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Uniting *Tricholoma sulphureum* and *T. bufonium*

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The taxonomic status and relationship of *Tricholoma sulphureum* and the similar *T. bufonium* were investigated using different sets of characters. These included morphological data on fruit bodies, ecological and chorological data, and analysis of the sequence data obtained for the ITS of basidiomes of different ecological and geographic origin. Moreover, the ectomycorrhizas formed by *T. bufonium* on *Abies alba* and *Quercus* sp. were characterised, and anatomical features compared with those of *T. sulphureum* mycorrhizas on coniferous and broad-leaved host trees. Our results revealed extensive ITS variation in members of the *T. sulphureum* group, but this variation was not correlated with morphology, ecology, or geographical distribution. We conclude that *T. bufonium* cannot be maintained as an autonomous taxon and should be treated as an infraspecific variant of *T. sulphureum*.

INTRODUCTION

In the last few years, we have undertaken a study of the hitherto little-known ectomycorrhizas of silver fir (*Abies alba*) (Comandini, Pacioni & Rinaldi 1998, 2001, Eberhardt *et al.* 2000), an ecologically important tree found in many mountainous regions of central and southern Europe. During our investigations, we distinguished and characterised more than 60 ectomycorrhizal morphotypes on silver fir, the vast majority of which had never been described before and were formed by unidentified mycobionts (Comandini *et al.* 2001). During this study we repeatedly collected fruit bodies that were morphologically identified as *Tricholoma bufonium*, fruiting, in a natural silver fir wood and in an artificial silver fir plantation both located in central Italy, and we also isolated their ectomycorrhizas.

T. bufonium is generally considered rare, and its taxonomic status has for a long time been a matter of discussion. Some authors question the validity of the taxon and claim that it is only a variety of the closely related *T. sulphureum* (Noordeloos & Christensen 1999). Others accept *T. bufonium* as a species on the basis of the constancy of the features distinguishing it from *T. sulphureum* (Bon 1984, Riva 1988), although, admittedly, intermediary forms exist. The two 'species' are clearly depicted in colour by Breitenbach & Kränzlin (1991). Both share the peculiar pungent odour (described as gas-like or coal tar-like) and the large amygdaliform spores.

Here we provide an extensive morpho-anatomical characterisation of the naturally occurring ectomycorrhizas formed on silver fir by *T. bufonium*. The species was classified based on basidiome features, and the identity of the ectomycorrhizas was confirmed by molecular methods. Molecular information has also been obtained from fruit bodies of *T. bufonium* and *T. sulphureum*. Our observations, together with already published information, notably the morpho-anatomical characteristics of *T. sulphureum* ectomycorrhizas on spruce (Agerer 1987), allow a better judgement to be made as to the taxonomic status and relationships of these taxa.

MATERIALS AND METHODS

Collection and morpho-anatomical characterisation of ectomycorrhizas

The *Tricholoma* basidiomes and ectomycorrhizas used in this study are listed in Table 1. Fruit bodies

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Table 1. Overview of *Tricholoma sulphureum*/*bufonium* basidiomes and ectomycorrhizas used in this study.

Species		Host	Geographic origin	GenBank accession no. *ITS #LSU	Reference material	Code in phylogram
T. bufonium	(b)[a]	*Abies alba*	Fonte Gelata, Italy	AY462029* AY462031#	AQUI[b]	OC01-51
T. bufonium	(ecm)[c]	*Abies alba*	Fonte Gelata, Italy	AY462028*	AQUI	not shown
T. bufonium	(b)	*Abies alba*	Colle Pelato, Italy	ns[d]	AQUI	
T. bufonium	(ecm)	*Abies alba*	Colle Pelato, Italy	ns	AQUI	
T. bufonium	(b)	*Quercus* sp.	Pescocanale, Italy	AY462030*	AQUI	OC01-131
T. bufonium	(ecm)	*Quercus* sp.	Pescocanale, Italy	ns	AQUI	
T. sulphureum	(b)	*Picea abies*	Tübingen, Germany	AY462032* AY462040#	TUB	TUB011629
T. sulphureum	(b)	*Castanea sativa*	Marana, Italy	AY462033*	AQUI	OC01-77
T. sulphureum	(ecm)	*Castanea sativa*	Marana, Italy	ns	AQUI	
T. sulphureum	(b)	*Picea abies*	Brunflo, Sweden	AY462034*	C	MC97-101
T. sulphureum	(b)	*Fagus sylvatica*	Løvenholm Skovene, Denmark	AY462035*	C	MC96-162
T. sulphureum	(b)	*Quercus* sp./*Populus tremula*	Kås egekrat, Denmark	AY462036*	C	MC94-023
T. sulphureum	(b)	*Quercus* sp.	Øjesø, Denmark	AY462037*	C	MC96-245
T. sulphureum	(b)	*Fagus sylvatica*	Enemaerket, Denmark	AY462038*	C	MC95-188
T. sulphureum	(b)	*unknown*	Le Brassus, Switzerland	AF377244*[e]	SFSU	AF377244
T. sulphureum	(b)	*Fagus sylvatica*	Arendal, Norway	AF377245*[e]	O	AF377245
T. sulphureum	(b)	*Quercus* sp./*Pinus* sp.	Gainesville, Florida	AY462039*[f]	SFSU	DED4539
Outgroup						
T. lascivum	(b)			AF377205*[e]		AF377205
T. lascivum	(b)			AF377206*[e]		AF377206
T. lascivum	(b)			AF241513*[e]		AF241513

[a] Basidiome.
[b] Vouchers: AQUI, University of L'Aquila; TUB, University of Tübingen; C, University of Copenhagen; SFSU, San Francisco State University; O, University of Oslo.
[c] Ectomycorrhiza.
[d] Not sequenced.
[e] Previously available in database.
[f] Sequence provided by Martin Bidartondo (University of California, Berkeley).

and ectomycorrhizas of *T. bufonium* and *T. sulphureum* were collected at various localities in central Italy and around Tübingen, Germany. After collection, basidiomes were dried and deposited in the Herbarium Mycologicum Aquilanum (AQUI) and in the Institute of Botany, Spezielle Mykologie, Tübingen (TUB). Additional reference material of *T. sulphureum* collected in northern Europe was obtained from the Botanical Museum of the University of Copenhagen (C). For ectomycorrhizas, soil cores were excavated just below the basidiomes, kept in the refrigerator until use, soaked in tap water overnight, and mycorrhizal roots carefully separated under a dissecting microscope. Several tips were immediately transferred into 50% ethanol and stored at −20 °C for subsequent DNA analysis. The general methodology and terminology used to characterise ectomycorrhizas follows Agerer (1986, 1987–1998, 1995). *Munsell Soil Color Charts* (1975) were used as a reference for the descriptions of colours of ectomycorrhizas. Voucher specimens of ectomycorrhizas were deposited in AQUI as dried and fixed/preserved material (4% glutaraldehyde or ethanol 50%), respectively, together with slides. For microscopy, mantle preparations of fresh ectomycorrhizas were fixed on slides with polyvinyl lactophenol and observed with a Zeiss Axioplan 2 microscope. For longitudinal sections (2.5 μm thick), ectomycorrhizas were embedded in LR White resin (Multilab, Dalmuir, UK), cut with a Leica Ultracut R ultramicrotome, and stained with toluidin blue in 1% sodium borate for 15 s at 60 °.

DNA extraction, amplification, and sequencing

DNA was isolated according to the manufacturer's instructions from dried fruit bodies (lamellae and cap) using the DNAeasy Plant Mini Kit (Qiagen, Hilden). The internal transcribed spacer (ITS) within the ribosomal RNA genes was amplified using the primers ITS1F and ITS4 (White *et al*. 1990, Gardes & Bruns 1993). PCR conditions were chosen as in Haug (2002). PCR reaction volume was 50 μl, with concentrations of 1.5 mM $MgCl_2$, 200 μM of each dNTP (Life Technologies, Eggenstein, Germany), 0.5 μM of each of the primers (MWG-Biotech, Ebersberg, Germany), 1U Taq-polymerase (Life Technologies), 10% amplification buffer (Life Technologies), and an empirically determined dilution of the DNA extract. The PCR products obtained were purified using the QIAquick protocol (Qiagen). Cycle sequencing was conducted using the ABI PRISM Dye-Terminator

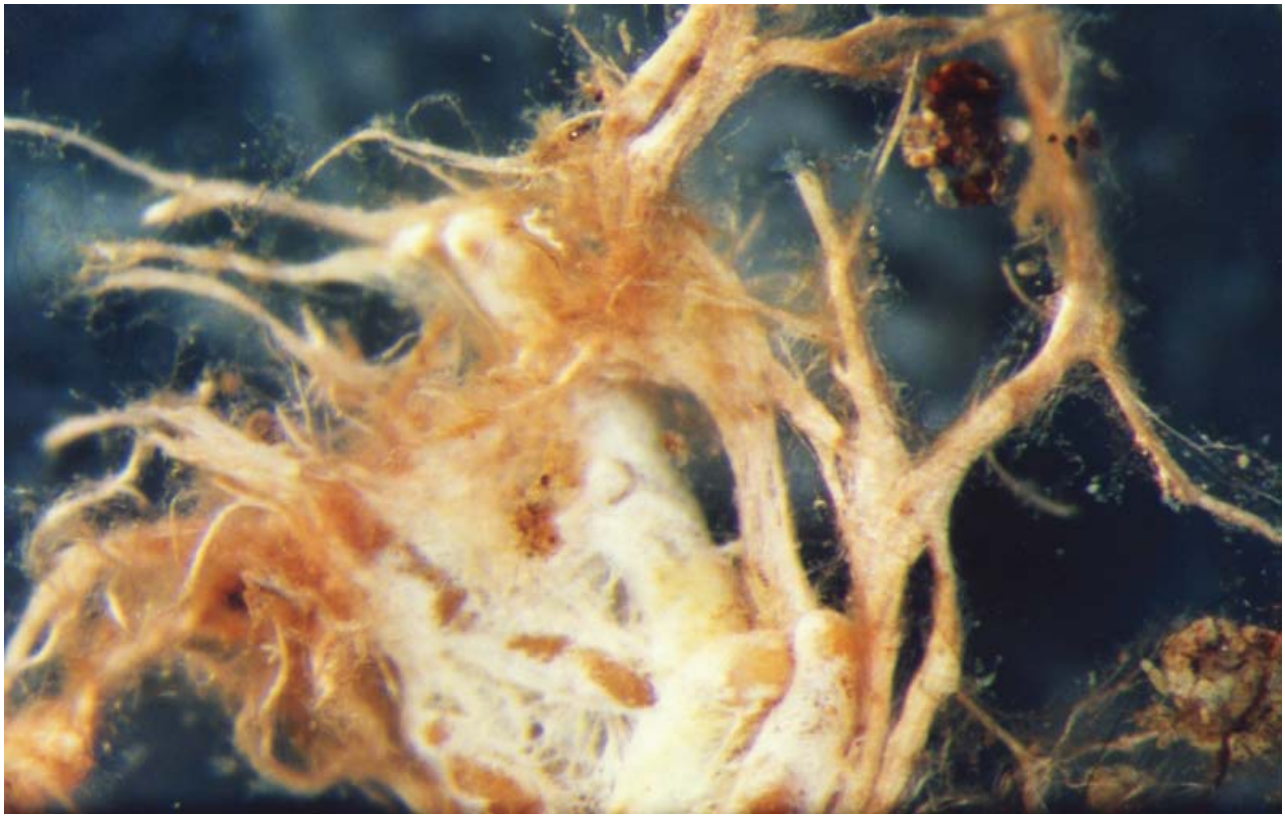

Fig. 1. Typical habit of *Tricholoma bufonium* ectomycorrhiza on *Abies alba* with typical reddish yellow rhizomorphs growing off in flat angles and forming hyphal fans. Magnification 20×.

Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Foster City, CA) followed by electrophoresis on an automated sequencer (ABI 373A Stretch, Applied Biosystems). Sequencing was carried out according to the protocols supplied by the manufacturer, except for reducing the cycle sequencing reaction volumes by half. Both strands of DNA were sequenced. Sequence editing was performed using Sequencher TW Version 4.1 (Gene Codes, Ann Arbor, MI).

Sequence analysis

The obtained sequences were used as probes in BLAST searches (Altschul *et al*. 1997) in GenBank in order to retrieve the most similar available sequences for inclusion in the phylogenetic analyses. The ITS sequences were aligned in the computer program ClustalX (Thompson *et al*. 1997). The alignment was manually revised with Se-Al version 2.03a (Rambout 1996). Since aligning of the end of the ITS2 region was not possible without conspicuous uncertainties, an ITS alignment of 596 sites was used for phylogenetic analysis with PAUP version 4.0b10 (Swofford 2002): a neighbor-joining analysis (Saitou & Nei 1987) was done with Kimura-2-parameter genetic distances (Kimura 1980), combined with a bootstrap analysis (Felsenstein 1985) from 1000 replicates. The sequences have been deposited in GenBank under accession nos AY462028–AY462040.

RESULTS

Description of ectomycorrhizas

[*Tricholoma bufonium* + *Abies alba*]

Morphological characters

Mycorrhizal systems (Fig. 1): from 3.2 to 5.8(–8) mm long, rarely simple, mostly monopodial-pinnate or irregularly-pinnate, orders of ramification 0–2. *Main axes*: 0.4–0.6 mm diam. *Unramified ends*: straight to bent with cylindrical tips, up to 3 mm long and 0.4–0.5 mm diam; mycorrhizas silvery white with reddish-yellow tones mostly due to the presence of abundant rhizomorphs. *Surface of unramified ends*: matted and stringy, frequently covered with soil particles; mantle not transparent, mycorrhizas not carbonizing. *Rhizomorphs*: abundant, growing off in flat angles and forming hyphal fans, emanating from all parts of mycorrhizas, to 0.35 mm diam, more or less flat in section, with smooth to hairy margins. Colour from reddish yellow (5 YR 6/6) to reddish brown (2.5 YR 5/4), very similar to the cap colour of the mycobiont. In young samples the tips have

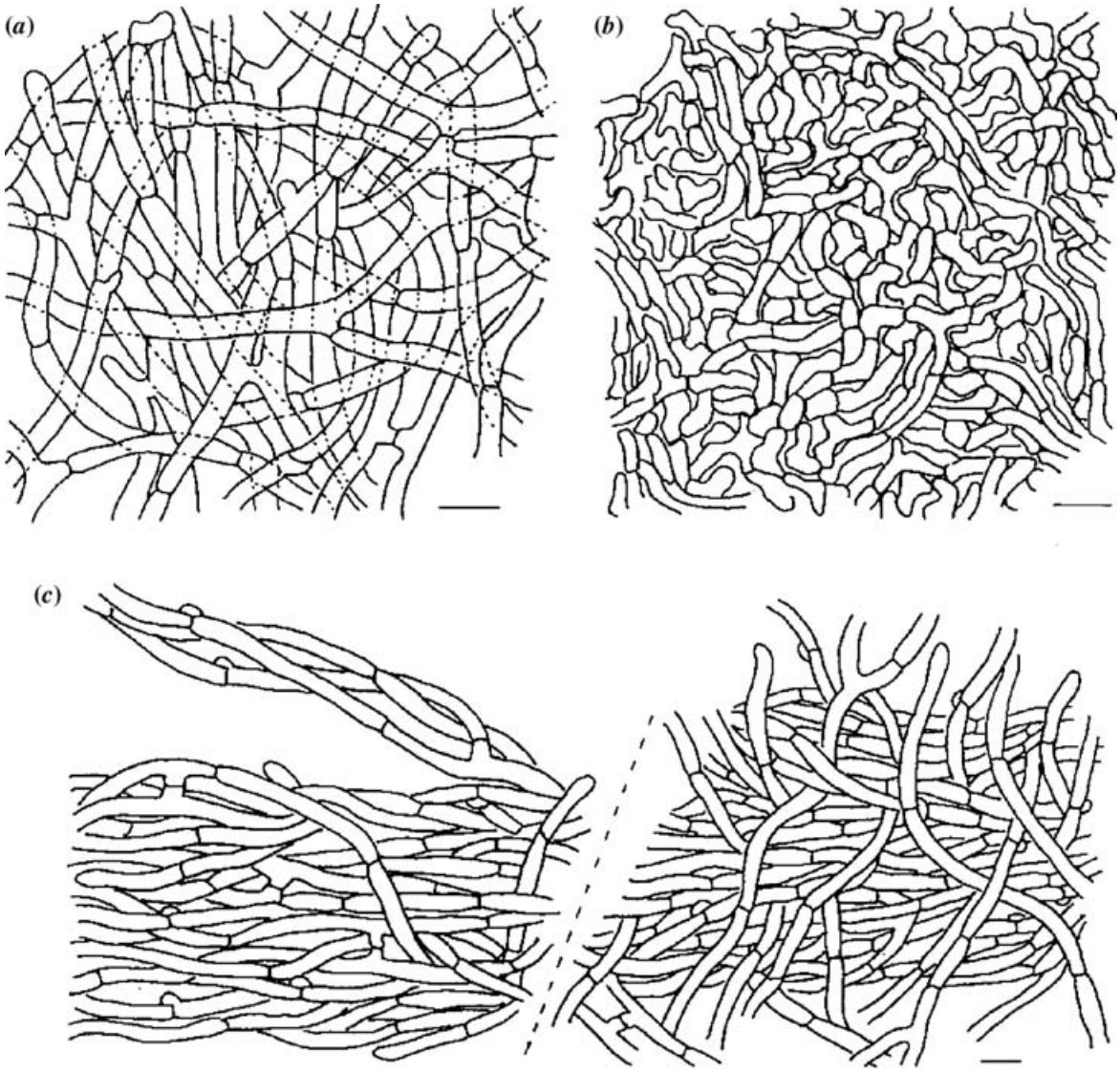

Fig. 2. Anatomical characters of *Tricholoma bufonium* ectomycorrhizas on *Abies alba*. (*a*) Outer mantle layer with plectenchymatous structure; hyphae are irregularly arranged without any special pattern. (*b*) Inner mantle layer with densely plectenchymatous structure without a discernible pattern; hyphae are irregularly shaped. (*c*) Surface view of thicker rhizomorphs with hyphae rather loosely interwoven and growing out of the margins (right side), and surface view of thinner rhizomorphs with smooth margins and hyphae compactly arranged (left side). Bars = 10 μm.

yellow tones similar to the stipe color of the mycobiont.

Anatomical characters of mantle in plan views

Outer mantle layers (Figs 2a and 3a): plectenchymatous, hyphae irregularly arranged without any special pattern. Nevertheless, in some parts, frequently branched hyphae that form a ring-like arrangement are present. Hyphae undifferentiated, mostly constricted at septa, from hyaline to slightly yellow, sometimes with a sort of incrustation on the surface, scarcely clamped, elbow-like protrusions sometimes present. Hyphae generally 3.5–4.5 μm diam, walls about 0.5 μm thick; septa as thick as walls, their distance ranging from 10–15 to 30–50 μm. Anastomoses present, closed by a contact-septum or by a simple septum (hyphal bridge). Short and sometimes distorted end-cells can occasionally be observed. *Middle mantle layers*: densely plectenchymatous, hyphae 3.5–4 μm diam, with the same features as those of the outer mantle, walls about 0.5 μm thick, hyaline or slightly yellow, clamps not observed. *Inner mantle layers* (Figs 2b and 3b): densely plectenchymatous, without discernable pattern, hyphae irregularly shaped, 2.5–3.5 μm diam, without clamps, walls about 0.5 μm thick. *Extreme tip*: plectenchymatous, more densely arranged than the other parts, hyphae 3.5–4 μm diam.

Anatomical characters of emanating elements

Rhizomorphs (Figs 2c and 3c): to 0.35 mm diam, undifferentiated (type A-B), hyphae straight to sinuous,

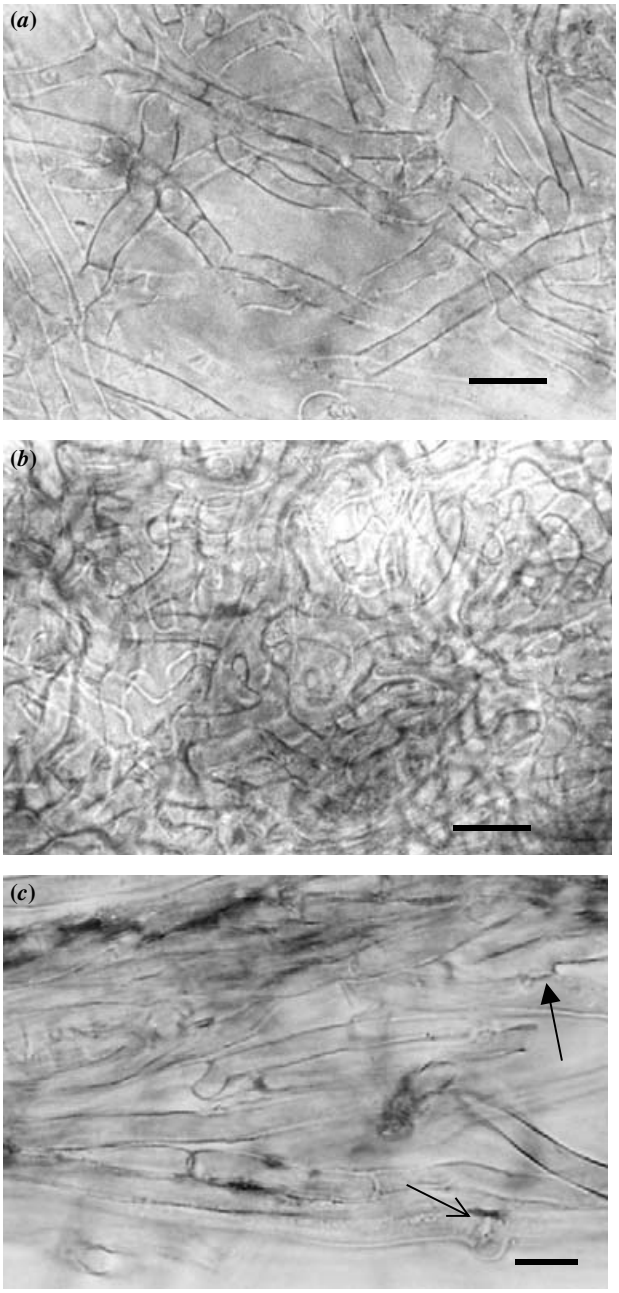

Fig. 3. Anatomical characters of *Tricholoma bufonium* ectomycorrhizas on *Abies alba*. (*a*) Outer mantle layer. (*b*) Inner mantle layer. (*c*) Surface view of rhizomorphs, with a clamp connection (arrow) and a contact septum anastomosis (solid arrow) in evidence. Bars = 10 μm. See legend to Fig. 2 for further details.

3–4.5(–6) μm diam, walls about 0.5 μm thick, hyaline to slightly yellow, frequently branched and with anastomoses such as in the mantle (contact-septum), clamps quite abundant. The thinner rhizomorphs have smooth margins and hyphae are compactly arranged, while the thicker ones possess hyphae rather loosely interwoven growing out of the margins.

Anatomical characters in longitudinal section

Mantle: 20–35 μm thick, the outer part (10–15 μm thick) loosely plectenchymatous, with hyphae 4–5 μm diam, hyphae of the inner part (10–20 μm thick) more compactly arranged, dimensions ranging from 7–15 μm tangentially and from 3–3.5 μm radially. *Tannin cells*: 1–2 rows of frequently collapsed cells tangentially elliptic, 30–50 μm tangentially and 5–8 μm radially. *Cortical cells*: tangentially elongated, 60–70(–90) μm tangentially and (14–)18–24(–30) μm radially. *Hartig net*: surrounding 2(–3) rows of cortical cells, 50–60 μm in depth, forming palmetti-like lobes 2–4 μm diam; in section, the Hartig net is formed by roundish or more cylindric cells (2–3 × 2–3 μm).

The ectomycorrhizas formed by *T. bufonium* on *Quercus* sp., and by *T. sulphureum* on *Castanea sativa* were also collected (Table 1) and observed. The main features of these morphotypes were compared with those of *T. bufonium* on *A. alba* (this study) and *T. sulphureum* on *Picea abies* (Agerer 1987), as reported in the Discussion.

ITS sequence analysis

Using three *Tricholoma lascivum* sequences as outgroup, the phylogram (Fig. 4) showed the the *T. sulphureum* group is very well supported, and includes collections that had been referred to *T. sulphureum* and *T. bufonium*. Within that group, two well supported clades were noted. Both clades contained collections morphologically referable to *T. sulphureum* and *T. bufonium*, and also collections from broad-leaved and coniferous trees. While clade 2 was quite homogeneous, clade 1 contained far more molecular variation (Table 2). This variation was partly due to a stretch of 17 bp (between bp 157 and 173 in ITS1) with eight variable positions (Table 3). All variable positions of MC97-101 in this stretch were identical with the sequences of the accessions of clade 2, and seven of eight variable positions of AF377245 were identical with the sequences of the accessions of clade 2. MC97-101 shared a further ten synapomorphies with the accessions of clade 2 (data not shown). Small molecular differences were noted between a North American collection of *T. sulphureum* (DED4539) and Danish and German collections of that species (MC96-162, MC95-188, TUB011629), but differences between different collections from Denmark were much larger than this intercontinental difference (Table 2).

DISCUSSION

While the application of the name *Tricholoma sulphureum* has always been unambiguous, much confusion has surrounded the application of the name *T. bufonium*. Before evaluating the various data with regard to the question whether this latter taxon may better be considered an autonomous (morpho)species or an infraspecific (formally named) taxon or variant (without a formal rank) of *T. sulphureum*, a short nomenclatural excursion is necessary.

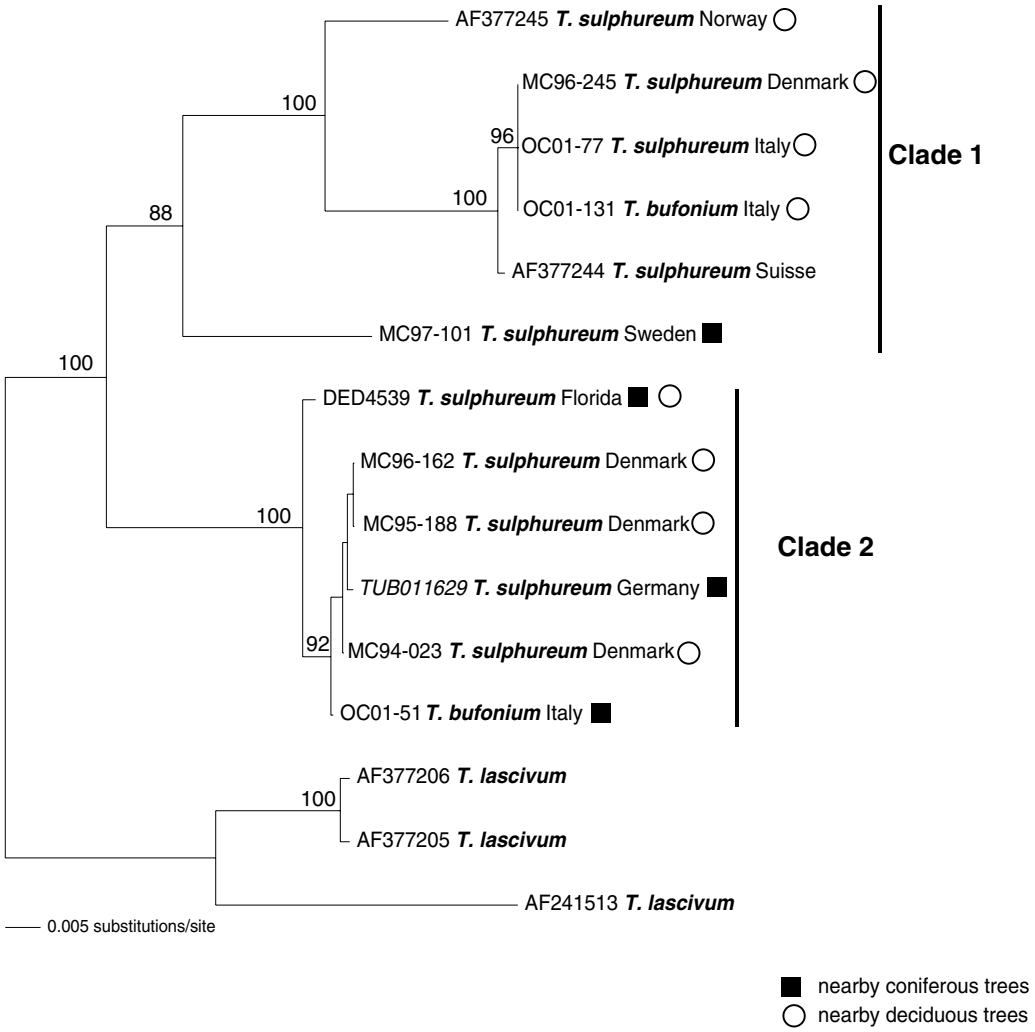

Fig. 4. Phylogenetic relationships of *Tricholoma sulphureum* and *T. bufonium* derived from ITS sequences: Neighbor-joining analysis of an ITS alignment of 596 sites. Genetic distances were computed according to the Kimura-2-parameter model. Branch lengths are scaled in terms of expected numbers of nucleotide substitutions per site. Numbers on branches are bootstrap values (1000 replicates). The topology was rooted with *T. lascivum*. The specimens with circles were found close to broad-leaved trees, with squares close to coniferous trees.

Persoon (1801: 359) described *Agaricus bufonius*, but the description suggests a fungus that is rather different from what is now called *T. bufonium*. It is remarkable that Persoon (1801: 322) also provided a description of *A. sulphureus*, but did not suggest a similarity between both species. Fries (1821: 88) accepted Persoon's species, without having observed it himself, and provided a description that was almost directly copied from Persoon. Gradually, Fries must have felt at unease with that species as in a re-evaluation of species described by Persoon, Fries (1830) suggested that *A. bufonius* could be an aberrant form of *Hygrophorus hypothejus* and subsequently (Fries 1832: 10) explicitly confirmed the synonymy between *A. bufonius* and *H. hypothejus*, a taxon notably lacking in Persoon (1801).

While this proposal as to the identity of *A. bufonius* could well have settled the issue, and have the name fall into oblivion, the name *A. bufonius* turned out to be far more recalcitrant. Fries (1838: 40) redescribed *A. bufonius*. Three aspects are noteworthy in that description: (1) Fries still considered it likely that *A. bufonius* referred to dried out specimens of *Hygrophorus hypothejus*; (2) Fries considered his earlier description (Fries 1821) as accurately representing the species, without realising that that description was almost literally copied from Persoon; and (3) the species was now listed next to *A. sulphureus*, even though nothing was said about the odour of *A. bufonius*.

We do not wish to discuss the nomenclatural implication of the contradictions between the sanctioning description (Fries 1821) and this later description (Fries 1838), but only remark that the liberal wording of Art. 7.8. of the *Code* allows us to maintain nomenclatural continuity between Persoon's name and the later interpretations through the sanctioning description. We opt for that possibility, because we believe that the taxonomic issues concerning *T. bufonium* should not be explained away by nomenclatural arguments.

Table 2. Proportion of sites differing between different collections of *Tricholoma sulphureum* and *Tricholoma bufonium* (based on an ITS alignment of 596 sites).

	MC96-245 OC01-77 OC01-131	AF377244	AF377245	MC97-101	DED4539	MC96-162 MC95-188 TUB011629	MC94-023
AF377244	1/557						
AF377245	23/549	22/550					
MC97-101	37/516	35/515	30/514				
DED4539	45/549	43/549	39/546	33/513			
MC96-162 MC95-188 TUB011629	47/549	45/549	43/547	35/511	5/554		
MC94-023	46/549	44/549	42/547	34/511	5/556	1/559	
OC01-51	45/548	43/548	40/546	33/510	3/555	2/558	1/560

Table 3. Variable positions in ITS1 between bp 157 and 173. Both AF377245 and MC97-101 belong (with MC96-245) to clade 1 in the phylogram (Fig. 4), although they share synapomorphies in this stretch of DNA with DED4539, which belongs to clade 2.

	ITS1							
Nucleotide	1	1	1	1	1	1	1	1
	5	6	6	6	6	7	7	7
	7	1	5	6	8	0	1	3
Taxon								
MC96-245	A	A	T	A	A	A	A	T
AF377245	C	T	C	G	A	–	G	C
MC97-101	C	T	C	G	G	–	G	C
DED4539	C	T	C	G	G	–	G	C

When Fries (1838) established the name for a taxon close to, but different from, *A. sulphureus*, the foundation was laid for subsequent taxonomic disagreements as to whether *T. bufonium* was a separate autonomous species or an infraspecific taxon of *T. sulphureum*. That latter view was stated most explicitly by Nüesch (1923: 89), who explicitly claimed that a separation between *T. sulphureum* and *T. bufonium* was unjustified. Kühner & Romagnesi (1953) also interpreted *T. sulphureum* in a wide sense and included variants with sulphur-yellow and with red-brown colours of the pileus, without even mentioning the name *T. bufonium*.

Authors who accepted both taxa on the species level seemed to find it quite hard to present their taxonomic decision in unambiguous terms. Riva (1988: 191) stated that *T. sulphureum* and *T. bufonium* were connected by a chain of forms or varieties, although he still maintained them as separate species. Bon (1984: 104) also expressed ambiguity, noting that these two varieties are possibly the links in a chain that connects *T. bufonium* with *T. sulphureum*, and Noordeloos & Christensen (1999: 147) suggested that *T. bufonium* might be considered a variety of *T. sulphureum* with a different ecology and distribution.

Four sets of characters can be used to evaluate the taxonomic position of *T. bufonium*, *viz*. (1) morphological data of fruit bodies; (2) anatomical data of the ectomycorrhizas; (3) ecological and chorological data; and (4) molecular data.

Morphological data

Recent treatments all agree that *Tricholoma sulphureum* and *T. bufonium* are identical in microscopical characters. Usually, only one macroscopical character has been used to separate them, pileus colour being the decisive one; predominantly bright yellow in *T. sulphureum*, and red-brown in *T. bufonium*. However, darker variants of *T. sulphureum* are accepted by Bon (1984; purplish brown squamulose in centre; named var. *coronarium*), Noordeloos & Christensen (1999; brownish tinges in the central part of the pileus; an unnamed variant), and Riva (1988; pileus intensely yellow, but with the centre finely purplish brown squamulose; as var. *coronarium*). The pigments of *T. sulphureum* have been investigated by Gluchoff & Steglich (1974) and Oertel (1984). Unfortunately, we found no data on the pigments of *T. bufonium*. The yellow pigments of *T. sulphureum* are formed by a number of flavomannin dimethyl derivatives and the anthraquinone endocrocin. The dominant compound was found to be trans-4-hydroxy-FDM (flavomannin-6,6′-dimethylether), both in the B-series (dominant) and in the A-series (in trace amounts). The same component, but with the A-series dominating, is found in *Cortinarius splendens* and *C. vitellinus* (Oertel 1984). These latter two species are described as having a bright yellow pileus, partly to completely brown-spotted, as shown in the coloured illustrations by Moser (1960). It is therefore possible that the brownish pigments are further derivates from the main pigments and without much taxonomic value in these *Tricholoma* and *Cortinarius* species.

Variants with reddening lamellae have been described in both *T. sulphureum* and *T. bufonium*, which further supports the suggestion that both taxa intergrade. A further difference between *T. sulphureum* and

T. bufonium was suggested by Breitenbach & Kränzlin (1991), with the latter species having much more crowded lamellae. However, that character has not been mentioned by other authors.

Anatomy of ectomycorrhizas

The mycorrhizas formed by several *Tricholoma* species in association with deciduous and coniferous trees have been extensively described (e.g. Agerer 1987, Uhl 1988, Waller & Agerer 1993, Cripps 1997, Nezzar-Hocine *et al*. 1998, Gill *et al*. 2000) and illustrated in full colour in some instances (Agerer 1987–99, Gill *et al*. 2000). Agerer (1995) also reviewed the key characteristics of *Tricholoma* ectomycorrhizas described so far. Features common to many of the *Tricholoma* mycorrhizas are a stringy, silvery mantle, surrounded by conspicuous extramatrical hyphal fans and rhizomorphs, and a plectenchymatous outer mantle layer. These characters were also observed in *T. bufonium* ectomycorrhizas on *A. alba*.

In an attempt to define functional groups of ectomycorrhizas, Agerer (2001) proposed a classification of ectomycorrhizal mycelial systems based on the amount of emanating hyphae and the presence and differentiation of rhizomorphs. Indeed, these features may be relevant to the exploration of soil, thus being predictive for distinctive foraging strategies adopted by a group of mycobionts. According to this classification system, members of the genus *Tricholoma* form both medium- and long-distance exploration type ectomycorrhizas, characterised by the constant presence of rhizomorphs that extend to a significant distance from the root tip and greatly increase the volume of exploited soil, thus representing an important extension of the host's root system. Interestingly, several *Tricholoma* species have been found to be associated with epiparasitic *Monotropoideae*, non-photosynthetic plants that obtain fixed carbon from other plants *via* a shared mycorrhizal fungus (Martin 1985, Bidartondo & Bruns 2001, 2002). It is tempting to speculate that it might be inherently advantageous for the epiparasitic plants to establish mycorrhizal connections with fungal symbionts able to spread in the soil over larger distances by means of extramatical mycelia with transport function. To support this view, it could be stressed that, with very few exceptions (mostly *Russula* spp.), the known mycobionts of monotropoid mycorrhizal symbiosis (besides *Tricholoma*, *Gautiera*, *Hydnellum*, and *Rhizopogon*; Bidartondo & Bruns 2001, 2002) all form medium- and long-distance exploration types (Agerer 2001).

During this study, mycorrhizas of *T. bufonium* on *Quercus* sp. were also collected (Table 1) and compared with those on *Abies alba*. The main features of both mycorrhizal types (mantle structure, hyphal shape and dimensions) were identical. The only notable differences were the colour of the mycorrhizas and their rhizomorphs, which in the *Quercus* mycorrhizas were whitish or very pale brown (10 YR from 8/2 to 8/4) and not reddish yellow (5 YR 6/6) to reddish brown (2.5 YR 5/4; similar to the cap colour of the fungal symbiont) as those on *A. alba*. Other observed differences, such as the shape and dimensions of mycorrhizal systems and the depth of the Hartig net, are known to be dependent on the different host species (Pillukat & Agerer 1992).

Mycorrhizas of *T. sulphureum* on *Picea abies* have been fully characterised by Agerer (1987). We collected *T. sulphureum* ectomycorrhizas on *Castanea sativa* (Table 1) for a comparison with those already described. All the main macroscopic and microscopic features were identical in both types (colour of ectomycorrhizas, hyphal characters such as dimensions, shape, presence of contact-septum between hyphae, and rhizomorph structure). A comparison between *T. sulphureum* and *T. bufonium* mycorrhizas yielded only very slight differences, regardless of the specific host tree: (1) Morphological characters: smaller dimensions of *T. sulphureum* mycorrhizas (unramified ends up to 0.2 mm long and up to 0.35 mm diam, *vs* 3 mm long and 0.5 mm diam); different colours of mycorrhizas and their rhizomorphs (white, sometimes yellow in *T. sulphureum vs* reddish-yellow/reddish-brown or whitish in *T. bufonium*); (2) Anatomical characters: mantle structures are very similar between the two types; hyphal dimensions and characters (diam, cell wall thickness) are also very similar; rhizomorphs are undifferentiated in both types and possess contact-septum anastomoses; clamp connections are rarely seen in *T. sulphureum* but are more abundant in *T. bufonium*; in longitudinal section mantle is thinner in *T. sulphureum* than in *T. bufonium* (3–20 μm *vs* 20–35 μm); in both the ectomycorrhizal types, the outer part of the mantle is formed by very loosing hyphae.

In conclusion, no convincing diagnostic anatomical features have been found to distinguish *T. bufonium* from *T. sulphureum* ectomycorrhizas on both coniferous and broad-leaved host trees. As in the basidiomes, only colour seems to be the main character for separating the two morphotypes. Again, this character difference is not absolute.

Ecological and chorological data

Tricholoma bufonium is said to be restricted to conifer forests in the montane zone, while *T. sulphureum* has a much wider ecology and chorology, occurring both in deciduous and coniferous forests, from sea level to the alpine zone. Trappe (1962) stated that *T. sulphureum* was linked to *Fagus sylvatica* and *Quercus* spp., but did not mention *T. bufonium*. More recently, the co-occurrence of both *T. bufonium* and *T. sulphureum* (var. *coronarium*) in the same habitat, a pure *Abies boriisii-regis* stand in Greece, has been reported (Athanassiou & Theochari 2001). Variants of *T. sulphureum* with brownish caps, growing under deciduous trees in the lowlands, have been regularly reported. However, there is a serious risk of circular reasoning

if these variants are referred to *T. sulphureum* on the basis of ecological and chorological arguments. Colour variation in fruit bodies and ectomycorrhizas (see above) suggests that morphological criteria do not allow independent assignment of such collections to either species.

Molecular data

The ITS data showed extensive molecular variation in members of the *Tricholoma sulphureum* group. Within the group two well supported basal clades could be recognised, and one of these clades (clade 1) consisted of reasonably well supported subclades. Specimens referable to *T. sulphureum* and *T. bufonium* occurred in both clades. Likewise, mycorrhizal associations with broad-leaved and coniferous trees were noted in both clades. Finally, the phylogram revealed no geographical structure, as sympatric populations from Denmark were very different from a molecular point of view, and allopatric (intercontinental) populations were sometimes almost similar. Interpretation of the four subclades of clade 1 in terms of morphological or ecological characteristics was not possible. The phylogram clearly showed that dark-coloured fruit bodies, as diagnostic for *T. bufonium*, occurred in both clades. We conclude that from a molecular point of view there is no reason to maintain *T. bufonium* and consider *T. bufonium* as an infraspecific variant of *T. sulphureum* without formal taxonomic status.

Substantial intraspecific sequence variation in ITS is rarely encountered in the *Agaricales*. Aanen, Kuyper & Hoekstra (2001) noted large sequence variation in a biological species of the *Hebeloma velutipes* clade, where ITS sequence differences of 20 bp were noted. Individuals having either type and individuals (dikaryons) having both types were encountered. Kretzer *et al*. (1996) investigated ITS sequences of morphospecies of the ectomycorrhizal genus *Suillus* and observed large intraspecific sequence variation in allopatric populations of the same species, while sequence variation was minor to absent in cases of sympatric populations or in cases of allopatry where species were recently introduced in a geographically different area. In cases of large sequence variation, the authors suggested the possibility that different morphospecies could in the future be recognised.

Curiously, extensive ITS divergence is not uncommonly encountered in *Tricholoma*. Mankel, Kost & Kothe (1999) investigated six isolates of *T. terreum*, and found substantial ITS variation ranging from 2 to 32 bp. Horton (2002) noted substantial variation (3.8–6.2%) in *T. flavovirens* (often considered a younger synonym of *T. equestre*). The sequence differences in the *T. sulphureum* group ranged from 0–47 bases (in the used alignment with 596 sites; Table 2), and from 0–31 bases in the three collections of *T. lascivum*. Several explanations have been forwarded to explain this variation. Horton (2002) mentioned the possibility of cryptic species, but no morphological support for this claim has been published. In the *T. sulphureum* group, we did not see morphological characters that support the division into two clades. Large molecular variation in clade 1 (due to MC97-101 and AFF377245, whose ITS contains a stretch of DNA with synapomorphies with accessions of clade 2), is consistent with a hypothesis of a former intraspecific hybridisation event between members of both clades followed by extensive introgression. Extensive sequence divergence combined with intraspecific hybridisation in a biological species of the *Hebeloma velutipes* clade was explained by molecular divergence in allopatry without the origin of sterility barriers (Aanen *et al*. 2001). Allopatric divergence in the *T. sulphureum* group could have originated in the various refugia of broad-leaved trees such as oaks during the last glacial periods in Europe. More detailed population studies of *T. sulphureum* in the Iberian peninsula, Italy, and the Balkan are needed to address this question.

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