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The population of the hypogeous fungus *Tuber aestivum* syn. *T. uncinatum* on the island of Gotland

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Abstract The aim of our study was to examine the genetic variation within *Tuber aestivum* on the Baltic island of Gotland, Sweden. Variation in such a limited geographical area should help illuminate the dispersal abilities of *T. aestivum*. Knowledge of the genetic variation in this northern outpost could also be useful in the selection of inoculum for the establishment of truffle orchards. Genetic structure and homogeneity of the population were studied using principal component and parsimony analyses of randomly amplified polymorphic DNA data. Our inventories showed that *T. aestivum* is abundantly distributed in suitable habitats on Gotland. The genetic variation observed suggests sexual reproduction and slow dispersal on the island. It is possible that the present population was established from one introduction, which may be due to ability to survive in this habitat rather than to rare colonising events. The *T. aestivum* population on Gotland may be an ecotype adapted to the climate and soil conditions on the island.

Keywords *Tuber aestivum* · *Tuber uncinatum* · Population · Dispersal · Island biogeography

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Introduction

Tuber aestivum Vitt. is an hypogeous ascomycete that grows in ectomycorrhizal symbiosis with e.g. *Quercus robur* and *Corylus avellana* (Chevalier and Frochot 1997). *T. aestivum* (Wedén and Danell 1998) and *T. mesentericum* Vitt. (Wedén et al. 2001) are the only black truffles reported from Sweden, both from the island of Gotland. *T. aestivum*, like many other hypogeous fungi, is dependent on mammalian vectors for spore dispersal (Miller 1985; Trappe 1988; Trappe and Castellano 1991; Trappe et al. 2001).

Tuber aestivum, described by Vittadini (1831), and *T. uncinatum*, described by Chatin (1887), were indistinguishable in several molecular studies (Pacioni et al. 1993; Gandeboeuf et al. 1997; Wedén and Danell 1998). Furthermore, the morphological differences are not diagnostic (Chevalier and Frochot 1997; Montecchi and Sarasini 2000). Recently Mello et al. (2002) attempted to separate *T. aestivum* and *T.uncinatum* by neighbour joining analysis of internal transcribed spacer (ITS) and microsatellite sequences. The analyses were made on material microscopically identified as either *T. aestivum* or *T. unciantum* based on depth of spore reticulum. The differences obtained may reflect intraspecific variation, since their clusters represent different countries. Furthermore, microscopically intermediate taxa were excluded and the number of *T. aestivum* samples was low. Differences in taste and scent have been used as the main characters for discrimination of *T. aestivum* from *T. uncinatum*. In a blind test performed at the French truffle museum in Sorges (France) in October 2001, French *T. uncinatum* and Swedish *T. aestivum* syn. *T. uncinatum* could not be distinguished. Since it is very probable that *T. aestivum* and *T. uncinatum* are the same species and the oldest name still has priority, we will only use the name *T. aestivum* in this article.

The Swedish *T. aestivum* localities are all on the island of Gotland in the Baltic Sea, 90 km from the Swedish east coast. Gotland has a total area of 3,140 km^2 and mainly consists of sedimentary Silurian calcareous rock. Gotland

rose from the sea after the last ice age, 11,600 years ago and has remained unconnected to the main land (Fredén 1998). This is the first scientific publication on *T. aestivum* from Sweden, which can be regarded as its northern outpost.

Island biogeography has yielded insights into evolution of many groups of organisms, but has not been applied much to fungi, especially not to fungi depending on vectors for spore dispersal (Trappe et al. 2001). Two population studies on *T. aestivum* have shown pronounced heterogeneity within the species, even though rather coarse methods such as allozyme analysis were used (Pacioni and Pomponi 1991; Pacioni et al. 1993). Guillemaud et al. (1996) found restriction fragment length polymorphism of the ITS sequence not to be useful for studying intraspecific variation in *T. aestivum*. Gandeboeuf et al. (1997), using randomly amplified polymorphic DNA (RAPD), also showed that *T. aestivum* is highly heterogeneous compared with other closely related species, but they only included 10 specimens of *T. aestivum* and *T. uncinatum* from three countries. The aim of our study was to use reliable methods to investigate genetic variation among 21 localities in an outpost island population.

Materials and methods

Sampling

The truffle localities on Gotland were found using trained truffle dogs in collaboration with Gérard Chevalier, INRA Clermont-Ferrand, France. Inventories were made in September 1999 and October 2000. A locality is defined here as a *Q. robur* and/or *C. avellana* stand disconnected from other localities by e.g. roads or arable land. In this study, all localities were represented by at least one fruit body, three localities by two or more fruit bodies, each fruit body forming one sample. The sampling localities are referred to as T1–T13 and T15–T22 from south to north Gotland. Four foreign samples were also included as an outgroup in the analysis. These were samples T23/DK (Møn) and T24/DK (Aarhus) from Denmark, sample T25/GB from England (Oxford) and sample T26/F from France (Burgundy). The samples were kindly supplied by Christian Lange (T23/DK, T24/DK), Kew Gardens (T25/GB) and Michel Jalade (T26/F). Samples T28–T30, T31–T32 and T33 originated from localities T5, T2 and T11, respectively. Samples T31 and T32 originated from the same fruit body.

RAPD

RAPD is a powerful tool for studying populations when mating systems and genetic markers for accurately studying intraspecific variation are unknown (Tommerup et al. 1995; Gandeboeuf et al. 1997; Bertault et al. 1998). DNA was extracted from 27 samples from Gotland and four foreign samples using a modified cetyltrimetylammonium bromide (CTAB) protocol (Gardes and Bruns 1993; Danell 1994). Samples of approximately 25 mm^3 of dried or frozen fruit body material (gleba) were homogenised with a plastic pestle in a microcentrifuge tube (1.5 ml) with 750 μl lysis buffer (100 mM Tris-HCl pH 9; 1.4 M NaCl; 20 mM EDTA; 2% CTAB; 0.2% mercaptoethanol) and incubated at 65°C for 1 h. After incubation, 600 μl chloroform was added and the tube was briefly vortexed and centrifuged for 15 min. The aqueous phase was removed to a new tube, purified by ethanol/isopropanol precipitation and resuspended in 50 μl TE buffer (10 mM Tris-HCl pH 8; 1 mM EDTA). The DNA extractions were diluted 1:1 to 1:20 with distilled and filtered H_2O to a concentration of 5 ng DNA/μl for use in the RAPD-PCR reactions. DNA was amplified with the 9-bp primers 152G and 152C (Selosse et al. 1998; Cybergene AB) and the 10-bp primers OPS-01, OPS-03, OPS-04 and OPS-07 (Operon Technologies, Inc.). The reaction mixture consisted of 2 μl of diluted DNA (containing 10 ng DNA) and 23.4 μl master mix containing 39.4 μM dNTP, 15 pmole of one of the six primers, 2.5 units of *Taq* DNA polymerase and 2.5 μl 10x PCR buffer with $MgCl_2$. The RAPD-PCR was performed in a PC-960G Gradient Thermal Cycler (Corbett Research) as follows: 30 s at 94°C, 45 cycles of 5 s at 94°C, 45 s at 40°C, 2 min at 72°C, a final synthesis at 72°C for 7 min, and then stored at 4°C. The RAPD products were run on a 1.8% agarose gel, stained with ethidium bromide, photographed under UV light and subsequently analysed visually. In order to confirm RAPD reproducibility, two samples taken from the same fruit body (T31 and T32) were included in the study and samples T1, T2, T6, T8, T9, T18, T21 and T25/GB were analysed five times using the same primer (152G). These eight samples were also analysed twice with the other five primers.

RAPD data analysis

Parsimony analysis of the RAPD data matrix was performed using PAUP 4b10 (Swofford 2002) under the assumption of Fitch parsimony (Fitch 1971). The rational for using parsimony analysis are twofold. The primary question for this analysis was not to elucidate a 'phylogeny' of *T. aestivum* on Gotland but to interpret the pattern of co-variation in the RAPD data. In this case, parsimony analysis has several advantages over various distance-based methods, as there is a direct linkage between a specific node in the resulting tree and presence or absence of bands in the RAPD data. Furthermore, for the interpretation of the ancestry of *T. aestivum* on Gotland, parsimony has become widely accepted recently as the method of choice for phylogenetic analysis, together with maximum likelihood. The latter, however, suffers from the methodological drawback that a specific evolutionary model is required for the analysis. Such models are rare and at present none is available for interpreting RAPD data from fungi. An initial heuristic search with 1,000 random addition sequence replicates followed by the tree bisection-reconnection (TBR) branch-swapping algorithm formed the starting point for subsequent successive approximations reweighting (Farris 1969). Tree stability was estimated with a bootstrap analysis (Felsenstein 1985) of 1,000 replicates, sampling characters with equal probability, but applying weights as obtained from the successive approximations reweighting.

Principal component analysis (PCA) was used to make a multivariate statistical analysis of the RAPD data. The PCA was performed with PC-ORD (McCune and Mefford 1999).

Results

Sampling

During the inventories of 1999 and 2000, *T. aestivum* was found at 21 localities distributed over the main island of Gotland. *T. mesentericum* was found at three of the *T. aestivum* localities and at six additional localities (Wedén et al. 2001).

RAPD data analysis

The analysis of the RAPD electrophoresis patterns resulted in 189 unambiguous characters. The repro-

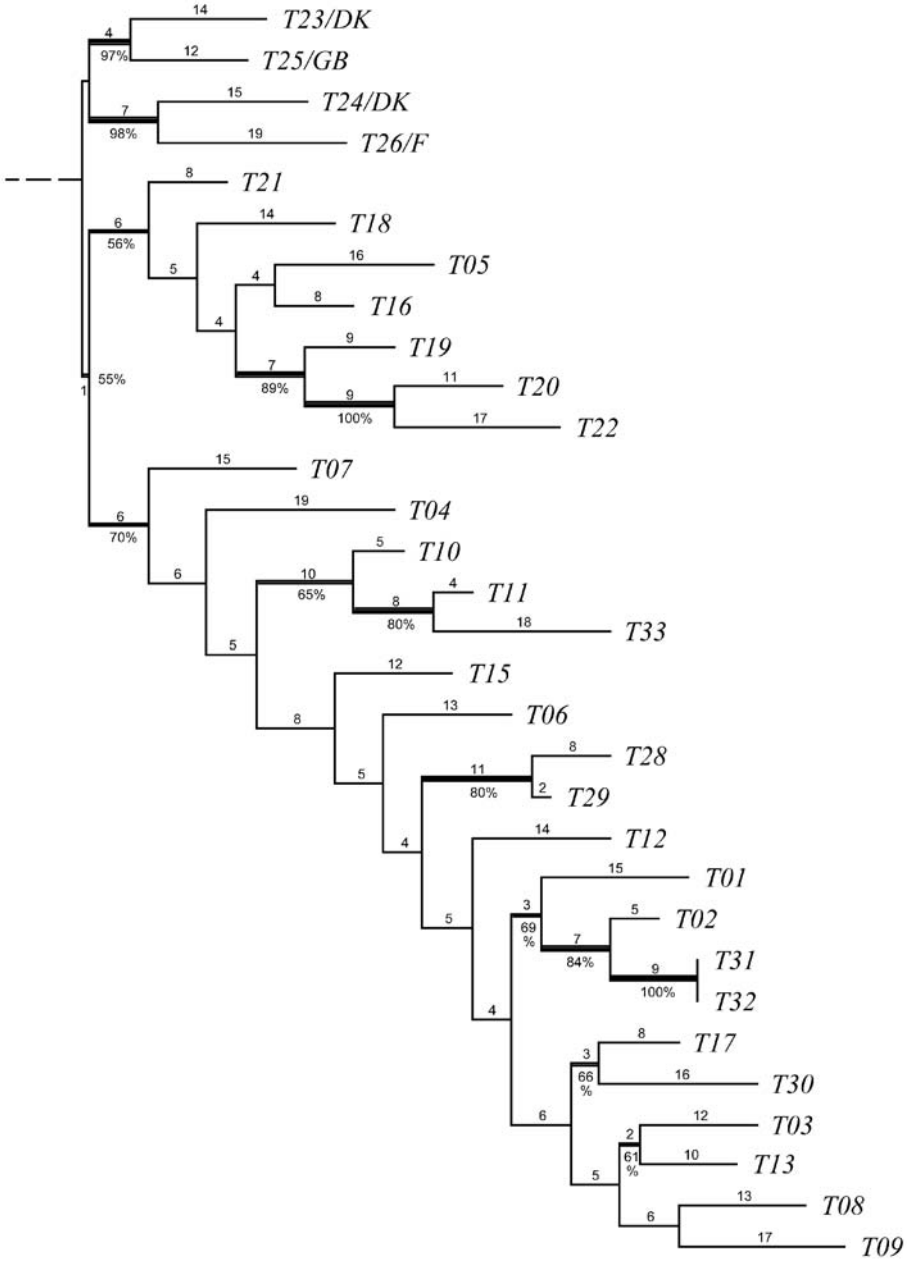

Fig. 1 The unweighted pair group method with arithmetic mean (UPGMA) phylogenetic tree based on randomly amplified polymorphic DNA (RAPD) analysis of *Tuber aestivum* samples from Gotland (Sweden), Denmark, Britain and France. The 27 Gotland (Sweden) samples (T01–T13, T15–T22, T28–T33) form a clade, while the two Danish samples (T23, T24), the British sample (T25) and the French sample (T26) differ at least as much from each other as from the Gotland clade

ducibility of the RAPD analysis was 100%. The two samples (T31 and T32) originating from the same fruit body, were identical in all characters. The negative control (water) never generated characters corresponding to the samples. The initial heuristic search in the parsimony analysis of the RAPD data matrix resulted in two equally parsimonious trees of 452 steps each with a consistency index (Kluge and Farris 1969) of c_i=0.26 and a retention index (Farris 1989) of r_i=0.42. After three rounds of successive approximations reweighting, a stable result was obtained consisting of one single tree (Fig. 1) based on 118 phylogenetically informative characters out of the 189 scored characters. This tree was dissimilar to the two initial trees, and its corresponding values were tree length 460 steps (normalised, all characters with weight 1), c_i=0.26 and r_i=0.40. Normalised branch lengths with each step corresponding to observations on one RAPD fragment and bootstrap support values above 50% are indicated in Fig. 1.

The PCA (Fig. 2), based on 133 informative characters out of the 189 characters scored, resulted in a tight cluster of the Gotland samples, clearly separated from the Danish, English and French samples.

Discussion

The results from our inventories on Gotland show that *T. aestivum* is spread all over the island and is, thus, well adapted to the local soil and climatic conditions. Several

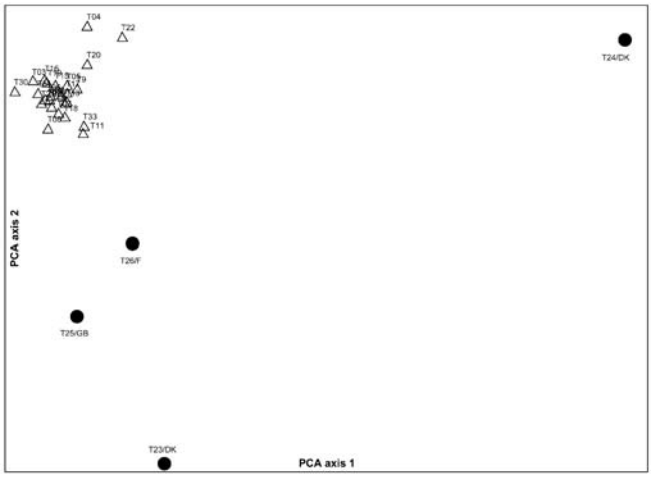

Fig. 2 The principal component analysis graph based on the RAPD analysis of *T. aestivum* samples from Gotland (Sweden), Denmark, Britain and France. The Eigen values were 10.884 and 6.994 for axes 1 and 2, respectively. The percentage of variance of axes 1 and 2 were 17.276 and 11.101, respectively. The 27 Gotland (Sweden) samples (T01–T13, T15–T22, T28–T33) cluster together, while the two Danish samples (T23, T24), the British sample (T25) and the French sample (T26) differ at least as much from each other as from the Gotland cluster

features appear to be congruent from both the parsimony analysis (Fig. 1) and the PCA (Fig. 2). In both cases, the assigned outgroup consisting of two Danish, one English, and one French specimen is clearly distinguishable from the ingroup from Gotland. It can be seen from the parsimony analysis that one of the Danish samples, T23/DK, groups together with the English sample T25/GB. A corresponding pair is formed with T24/DK and T26/F. These pairs are very strongly supported by bootstrap analysis with 97% and 98% support, respectively. Although the Danish *T. aestivum* apparently has at least two different origins, no further conclusions about these origins can be drawn, since only one English and one French specimen were included in this study.

Multiple founding events on Gotland are a possibility, as there are another 11 recorded species of hypogeous fungi on the island (Bohus-Jensen 1988; Wedén et al. 2001) that may have been introduced by the same vectors as *T. aestivum*. Genetic homogeneity estimated from branch lengths obtained from the parsimony analysis can be interpreted to suggest that *T. aestivum* on Gotland results from one single introduction, but that a very early separation was established between two lineages. Another possible interpretation is that there were in fact two early introductions from a similar source at approximately the same time. These two introductions gave rise to the two major groups, supported by 56% and 70% bootstrap values in the parsimony analysis (Fig. 1). Both these hypotheses correspond well with rough estimates from branch lengths, but would require a source different with reference to each of the four outgroup samples included in this study. If Gotland is considered as the northern outpost for *T. aestivum*, with climate and soil conditions demanding a very specific *T. aestivum* ecotype, new introductions may have occurred several times, but the majority became extinct due to lack of adaptation. Thus, the selection of indigenous inoculum is recommended for future truffle seedling production aimed at establishing truffle orchards on Gotland and other areas in the world with similar conditions.

The result of our parsimony analysis suggests ongoing dispersal within Gotland, since geographically adjacent samples may sometimes be more different from a genetic point of view than geographically distant samples (Fig. 1). There are no apparent barriers, such as mountains, large lakes or cities, that would prevent gene flow on Gotland. The results of a pair-wise comparison of the RAPD data from all our samples plotted in a histogram showed a normal distribution of the comparisons (data not shown). This normal distribution indicates that sexual reproduction within the Gotland population is likely, since the occurrence of clones would have created a more asymmetrical distribution. A similar assumption was made by Redecker et al. (2001), who used AFLP.

It is difficult to estimate when *T. aestivum* first colonised Gotland. *C. avellana* was the first potential symbiont of *T. aestivum* to colonise Gotland after the last ice age, as evidenced by pollen from 9,000 years ago (Påhlsson 1977). The importance of this early colonisation to the introduction of *T. aestivum* is controversial, since edaphic and climatic interactions in Europe seem more important than the distribution of host species for the distribution of many fungal species (Trappe et al. 2001). Since *T. aestivum* (i.e. *T. uncinatum*) is an economically valuable species, a record would probably have been kept had it been used or introduced deliberately. There is no known tradition of consuming truffles in Sweden, including Gotland, and there are no historical Swedish records of *T. aestivum* (I. Svanberg, Uppsala University and O. Hoffman, Gotland University College, personal communication). Carl Linnaeus visited Gotland in 1741 to record the presence and uses of minerals, plants and other natural resources (Linnaeus 1742). He does not mention truffles or the use of truffles on Gotland, but his excursion was made in July before the truffle season. During his stay on Gotland, he met with scholars who would have known about the use of truffles. Clearly, from our data we can not rule out that *T. aestivum* was unknowingly introduced via e.g. seedlings (root or soil), tools or domestic animals, brought to the island from the European mainland. Mammals are vectors for spores of hypogeous fungi (Miller 1985; Trappe 1988; Trappe and Castellano 1991; Trappe et al. 2001), but other vectors should be considered for long-distance dispersal over seas.

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References

Bertault G, Raymond M, Berthomieu A, Callot G, Fernandez D (1998) Trifling variation in truffles. Nature 394:734

Bohus Jensen E (1988) Om trytflar på Gotland. Rindi 8:15–18

Chatin AD (1887) Une novelle espèce de truffe. C R Acad Sci 104:1132

Chevalier G, Frochot H (1997) La truffe de Bourgogne. Pétrarque, Levallois-Perret, France

Danell E (1994) *Cantharellus cibarius*: mycorrhiza formation and ecology. PhD thesis, University of Uppsala, Sweden

Farris JS (1969) A successive approximations approach to character weighting. Syst Zool 18:374–385

Farris JS (1989) The retention index and the rescaled consistency index. Cladistics 5:417–419

Felsenstein J (1985) Confidence limits on phylogenies: an approach using the bootstrap. Evolution 39:783–791

Fitch WM (1971) Toward defining the course of evolution: minimum change for a specific tree topology. Syst Zool 20:406–416

Fredén C (1998) Berg och jord — Sveriges nationalatlas. Sveriges geologiska undersökning, Uppsala, Sweden

Gandeboeuf D, Dupré C, Roeckel-Drevet P, Nicolas P, Chevalier G (1997) Grouping and identification of *Tuber* species using RAPD markers. Can J Bot 75:36–45

Gardes M, Bruns TD (1993) ITS primers with enhanced specificity for Basidiomycetes: application to the identification of mycorrhizae and rusts. Mol Ecol 2:113–118

Guillemaud T, Raymond M, Callot G, Cleyet-Marel J-C, Fernandez D (1996) Variability of nuclear mitochondrial ribosomal DNA of a truffle species (*Tuber aestivum*). Mycol Res 100:547–550

Kluge AG, Farris JS (1969) Quantitative phyletics and the evolution of the anurans. Syst Zool 18:1-32

Linnaeus C (1742) Carl Linnaeus öländska och gotländska resa år 1741. Wahlström and Widstrand, Stockholm, Sweden (1991)

McCune B, Mefford MJ (1999) Multivariate analysis of ecological data, version 4. MjM Software Design, Gleneden Beach, Ore, USA

Mello A, Cantisani A, Vizzini A, Bonfante P (2002) Genetic variability of *Tuber uncinatum* and its relatedness to other black truffles. Environ Microbiol 4:584–594

Miller SL (1985) Rodent pellets as ectomycorrhizal inoculum for two *Tuber* spp. In: Molina R (ed) Proceedings of the 6th North American Conference on Mycorrhizae, 25–29 June 1984, Corvallis, Ore, USA

Montecchi A, Sarasini M (2000) Funghi ipogei d'Europa. Associazione Micologica Bresadola, Trento, Italy

Pacioni G, Pomponi G (1991) Genotypic patterns of some Italian populations of the *Tuber aestivum-T. mesentericum* complex. Mycotaxon 42:171–179

Pacioni G, Frizzi G, Miranda M, Visca C (1993) Genetics of a *Tuber aestivum* population (Ascomycotina, Tuberales). Mycotaxon 47:93–100

Påhlsson I (1977) A standard pollen diagram from the Lojsta area of Central Gotland. Striae 3, Societas Upsaliensis Pro Geologia Quarternaria, Uppsala, Sweden

Redecker D, Szaro TM, Bowman RJ, Bruns TD (2001) Small genets of *Lactarius xanthogalactus*, *Russula cremoricolor* and *Amanita francheti* in late-stage ectomycorrhizal successions. Mol Ecol 10:1025–1034

Selosse MA, Martin F, Le Tacon F (1998) Survival of an introduced ectomycorrhizal *Laccaria bicolor* strain in a European forest plantation monitored by mitochondrial ribosomal DNA analysis. New Phytol 140:753–761

Swofford DL (2002) PAUP. Phylogenetic analysis using parsimony (and other methods), version 4. Sinauer, Sunderland, Mass, USA

Tommerup IC, Barton JE, O'Brien PA (1995) Reliability of RAPD fingerprinting of three basidiomycete fungi, *Laccaria*, *Hydnangium* and *Rhizoctonia*. Mycol Res 99:179–186

Trappe JM (1988) Use of truffles and false truffles around the world. In: Bencivenga M, Granetti B (eds) Atti del Secondo Congresso Internazionale sul Tartufo. Comunità montana dei monti martani e del serano, Spoleto, Italy, pp 19–30

Trappe JM, Castellano MA (1991) Keys to the genera of truffles (Ascomycetes). McIlvainea 10: 47–65

Trappe J, Castellano M, Claridge A (2001) Continental drift, climate, mycophagy and the biogeography of the hypogeous fungi. In: Fédération Française des Trufficulteurs. Actes du Ve Congrès International Science et Culture de la Truffe, 4–6 March 1999, Aix-en-Provence, France

Vittadini C (1831) Monographia tuberacearum. France

Wedén C, Danell E (1998) *Tuber aestivum* and other truffles in Sweden. Sven Bot Tidskr 92:65–80

Wedén C, Ericsson L, Danell E (2001) Research on *Tuber aestivum* syn. *T. uncinatum*, and *T. mesentericum* reported from Sweden for the first time. Sven Bot Tidskr 95:205–211