulting mycelia powder was collected and conserved in a freezer at -80°C. Then, nucleic acids were extracted by using the CTAB (N-cethyl-NNN-trimethyl ammonium bromide) protocol[7]. 700 µl of boiling extraction buffer 2X (2 % CTAB, 10 mM Tris-HCl, pH = 8, 20 mM EDTA, 1.4 M NaCl) with 2 % βmercaptoethanol were added to the mycelia powder. The mixture was incubated at 65°C for 20 minutes. After return at room temperature, 700 µl of chloroform-isoamylalcohol (24/1, v/v) were added to the mixture. Then, the mixture was stirred by inversion and centrifuged at 12,000 g, 20°C for 5 minutes. Aqueous phase containing the nucleic acids was recovered. A second extraction with chloroform-isoamylalcohol was realized. DNA was precipitated with 700 µl precipitation buffer (1 % CTAB, 50 mM Tris-HCl, pH = 8; 10 mM EDTA) at room temperature for 30 minutes. The pellet obtained by centrifugation (12,000 g, 20°C for 15 minutes) was dried and suspended in 1 M NaCl (500 µl). Nucleic acids were precipitated by adding two volumes of absolute ethanol. The pellet was recovered by centrifugation (12,000 g, 30 minutes and 20°C). After two successive washings with 1 ml ethanol 70 % (v/v), the pellet was dried then dissolved in 100 µl sterile distilled water containing RNase A (20 µg/ml).

PCR and Sequencing

PCR amplifications were carried out using ITS4 and ITS5 universal primers[8]. Reaction mixes contained 10 µl Colorless Go Taq flexi buffer 5X (Promega), 1 µl dNTP 10 mM (Eurobio), 0.5 µl of each primer pairs (50 nm), 1 µl fungal DNA (around 100 ng) and 0.5 µl Gotaq flexi DNA polymerase (Promega). The mixture was completed to 50 µl with sterile distilled water. PCR reactions consist in an initial denaturation at 95°C for 3 minutes; followed by 35 cycles composed of a denaturation step at 95°C for 30 s, an hybridization step at 55°C for 30s and elongation step at 72°C for 1 minute 30 s. A final elongation was carried out at 72°C for 10 minutes. PCR products were sequenced using the same primers by Beckman Coulter Genomics

Sequence analysis

The sequences were compared, with the help of the Blastn algorithm[9], with those of the GenBank to identify homologous sequences. Sequences were aligned with those from clearly identified taxa with the Muscle software ver. 3.7[10]. Alignment corrections was achieved by using the software Gblocks ver. 0.91 b and the phylogenetic analyzes were performed by the maximum likelihood (ML) method using Phy ML software ver. 3.0 Alrt[11,12]. This software estimates the maximum likelihood phylogenies from the alignment of nucleotide or amino acid sequences. The phylo genetic trees were visualized using the software Tree Dynn ver. 198.3[13]. All these programs are available on line at: http://www.phylogeny.fr/ and described by Dereeper A. and *al*[14,15].

RESULTS

Phylogenetic analysis of the ITS1/5.8S/ITS2 region of the ribosomal unit of three Senegalese vouchers belonging or related to the genus *Agaricus*

The sequences of ITS1/5.8S/ITS2 region of the nuclear ribosomal unit from the three collected Senegalese vouchers ICS031 (GenBank Accession N° KJ510528), ICS002 (GenBank Accession N° KJ510529) and ICS032 (GenBank Accession N° KJ510530) were aligned (Muscle software) with orthologous sequences of the GenBank (Table 1) representing 39 different *Agaricus* species and one *Hymenagaricus tawanensis* strain used as out group. This alignment was used to construct a phylogenetic tree by Maximum-likelihood method (Figure 2). The branches having SH-like support values lower than 50 % were collapsed; those above 50 % were supported.

Phylogenetic analysis of the ITS1/5.8S/ITS2 region of the ribosomal unit of four Senegalese vouchers belonging to the genus *Ganoderma*

In the same, the sequences of the ITS1/5.8/ITS2 region of the nuclear ribosomal unit sequences from the four collected Senegalese vouchers ICS004 (GenBank Accession N° KJ510531), ICS026 (GenBank Accession N° KJ510532), ICS027 (GenBank Accession N° KJ510533 and ICS037 (GenBank Accession N° KJ510534) were aligned with orthologous sequences of the (Table 1) from 24 different *Ganoderma* species and one sequence from the phylogenetically distant *Lentinus striatulus* species choosen as out group. This alignment was used to construct a phylogenetic tree by the Maximum-likelihood (ML) method (Figure 3).

Phylogenetic analysis of the ITS1/5.8S/ITS2 region of the ribosomal unit of a Senegalese voucher belonging to the genus *Termitomyces*

As above, for vouchers belonging to the *Agaricus* and *Ganoderma* genera, the Senegalese voucher ICS001 (GenBank Accession N° KJ510535) was aligned with orthologous sequences of 18 described species belonging to the *Termitomyces* genus and strain of *Lyophyllum decastes* representing the outgroup species (Table 1). The resulting Phyl ML tree obtained from the alignment is shown in Figure 4.

DISCUSSION

One the phylogenetic tree of Figure 2, the *Agaricus* related ICS032 voucher was shown to form a clade with eight *Agaricus* species, precisely *A. cupressicola, A. bridghami, A. devoniensis, A. subperonatus, A. subfloccosus, A. bisporatus, A. bisporatus* and *A. martinezianus.* The clade was supported by a low but significant SH-like branch value of 72 %. Interestingly, it is to be noted that, in this clade, this Senegalese voucher appears distant (i.e. isolated on a long branch) from all the other strains and shares this feature with *A. martinezianus* which is also a tropical species identified by Zhao and *al*[16]. This suggests that Voucher ICS032 could represent a new and still undescribed *Agaricus* species from Senegal. On the contrary, the *Agaricus* voucher ICS002 was found phylogenetically related (SH-like branch value of 66 %) to a single previously described species *A. inoxydabilis,* an *Agaricus* species described by Heinem in 1980. As the length of the branches separating both species in the clade was quite long, it can also be hypothesized that both sequences represent two different species and consequently, the voucher ICS002 could also be representative of a new, still unknown *Agaricus* species from Senegal. The third *Agaricus* voucher ICS031 ranges in an out-group position of all the other 39 *Agaricus* species of our analysis and could be related to the genus *Hymenagaricus*, close related to the *Agaricus* genus. All these strains were separated of others species of *Agaricus* like that strain (thoen7297) of *Agaricus* collected in Dakar market by Thoen in 2010[17]. Finally, the phylogenetic analysis of the three *Agaricus* related vouchers collected in Senegal are in favour of three new species, two (ICS 032 and ICS002) belonging to different section of the *Agaricus* genus and one (ICS031) belonging to a genus different but close related to the *Agaricus* one such as the *Hymenoagaricus*. It should be noted that *Agaricus* genus is an economically important genus. For example, *A. bisporus* has an annual production of two million tons and is the worldwide most eaten mushroom [18]. The phylogenetic tree of Figure 3 clearly shows that the Senegalese vouchers ICS004, ICS026, ICS027 and ICS037 belong to the *Ganoderma* genus. Among these four vouchers, three form a clade with *G. mirabile, G. tornicatum* and *G. neojaponicum*. Moreover, vouchers ICS026 and ICS027 strains are grouped in a separate clade with a SH-like branch value of 71 %. However the branch length separating both sequences suggests that they could represent two related but different species. In the same way, ICS004 voucher appears also related to the vouchers ICS026 and ICS027 representing Senegalese's *Ganoderma* but could represent a new species phylogenetically closer to *G. mirabile* and *G. tornicatum.* On the tree, the ICS0037 sequence ranges in an out-group position with *G. Flexipes.*

Table 1: Collection of fungi available in Genbank

Species	Strains	Genbank Acc. N°	Geographical origin	Références
Agaricus pseudopratensis	LAPAG 20	DQ 182 526		Kerregan R.W. and *al* (2005)
A. xanthosarcus	Goossens 5415	JF 514 523		Raspe O.J.M. and Karunarathna S.C. (2011)
A. bisporis	ABP 1	HM 561 978	Malaisie	Ahmadi Avin Fi and *al* (2010)
A. boisseleltii	CA 123	DQ 182 531		Kerregan R.W. and *al* (2005)
A. fiardii	F 2285	JF 797 201	Martinique	Callac P. and Kanunaratha S. (2011)
A. fissuratus	LAPAG 488	JQ 824 135	Espagne	Foulongne Oriol M. and *al* (2012)
A. arvensis	CA 640	JF 797 194	France	Callac P. and Moinard M. (2011)
A. lanipes	CA 406	JF 797 190	France	Callac P. and Moinard M. (2011)
A. aridicola	CA 101	JF 797 195	France	Callac P. and Moinard M. (2011)
A.viridopurpurascens	Horak 681 79	JF 514 525		Raspe O.J.M. and Karunarathna S.C (2011)
A. pseudolutosus	LAPAG 77	JF 727 868	Espagne	Callac P. and Moinard M. (2011)
A. heinemannianus	LAPAG 111	JF 797 182	Espagne	Callac R. and Parra L.A. (2011)
A. deserticola	RWK 2019	JF 896 228	BSA	Kerrigan R.W. and Callac P. (2011)
A. cupressicola	Cp2	AF 432 904		Challen M.P. and *al* (2003)
A. denoniensis	DV6	AF 432 896		Challen M.P and *al* (2003)
A. laskibarii	LAPAG 115	AY943975		Kerrigan R.W. and *al* (2005)
A. pocillator	TENN61603	FJ596846		Hughes K.W. and *al* (2009)
A.menieri	LAPAG237	DQ182520		Kerrigan R.W. and *al* (2005)
A. nevoi	408	AJ884638	Israël	Didukh M. and al (2005)
A. subfloccosus	FS5 lowland	AF432888		Challen M.P and *al* (2003)
A. bridghami	RKW1899	AF432891		Challen M.P and *al* (2003)
A. bisporatus	contu1	AF432882		Challen M.P and *al* (2003)
A. subperonatus	PDD68573	AF432889		Challen M.P and *al* (2003)
A. brasiliensis		AY818650		Kerrigan R.W. (2005)
A. pequinii	Hai herbarium 1059	AJ884639		Didukh M. and *al* (2005)
A. bernadiiformis	10	AF884632	Hungary	Didukh M. and *al* (2005)
A. litoralis	CA 829	JF 727 867	France	Callac P. and *al* (2011)
A. andrewii	RWK 1917	AF 432 877		Kerrigan R.W. (2001) Challen M.P. and *al* (2003)
A. microvolvatulus	Grinling70 199	JF 514 524		Raspe O.J.M. and Karunarathna S.C. (2011)
A. campestris	W1H	DQ182533		Kerrigan R.W. and *al* (2005)
A. cupreobrunneus	LAFAG 322	JQ 824 136	Espagne	Callac P. (2012)
A. gennadii	CA 387	JF 797 188	France	Callac P. and Moinard M. (2011)
A. martinezianus	Voucher SP 307818	JF896227	Brazil	Kerrigan RW and *al* (2011)
A. inoxydabilis	Voucher LAPAF 1	JF 727841	Togo	Callac P. and *al* (2011)
Agaricus sp	ICS031	KJ510528	Sénégal	This work
Agaricus sp	ICS002	KJ510529	Sénégal	This work
Agaricus sp	ICS032	KJ510530	Sénégal	This work
A. pattersonae	RWK 1415	AY 943 974		Kerrigan R.W et *al* (2007)

Species	Strains	Genbank Acc. N°	Geographical origin	Reference
Ganoderma tropicum	Dai 9724	JQ 781 879	China	Cao Y. and *al* (2012)
G. hoehnelianum	GDGM 25735	JX 195 203	China	Li T. (2012)
G. pfeifferi	CBS 747.84	JQ 520 198		Park Y.J. and *al* (2012)
G. lobatum	ASI 7061	JQ 520 166		Park Y.J. and *al* (2012)
G. tornatum	CBS 109679	JQ 520 217		Park Y.J. and *al* (2011)
G. annulaire	KCTC16803	JQ 520 160		Park Y.J. (2012)
G. neojaponicum	AS5.541 type 4	AY 593 867	China (Taïwan)	Wang D.M. and Yao Y.J. (2005)
G. lucidum	ATCC 46755	JQ 520 185		Park Y. J. and *al* (2012)
G. oregonense	ASI 762	JQ 520 195		Park Y.J. and *al* (2012)
G. carnosum	GCR1	JN 222 419		Siwulki M. and *al* (2011)
G. mastoporum	GDGM25720	JX195 201	China	Li T. (2012)
G. meredithae	ATCC64492	JQ520 190		Park Y. J.and *al* (2012)
G. japonicum	AS5.69	AY593864	China	Wang D.M. and Yao Y.J.
G. flexipes	Wai 5491	JQ 781 850	China	Cao Y. and *al* (2012)
G. australe	HMAS86596	AY884180	England	Wang D. M. and Yao Y.J. (2005)
G. tsugae	Dai 3937	JQ781853	China	Cao Y., Wu Sh. and Dai Y. C. (2012)
G. lingzhi	Dai 12443	JQ 781 866	China	Cao Y., Wu Sh. and Dai Y. C. (2012)
G. orbiforme	BCC 22324	JX 997 990	Thailand	Isaka M. (2012)
G. gibbbosum	AS5.624 type 4	AY593857	China	Wang D.M. and Yao Y.J.
G. mirabile	CBS218.36	JQ520192		Park Y.J. and al (2012)
G. fornicatum	AS5.539 type2	AY598860	China (Taiwan)	Wang D.M. and Yao Y.J
G. mutabile	Voucher Yuan 2289	JN 383977	China	Cao Y and Yuan HS (2013)
G. valesiacum	CBS 428.84	JQ520218		Park Y.J. and al (2012)
Ganoderma sp	ICS004	KJ510531	Sénégal	This work

Ganoderma sp	ICS0026	KJ510532	Sénégal	This work
Ganoderma sp	ICS027	KJ510533	Sénégal	This work
Ganoderma sp	ICS037	KJ510534	Sénégal	This work
Hymenagaricus taiwanensis	AFTOL-ID	DQ 490633		Matheny PB and *al* (2006)
Lentinus striatulus	Isolate Tage-Roland MO 135	GU 207311	Costa Rica	Grand EA and al (2011)

Species	Strains	Genbank Acc. N°	Geographical origine	Reference
Termitomyces heimii	PUN4243	JQ928938	Inde	Atri N.S. and *al* (2012)
T. auranticus	CB55	JQ228252		He Y. and *al* (2011)
T. clypaetus	MU19-50	FJ147329	Thaïlande	Sawhasan P. and *al* (2008)
T. symbiote Macrotermes subhyalinus	MS6	HQ902240		Nobre T. and *al* (2011)
T. symbiote M. bellicosus	Mb 39	HQ902232		Nobre T. and *al* (2011)
T. symbiote M. muelleri	Dka 367551	GQ922688		Nobre T. and *al* (2009)
T. symbiote Ancistrotermes crucifer	Dka 69	GQ922656		Nobre T. and *al* (2010)
T. symbiote Synacanthotermes heterodon	Dka 119	GQ922654		Nobre T. and *al* (2010)
T. symbiote Protermes minutis	Dka 367567	GQ922689		Nobre T. and *al* (2010)
T. symbiote M. nobilis	Dka 367569	GQ922687		Nobre T. and *al* (2010)
T. symbiote Ancistrotermes cavithorax	Dka 46	GQ922655		Nobre T. and *al* (2010)
T. microcarpus	PRU3900	AF357023		Hofstetter V. and *al* (2002)
T. striatus	171348	AF321367	Congo	Rouland Le Fève and *al* (2002)
T. eurhizus	171347	AF321366	Congo	Rouland Le Fève and *al* (2002)
T. symbiote M. bellicosus	Dka 15	CQ922683		Nobre T. and *al* (2009)
T. symbiote M. subhhyalinus	Dka 5	GQ922686	Sénégal	Nobre T. and *al* (2009)
Termitomyces DKA2007	Symbiont 18	EF 636920		Aanen DK and *al* (2007)
T. symbiont of M. bellicosus	Isolate dka8	GQ922631		Nobre T. and *al* (2010)
Termitomyces DKA2007	Symbiont 17	EF 639919		Aanen DK and *al* (2007)
Termitomyces sp	ICS001	KJ510535	Sénégal	This work
Lyophyllum decastes	JZB115005	JQ293099		Wang S.X. and *al* (2011)

Figure 1: Morphology of sporophores from Senegalese strains, A: ICS031, B: ICS001, C: ICS032, D: ICS002, E: ICS004, F: ICS026, G: ICS027, H: ICS037

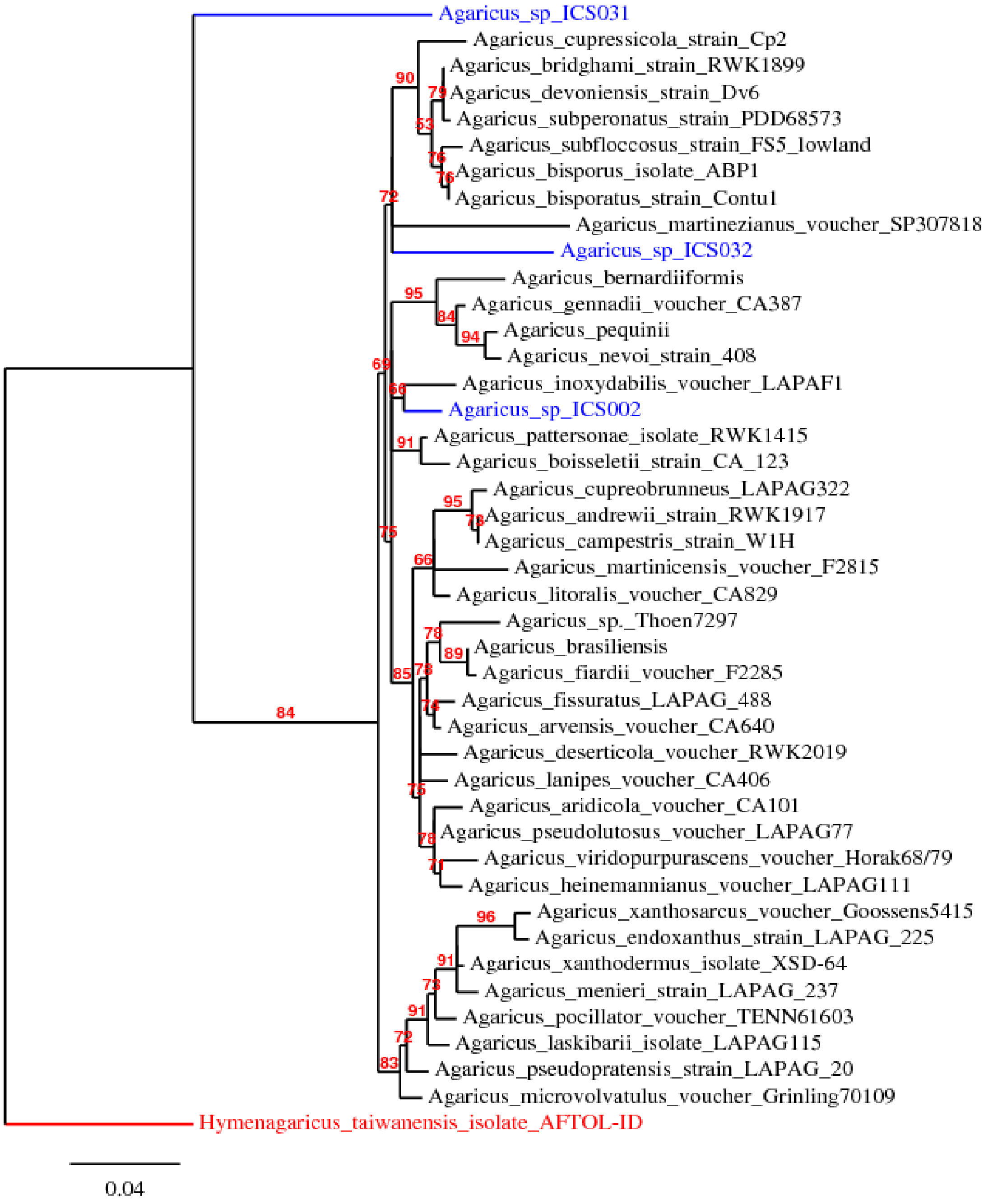

Figure 2: Most likely ML phylogram based of ITS1 + 2 sequences of Senegalese *Agaricus* strains.
The maximum likelihood method was used to construct the trees. Branch supports were computed by the aLRT statistical test alogorithm

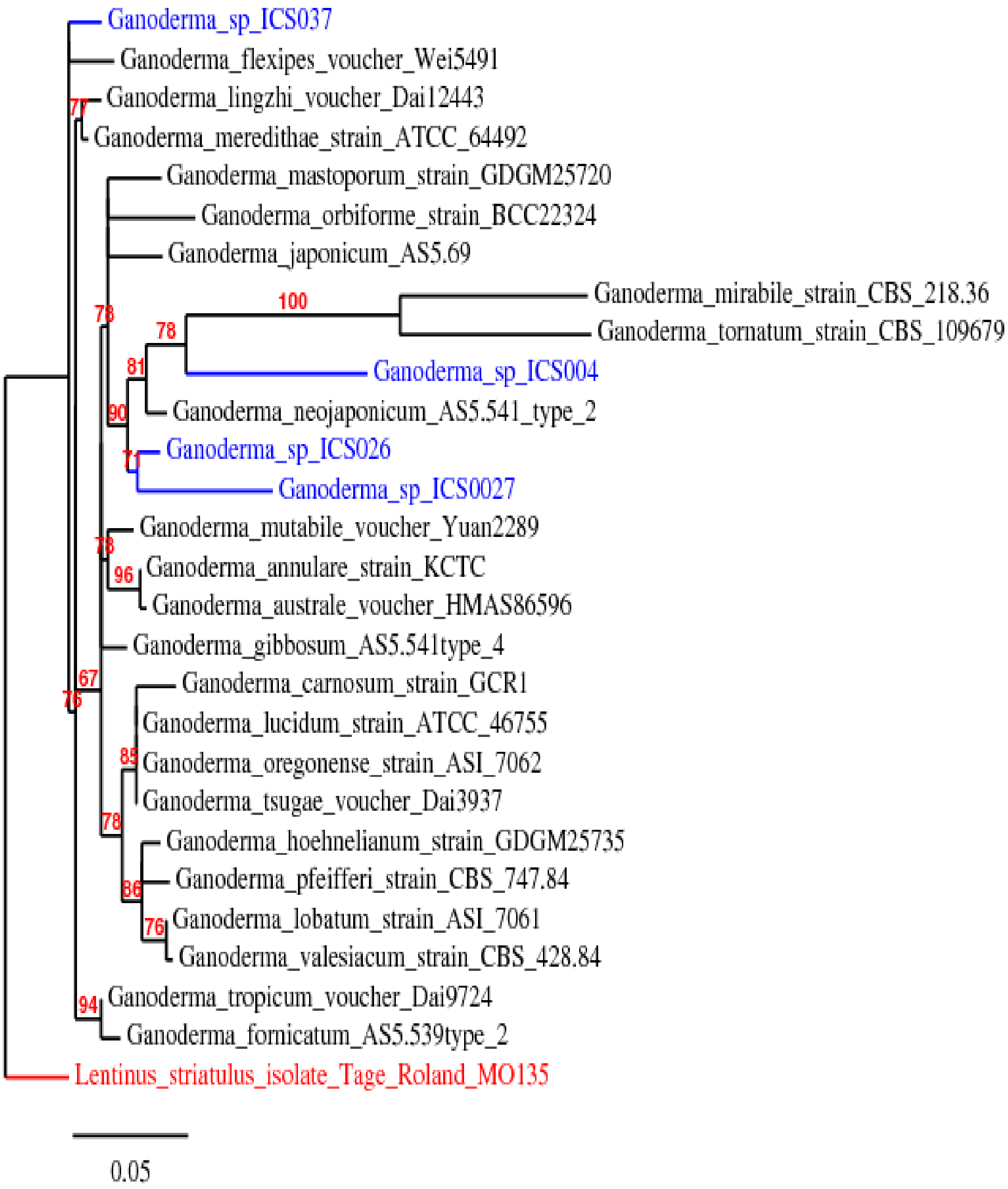

Figure 3: Most likely ML phylogram based of ITS1 + 2 sequences of Senegalese *Ganoderma* strains.
The maximum likelihood method was used to construct the trees. Branch supports were computed by the aLRT statistical test alogorithm

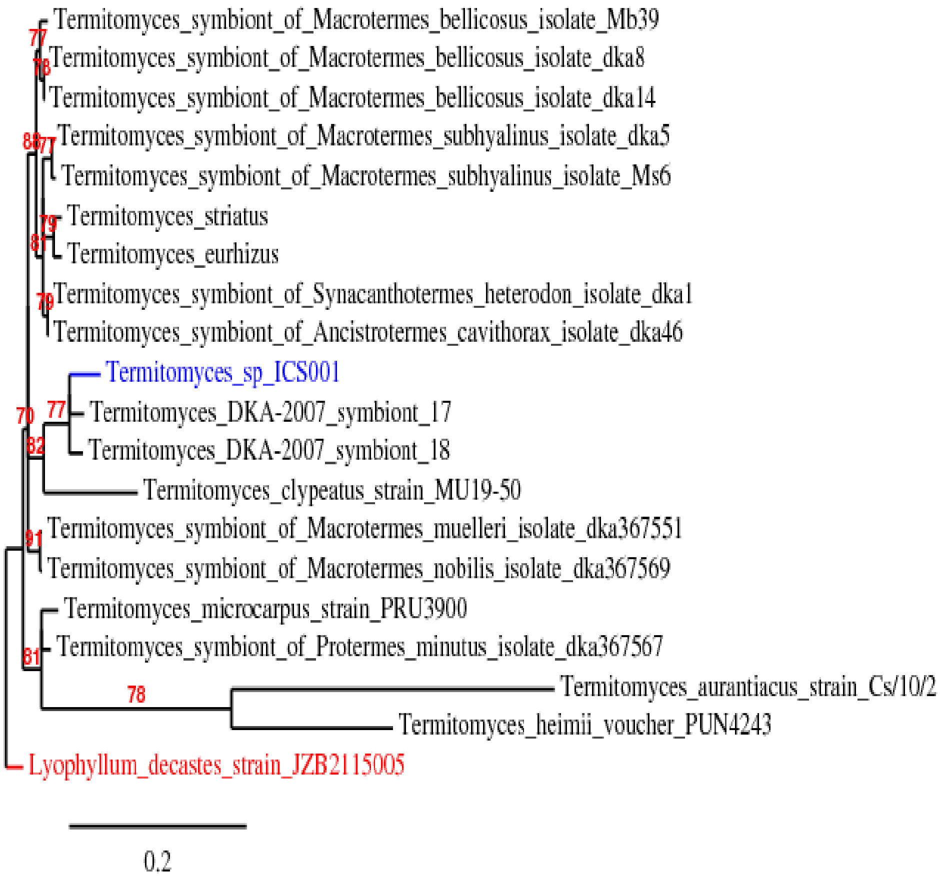

Figure 4: Most likely ML phylogram based of ITS1 + 2 sequences of Senegalese *Termitomyces* strains.
The maximum likelihood method was used to construct the trees. Branch supports were computed by the aLRT statistical test alogorithm

As previously observed with the *Agaricus* samples, all the four Senegalese *Ganoderma* vouchers could constitute new and still unknown *Ganoderma* species. In a recent *in vitro* study, more than 75 % of polypore species surveyed showed antimicrobial property[19]. Thus, the group of *Ganoderma* seems to possess medicinal property for treatments of cancer, diabetes and diseases of liver[20-22]. These medicinal properties were due to their polysaccharides content that have antineoplastic and immunologic effects[23]. In this context, it is interesting to note that *G. neojaponicum,* belonging to the same clade that Senegalese's *Ganoderma* ICS004, ICS026 and ICS027 has been used for medicinal purposes in China and as a traditional food ingredient in Taiwan[24,25]. *Ganoderma* genus has other properties. Thus, *G. cupreum* AG-1 has an ability to decolorize dyes because of its high laccase activity. Its use in treatment of effluents has also been reported[26]. Interestingly, the ICS001 voucher forms a group (SH-like branch value of 82 %) with *T. clypeatus* which is an edible species and two *Termitomyces* (DKA 2007 symbiont 17 and 18) collected by Aanen and *al*[27]. This species is probably edible like others species of *Termitomyces*. Indeed *Termitomyces* species are also highly appreciated in gastronomy in sub-Saharan Africa and are sought after the rainy season for consumption and sale[28].

CONCLUSION

Sequence analysis of different vouchers collected in Senegal allowed the establishment of phylogenetic relationships with sequences of references species available in GenBank. These phylogenetic analyses act in favour of the idea that these vouchers could represent new taxonomic entities and species. These collected Senegalese mushroom belong to three important genera and it would be important to study their socio-economic importance before large scale use.

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