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Detection of species within the *Xerocomus subtomentosus* complex in Europe using rDNA–ITS sequences☆

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ARTICLE INFO

Article history:
Received 24 June 2005
Received in revised form
19 October 2005
Accepted 6 November 2005
Published online 17 February 2006
Corresponding Editor: David L. Hawksworth

Keywords:
Basidiomycota
Boletaceae
Boletus
Cryptic species
Molecular phylogeny

ABSTRACT

Identification of species within the boletoid genus *Xerocomus* has relied heavily upon the macromorphological features of the basidiomes. However, the phenotypic plasticity of these features has resulted in considerable confusion over the delimitation of taxa. In this study, we examined collections attributed to the *X. subtomentosus* complex in Europe using morphological and rDNA–ITS sequence data. In total, 45 European collections from a wide range of geographical areas and ecological conditions were included in the study. In spite of detecting considerable genetic variation, even within individual basidiomes of *X. subtomentosus*, molecular data, spore size, flesh colour, and the colour of the basal mycelium allow for the recognition of four distinct taxa: two correspond to *X. subtomentosus* (13 collections) and *X. ferrugineus* (20); one *X. chrysonemus* sp. nov. (10), to date only found in the UK, is described as new; and the existence of another taxon (two; Italy and UK) is noted but left undescribed owing to lack of material. Eight collections from North America were also included in the study, from which two taxa with a close affinity to *X. ferrugineus* were recognised.

Introduction

The confusion and controversy associated with the taxonomy of the boletoid genus *Xerocomus* in Europe is remarkable considering the small number of species (16–22) involved (Engel *et al.* 1996; Ladurner & Simonini 2003). The variability of the macroscopic characters that have been used extensively to delimit species within *Xerocomus* has led to a proliferation of taxa (Oolbekkink 1991; Engel *et al.* 1996) and the incorporation of multiple taxa within broader concepts (Hansen & Knudsen 1992). It is also clear that many *Xerocomus* taxa have a southerly distribution (Ladurner & Simonini 2003) and that this has led to a strong regional element in the recognition of taxa within the genus.

The existence of two species complexes, *Xerocomus chrysenteron* and *X. subtomentosus*, has contributed considerably to the

☆ We dedicate this paper to Roy Watling.

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doi:10.1016/j.mycres.2005.11.013

taxonomic problems within *Xerocomus*. Recently, Peintner *et al.* (2003) critically examined the first of these complexes using detailed microscopical analyses and rDNA–LSU sequence data, and demonstrated that it was possible to clearly define species limits within this group. Eight taxa were recognised. The study also showed that, although useful, the colour of the pileus was not a reliable character for distinguishing species. Spore size and ornamentation, length of the pileipellis end cells, and the presence of 'pruinatus' hyphae (Ladurner & Pöder 2000) were the most useful morphological characters for taxon delineation.

The other species complex around *X. subtomentosus* is less species rich but no less confused. Singer (1945) split the complex into two sections: those with a blue–green colour reaction to ammonia on the pileipellis (sect. *Pseudophyllopori*) and those without the reaction (sect. *Subtomentosi*). Engel *et al.* (1996) used this arrangement in a monograph of *Xerocomus* in Europe, and included five taxa in the former and six in the latter section. Excluding those taxa that are now known to be more closely related to *X. chrysenteron* (Peintner *et al.* 2003), would leave three taxa in sect. *Pseudophyllopori* (*Xerocomus ferrugineus*, *Xerocomus flavus*, *Xerocomus lanatus*) and two taxa in sect. *Subtomentosi* (*X. subtomentosus*, *Xerocomus xanthus*). Within these sections, Engel *et al.* (1996) differentiated taxa using pileus colour and the degree of development of raised lines or ridges on the upper part of the stipe. However, the separation of taxa into these sections is artificial. Redeuilh (1994) demonstrated that the intensity of the blue–green reaction of the pileus cuticle to ammonia is dependent upon the amount of brown pigment in the cuticle, and that the reaction is actually a character for all taxa around *X. subtomentosus*. In the most recent monograph on *Xerocomus* in Europe, Ladurner and Simonini (2003) maintained only two species, *X. subtomentosus* and *X. ferrugineus* within the *X. subtomentosus* complex. This monograph was based primarily on material from central and southern Europe. In northern Europe, only a single taxon is recognised within the complex, with *X. ferrugineus* treated under *Boletus subtomentosus* (Hansen & Knudsen 1992).

The aim of the present study was to examine the complex of taxa around *X. subtomentosus*, with a view to establishing the number of taxa present, their morphological limits, and potential geographic distributions. We chose to use the generic name of *Xerocomus* in this study as it seems likely that molecular studies will show that the *X. subtomentosus* group is distinct from *Boletus* (Binder 1999); *X. subtomentosus* is also the type species of *Xerocomus*. We examined collections attributable to the complex from widely distributed locations in Europe, and also some from North America to examine conspecificity of European *X. subtomentosus* and *X. ferrugineus* with their North American counterparts. These collections are referred to as '*Xerocomus spadiceus*', following North American adoption of this Friesian name. In Europe (e.g. Ladurner & Simonini 2003), *X. spadiceus* is commonly interpreted as a synonym of *X. ferrugineus*.

Sequence data from the rDNA–ITS region were generated from each collection. This region has been used extensively to examine species limits and has been shown to correspond well with morphological species concepts in a wide range of fungal groups (e.g. Eberhardt 2000; Garnica *et al.* 2003; Leonardi *et al.* 2005). Here, we present the findings of this investigation, providing morphological descriptions for *X. subtomentosus s. str.*, *X. ferrugineus*, and *Xerocomus chrysonemus* sp. nov., including a table summarizing their distinguishing features. A fourth taxon differentiated is known from only two collections (one Italian and one from UK), and remains undescribed owing to a lack of material.

Materials and methods

Collection of material and morphological analysis

We studied 45 collections of the *Xerocomus subtomentosus* complex from Europe, including Italy, the UK (England, Scotland), and Sweden (Table 1). The initial identification of the collections was based upon local guides; Italy (Alessio 1985), Sweden (Hansen & Knudsen 1992), and the UK (Watling 1970). Eight North American collections from the complex were also included in the study (Table 1). Of these, seven originated from the eastern USA and one was from California. Nomenclature for North American material follows Snell and Dick (1970) and Arora (1986).

Microscopical analysis of spores followed that of Ladurner (2001) and Peintner *et al.* (2003). Briefly, spores were examined from either deposits at the stipe apex or associated with hymenophoral material. Measurements were made of 30 spores from each collection mounted in 3 % potassium hydroxide aqueous solution. Care was taken to ensure that only mature spores were measured. These usually contain one to three guttules and have darker walls than immature spores (Ladurner & Simonini 2003). Spore measurements are given as (minimum) mean $\pm$ S.D. (maximum). The spore quotient (Q) is the ratio of spore length to breadth (Q = l/b). Comparisons of spore characteristics among taxa were carried out using one-way analysis of variance (Minitab, Version 12©).

Species descriptions given here for *X. subtomentosus* and *X. ferrugineus* are adapted from Ladurner and Simonini (2003). The description of *X. chrysonemus* was prepared by A.H. and is based on fresh and dried material. Colour codes refer to Kornerup and Wanscher (1963).

Molecular analysis

The molecular analyses were based on DNA sequence data from the ITS region of nucRNA genes, including the two spacer regions ITS1 and ITS2, enclosing the highly conserved 5.8 S ribosomal gene. Sequencing was carried out between 1998 and 2004, using a number of protocols. Details of DNA extraction, primers, and PCR conditions have been described previously (Kårén *et al.* 1997; Eberhardt 2002; Rosling *et al.* 2003). In addition, some sequences were generated using a Beckman Coulter CEQ 2000 automated sequencer (Beckman Coulter, Fullerton, CA), using the CEQ dye terminator cycle sequencing kit (Beckman Coulter, Fullerton, CA) or an ABI PRISM 3700 DNA Analyser (Applied Biosystems, Foster City, CA) according to the manufacturers instructions.

Raw sequence data were edited in Sequencher (v. 4.1, Gene Codes Corporation, Ann Arbor, MI). In a number of *X. subtomentosus s. str.* specimens, two different copies of the ITS region could be recognised by comparing

Table 1 – Material included in the phylogenetic analysis of the *Xerocomus subtomentosus* complex, accession numbers of rDNA–ITS data, origin and host tree where known

Taxon	Initial ID, collection code and herbarium number[a]	GenBank accession no.	Origin	Associated tree
European material				
X. chrysonemus	IMP0002	DQ066380	UK, Hampshire, New Forest, Mystock Bridge	*Quercus* sp.
	IMP0056	DQ066377	UK, Kent, North Bishopden Wood, Blean woods	*Quercus* sp.
	B. lanatus AH97063	DQ066378	UK, Hampshire, New Forest, Vinney Ridge	*Quercus* sp.
	AH1999083	DQ066379	UK, Hampshire, Rufus Stone, New Forest	*Quercus robur*
	AH2000037	DQ066381	UK, Hampshire, New Forest, Gritnam Wood	*Q. robur*
	AH2000054	DQ066382	UK, Hampshire, New Forest, Gritnam Wood	*Q. robur*
	AH2000071	DQ066383	UK, Hampshire, New Forest, Vinney Ridge	*Q. robur*
	AH2001087	DQ066384	UK, Hampshire, New Forest, Vinney Ridge	*Q. robur*
	B. lanatus AH2001095	DQ066385	UK, Kent, North Bishopden Wood, Blean woods	*Quercus* sp.
	AH2003040 (K(M) 123243 – holotype)	DQ066376	UK, Hampshire, New Forest, Pig Bush	*Q. robur*
X. ferrugineus	AH2000024	DQ066396	UK, Gloucester, Forest of Dean	*Quercus* sp., *Fagus sylvatica*
	AH2000108	DQ066400	UK, Hampshire, New Forest, Vinney Ridge	*Pseudotsuga menziesii*
	AH2001020	DQ066401	UK, Berkshire, Windsor Great Park, Bishopsgate	*F. sylvatica*
	AH2001108	DQ066392	UK, Aberdeenshire, Morrone Birkwood, nr. Braemar	*Betula pubescens*
	AH2001114	DQ066395	UK, Perthshire, Kindrogan	*F. sylvatica*
	B. subtomentosus AT1999098	DQ066398	Sweden, Uppsala, City Forest	Mixed woodland
	B. subtomentosus AT2000107	DQ066390	Central Sweden, Riddarhyttan	*Pinus sylvestris*
	B. subtomentosus AT2000113	DQ066386	Sweden, Uppsala, Hammarby Graveyard	Mixed woodland
	B. subtomentosus AT2000118	DQ066397	Sweden, Uppsala, Hammarby Graveyard	*P. sylvestris*
	B. subtomentosus AT2000123	DQ066388	Sweden, Uppsala, Kvarnbo Mill	Mixed woodland
	B. subtomentosus AT2001071	DQ066402	Sweden, Umeå	*P. sylvestris*
	AT2004283	DQ066391	Sweden, Lammö	Mixed woodland
	GS0898	DQ066403	Italy, Reggio Emilia, Villaminozzo	*F. sylvatica*
	GS1215	DQ066399	Italy, Reggio Emilia, Passo della Stalucchia	*Picea abies*
	GS1236	DQ066404	Italy, Reggio Emilia, Lapo del Ventasso	*F. sylvatica*

Table 1 – (*continued*)

Taxon	Initial ID, collection code and herbarium number[a]	GenBank accession no.	Origin	Associated tree
X. ferrugineus var. *citrinovirens*	*Boletus citrinovirens* E 00099202 – holotype	DQ066389	UK, Aberfeldy	*F. sylvatica*
	B. citrinovirens AH1999103	DQ066394	UK, Berkshire, nr Ascot, Silwood Park	*Q. robur*
	B. citrinovirens AH2001110	DQ066393	UK, Perthshire, Kindrogan	*Salix repens*
	B. citrinovirens AT1998118	DQ066397	UK, Perthshire, Kindrogan	*P. sylvestris*
	GS1920	DQ066405	Italy, Trentino, Arco, Monte Velo,	*F. sylvatica, Picea abies*
Xerocomus. sp.	AH2004074	DQ066374	UK, Berkshire, nr Ascot, Silwood Park	*Populus* × *canescens*
	X. cfr *ferrugineus* GS1959	DQ066375	Italy, Reggio Emilia, Carpineti, Marola	*Castanea sativa*
X. subtomentosus	*B. lanatus* AH1997028	DQ066370	UK, Hampshire, New Forest, Gritnam Wood	*Quercus* sp.
	B. lanatus AH2001082	DQ066365	UK, Stirling, Rowardennan Forest	*Carpinus betulinus, Q. robur*
	AH2000001	DQ066369	UK, Bucks, Bernwood Forest	*Quercus petraea*
	AH2000014	DQ066368	UK, Berkshire, Windsor, Ascot Gate	*C. sativa*
	AT1998075	DQ066371	UK, Derbyshire, Chatsworth House	*Q. robur*
	AT2002025	DQ066361 DQ066362	Sweden, Uppsala, Slott Vik	*Q. robur*
	AT2004282	DQ066363 DQ066364	Sweden, Lammö	Mixed woodland
	X. subtomentosus f. *rubrotinctus* GS1284	DQ066367	Italy, Reggio Emilia, Casino, Peconte	Mixed broad leaved forest
	X. flavus GS1135	DQ066359 DQ066360	Italy, Reggio Emilia, Pulpiano, Viano	*Q. cerris*
	X. flavus GS2048	DQ066357 DQ066358	Iatly, Sardinia, Biasi, Pedru	*Q. suber*
	X. xanthus GS1681	DQ066372	Italy, Portanova di Ancona	*Q. ilex, Arbutus unedo*
	X. xanthus GS1796	DQ066355 DQ066356	Italy, Reggio Emilia, Reggio Emilia	*Quercus* sp.
	X. xanthus IMP0003	DQ066373	France, Belleme	Unknown
North American material				
X. ferrugineus s. lat.	*B.* cfr *lanatus* Both4193	DQ066412	USA, NY, Hamilton Co.	*Pinus strobus*
	B. cfr *lanatus* Both4214	DQ066408	USA, NY, St Lawrence Co., Star Lake	*Pinus* sp.
	Boletus spadiceus Both3551	DQ066407	USA, NY, Hamilton Co., Raquette Lake	*P. abies*
	B. spadiceus Both4079	DQ066410	USA, NY, Erie Co., North Collins	*P. sylvestris, P. strobus*
	B. spadiceus Both4086	DQ066411	USA, MA, Norfolk Co., Hingham	Unknown
	B. subtomentosus Both3312	DQ066413	USA, NY, Erie Co., Orchard Park	*Fagus grandifolia, Tsuga canadensis*
	B. subtomentosus Both3704	DQ066409	USA, NY, Hamilton Co., Raquette Lake	*P. abies*
	B. subtomentosus DA00-120	DQ066406	USA, California	Unknown

a Initial identity given to collections where this differed from the final analysis. Collection codes: AH & IMP, A. Hills; AT, A. F. S. Taylor; GS, G. Simonini; Both, E. E. Both; and DA, D. Arora.

complementary patterns of double peaks in sequencing results from different ITS primers (ITS2, ITS3, ITS4, White *et al.* 1990; ITS1f, Gardes & Bruns 1993). In these cases, both sequences were included in the analyses. In some sequences, there were even indications for the presence of additional deviating ITS copies. Sequences with more than two multiple copy sequence parts could not be retrieved and were accordingly coded as missing data.

Sequence alignment was initially carried out by ClustalX (version 1.81; Thompson et al. 1997) with standard settings and later edited by hand. Alignment was attempted, including ITS sequences, from all other known European *Xerocomus* spp. (published and unpublished data) without success. This was undertaken to ensure that all relevant taxa were included in the present analysis. As a result of difficulties in the alignment, no outgroup taxon was included. Gaps were introduced in the alignment where unambiguous alignment of sequences of different taxa could not be accomplished. This was performed in order to maintain existing intraspecific variation. Analyses were then run on the complete alignment. In addition, further analyses, excluding all gapped positions, were carried out to assess the overall phylogenetic resolution and topology.

Neighbour joining (NJ; Saitou & Nei 1987) and maximum parsimony (MP; Fitch 1971) analyses and the respective bootstrap analyses (Felsenstein 1985) were carried out in PAUP* (version 4.0b10; Swofford 2002). Modeltest (version 3.6; Posada & Crandall 1998) were used to give an indication of how many and which parameters should be considered in likelihood models. The suggested model was then used in a NJ analysis, complemented by a bootstrap analysis (1000 replicates) under the same model. To further explore the phylogenetic resolution of the data, MP analyses were performed in 100 and 1000 replicates (random addition of sequences) of heuristic search employing the default settings of PAUP*, and including and excluding gapped positions. If included, gaps were treated as unknown data. MP bootstrap analyses were done in 10 000 replicates with the fast bootstrap option.

Pairwise sequence comparisons were computed using PAUP*, with data settings in effect such that the absolute number of character differences were counted including gaps and excluding or including ambiguous base pairs.

Results

Molecular analyses

The alignment (TREEbase accession no. M2314) of the ITS spacer regions of 53 specimens revealed a pattern of sequence stretches that were reasonably conserved among all sequences, interspersed with stretches that could be widely divergent between specimens, but similar within groups of sequences. These groups are referred to as *Xerocomus subtomentosus s. str.*, *Xerocomus ferrugineus s. lat.* (including European *X. ferrugineus* and American *X. spadiceus*), *Xerocomus chrysonemus* and *Xerocomus* sp. (see below and Fig 1). The European *X. ferrugineus* and American *Xerocomus spadiceus* sequences shared the same ITS structure. By contrast, considerable length variation was encountered within the *X. subtomentosus s. str.* sequences. In addition to the variation found between specimens in *X. subtomentosus s. str.*, the raw sequence data of some of the collections revealed intragenomic length variation in the ITS, located in several places in the ITS1 and ITS2 (Fig 2). None of the other species showed evidence of similar intragenomic ITS length variation. Several taxa showed possible indications of intragenomic variability with respect to point mutations; e.g. position 82 in *X. chrysonemus*, positions 585 and 697 in European *X. ferrugineus*, and positions 271 and 872 in *X. subtomentosus s. str.* (position numbers refer to alignment TREEbase accession no. M2314).

Pairwise sequence comparisons

Table 2 summarizes the results of pair-wise sequence comparisons. Only sequences spanning at least 95 % (94 % in *X. subtomentosus s. str.*) of the entire ITS1 – 5.8 S – ITS2 were considered. The large number of intraspecific and intragenomic differences in *X. subtomentosus* is mainly due to four indels of varying lengths (Fig 2). The end of the ITS2 is intragenomically very variable in some specimens and is not assessable by direct sequencing. Missing data in the end of ITS2 (see Fig 2, downstream from bp 875) indicate that two, if not more than two, variants of this sequence stretch exist within the respective genomes. The high variability within *X. subtomentosus s. str.* contrasts sharply with the sequence homogeneity of the European collections of *X. ferrugineus* (Table 2). In terms of direct counts of base pair differences, the maximum sequence variation among members of the *X. subtomentosus* clade is larger than the maximum sequence variation among members of the *X. ferrugineus s. lat.* group. A crude comparison of the means and standard deviations (Table 2; not corrected for sequence numbers) shows that the ITS region of each of the three sub-divisions of *X. ferrugineus s. lat.* group (European *X. ferrugineus*, American *X. spadiceus* 1 and 2) is remarkably homogeneous. The *X. chrysonemus* group shows a moderate level of variability. The two sequences from the unnamed *Xerocomus* sp., one from the UK, the other from Italy, are identical.

The ITS1 sequence (252 bp, including one ambiguous base pair; spanning partial SSU and partial 5.8 S) was obtained from the type specimen of *Boletus citrinovirens*. The sequence falls within the European *X. ferrugineus* cluster (Fig 1) and differs by 0 bp (excluding ambiguous basepairs) from the other 19 collections in this group.

Phylogenetic analyses

Hierarchical likelihood ratio tests suggested a likelihood model incorporating unequal base frequencies, different substitution rates for transitions and transversions, and approximating the distribution of variable sites across the alignment by a gamma distribution with $P = 0.14$ (HKY + G). The result of a NJ analysis under this model is shown in Fig 1. MP analyses, including and excluding gapped position (results not shown), supported the same overall topology and indicated four major clades. These represent *X. subtomentosus s. str.*, *X. ferrugineus s. lat.*, *X. chrysonemus*, and a fourth branch, representing an undescribed taxon. The topology within these four groups was not fully resolved using MP analyses. However, the internal topology and the division of the *X. ferrugineus s. lat.* clade into one European group and two American clusters is supported in all analyses. MP bootstrap support is lower for some branches than the NJ values shown in Fig 1. In particular, when excluding the gapped positions, bootstrap support ≥ 75 % cannot be obtained for (a) the branch connecting the *X. ferrugineus s. lat.* and the *X.* sp. clade and (b) the branch separating the European *X. ferrugineus* sequences from the two American *X. spadiceus* groups.

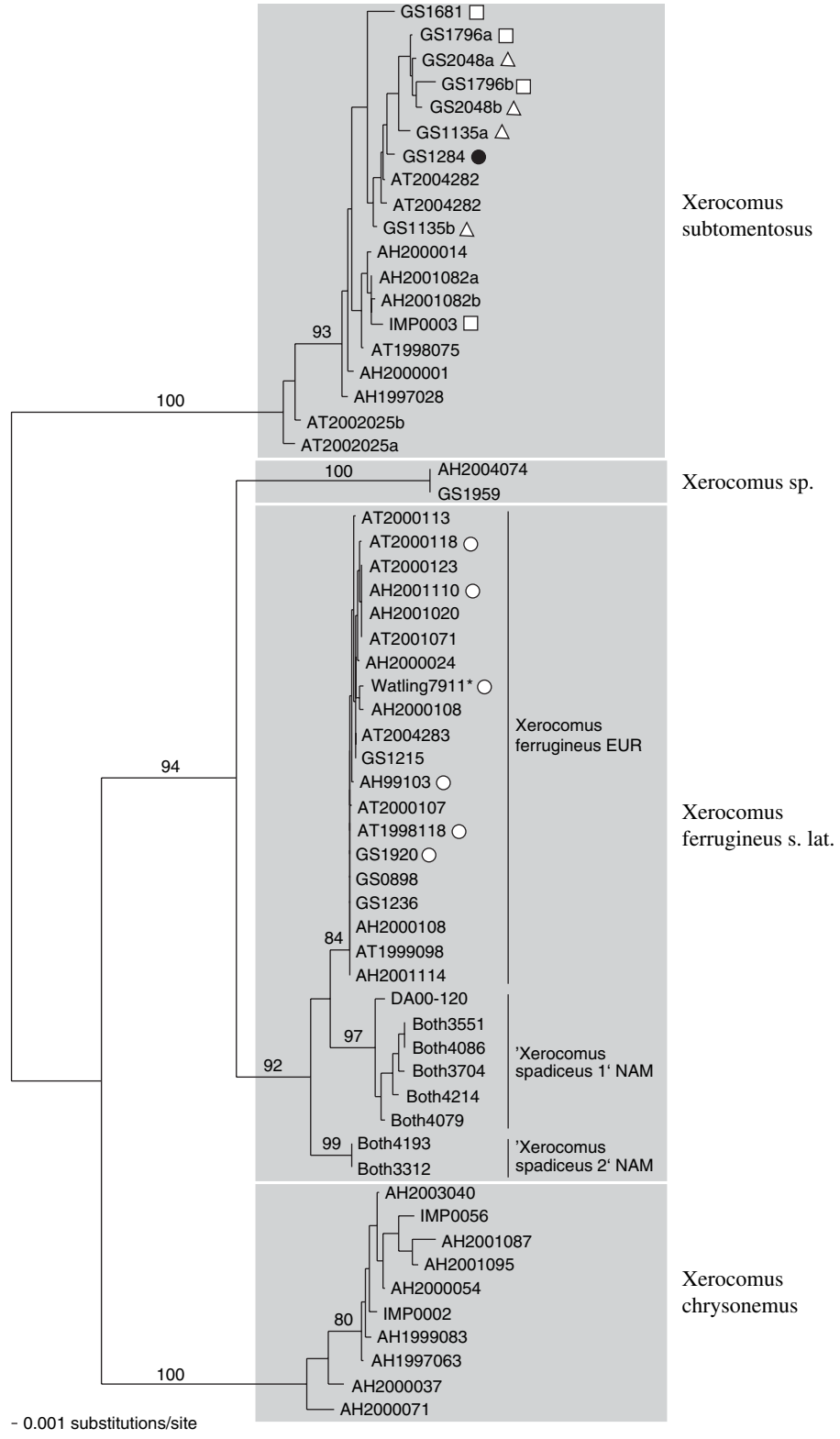

Fig 1 – Unrooted NJ topology of ITS sequences from 54 collections from the *X. subtomentosus* complex, complemented by a bootstrap analysis with 1000 replicates. Collection numbers refer to Table 1. Some collections harboured two different copies of the ITS, indicated by 'a' and 'b' following the collection no. Open squares indicate specimens identified as *X. xanthus*, open triangles *X. flavus*, closed circles *X. subtomentosus* f. *rubrotinctus*, and open circles *X. ferrugineus* f. *citrinovirens*. AT2002025 was a yellow form of *X. subtomentosus s. str*. The asterisk indicates the type collection of *Boletus citrinovirens* (Watling 7911E).

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                88        273       526        876
                |         |         |          |
AH2000014       CCTA--    -------   ------     GATCTTGATCTT-GATCTTGATCTTT
IMP0003         CCTA--    -------   ------     GATCTTGATCTT-GAT------CTTT
AT1998075       CCTA--    -------   ------     GATCTTGATCTTTGAC------CTTT
AH2000001       CCTA--    -------   ------     GATCTTGATCTTT-------------
AH1997028       CCTA--    -------   ------     GATCTTGATCTTT-------------
AH2001082a      CCTA--    -------   ------     GATCTTGATCTTT-------------
AH2001082b      CCTA--    -------   ------     GATCTTGATCTTTGAT------CTT-
AT2002025a      CCTA--    -------   ------     ????????????????????????????
AT2002025b      CCTA--    -------   GACTGA     ????????????????????????????
AT2004282a      CC----    -------   AACTGA     GATCTTGA???????????????????
AT2004282b      CCTACC    -------   AACTGA     GATCTTGA???????????????????
GS1284          CCTACC    CAATGAT   AACTGA     GATCTTGATCTTT-------------
GS1796b         CC----    CAATGAT   AACTGA     CATCTTGATCTTT-------------
GS1796a         CC----    -------   AACTGA     CATCTTGATCTTT-------------
GS2048a         CC----    -------   AACTGA     SATCTTGATCTTTGA-------CTTT
GS2048b         CC----    CAATGAT   AACTGA     SATCTTGATCTT--------------
GS1135b         CC----    -------   AACTGA     ????????????????????????????
GS1135a         CC----    CAATGAT   AACTGA     ????????????????????????????
GS1135c*        ??????    ???????   ------     ????????????????????????????
GS1681a         ---ACC    -------   AACTGA     GATCTTGATCTT-GAT------CTTT
GS1681b*,#      CCTACC    ???????   ??????     ????????????????????????????
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Fig 2 – Multiple base pair Indels in *X. subtomentosus* s. str. ITS sequences. Numbers refer to positions in alignment M2314 (TREEbase). Pairs or triplets of sequences derived from the same specimen are highlighted. *Not included in the phylogenetic analyses. #Only present in a minority of the amplicons.

The results of NJ and MP analyses support the conclusion that *X. xanthus*, *X. flavus*, and *X. subtomentosus* f. *rubrotinctus* are simply colour variants of *X. subtomentosus* s. str. Similarly, green and brown forms of *X. ferrugineus* are not distinct with respect to their ITS sequences. In *X. subtomentosus*, the intraspecific variation is suggestive of some degree of geographic variation, with the UK (including one French) and Italian specimens divided into different groups, with Swedish specimens breaking the pattern and uniting the groups.

In addition to *X. ferrugineus* and *X. subtomentosus* s. str., molecular data strongly supports the existence of two cryptic species in Europe that have not been previously recognized (Fig 1). One of these is described below as *X. chrysonemus*. So far, it has only been found in England. In contrast, the other species seems to be more widespread, with single collections from Italy and the UK.

A strong geographic pattern is clear in the *X. ferrugineus* s. lat. group. The sequenced European collections are very

Table 2 – Base pair differences (rDNA–ITS) among and within collections from the *Xerocomus subtomentosus* complex

Taxa	No. sequences	No. collections	No. of bp in alignment	Total no. differences	Difference (excluding ambiguous bp)	Mean	S.D.
X. ferrugineus, Europe	19	19	612	0–8	0–3	0.6	0.8
X. spadiceus 1, North America	5	5	610	0–5	0–2	0.7	0.8
X. spadiceus 2, North America	2	2	612	0	0	0	0
X. spadiceus 1 *vs X. spadiceus* 2	na	na	613	17–19	15–17	16.3	0.9
X. ferrugineus vs X. spadiceus 1&2	na	na	614	8–22	6–18	10.3	3.6
X. subtomentosus, intragenomic only	12	6	738	5–18	5–16	8.7	3.6
X. subtomentosus, intraspecific only[a]	19	12	738	0–39	0–37	15.2	7.7
X. chrysonemus	10	10	662	0–7	0–7	2.2	1.8
Xerocomus sp., Europe	2	2	667	0	0	0	0

na, Not applicable.
a Intraspecific variation, excluding intragenomic variation.

homogeneous over a wide geographical range, from northern Sweden to Italy. The differences between European and North American collections are relatively few compared with the intraspecific differences in *X. subtomentosus*, but they are remarkably constant. The American sequences of *X. spadiceus* fall into two clusters, both of which are more or less equally distant from the European *X. ferrugineus*, but clearly distinct from each other. *X. spadiceus* 1 includes specimens from the eastern States and from California. *X. spadiceus* 2 collections are only from Eastern North America.

Spore characteristics

The spores of *Xerocomus chrysonemus* were the most divergent in the taxa analysed (Table 3). In general, they were shorter and wider than those in other taxa. This was reflected in the spore quotient, which was significant lower than in any other taxon. Spore length showed the least variation among the taxa with only *X. chrysonemus* being shorter than *X. subtomentosus*. The width of the spores divided the taxa into two clear groups, with the spores of *X. ferrugineus* s. *lat.* being significantly narrower than in the other two taxa. In European taxa, spore quotient declined significantly in the following series *X. ferrugineus* (Eur) > *X. subtomentosus* s. *str.* > *X. chrysonemus*. The spore quotient of *X. spadiceus* (North America) differed significantly from *X. chrysonemus*. There was considerable variation in the length, of spores in European *X. ferrugineus*. A comparison of average spore length, based on whether collections were associated with broad-leaved or conifer hosts, showed that collections from conifers ($L = 11.5 \pm 0.5$ μm, $n = 8$) had significantly ($F = 7.16$, $P = 0.017$) shorter spores than those from broad-leaved hosts ($L = 12.0 \pm 0.5$ μm, $n = 9$).

Taxonomy

Spore characteristics were determined from material sequenced in this study; values in squared parentheses are from Ladurner and Simonini (2003).

Xerocomus subtomentosus (L. 1753 : Fr.) Quél. 1888

Syn.: Xerocomus subtomentosus var. xanthus Gilbert 1931

Xerocomus xanthus (Gilbert) Contu 1989

Xerocomus flavus Singer & Kuthan 1976

Xerocomus subtomentosus f. *rubrotinctus* Simonini & Contu 2001

Boletus lanatus Rostk. 1844

Illustrations: Ladurner and Simonini (2003: figs 105–118)

Pileus 20–130 (–250) mm broad, young nearly hemispherical then applanate, felty or tomentose. Very variable in colour: typically yellow–olivaceous, olivaceous to olive–brown in dry weather conditions, but becoming brown–reddish in wet conditions, more rarely pure yellow, garnet or blood-red. *Pileipellis* composed of entangled short hyphae, with no or at most with very fine, scattered incrustations, end cells 7 – 11 (16) μm wide. *Pores* yellow, weakly to distinctly bluing when damaged. *Stipe* cream coloured to yellow, slender, scurfy, smooth or with ridges or even with a rough net. *Flesh* pale yellow, pinkish or brown–reddish and doughy at the stipe base, slowly turning blue especially in the pileus. *Spores* (11-) 12.5 ± 0.5 (-13) × (4.5-) 5 ± 0.5 (-5.5) μm, Q - (2-) 2.5 ± 0.2 (-2.5): [(9.5-) 12 ± 1 (-17) × (4-) 5 ± 0.5 (-6) μm, Q - (2-) 2.5 ± 0.2 (-4)].

Observations: Under broad-leaved trees and conifers.

Xerocomus ferrugineus (Schaeff. 1774) Bon 1985

Syn.: *Boletus citrinovirens* Watling 1969

Illustrations: Ladurner and Simonini (2003: figs 119–131)

Pileus 20–130 (–250) mm broad, young nearly hemispherical then applanate. Surface dry tomentose, the felty surface tissue is rapidly restored on areas that have been eaten or damaged (a feature not observed in *X. subtomentosus*). Pileus colour variable: greenish yellow to yellowish–olive, olive–brownish to olive–green in dry weather conditions, but brown–reddish in wet conditions, also pure green. *Pileipellis* composed of filamentous hyphae, with no or at most with very fine, scattered incrustations; end cells (5–) 7 – 11 μm wide. *Pores* yellow, not or only weakly bluing. *Stipe* cream to pale brown, slender, rarely roughly scurfy, often with a rough net, especially in the upper part. *Flesh* whitish, unchanging. Basal mycelium often yellow. *Spores* (10.5-) 12 ± 0.5 (-13) × (4-) 4.5 ± 0.5 (-5) μm, Q - (2.5-) 2.5 ± 0.2 (-3): [(9-) 11.5 ± 1 (-14.5) × (3.5-) 4 ± 0.3 (-5) μm, Q - (2-) 3 ± 0.2 (-3.5)].

Observations: Under deciduous (particularly *Fagus sylvatica*) and coniferous trees on acid soils. A pure green coloured form exists (*X. ferrugineus* f. *citrinovirens*), where the margin is often markedly inrolled, the hymenial surface is uneven and the context has silvery streaks.

Xerocomus chrysonemus A. E. Hills & A. F. S. Taylor **sp. nov.**

Etym.: *chryso*, golden; *nema*, mycelium

Illustrations: Figs 3–4

Pileus 25–70 mm, flavus sinapis vel olivaceo-flavus, mutabilis. *Poris* e aureo-luteo mox leviter viridi-flavis, postremo viridi-flavis,

Table 3 – Spore characteristics (mean ± SD) of taxa within the *Xerocomus subtomentosus* complex

Taxon	*n*[a]	Length (μm)	Breadth (μm)	Ratio (l/b)
X. subtomentosus	12	12.3 ± 0.7 b[b]	5.1 ± 0.3 b	2.4 ± 0.2 b
X. chrysonemus	9	11.3 ± 0.5 a	5.1 ± 0.2 b	2.2 ± 0.1 a
X. ferrugineus (Europe)	18	11.8 ± 0.7 a,b	4.5 ± 0.3 a	2.7 ± 0.2 c
X. spadiceus (NorthAmerica)	7	11.8 ± 0.4 a,b	4.5 ± 0.2 a	2.6 ± 0.1 b,c
F-value		4.53	19.71	21.74
Significance (*P*)		0.008	<0.001	<0.001

a *n* refers to the number of collections included in the analysis. Values within collections are based on 30 spores.
b Values within columns not sharing the same letter differ at $P \leq 0.01$.

haud cyanescentibus. *Contextus* albidus vel pallido-citrinus, stipes basi aureus, immutabilis ubi scissus vel contusus. *Mycelium* profunde aureum vel flavum sinapis, color exsiccatorum similis. *Basidiosporae* (9-) 11.5 (-14.5) × (4.5-) 5 (-7.0) μm, laeves, ellipsoideae vel lato-subfusoideae, crassotunicatae.

Typus: **UK**: *Hampshire*: New Forest, Pig Bush, 13 Aug. 2003, *S. Kelly* (K(M) 123243 – holotypus).

Pileus 25–70 mm diam, convex becoming applanate, at times plano-concave. Initially finely tomentose becoming glabrous with age, margin inrolled when young. Greyish yellow–golden (4C6) to brown (6E4) becoming more yellow–golden after collecting. Often very variable in colour, mustard to olivaceous–yellow, fulvous, with age becoming darker, sepia, sienna with a hint of red–brown or toward rich copper in damp weather, ± somewhat mottled in the centre, colour uniform around margin. *Pileipellis* somewhat intricate trichoderm of fairly short cells 4.5–18.0 μm diam, having little or no ornamentation, some at times branching, end cells rounded to bullet shape, rarely tapering, ± disarticulating (Fig 4). *Hymenophore* tubulate, adnexed, up to 13 mm in length, bright golden yellow when young, soon showing a greenish tinge and becoming greenish-yellow, not bluing or changing with pressure. *Pores* cadmium yellow (4A8) when young with closed pores, soon butter yellow (4A5) then amber yellow (4B6) with age, becoming large and angular, unchanging with pressure. *Basidia* narrowly clavate to clavate, bearing two, three, and four sterigmata, mostly two. In mature sporocarps, basidia 37–59 × 8–14 μm (up to 15.5 μm in 4-spored basidia). Sterigmata noticeably elongated. *Cheilocystidia* grouped, very scattered and variable in shape, tapering from the narrow base to become fusiform to very broadly fusiform, many becoming mucronate or somewhat rostrate. 57–88 × 13–36.5 μm. *Pleurocystidia* similar to but smaller than the cheilocystidia but never broadly fusiform, 29–62 × 9–15 μm. *Caulocystidia* mostly capitate. *Clamp connections* not observed. *Stipe* 30–50 × 5–18 mm, slender when young, becoming ± fairly robust, generally distinctly tapered, with a strikingly bright yellow appearance when very young, resembling the pores, soon toning down, congruent with the pore colour, finally dull straw with a minute foxing of reddish-brown flecks, striate, at times the flecks forming an incomplete, stretched reticulate pattern. *Flesh* off-white to pale lemon yellow in the pileus, brighter yellow in the stipe to golden at the very base, the yellow is vibrant in very young sporocarps. Normally unchanging with cutting or bruising. *Mycelium* deep golden-yellow to mustard yellow. In water-saturated collections the mycelium looks yellow, but becomes more golden–yellow after drying. *Spores* (Fig 4) smooth, ellipsoid, to ± broadly subfusiform, thick-walled, (9-) 11.5 (-14.5) × (4.5-) 5 (-7) μm, Q = 2.1–2.3 (spore measurements from material included in molecular analysis (10-) 11.5 (-12) × (5-) 5.5 (-6) μm, Q = (2.1-) 2.2 (-2.3)). Spores deposit olive brown 4 E 7.

Chemical reactions: *On pileus*: ammonia—blue–green, soon fading to leave a blue green ring that fades to purple and disappears with time. *On context*: ammonia—pearl; iron sulphate—golden–yellow; potassium hydroxide (10 %)—pale yellow

Taste and odour: Mild. Smell indistinct, not unpleasant.

Habitat: Fruiting in damp, well-rotted leaf litter within small hollows in the forest floor, or on moss covered shaded banks. Soil pH approx. 7.

Observations: To date found only in Hampshire and Kent, **UK**, associated with *Quercus* in shady positions. Field identification can be made on the bright golden mycelium and the intense yellow exposed context on cutting. It could be mistaken for *Xerocomus moravicus* when young, in later stages it somewhat resembles *X. ferrugineus*.

Additional specimens examined: **UK**: *Kent*: North Bishopden wood, Blean woods, *Quercus*, 12 Sept. 2001, *M. Allison* (K(M)123244); *loc. cit. Quercus*, 15 Sept. 1999, *M. Allison* (A. E. Hills IMP0056); *Hampshire*: New Forest, Gritnam wood, *Quercus*, 31 Aug. 2000, *A. E. Hills* (K(M)123245); *loc. cit.* Gritnam wood, *Quercus*, 21 Aug. 2000, *S. Kelly* (A. E. Hills AH2000037); *loc. cit.* Rufus Stone, *Quercus*, 17 Sept. 1999, *A. E. Hills* (A. E. Hills AH1999083); *loc. cit.* Rufus Stone, *Quercus*, 20

Fig 3 – Basidiomes of *X. chrysonemus* in their natural habitats (A, AH2004071; B, AH2004135).

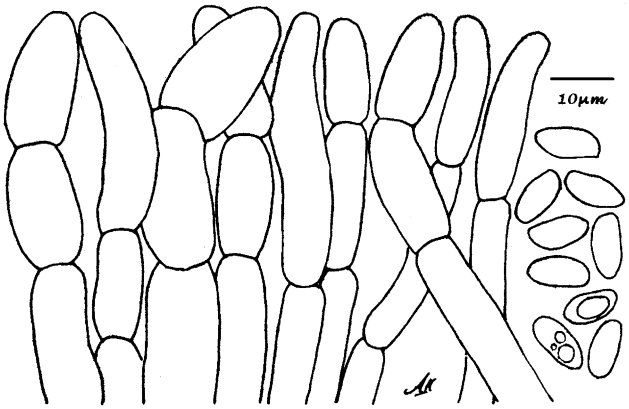

Fig 4 – Pileipellis end cells and basidiospores of *Xerocomus chrysonemus* (K(M)123243 – holotypus).

Sept. 2004, *A. E. Hills* (A. E. Hills AH2004155); *loc. cit.* Vinney Ridge, *Quercus*, 3 Oct. 2000, *A. E. Hills* (E(M)196160); *loc. cit.* Vinney Ridge, *Quercus*, 9 Aug. 2004, *A. E. Hills* (A. E. Hills AH2004071); *loc. cit.* Vinney Ridge, *Quercus*, 12 Aug. 2004, *A. E. Hills* (A. E. Hills AH2004072); *loc. cit.* Millyford Bridge, *Quercus*, 5 Oct. 1998, *A. Henrici* (A. E. Hills IMP0002); *loc. cit.* Denny wood, *Quercus*, 20 Sept. 2004, *A. E. Hills* (A. E. Hills AH2004157); *loc. cit.* Busketts Wood, *Quercus*, 7 Sept. 2004, *A. E. Hills* (A. E. Hills AH2004140); *loc. cit.* Stubbs wood, *Quercus*, 6 Sept. 2004, *A. E. Hills* (A. E. Hills AH2004134); *loc. cit.* Pig Bush, *Quercus*, 6 Sept. 2004, *A. E. Hills* (A. E. Hills AH2004132); *loc. cit.* Ashurst wood Area, *Quercus*, 20 Sept. 2004, *A. E. Hills* (A. E. Hills AH2004156).

Discussion

It is clear from the present study of the *Xerocomus subtomentosus* complex in Europe that neither pileus colour nor the degree of development of ridges on the stipe are taxonomically informative. However, it is evident from the ITS data that several taxa do exist within the complex in Europe and that the colour of the context and the basal mycelium and spore characteristics are the most useful morphological characters for distinguishing three of these entities (Table 4). However, it is also clear that other, so far undetected, taxa may exist, as indicated by the undescribed taxon that clearly falls within this complex.

X. subtomentosus s. str. and *Xerocomus ferrugineus* appear to be taxa with wide geographic and ecological aptitudes. Both may display very variable pileus colours, particularly *X. subtomentosus*. With the exception of a slight geographic segregation, the genetic variation found in *X. subtomentosus s. str.* (see also below) could not be correlated with either morphology or ecology. In contrast, even though the variation in *X. ferrugineus* was remarkably small, there was a clear indication for ecotypic variation in spore length linked to broad-leaved and coniferous host selection. *Xerocomus chrysonemus* is also variable with respect to pileus colour, but, so far has a more restricted geographic distribution. The distinctive yellow colouration of the context and the shorter, wider spores of this species should enable a positive identification.

The ITS region used in this study has been found to correspond well with morphological species concepts in a wide range of fungal groups. However, interspecific ITS variation can vary greatly within different genera. Within *Xerocomus* (A. T. & U. E., unpubl.), ITS sequence variation is so extensive that different species normally possess distinctly different sequences. When considering all European species of *Xerocomus* (Ladurner & Simonini 2003), it is not possible, with the exception of a few conserved regions, to successfully align ITS sequences. Easily alignable ITS sequences imply that the group of taxa under study, in this case the *X. subtomentosus* complex, are closely related with each other, and probably constitute a natural unit (i.e. are monophyletic).

In the present study, molecular data from the ITS region were very effective in delimiting species in the *X. subtomentosus* complex. Two new species (*X. chrysonemus* and *Xerocomus* sp.) were distinguished from molecular data that would likely to have gone unnoticed in the considerable confusion of morphological characters used to delimit taxa in the complex.

In addition, cryptic taxa were also found in *X. ferrugineus s. lat.* The structural similarities within the ITS sequences of this group are greater than between any two other taxa in the *X. subtomentosus* complex. This suggests a common ancestor for North American and European *X. ferrugineus*. In comparison with the normal level of infrageneric ITS variation among the other species treated here, this structural similarity argues against considering the taxa within *X. ferrugineus s. lat.* as independent species. However, based on the available evidence, within each taxon the sequences are very homogeneous and constant, suggesting distinct evolutionary lineages, which may deserve species recognition. This question and the other American taxa of the *X. subtomentosus* group will be dealt with in a future publication.

The hyphae of *Xerocomus* are dikaryotic, and the nuclear ribosomal genes are multicopy genes, suggesting that there is potential for polymorphism in the ITS region. The occurrence of different ITS copies within the same organism has been reported in a variety of fungi (e.g. Eberhardt 2000; Aanen *et al.* 2001; Nuytinck & Verbeken in press; Leonardi *et al.*

Table 4 – Distinguishing characters of taxa within the *Xerocomus subtomentosus* complex in Europe

Character/taxon	*X. chrysonemus*	*X. ferrugineus*	*X. subtomentosus*
Predominant colour of flesh	Pale to intensely yellow	White or whitish	Pale to distinctly yellow
Basal mycelium	Golden yellow, at least when dry	Yellowish	Whitish
Spore Q value (l/b)	≤2.3	≥2.5	Usually > 2.3

2005). This phenomenon is possibly more common but may be either unobserved or ignored: in direct sequencing, only those variants occurring in at least a third of the amplicons will be apparent in the sequence raw data (ABI PRISM Big Dye Terminator Cycle Sequencing Ready Reaction Kit, documentation); intragenomic (length) variation that shows no regularity and which is not restricted to very few positions between two priming sites renders the raw sequence data unreadable.

The occurrence of intragenomic ITS variation in some species within a species group or complex, but not in others, has also been observed in the *Boletus edulis*-group (Leonardi *et al.* 2005). It is also found in other *Xerocomus* spp., *Russula* spp. and *Hebeloma* spp. (A. T. and U. E., unpubl.). Intragenomic ITS variation has been interpreted as an indication of speciation in progress (Odorico & Miller 1997) or recent mixing of previously separated populations (Aanen *et al.* 2001). These interpretations are based on the assumption that mechanisms, referred to as concerted evolution (Arnheim *et al.* 1980), would homogenize the ITS copies in a biological species over time. However, depending on the effectiveness of past and present barriers to gene exchange, the efficiency of molecular drive and, assuming that molecular drive does not necessarily favour the same version of the ITS in every individual organism or even individual cell, it is possible that different (functional) copies of the ITS persist within one species, without necessarily indicating ongoing speciation.

The various indels occurring in the ITS of *X. subtomentosus s. str.* (Fig 2) do not seem to be linked in their presence/absence to each other, suggesting that molecular drive may indeed not always favour the same ITS version. Even though intragenomic ITS variation was encountered in more of the Italian than of the UK collections, there were no constant patterns in the variation. Therefore, there is no indication in the data that *X. subtomentosus s. str.* consists of more than one species.

The large variation in the ITS data within European *Xerocomus* (*cfr* UNITE database; Kõljalg *et al.* 2005) does not allow any meaningful comparisons to be made with regard to evolutionary history of the genus. Binder (1999), using LSU data, suggested that *Xerocomus* is paraphyletic and recognised *Xerocomus*, *Paraxerocomus*, and *Pseudoboletus* and, perhaps other groups, as potential genera. According to Binder, *Xerocomus s. str.* in Europe would include *X. subtomentosus* (type species), *X. moravicus*, *Boletus impolitus*, *B. depilatus*, and *Phylloporus pelletieri*. However, comparisons made in the present study using the ITS data (data not shown) suggest that the *X. subtomentosus* complex has a rather isolated position compared with these other taxa. Further work using multi-gene analyses are required to more clearly resolve the evolutionary history of xerocomoid fungi within the *Boletales*.

Acknowledgements

We thank Katta Ihrmark, Lena Jonsson, Maria Jonsson and Ylva Lennhed for help with sequencing. Thanks also to David Arora, Steve Kelly, and Birgitta Wasstorp for contributing material, and Roy Watling for providing the Latin description of *Xerocomus chrysonemus*.

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