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A new record of *Tricholoma caligatum* (Tricholomataceae) from North Africa with a discussion of related species

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Abstract

Among the Basidiomycota, matsutake are the most appreciated mushrooms in Japan. Some *Tricholoma* species belonging to matsutake group are exported from North Africa to Japan. Until the beginning of the 21st century, the North African 'matsutake' was identified as *T. caligatum*, which is a circum-Mediterranean species described in 1834. However, recent molecular analyses uncover some North African isolates as *T. anatolicum*, which is a species described from Turkey in 2003. As a result, the presence of *T. caligatum* in North Africa remained to be confirmed. We analyzed a recent specimen collected in Algeria from mixed forest and based on molecular and morphological data, we found that it belongs to *T. caligatum*, indicating the existence of two species in North Africa. Morphological traits and molecular markers are proposed here to easily distinguish these two species from each other. The concept of both the species and their respective geographic distributions are discussed.

Key words: fungal taxonomy, phylogeny, polyphyly, species hypothesis

Introduction

Tricholoma matsutake (S. Ito & S. Imai) Singer and its allied species are ectomycorrhizal fungi that are the most economically important edible mushrooms in Japan (Ota *et al.* 2012), where they are a delicacy popular as black truffle in Europe. The collection of these fungi constitutes a valuable resource as a non-timber forest product (NTFP) with an economic value that can outstrip that of timber production in the same forest (Chapela & Garbelotto 2004). In North Africa, collection of *Tricholoma* species from matsutake group is very significant economically with an average of 61 tons (t) collected each year (peaking at 80 t in 1997) in Morocco (under *Cedrus atlantica* (Endl.) G.Manetti ex Carrière and *Pinus halepensis* Mill.), and exported to Japan (Harki & Hammoudi 2008, Abourouh 2013). A *Tricholoma* identified as matsutake was also exported from Algeria to Japan Boa (2004). The North African matsutake is actually not *T. matsutake* and was considered to be *T. caligatum* (Viv.) Ricken (1914) until the beginning of the 21st century. It is also consumed by local populations.

Tricholoma caligatum was originally collected in Chiavari (Italy) and described by Viviani (1834). It was reported as southern species occurring most commonly around the western Mediterranean Sea, especially in Southern France, Eastern and South-Eastern Spain and in adjacent northwestern Africa. Maire (1915) reported Algerian specimens under *Cedrus atlantica*. This description was complemented by Malençon & Bertault (1975) who found this species under *Cedrus* and *Pinus halepensis*. Kytövuori (1988) considers the species found under *Cedrus atlantica* in North Africa to be *T. nauseosum* (A. Blytt) Kytöv., a species described by Blytt as *Armillaria nauseosa* A. Blytt in 1904 and transferred to genus *Tricholoma* by Kytövuori in 1988 (*Tricholoma nauseosum*). Later, Iwase, (1994) distinguished three types of *T. caligatum: T. caligatum*1 distributed in North Africa and Southern Europe, *T. caligatum*2 found in western North America and associated with conifers, and *T. caligatum*3 found in eastern North America and associated

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with oak trees. Matsushita *et al.* (2005), based on a molecular analysis, suggested that *T. nauseosum* and *T. matsutake* were the same species (current name in Index fungorum: *T. matsutake*). Chapela & Garbelotto (2004), using ITS polymorphism, considered *T. caligatum* as a polyphyletic group. Their analyses demonstrate that ITS region (ITS1, ITS2 and 5.8S) alone was insufficient to clarify relationships between *T. matsutake, T. bakamatsutake* Hongo*, T. fulvocastaneum* Hongo and *T. caligatum*. The addition of two other markers, tef and megB1, (Ota *et al*. 2012), allowed to identify the clade corresponding to *T. caligatum.* This clade included two specimens morphologically identified from Spain (AB699667) and Italy (AB699665, where the species was initially described).

Intini *et al*. (2003) identified a new species of *Tricholoma* from Turkey, *T. anatolicum* H.H. Doğan & Intini. Dogan & Akata (2011) described the ecological features of this species and compared its characteristics with *T. caligatum* and other species. He noticed that "*T. anatolicum* has been known erroneously as *T. caligatum* somewhere in Turkey". Two strains from Morocco found under *Cedrus atlantica* in the Atlas Mountains (ITS sequence: AF309532, collected by T. Nakai, and D86572, strain MO1) were initially identified as *T. caligatum*, but were considered identical to *T. anatolicum* by Chapela & Garbelotto (2004). Primers used by Murata & Yamada (2000) amplified DNA of several *T. matsutake* and of two other strains from Morocco, MC1 and TM5 (collected in 1998 and 1992, corresponding to AB699645 and AB699646 respectively) and not *T. caligatum*. In their analysis Ota *et al*. (2012) confirmed, based on multilocus dataset, that these samples from Morocco belong to the clade *T. anatolicum*. Morphological characteristics of *T. caligatum* are difficult to differentiate from that of *T. anatolicum* and these species might have been confused for a century in North Africa. On the other hand, due to systematic uncertainties and probable misidentifications, the sequences in public databases are confusing. Each time a molecular check was done from North African *T. caligatum* specimens, it was found that they had similar sequences as *T. anatolicum* and differed from *T. caligatum* whose phylogenetic position was specified by Ota *et al* (2012).

The objectives of the present work are to clarify the circumscription of the species previously confused with *T. caligatum*, to formally confirm if *T. caligatum* is currently present in North Africa and to propose easy ways to differentiate this species from *T. anatolicum* without ambiguity.

Materials and methods

Material and Study area

The basidiocarps used for this analysis were collected in December 2012 in Northwest Algeria in the 500 ha « Parc Zoologique » which is part of the 1570 ha M'Sila Forest. The forest is a mixture of *Quercus suber* L. and *Pinus halepensis*. This forest (elev. 90–380 m) is located in the municipality of Boutlelis, Wilaya of Oran, 7 km far from the Mediterranean Sea and 11 km west of the city of Oran. The climate is typical of Mediterranean coasts (Berriah 2015) with a mild winter; temperatures ranging between 7 and 18°C, with August being the hottest month with an average temperature of 24.8°C. The area is known as semi-arid with rainfalls averaging 400 mm / year. However, the lack of rain is compensated by the contribution of fresh sea breezes (Aimé 1991). Soil texture is clayey or siliceous, sand to clay-loam in different places (Bouhraoua 2003). The pH of the soil is slightly alkaline, approximately between 7.2 and 7.9. Alkaline pH is characteristic of semi-arid zones in the west of Algeria (Aimé 1991). The soil has very low limestone content, following a decarbonatation (Aimé 1991). This low limestone content explains the presence of the cork oak, a calcifuge tree. However, it is present only in the form of relic forests insulated in the northwest of Algeria. The specimen (voucher number MPU028328) was deposited in Herbarium MPU Montpellier, France, http://www.collections.univ-montp2.fr/herbier-mpu-presentation/base-de-donnees-botanique-herbier-mpu. The other specimens mentioned in this study are named with their ITS GenBank number which are indicated in the phylogenetic tree of Fig. 1.

Taxonomy

The basidiocarps were photographed with a Kodak M1033 (10 MEGAPIXELS) camera in their natural habitat, brought back to the laboratory where their morphological characteristics were recorded. Dried specimen (number CM030) were kept in a zip lock bag. Identification was made according to Viviani (1834), Bigelow (1979) and Dogan & Akata (2011). For micromorphological features, sections of lamellae from dried specimens were mounted in 3% KOH and ammoniacal Congo red to observe spores and basidia using a HB-LUX microscope at X 1000 magnification and a camera (TOUPCAM CMOS 5.2MP). Basidiospores and basidia were measured with Piximètre software v.5.9. Size values reported for basidiospores were based on at least 30 measurements and included the mean length × mean width ± SD; Q, the quotient of basidiospore length to width, and Qm, the mean of Q-values.

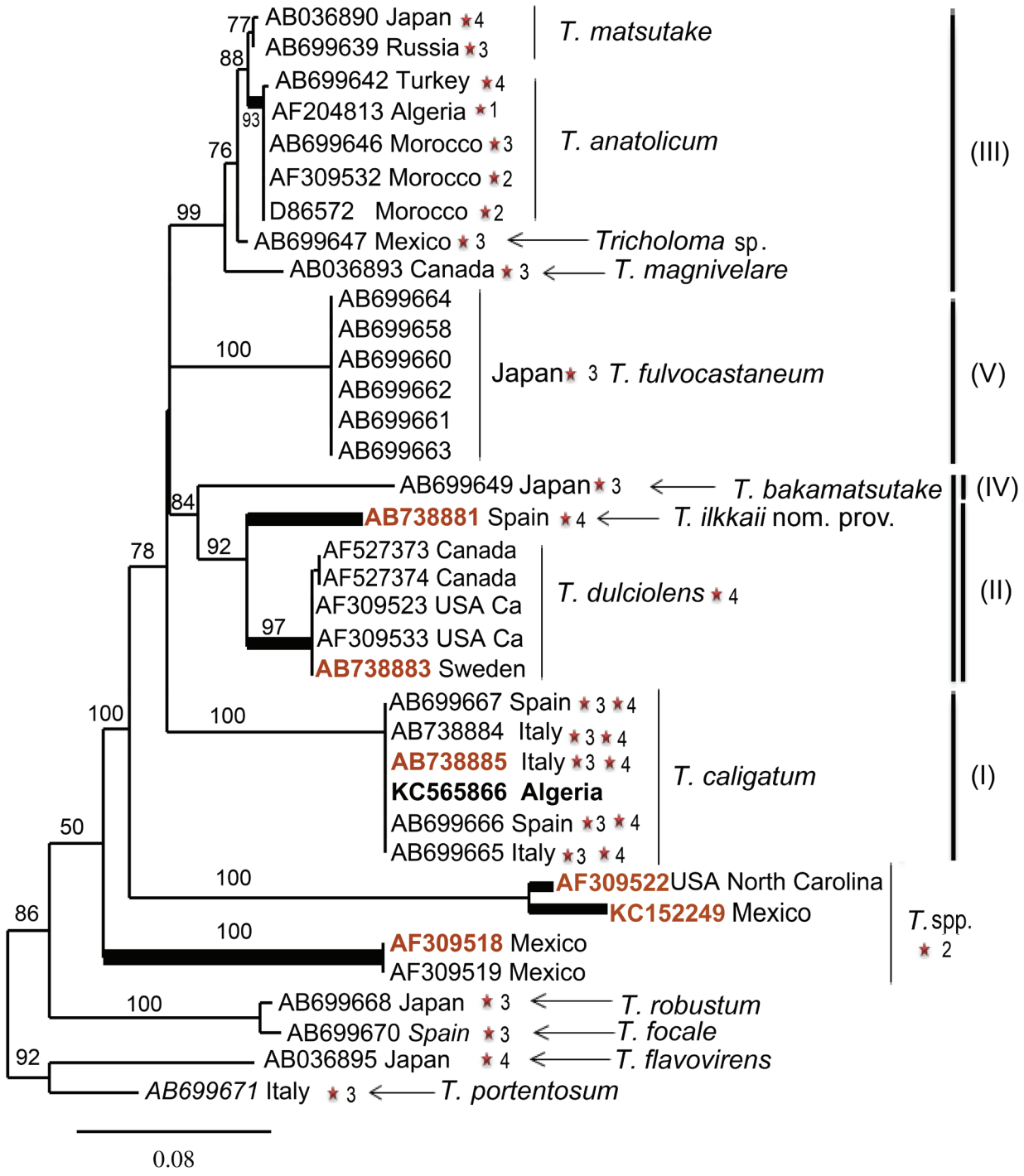

FIGURE 1. PhyML Phylogram of *Tricholoma caligatum* sensu strico, *Tricholoma* identified in the past as *T. caligatum*, *T. anatolicum* and similar species. The tree was obtained after analyzing rDNA ITS (ITS1, 5,8S and ITS2) sequences. *T. flavovirens* (AB036895) and *T. portentosum* (AB699671) (Ota *et al*. 2012) are used as outgroup to root the phylogeny. Thickened black branches refer to species that were initially identifed as *T. caligatum* but correspond to different species. The origins of the specimens are indicated. Clade numbering follows Murata *et al*. 2013a. Bootstrap values >50% are shown above or left of branches. Sequence in bold corresponds to the Algerian sample collected for this study. In red are RefSeq or RepSeq for SH in Unite database, corresponding to different clades in this phylogram. Sequences used in articles updating phylogeny of Tricholomatoid clade and *Tricholoma* genera are indicated (*1 Kikuchi *et al.* 2000; *2 Chapela & Gabelotto 2004; 3* Ota *et al.* 2012; *4 Murata *et al*. 2013a).

DNA extraction, PCR amplification and sequencing

DNA extraction and amplification from dried specimens were performed following Doyle (1987) and Noël & Labarère (1987) with minor modifications. Mass of 25 mg of dried basidiocarps was ground in liquid nitrogen before extraction. The resulting powder was re-suspended in 700 µl of hot lysis buffer (CTAB 2 %, NaCl 1.4 M, EDTA pH 8.0 20 mM, Tris–HCl pH 8.0 100 mM) supplemented with 14 µl of β-mercapto ethanol. The resulting mixture was incubated for 20 min at 65°C, mixed by regular inversion and cooled to room temperature. One volume of chloroform:isoamyl alcohol (24:1) was added and gently mixed with the samples until they emulsified. The mix was centrifuged for 10 min at 20 000 g. The aqueous portion containing rDNA was transferred to a new tube. A second extraction was carried with 700 µl of chloroform:isoamyl alcohol (24:1) and 5 min centrifugation at 20 000 g. DNA present in the supernatant was precipitated with 700 µl of precipitation buffer (CTAB 1 %, NaCl 0.04 M) and incubated at room temperature for 30 min. The supernatant was discarded and pellet was washed with 500 µl of NaCl 1M and 1 ml of absolute ethanol. The suspension was carefully mixed, incubated at room temperature for 30 min and centrifuged 10 min. The pellet was washed three times with 1 ml 70% ethanol; for each rinse, the suspension was centrifuged for 10 min at 20 000 g and the supernatant was discarded. The pellet was dried in a Speed Vac (Thermosavant) and finally resuspended in 50 µl of UltraPure DNase/RNase-Free distilled water. DNA quantification was performed using a NanoDrop Spectrophotometer ND-1000.

Amplification of ITS1 and ITS2 regions and 5.8S rDNA was carried out in PCR reaction mixture containing 50–100 ng of fungal DNA, 2 µl of each primer solution (10 mM): ITS1(5′-TCCGTAGGTGAACCTGCGG-3′) (White *et al*. 1990), and ALR0 (5′- CATATGCTTAAGTTCAGCGGG-3′) (Collopy *et al*. 2001), seven micro litre of mix dNTP (10 mM) (GAE PCR 11–5D, Amersham Pharmacia Bioth inc.), 10 µl of *GoTag* buffer (5X), 0.4 µl of *GoTag* DNA polymeras (Promega Corp., Madison, Wis, USA), and adjusted to a final volume of 50 µl.

Reactions were performed in a thermocycler (Eppendorf Mastercycler) using the following program: (a) denaturation at 95°C for 5 min; (b) 38 cycles of denaturation at 95°C for 40 s, annealing at 57°C for 45 s and elongation at 72°C for 1 min; (c) final extension 5 min at 72°C. PCR products were checked in 1 % agarose gels, and DNA was sent to Beckman coulter Genomics for sequencing of both stands (Sanger dideoxy sequencing method). CAP (v.1991) was used to assemble sequences.

Phylogenetic analyses

Samples from different geographic regions, with sequence data published in GenBank and originally identified as *T. caligatum* were used in the analyses. These sequences were derived from mushrooms originating from Spain (AB738881), Costa Rica (AF309520), Mexico (AF309518, AF30952219) and the USA, North Carolina (AF309522). We also added five sequences derived from samples originally identified as *T. caligatum*, but belonging to *T. dulciolens* according to Murata *et al*. (2013a) who sequenced the type specimen (AB738883) and positioned it in the phylogeny of *Tricholoma* spp. producing "matsutake" mushrooms.

The phylogenetic tree was constructed using the maximum likelihood method implemented in the PhyML program (v3.0 aLRT). First sequences were aligned with MUSCLE (v3.7) and then ambiguous regions were removed with Gblocks (v0.91b) with default settings. In ML analysis (http://www.phylogeny.fr/), the default substitution model was selected assuming an estimated proportion of invariant sites and four gamma-distributed rate categories to account for heterogeneity rate across sites. The gamma shape parameter was estimated directly from the data. Reliability for internal branch was assessed using the bootstrapping method (100 bootstrap replicates). Graphical representation and editing of the phylogenetic tree were performed with TreeDyn (v198.3). Editing of sequence alignment was performed with BioEdit software (v7.0.9.0) Hall (1999).

Results and discussion

Phylogenetic position of North African specimens

The ITS sequence (KC565866) of the specimen recently isolated in Algeria (MPU028328) was identical to sequences of two specimen vouchers of *T. caligatum*: AB738884 from Calabria (Italy) and LT000152 from Valencia (Spain).

In the phylogenetic analyses (Fig. 1), the sequences of the recent Algerian sample clustered in the same clade as AB699665 from Italy, as AB699666 and AB699667 from Spain. These three samples were considered as the reference for *T. caligatum* by Ota *et al*. (2012) and Murata *et al*. (2013a). One more sequence, AB738885, from an Italian sample, considered as the reference sequence (RefSeq) for the species hypothesis (SH), corresponding to the southern

European *T. caligatum* (*Tricholoma caligatum*|AB738885|SH221552.07FU Threshold 1.5) in UNITE database, belong to the same clade. These five specimens were found under, *P. halepensis* (AB699666), *Pinus pinea* (AB738885 and AB699665) or *Pinus* sp. (AB699667), and M'Sila mixed forest (*P. halepensis* and *Quercus suber*) for KC565866. The present analyses confirm the presence of *T. caligatum* in Algeria. Other samples originally misidentified as *T. caligatum,* clustered together in different clades well supported by bootstraps values and different from clade I (*T. caligatum sensu stricto*).

In our phylogenetic ML tree (Fig. 1) we observe four strongly supported major clades (bootstrap support value > 80) corresponding to the previous clades of Murata *et al.* (2013a), except that clade II and IV cluster together in our study.

Taxonomy

Tricholoma caligatum: (Viv.) Ricken, Die Blätterpilze 1: 331 (1915)

Description based on the specimen CM030.

Macroscopic characteristics: Pileus 9–10 cm diam., broadly convex, covered with large appressed brown-ochraceous scales on a whitish background which is well-visible near the margin; Margin inrolled. Lamellae whitish, narrow, slightly adnexed, edges smooth. Stipe 9 X 3.5 mm; completely buried in the ground, sub-equal, slightly attenuated towards the base, with a boot opening at the top in a large white annulus, brown scales on the boot, giving ornamentation streaks or stripes across white background; stipe white above the ring. *Microscopic characteristics*: *Basidiospores:*—white (4.8–)5.5–6.1–6.5(–6.7) × (3.8–)4.4–4.8–5.3(–5.6) µm; Ovoid to ellipsoid; thin-walled, smooth, sometimes with a guttule. *Basidia:*—23.8–34.5 × 5.8–8.1 µm clavate, 4-spored. *Cystidia* and *Clamp connections* not observed.

Species-specific ITS markers:—Tricholoma caligatum possesses fourteen species-specific ITS markers among the whole species used in our phylogenetic analyses (Table 1).

Specimen examined:—ALGERIA. Wilaya of Oran, M'Sila forest, Parc zoologique, 35° 64' 36''N, 0° 89' 16''E (DD), elev. 90–380 m, December 2012, collector M. Benazza, CM030 (MPU 028328).

Note:—The main characteristics that allow to distinguish *T. caligatum* from *T. anotolicum* have been recalled and summarized in Table 2. Microscopic characteristics such as basidiospore size and basidia don't allow distinction between *T. anatolicum* and *T. caligatum*, except the maximum width of the hyphae on pileal surface (Table 2), a character not available in our dried specimen. Morphological criteria cannot be used for accurate species identification. Some of these specific characteristics, pileipellis, lamellae, odor and taste need to be confirmed in the future on fresh specimens from North Africa. According to its basic morphology, cytology and molecular specific traits (Fig. 2) the specimen isolated in M'Sila forest, Algeria, in 2012 corresponds to *T. caligatum*. This result is concordant with the phylogenetic analyses.

TABLE 1. The fourteen species-specific ITS markers that distinguish species *T. caligatum* from all species used in this study. Positions are measured from the first base of sequence KC565866 (AAGGATCATTA).

Species	Positions												
	ITS1							5.8S	ITS2				28S
	12	16	33	51	65	71	182	341	423	439	447	477	598
T. caligatum	C	G	C	A	T	G	C	A	T	T	T	T	A
Other species*	T	A	T	G	C	C	A	G	C	C	C	C	G

**T. matsutake* and allied species

Taste and fragrance

The diversity of opinions on the taste and fragrance of these mushrooms could be due to taxonomical misidentification, genetic diversity among populations of the same species and/or variations with the environment. With molecular tools, it will now be possible to determine the real species. For each population of *T. caligatum* and related species, and for *T. anatolicum* it will be possible to determine taste and fragrance in relation with host tree.

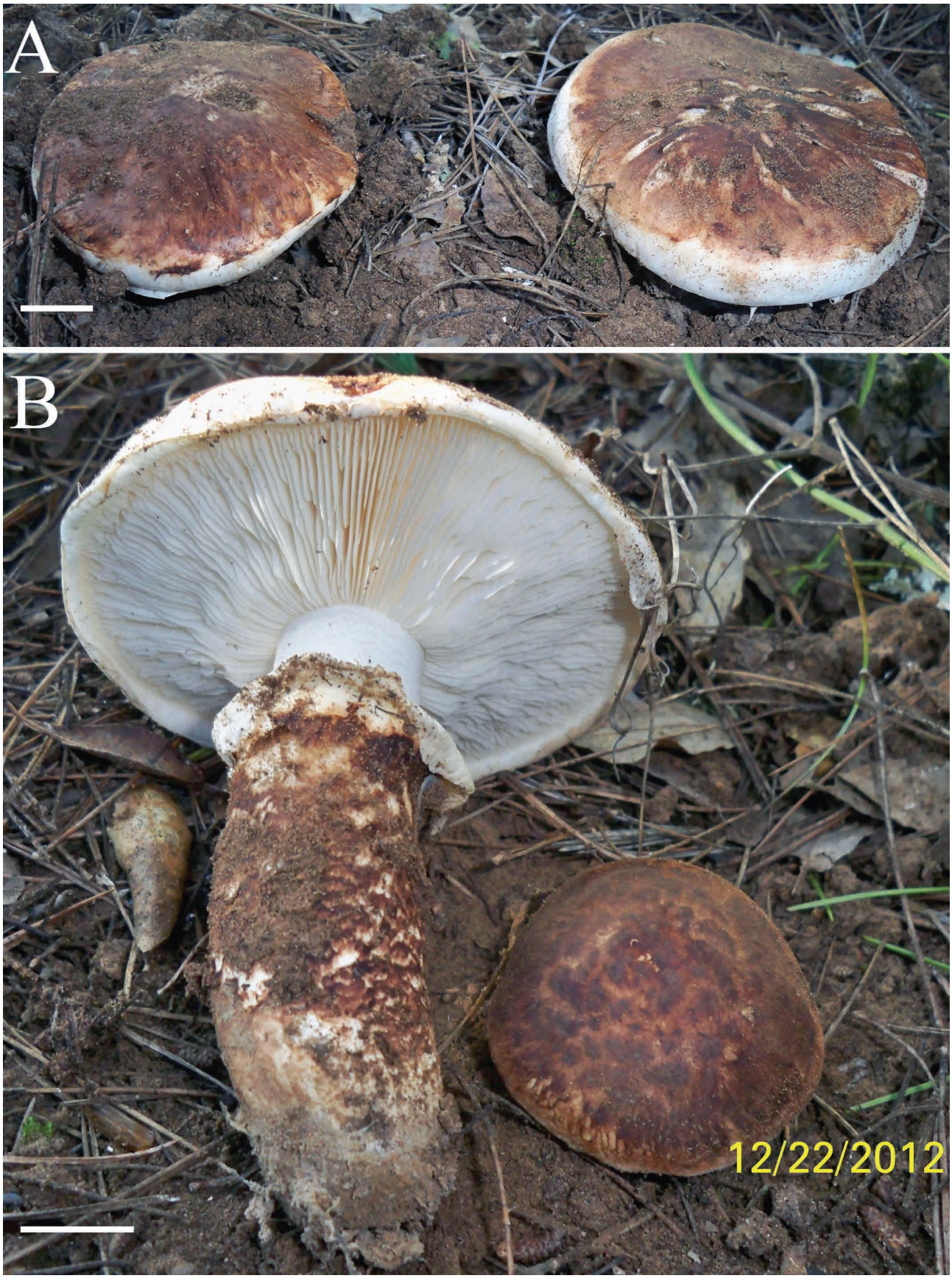

FIGURE 2. *Tricholoma caligatum* from M'Sila forest near Oran in Algeria. Bars: 2cm. A. mushroom buried in sandy soil, B. stipe with brown scales.

TABLE 2. Main characters, selected from Dogan & Akata (2011), which allow distinguishing *T. caligatum* from *T. anotolicum*.

	T. anatolicum	*T. caligatum*
Pileus	4 to 20 cm, hemispherical, convex to plane, **white to pale creamy when young**, brown to brownish-ochraceous with age.	3 to 12 cm, subumbonate, **blackish brown**, with **dark brown scales**.
Odour and taste	Fragrant, like that cedar of Lebanon (*C. libani*), taste very mild, pleasant	Strong, just like that *Inocybe corydalina*, taste sweetish-bitter to bitter
Lamellae	Narrow, adnexed	**Close**, broad, sinuate
Annulus	very close the lamellae	7 to 25 mm down from the lamellae
Stipe	above the annulus white, below the annulus ochraceous-brown zones	more or less transverse, blackish brown zones on a lighter background
Pileal surface	hyphae, 7 to **28** µm wide,	7 to **16** µm wide

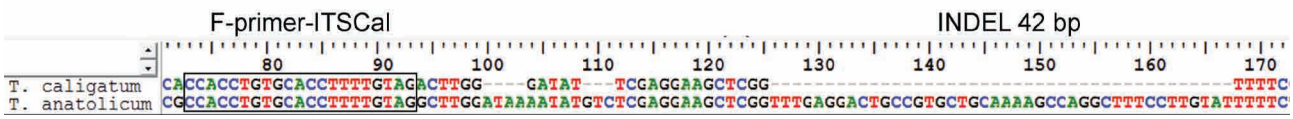

FIGURE 3. Indel (42bp) that distinguish ITS1 sequence of *T. caligatum* and *T. anatolicum*. The black box indicats the position of unspecific primer ITScalf used with ITScalr to amplify this region and identify the two species present in North Africa.

Clarification of T. caligatum species concept in UNITE database

Kõljalg *et al*. (2013) proposed the concept of species hypotheses (SHs) for taxa arising from two rounds of clustering of ITS sequences. In Unite database (http://www2.dpes.gu.se/project/unite/UNITE_intro.htm), clustering using different similarity threshold values are proposed as no single threshold "will demarcate intraspecific from interspecific variability in all fungi". One sequence is chosen to represent each SH; these sequences are called representative sequences (RepSeq) or reference sequences (RefSeq) depending on the sequence choice (https://unite.ut.ee/repository.php).

The 98.5 similarity threshold is sufficient to distinguish species without ambiguity as the four different species of the matsutake group, which are *T. matsutake, T. anatolicum, T.* sp. from Mexico and *T. magnivelare* (Peck) Redhead, *Trans. Mycol. Soc. Japan* **25**(1): 6 (1984), species confirmed by Ota *et al*. (2012) and Murata *et al.* (2013b), cluster in a single SH at this similarity cut-off. With this setting the present analysis shows that many samples initially identified as *T. caligatum* did not belong to the clade *T. caligatum s. str.* and these accessions correspond to species distinct phylogenetically from the European *T. caligatum* to which the original specimen collected in Italy belong. Sequences of the four Moroccan samples clustered in the *T. anatolicum* clade that were clearly circumscribed in the analyses done by Ota *et al*. (2012) using three partial genes and that have AB699644 as RefSeq in UNITE database. The Algerian sample Tc1(AF204813—Kikuchi *et al*. 2000), the fifth strain of the north African *T. "caligatum"* of which ITS sequence was obtained and released in GenBank before we released the sequence KC565866 in May 2013, is actually a *T. anatolicum* in the phylogenic tree (see Fig. 1). Among the five hypothetical species identified with the name *T. caligatum* in UNITE database in May 2016, only one should keep this identification (clade I in Fig. 1). The four others need to be described and re-named accordingly.

Finally, phylogenetic analyses and SH discrimination using similarity cut-off confirms that *T. caligatum* cannot be considered as a polyphyletic group. This species is well circumscribed by phylogenic analyses of rDNA ITS sequences. Among the eight different species that have been identified in the past as *T. caligatum*, three have been described and named (the two first correspond to *T. caligatum1* of Iwase 1994):

− *Tricholoma caligatum* s. str., described by Viviani in 1834; according to the current state of knowledge, its distribution is limited to south Europe and Algeria (SFig. 1). In North Africa this species must not be confused with *T. anatolicum*. The type has not been sequenced yet and AB738885 can be used as RefSeq.
− *Tricholoma anatolicum* (RefSeq AB699644); the holotype was sequenced (Intini *et al*. 2003) but the sequence is not yet available in INSDC.
− *Tricholoma dulciolens* (RefSeq AB738883 from the type), corresponding to *T. caligatum*2 of Iwase 1994, collected in Sweden and western North America.

Five are new species that should be described, named and a holotype designated:

− *Tricholoma* sp.1 (*Tricholoma sp*. | AB738881 | SH214407.07FU), sister species to *T. dulciolens*, found in Spain. It is found in literature as *Tricholoma ilkkaii nom. prov.* (Gulden *et al.* 2013, Christensen & Heilmann-Clausen 2013)

− *Tricholoma* sp.2 (*Tricholoma* sp. | AF309522 | SH202219.07FU) and *T.* sp.3 (*Tricholoma* sp. | KC152249/ SH202218.07FU) clustered in a same clade, the first one from eastern North America (corresponding to *T. caligatum*3 of Iwase 1994), the other one collected in Costa Rica and Mexico.

− *Tricholoma* sp.4 (*Tricholoma* sp. | AF309518 | SH187071.07FU) collected in Mexico

− *Tricholoma* sp.5 (*Tricholoma* sp. | AF319425 | SH460882.07FU) origin unspecified (not included in our phylogenetic analyses)

A large indel (42 bp) distinguishes ITS sequences from T. caligatum and T. anatolicum

To find a secure and easy method to distinguish these two species growing in North Africa, we examined the differences between their aligned sequences. A large indel of 42 bp was found (Fig. 3) that may be used to develop a fast and efficient discriminating molecular method for the distinction between the two North African *Tricholoma* species. A fast DNA extraction using microwave oven (Goodwin & Lee 1993) or Whatman FTA Cards (Borman *et al*. 2006), followed by PCR with the primers ITScalf (CCACCTGTGCACCTTTTGTAG) and ITScalr (ACTCAAACAGGCATGCTCCT) are expected to amplify two DNA fragments of 282 bp and 328 bp for *T. caligatum* and *T. anotolicum* respectively (Fig. 3). The size of the amplicon can be measured by electrophoresis on a concentrated gel (1.5 % agarose) or on a high resolution agarose gel (with MetaPhor Agarose LONZA WALKERSVILLE INC) with a benchtop DNA ladder beside the samples. Two other ways to distinguish these two species are (i) to digest an amplicon obtained with universal fungal rDNA primers (ex ITS1/ALR0) using an appropriate restriction enzyme (EcoRII (/CCWGG) by example), or (ii) to amplify the ITS fragment by qPCR, using a specific probe for the longest sequence. In these cases a good quality DNA extract is required.

Distribution of the 4 European and North African species of T. matsutake and allied species

Kytövuori (1988) has presented the distribution of *T. caligatum, T. dulciolens and T. nauseosum.* Specimens of *T. nauseosum* examined and identified by Kytövuori in Algeria in 1988 were found under *Cedrus atlantica.* This author considers that specimens collected under the same tree in Morocco by Malençon and Bertault also are *T. nauseosum*. All specimen of *T. nauseosum* found under this tree in Morocco actually belong to *T. anatolicum* as revealed with molecular studies (Ota *et al*. 2012). Then, we predicate that specimens collected in North Africa were misidentified and are *T. anatolicum* and not *T. nauseosum* because *T. nauseosum* is synonym of *T. matsutake* (Kytövuori 1988)), which is confirmed by molecular study by Bergius & Danell (2000) and Matsushita *et al.* (2005). The presence of *T. matsutake* in North Africa as not yet been demonstrated.

Based on molecular analyses, the distribution of the four Europeans and North African species of *T. matsutake* and allied species is the following:

− *Tricholoma caligatum* distribution (http://dx.doi.org/10.15156/BIO/SH221552.07FU) is illustrated by SFig. 1. In Unite databases the RefSeq is AB738885 and our sample (KC565866) is the first African specimen reported confirmed by molecular tools. Specimens of *T. caligatum* from Cyprus (Loizides 2008) and Switzerland (Breitenbach & Kränzlin 1991), should be sequenced to determine if they belong to the same species as *T. caligatum s. str*.

− *Tricholoma anatolicum* distribution is different. It was found in Turkey, Morocco, Algeria (AF204813) and one sample (AF458443) was collected in western United States.

− *Tricholoma matsutake* is present in North of Europe (Norway, Finland—Ota 2012, Sweden—Matsushita *et al*. 2005), South Europe (Italy, Switzerland—Matsushita *et al.* 2005) and on other continents.

− *Tricholoma dulciolens* was found in Sweden (Murata *et al.* 2013a)

Host

It is now necessary to confirm if, in Algeria and Morocco, *T. anatolicum* is specifically associated with *Cedrus atlantica* and *T. caligatum* with *Pinus halepensis* similar to the association of *T. anatolicum* with *Cedrus libani* (in Turkey) and *T. caligatum* with *Pinus* spp. in Southern Europe.

Conclusion

*Tricholoma caligatu*m is not a polyphyletic group as previously proposed, but a well delimited species, according to phylogenetic analyses of rDNA ITS sequences and the Species Hypothesis concept from UNITE database. Five samples from North Africa for which ITS sequences were available had actually been misidentified and finally correspond most likely to *T. anatolicum*. Nontheless, with a recent collection from the Algerian coast (Oran) in a mixed forest of *Quercus suber* and *Pinus halepensis,* the presence of *T. caligatum* in North Africa is reported for the first time using molecular tools. This work demonstrates that *T. caligatum* and *T. anatolicum* are both present in North Africa. Considering the economic importance of these species as non-timber forest products, it is necessary to have strong criteria for distinguishing them. Both morphological and molecular keys for an efficient and rapid distinction will be required.

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