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A global meta-analysis of *Tuber* ITS rDNA sequences: species diversity, host associations and long-distance dispersal

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Abstract

Truffles (*Tuber*) are ectomycorrhizal fungi characterized by hypogeous fruitbodies. Their biodiversity, host associations and geographical distributions are not well documented. ITS rDNA sequences of *Tuber* are commonly recovered from molecular surveys of fungal communities, but most remain insufficiently identified making it difficult to determine whether these sequences represent conspecific or novel taxa. In this meta-analysis, over 2000 insufficiently identified *Tuber* sequences from 76 independent studies were analysed within a phylogenetic framework. Species ranges, host associates, geographical distributions and intra- and interspecific ITS variability were assessed. Over 99% of the insufficiently identified *Tuber* sequences grouped within clades composed of species with little culinary value (Maculatum, Puberulum and Rufum). Sixty-four novel phylotypes were distinguished including 36 known only from ectomycorrhizae or soil. Most species of *Tuber* showed 1-3% intraspecific ITS variability and >4% interspecific ITS sequence variation. We found 123 distinct phylotypes based on 96% ITS sequence similarity and estimated that *Tuber* contains a minimum of 180 species. Based on this meta-analysis, species in Excavatum, Maculatum and Rufum clades exhibit preference for angiosperm hosts, whereas those in the Gibbosum clade are preferential towards gymnosperms. Sixteen *Tuber* species (>13% of the known diversity) have putatively been introduced to continents or islands outside their native range.

Keywords: biodiversity, biogeography, hypogeous fungi, invasive biology, ITS rDNA, phylogeny, *Tuber*

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Introduction

Truffles are icons of the fungal world because of the aromatic hypogeous fruitbodies of some species in the genus *Tuber*. The ecology and host associations of a few commercialized European *Tuber* spp. have been intensely studied (Murat *et al.* 2004; Wedén *et al.* 2005; Paolocci *et al.* 2006; Riccioni *et al.* 2008); however, at the global scale, little is known about their overall species diversity or ecology. Albert B. Frank first recognized the mycorrhizal symbiosis in 1885 when he showed that fungi in the truffle-forming genus *Tuber* were found growing on the roots of living plants (Frank 2005). It has since been established that *Tuber* is an obligate mycorrhizal lineage, unable to complete it's life cycle apart from a host and that *Tuber* spp. are important to the nutrition and drought tolerance of host plants (Nardini *et al.* 2000; Bradshaw 2005; Nuñez *et al.* 2009a). *Tuber* spp. form ectomycorrhizae with a broad diversity of gymnosperm and angiosperm hosts in a variety of habitats including subtropical cloud forests, temperate forests, boreal forests, floodplains, tree nurseries, restoration sites and Mediterranean woodlands (Ceruti *et al.* 2003; Bidartondo *et al.* 2004; Izzo *et al.* 2005; Menkis *et al.* 2005; Bergemann & Garbelotto 2006; Frank *et al.* 2006a; Ishida *et al.* 2007; Hrynkiewicz *et al.* 2008; Krpata *et al.* 2008; Leski *et al.* 2008; Morris *et al.* 2008; Taylor *et al.* 2008; Southworth *et al.* 2009; Bulman *et al.* 2010).

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Since the early 1990s, ITS rDNA sequence data have been used to analyse the composition and dynamics of ectomycorrhizal communities (Gardes *et al.* 1991; Horton & Bruns 2001). The rate of 'species' discovery resulting from molecular community ecology studies now outpaces that of modern taxonomy by orders of magnitude, and this trend is expected to increase exponentially as next-generation sequencing technology becomes widely available (Hibbett *et al.* 2009). Although ITS is a robust marker for identifying fungi and for discriminating most fungal species, species-level determinations are questionable unless the reference sequence can be verified from well-preserved publicly available specimen. In the case of *Tuber*, it is currently difficult to identify ITS sequences using BLAST because of species misidentifications, mislabelling of specimen packets, nomenclatural errors in the public database and because of the quantity of insufficiently identified submissions (Trappe 2004; Halász *et al.* 2005; Iotti *et al.* 2007). It is common for sequences to be submitted to GenBank as 'unidentified' when reference taxa are lacking. These have been termed *insufficiently identified* sequences to distinguish them from *fully identified* sequences (Nilsson *et al.* 2006). The number of insufficiently identified sequences of *Tuber* in GenBank (1924 on April 1, 2010) places the genus within the top 10 insufficiently identified mycorrhizal genera in GenBank (Ryberg *et al.* 2008). Greater insight into *Tuber*'s species diversity, phylogeny, ecology and distribution are expected to emerge from the synthesis of these data. We hypothesized that many insufficiently identified *Tuber* sequences are conspecific but that many also represent novel taxa.

The invasive biology of *Tuber* is of interest, because some of the economically important species are being intentionally introduced into ecosystems around the world (Hall *et al.* 2007; Bonito 2009a), yet the phenomena of human-mediated long-distance dispersal of mycorrhizal fungi is not well understood. Vellinga *et al.* (2009) have proposed a conceptual model of ectomycorrhizal species invasion that involves four stages: (i) *transport* of ectomycorrhizal propagules to a novel location; (ii) *establishment* of the ectomycorrhizal species within the landscape; (iii) *spread* beyond the point of introduction; and, (iv) ecological *impacts*, such as the displacement of native species or alterations to biogeochemical processes. However, discerning native ranges from areas of introduction is challenging, given the cryptic nature and lack of available biogeographical and natural history data for most ectomycorrhizal fungi (Vellinga *et al.* 2009). Molecular approaches have largely superseded morphological approaches owing to their increased sensitivity for assessing evolutionary units, fungal biogeography and introduction events (Stukenbrock *et al.* 2006; Hosaka *et al.* 2008; Matheny *et al.* 2009; Pringle *et al.* 2009). In the event of an introduction, founder populations are expected to exhibit less genetic variability than source populations. Ectomycorrhizal fungi that are host-generalists are hypothesized to have higher rates of establishment and spread because they have a greater likelihood of finding a suitable host (Vellinga *et al.* 2009). If hypogeous fungi are constrained by mycophagy for spore dispersal, their biogeography and invasive ecology may differ from that of epigeous fungi adapted for wind dispersal (Trappe & Claridge 2005; Hosaka *et al.* 2008; Nuñez *et al.* 2009b).

Current research aimed at resolving phylogenetic relationships within *Tuber* has resulted in DNA sequencing from a high proportion (~70%) of known species in the genus and to the discovery of many new *Tuber* species and lineages (Guevara *et al.* 2008; Bonito 2009b; Bonito *et al.* 2010). These data were used together with >2000 ITS sequences from insufficiently identified *Tuber* spp. generated from herbarium collections, fresh fruitbodies, mycorrhizae and soil clones in order to provide the most comprehensive molecular assessment of global *Tuber* biodiversity to date.

Materials and methods

Material studied

ITS sequence data were generated from ~270 collections of identified and unidentified truffles, including 14 type specimens (i.e. holotypes, isotypes and paratypes), made available from the Oregon State University Mycological Collections (OSC), the National Fungus Collections (BPI), Harvard University's Farlow Herbarium (FH), and the Herbarium of Università di Bologna, Italy (BOLO). Fresh specimens collected 2006–2010 in Europe, Asia and North America were also sequenced and are available from the Duke University Herbarium (DUK). Additional ITS sequences from verifiably identified *Tuber* species were downloaded from GenBank and combined with our sequence database to create a *Tuber* ITS phylogeny to illustrate the phylogenetic diversity and major clades in *Tuber*.

The genus search tool in *emerencia* (http://www.emerencia.org/) was used to retrieve insufficiently identified ITS sequences from GenBank, whose pairwise similarity was most similar to identified *Tuber* species (Nilsson *et al.* 2005; Ryberg *et al.* 2009). GenBank numbers for sequences and citations for the studies in which they were generated were compiled and are available as supporting information (Table S1). Metadata accompanying individual sequences (including geographical origin and host) were compiled when available.

Molecular methods

DNA from truffle ascomata was extracted by the CTAB miniprep (Gardes & Bruns 1993). For DNA extraction, glebal tissue was ground (dried or in CTAB) in sterile sand and large cubic zirconium beads in a Mini Beadbeater for 1–2 min (Biospec Products, Bartlesville, OK). The internal transcribed spacer region (ITS) was amplified with the primer set ITS5-ITS4 (White *et al.* 1990). Amplified fragments were viewed through agarose gel electrophoresis, cleaned with Qiagen Quick-Clean columns and sequenced with Big Dye chemistry v.3.1 (Applied Biosystems). DNA sequences were determined on an ABI3700 DNA sequencer (Applied Biosystems).

Sequence analyses

Generated DNA sequences were viewed and manually edited in Sequencher 4.0 (Gene Codes, Ann Arbor, MI). Sequence alignments were performed in MUSCLE (Edgar 2004). Ambiguous regions were excluded in Mesquite 2.5 (Maddison & Maddison 2009). Outgroup selection was based on previous phylogenetic studies of *Tuberaceae* (O'Donnell *et al.* 1997; Bonito 2009b). Insufficiently identified sequences were assigned to a specific *Tuber* clade based on initial parsimony analyses conducted in PAUP* 4d106 (Swofford 2002).

The data set of compiled ITS sequences was filtered by removing sequences of poor quality or short length as well as sequences not belonging to *Tuber* based on BLAST (Altschul *et al.* 1997). In a few cases, we excluded sequences that were determined to be chimeras based on independent BLASTing of the ITS1 and ITS2 regions. Redundant sequences and those with minor variation were removed a priori by assembling total ITS sequences into 98% similarity clusters with the dirty data assembly algorithm in Sequencher 4.0 (Gene Codes). Sequences comprising each 98% similarity cluster were recorded (Table S2, supporting information), and only one representative sequence from each was included in the final analyses.

The ITS1 region is too diverse to align unambiguously across the complete *Tuber* genus. To improve phylogenetic resolution on the placement of sequences from insufficiently identified *Tuber*, separate individual alignments and analyses were performed for each of the three *Tuber* clades that contained the majority of unidentified sequences. These clade designations are supported by multigene phylogenetic reconstructions of the *Tuberaceae* based on rDNA, elongation factor 1 alpha, and RNA polymerase 2 (Bonito 2009b). Appropriate models of nucleotide substitution were selected in PAUP* (Swofford 2002) by Akaike information criterion, penalizing more complex models by one likelihood unit per additional free parameter. Phylogenetic analyses were conducted with maximum likelihood (ML) in PAUP* (Swofford 2002) and Bayesian inference (BI) with MrBayes (Huelsenbeck & Ronquist 2001). Maximum likelihood bootstrap support based on 1000 bootstrap replicates was assessed with RAxML (Stamatakis *et al.* 2008) through the CIPRES web portal (http://www.phylo.org/).

We also analysed 1622 putative *Tuber* pyrosequences accessioned as non-redundant (99% sequence identity) examples from *Quercus* ectomycorrhizae (Jumpponen *et al.* 2010). Because these were short sequences (<250) from a single study, they were analysed separately from the rest of our data set. We followed the same methods described earlier except that singletons remaining after clustering at 98% similarity were discarded to avoid possible sequencing artefacts. We analysed the remaining sequences by performing a BLAST search against our local *Tuber* database and discarding sequences with a bit score <150. The phylogenetic placement of the remaining 1548 sequences was assessed as described earlier.

To determine an appropriate phylotype definition for *Tuber*, levels of intraspecific and interspecific ITS variation were assessed for 20 *Tuber* species including all commercialized species and representatives from the nine major clades. Values of intraspecific and interspecific ITS variation were assessed by aligning pairs of species (multiple sequences from the species of interest and its closest sister taxon) in MUSCLE (Edgar 2004). Alignments were then manually edited, but no regions were excluded and uncorrected *P* values resulting from these ITS alignments were calculated in PAUP* (Swofford 2002). Based on these analyses, we have defined *Tuber* phylotypes a posteriori as those having at least 96% ITS sequence similarity. We consider this threshold as a species approximation. In some cases, this phylotype definition may lump morphological or ecological species. Geographical origin of sequences was documented to assess the distribution of *Tuber* species. Putative introduction events were diagnosed either as phylotypes occurring outside of *Tuber*'s native range, such as the Southern hemisphere or as phylotypes showing large continental disjuncts and having <1% ITS variation, a level often exceeded at the local scale in native populations (Smith *et al.* 2007b; Bonito 2009b; Southworth *et al.* 2009; Jumpponen *et al.* 2010). Estimates of global species diversity for *Tuber* was calculated with incidence-based estimators (i.e. ICE, Chao2, Jackknife2) in EstimateS (Colwell 2005). Sequences generated in this study have been annotated and deposited in GenBank (HM485330–HM485429), and collection numbers and herbaria where these sporocarps are accessioned are provided for future taxonomic work (Table S5, supporting information).

Results

Mining ITS sequence metadata from GenBank with *emerencia* yielded 230 sequences (>300 bp) from 75 studies (Table S1, supporting information) and another 1622 short sequences (<250 bp) from a single pyrosequencing study (Jumpponen *et al.* 2010). ITS sequences from reference taxa ($n = 74$) and unidentified fruitbodies ($n = 196$) were combined with GenBank sequences for a total of 2122 sequences. By removing all sequences of poor quality, this data set was reduced to 1950 sequences. Assembling these into clusters of 98% sequence similarity resulted in 185 unique ITS types.

Phylogenetic analyses of ITS rDNA from *Tuber* reference taxa distinguished nine major clades (Fig. 1). The majority (98%) of unique unidentified *Tuber* ITS sequences grouped within three of the nine *Tuber* clades: Puberulum (44%), Maculatum (34%) and Rufum (20%). Of the nine sequences outside these three clades, two from *Epipactis microphylla* mycorrhizae were identified as *T. excavatum* group A (Excavatum clade); two from *Epipactis microphylla* and *Cephalanthera damasonium* mycorrhizae were identified as *T. aestivum* (Aestivum clade); two from angiosperm mycorrhizae were identified as *T. magnatum* (Aestivum clade); one from a *Pinus sabiniana* ectomycorrhiza and was identified as *T. gibbosum* (Gibbosum clade) (Fig. 1). Two sequences from an artificially established truffle orchard in North America grouped with *T. melanosporum*.

Intraspecific ITS variation differed among the species examined and was below 3% for all species except *T. aestivum* (<3.7%) (Table 1). *Tuber oregonense*, *T. puberulum* and *T. castellanoi* had the lowest levels of intraspecific ITS variation (0.2%). At least 4% interspecific ITS variation occurred between pairs of sister taxa examined, except for the morphological species *T. excavatum*, *T. gennadii*, and *T. candidum*, which appear to be species complexes. *Tuber* species also differed significantly in ITS length (Table 1). *Tuber brumale* had the longest ITS (859 bp), while *T. bellisporum* had the shortest (465 bp).

Based on our phylotype definition, 25 putatively undescribed species can be added to the Puberulum clade (Fig. 2), including 15 represented only by mycorrhiza or soil clone sequences (Table S3, supporting information). Plant hosts varied considerably within the Puberulum clade. Some phylotypes are only recorded from angiosperms and others only with gymnosperms (Table 2). Four phylotypes (*T. borchii*, *T. menseri* nom. prov., *Tuber* sp.19, and *Tuber* sp. 24) in this clade were recorded as ectomycorrhiza on both angiosperm and gymnosperm hosts. Asia, Europe and North America each contain endemic *Tuber* species belonging to this clade. *Tuber menseri* nom. prov., *Tuber* sp.19, and *T. borchii* (cultivated) have been documented in New Zealand (Table 3).

Twelve putatively undescribed species can be ascribed to the Maculatum clade (Fig. 3). Five of these are represented only by mycorrhiza or soil clone sequences (Table S3, supporting information). Only one sequence (AM418469) in the Maculatum clade came from a gymnosperm host (*Pinus nigra* var. *austriaca*) (D. Redecker *personal communication*). One species (*T. rapaeodorum*) appears to occur in Europe, North America and New Zealand and was recovered in 14 studies (Table 3).

Twenty-five putatively undescribed species belong to the Rufum clade (Fig. 4), including 13 represented only by mycorrhiza or soil clone sequences (Table S3, supporting information). There was only one sequence (FJ789634) in the Rufum clade that came from a gymnosperm host (*Pinus jeffreyi*) (D. Southworth, *personal communication*). Species in the Rufum clade are distributed across Asia, Europe and North America, and one species, *Tuber* sp.57, has been documented in New Zealand (Table 3).

Analysis of pyrosequencing data from *Quercus* ectomycorrhizae (Jumpponen *et al.* 2010) indicated that 1517 of the 1548 *Tuber* sequences already had counterparts in our data set. These sequences were comprised of 11 phylotypes within the Rufum and Maculatum clades, three of which are novel to this study (Table S6, supporting information). The three most abundant sequences belonged to *Tuber lyonii* ($n = 520$), *Tuber* sp. 40 ($n = 494$) and *Tuber* sp. 36 ($n = 342$).

In total, 123 distinct phylotypes (based on 96% ITS sequence similarity) are presented in this study, including 59 singletons and 24 doubletons. Rarefaction curves did not plateau and incidence-based coverage estimators predict a minimum of 180 (Jackknife 2) to 230 (ICE) *Tuber* species worldwide (Fig. 5). We provide molecular evidence for the introduction of 10 *Tuber* species outside of their native ranges (Table 3). These consist of commercial and non-commercial species belonging to five major *Tuber* clades.

Discussion

Phylotype definition

The ITS rDNA region is considered a DNA barcode for fungi (Nilsson *et al.* 2008). Though not appropriate for all species, our data demonstrate it is reliable for discerning species in *Tuber*. For the species examined, intraspecific ITS variation was typically <3% and interspecific variation was 4% or greater. Therefore, a phylotype definition of 96% ITS sequence similarity appears to be a valid species approximation for *Tuber*. Based on this criterion, which is less stringent than the 97% phylotype threshold commonly used in fungal community studies (Smith *et al.* 2007b; Peay *et al.* 2008;

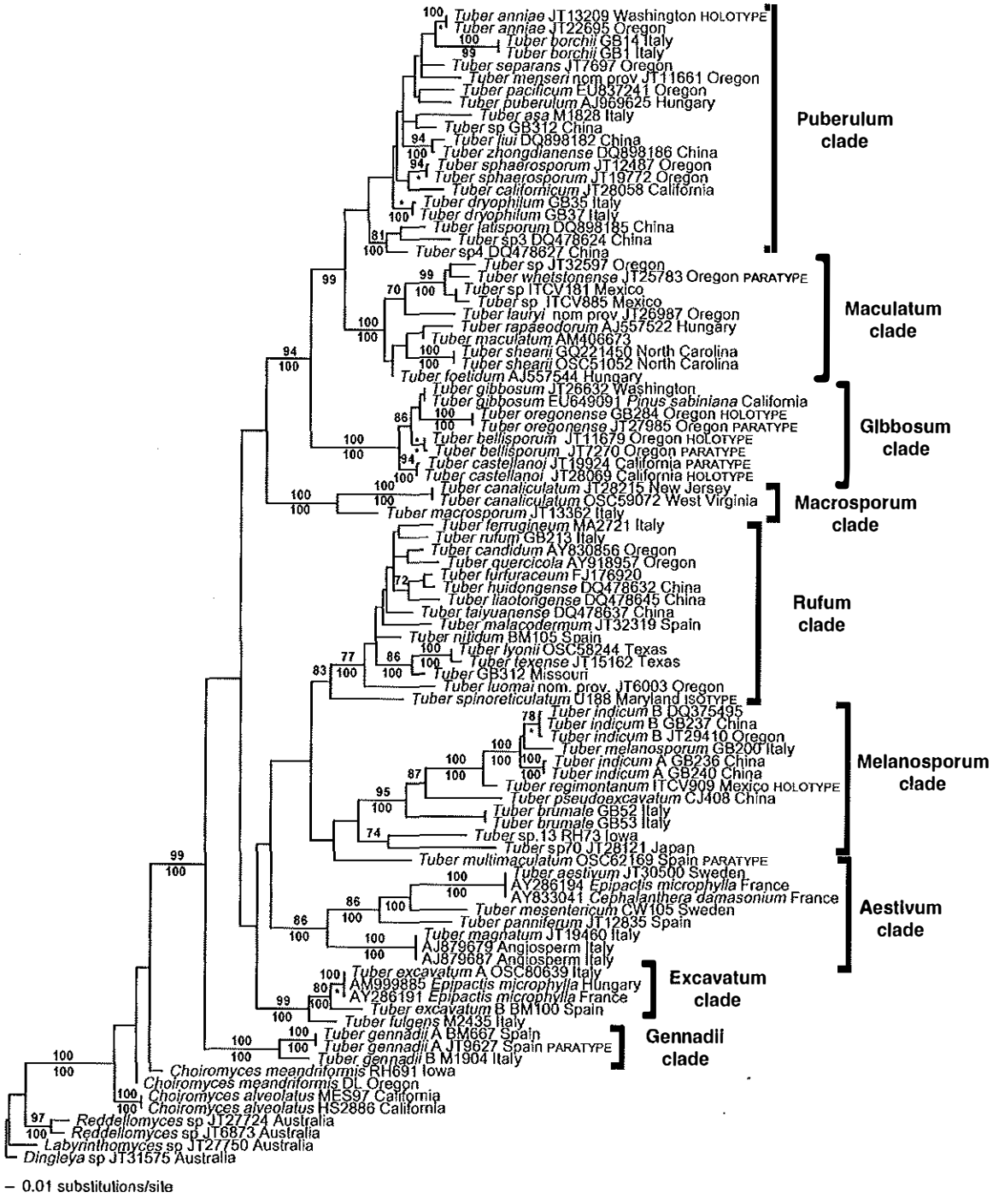

Fig. 1 *Tuber* ITS phylogeny showing the nine major clades in the genus. Taxa were chosen to demonstrate the phylogenetic breadth of *Tuber* and to represent as many described species as possible. Insufficiently identified sequences belonging in the Aestivum, Excavatum and Gibbosum clades have been included, while those belonging to the Maculatum, Puberulum and Rufum clades are shown in Figs 2–4. These analyses are based on ITS nuclear rDNA (323 included characters) and a GTR+G+I submodel of nucleotide substitution with three substitution rate classes. Maximum likelihood (ML) bootstrap values are shown above branches and posterior probabilities based on Bayesian inference (BI) below. Values of >70 for ML and ≥99 for BI are considered significant. Asterisks (*) denote support values of 100, when space was limited. Sequences are labelled with Latin binomials, GenBank accession or collection number and geographical origin. Other *Tuberaceae* genera were chosen as outgroups.

Table 1 Intraspecific and interspecific ITS variation of reference *Tuber* species. *Note*: limited geographical sampling or representative sequences for listed species could lead to an underestimate of intraspecific variation

Species	Number (*n*)	Maximum ITS length (bp)*	Intraspecific variation (%)	Interspecific variation (%)
Tuber aestivum	104	653	<3.7	>20.9
Tuber bellisporum	3	465	<0.4	>3.9
Tuber borchii	72	501	<2.2	>5.1
Tuber brumale	9	859	<0.5	>25.4
Tuber castellanoi	3	474	<0.2	>6.2
Tuber excavatum†	9	622	<14.7	>9.2
Tuber gennadii†	3	666	<16.7	>27.9
Tuber gibbosum	15	472	<0.6	>3.9
Tuber lyonii	26	537	<2.9	>5.3
Tuber indicum A	57	543	<2.5	>7.0
Tuber indicum B‡	30	541	<2.3	>7.0
Tuber macrosporum	4	587	<1.7	>9.1
Tuber magnatum	64	538	<0.4	>30.0
Tuber melanosporum	209	566	<1.6	>7.0
Tuber menseri nom prov	25	593	<2.3	>3.9
Tuber mesentericum	171	622	<1.0	>20.9
Tuber oregonense	34	473	<0.2	>8.9
Tuber puberulum	4	488	<0.2	>5.1
Tuber rapaeodorum	42	643	<1.6	>4.6
Tuber whetstoneuse	10	472	<2.5	>4.8

*Based on the number of basepairs between the end of the SSU - CATTA- motif, and the begiming 28s LSU -TAGGGT motif (if present).
†Represent species complexes (see Table S4, supporting information and Fig. 1).
‡Sequences accessioned as *T. formosanum* appear to be nested within *T. indicum* B, so are considered as *T. indicum* B in these calculations.

Hughes *et al.* 2009), the morphological species *T. excavatum*, *T. gennadii* and *T. candidum* are actually species complexes. *Tuber gennadii* is a rare species that has been described as a separate genus (*Loculotuber*) because of its morphology of chambers lined by asci (Alvarez *et al.* 1992). ITS places *T. gennadii* basal in the *Tuber* lineage and distinguishes two distinct phylotypes (Fig. 1). *Tuber excavatum* is often considered a single species, yet sequences cluster into three distinct phylotypes (Table S4, supporting information). *Tuber candidum* contains up to three phylogenetic species, which cannot be resolved by ITS alone (Fig. 4). The highest level of intraspecific ITS variation (<3.7%) was found for *Tuber aestivum* (=*T. uncinatum*). Mello *et al.* (2002) and Wedén *et al.* (2005) also report intraspecific variation of over 3% in *T. aestivum*. Morphological studies indicate *T. aestivum* occurs throughout Europe and Asia and has been recorded from Morocco in North Africa (Bucholtz 1901; Ceruti *et al.* 2003; Song *et al.* 2005). Although this economically important taxon is among the most studied *Tuber* species, there is still debate over whether this is a species complex (Mello *et al.* 2002; Paolocci *et al.* 2004; Wedén *et al.* 2004, 2005). Additional molecular studies will be needed to resolve the issue of cryptic species in these *Tuber* species complexes.

Distribution of Tuber species and introductions into non-native habitats

Relying on morphological and phylogenetic species concepts Vellinga *et al.* (2009) conclude that at least 200 species of ectomycorrhizal fungi (including eight *Tuber* species) have been introduced into novel habitats. In most cases, introduced fungi were associated with non-native host plant species and were likely introduced in conjunction with a plant host (either on roots or in the accompanying soil). With the inclusion of our molecular data, the number of *Tuber* species introduced to novel habitats is 16 (Table 3).

ITS analyses of reference taxa show that *Tuber* species are distributed regionally and are generally not shared between continents. Cases where individual *Tuber* phylotypes (ITS similarity >99%) are found on multiple continents appear to be examples of recent human-mediated introductions and include *T. aestivum*, *T. foetidum*, *T. indicum* B, *T. menseri* nom. prov., *T. melanosporum*, *T. rapaeodorum*, *Tuber* sp.19 and *Tuber* sp. 57. Clearly this is the case for the cultivated species *T. aestivum*, *T. borchii* and *T. melanosporum* (Hall *et al.* 2007; Pruett *et al.* 2008) but also for species reported from New Zealand (e.g. *T. borchii*, *T. brumale*, *T. foetidum*, *T. levissimum*, *T. maculatum*, *T. rapaeodorum*, *T. rufum*,

Table 2 The 10 most abundant insufficiently identified *Tuber* taxa, their geographical range and host plant mycorrhiza from which they have been sequenced. Species calls are based upon a 98% sequence similarity threshold across the complete ITS rDNA region and thus are likely to underestimate host and geographical ranges. Pyrosequence data have not been included in abundance scores. See (Table S2, supporting information) for a complete list of species and accompanying metadata on host, source, and accession number for each sequence

Rank	Abundance	Species	Geographical source	Host associates	Clade
1	42	*Tuber rapaeodorum*	Finland Netherlands Germany Hungary Poland Estonia Hungary USA, Oregon USA, California USA, Texas USA, New York Canada, Quebec New Zealand	*Epipactis helleborine* *Salix caprea* *Phragmites australis* *Populus alba* *Tilia cordata* *Alnus* *Cephalanthera damasonium*	Maculatum
2	19	*Tuber menseri* nom. prov.	USA, Oregon USA, Washington Canada, Quebec Lithuania Finland Poland Netherlands New Zealand	*Quercus garryana* *Salix caprea* *Betula pendula* *Salix alba* *Populus sp.* *Tilia cordata* *Pseudotsuga menziesii* *Pinus sylvestris* *Picea abies*	Puberulum
3	10	*Tuber* sp.19	Austria Germany Lithuania Sweden Quebec USA, Nebraska USA, California New Zealand	*Epipactis dunensis* *Populus tremula* *Betula pendula* *Pinus sylvestris* *Picea abies* *Pseudotsuga menziesii*	Puberulum
4	10	*Tuber lauryi* nom.prov.	USA, California USA, Oregon	*Epipactis helleborine* *Quercus wislizeni* *Notholithocarphus densiflorus*	Maculatum
5	10	*Tuber whetstonense*	USA, California USA, Oregon	*Quercus garryana* *Quercus wislizeni* *Quercus douglasii*	Maculatum
6	7	*Tuber* sp.36	USA, Missouri USA, Georgia USA, California	*Quercus* sp. *Carya illinoinensis* *Epipactis helleborine*	Maculatum
7	7	*Tuber borchii* (anamorphic)	Austria Spain Italy	*Pinus pinaster*	Puberulum
8	6	*Tuber separans*	USA, New York USA, North Carolina USA, Maryland USA, Oregon	*Castanea dentata* *Quercus* sp. *Quercus palustris*	Puberulum
9	6	*Tuber* sp.16	USA, New York USA, Michigan USA, California Canada, Quebec	*Populus* sp. *Epipactis hellborine*	Puberulum
10	6	*Tuber dryophilum* (anamorphic)	Italy Austria Germany Hungary		Puberulum

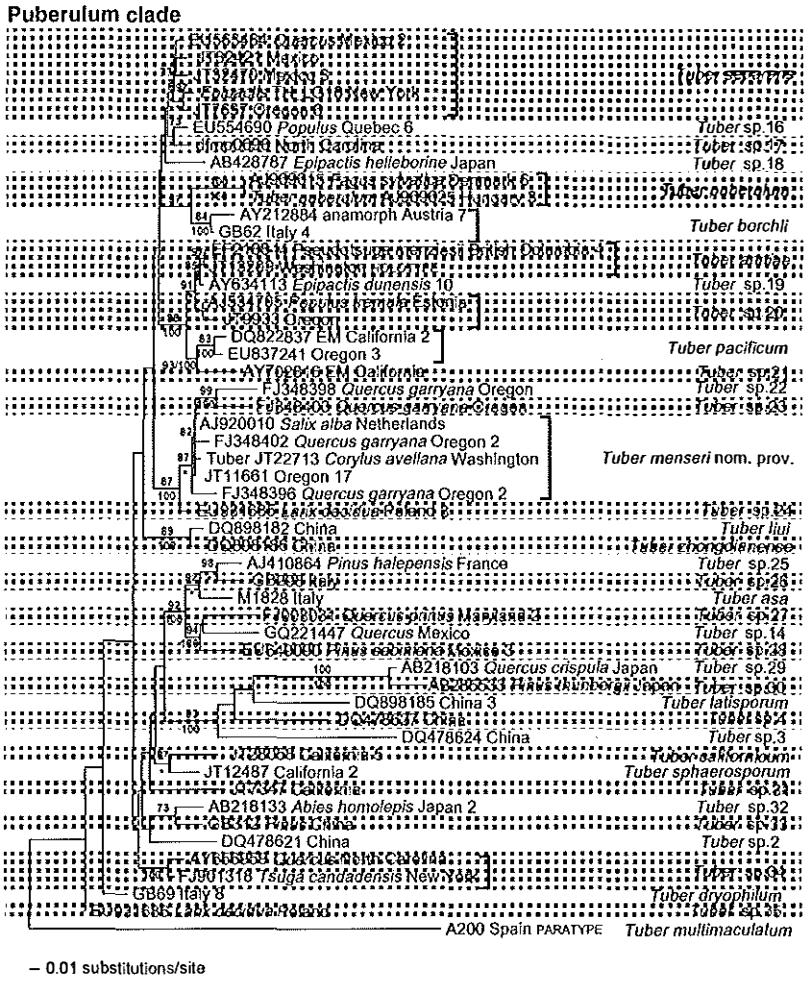

Fig. 2 Placement of insufficiently identified *Tuber* collections and sequences belonging in the *Puberulum* clade: most likely tree for *Puberulum* clade. The analysis is based on ITS nuclear rDNA (502 included characters) and a GTR+G+I submodel of nucleotide substitution with four substitution rate classes. Maximum likelihood (ML) bootstrap values are shown above branches and posterior probabilities based on Bayesian inference (BI) below. Values of >70 for ML and ≥99 for BI are considered significant. Asterisks (*) denote support values of 100, when space was limited. Sequences are labelled with Latin binomials, GenBank accession or collection number and geographical origin. Species are marked by alternating white and grey bands. *Tuber multimaculatum* was chosen as an outgroup.

T. separans, *Tuber* sp.19 and *Tuber* sp.57) and Argentina (*T. borchii*, *T. californicum* and *T. maculatum*) (Barroetavena *et al.* 2005, 2006; Bulman *et al.* 2010). *Tuber* is a Northern Hemisphere lineage and has only been found in New Zealand associated with introduced host plants and never in native forests (Chu-Chou & Grace 1983; Bulman *et al.* 2010).

Dispersal considerations

Epigeous fungi are dispersed by wind, and therefore less constrained than hypogeous forms dependent on small mammals and insects for spore dispersal (Trappe & Claridge 2005; Frank *et al.* 2006a). In cases where hypogeous fungi successfully establish after introduction, they may fail to spread across the landscape because of a lack of suitable dispersal agents (Nuñez *et al.* 2009b). There may be exceptions to this epigeous/hypogeous dichotomy however. Anamorphic states have been documented in the hypogeous ectomycorrhizal Pezizales genera *Tuber*, *Pachyphloeus* and *Ruhlandiella* and may be more common than is realized (Urban *et al.* 2004; Perry *et al.* 2007). The effect of anamorphic states on species distributions or invasion biology is unknown. The two known anamorphic *Tuber* species, *T. borchii* and *T. dryophilum*, do not appear to have exceptionally large ranges nor is there evidence for unintentional introductions of these species.

Table 3 Summary of *Tuber* species introduced into environments outside of their native range. Molecular determinations are based on ITS rDNA sequence similarities of >99%

Species	Host	Natural Distribution	Transported To	Determination	Reference
Tuber aestivum (=*T. uncinatum*)	*Quercus*	Europe	North America	Molecular (cultivated)	Pruett *et al.* (2008)
*Tuber borchii**	*Pseudotsuga menziesii*	Europe	Argentina	Morphological	Barroetaveña *et al.* (2006)
	Corylus sp.	Europe	New Zealand	Molecular (cultivated)	Bulman *et al.* 2010
Tuber brumale	Angiosperm	Europe	New Zealand	Molecular	Alexis Guerin-Laguette unpublished data
*Tuber californicum**	*Pseudotsuga menziesii*	North America	Argentina	Morphological	Barroetavena *et al.* (2006)
Tuber foetidum	*Epipactis helleborine*	Europe	Japan	Molecular	This study
	Angiosperms		New Zealand	Morphological	Bulman *et al.* (2010)
*Tuber gibbosum**	*Pseudotsuga menziesii*	North America	Italy	Morphological	Pomarico *et al.* (2007)
*Tuber levissimum**	Gymnosperms	North America	Spain	Morphological	Álvarez *et al.* (1993)
		North America	New Zealand	Morphological	Bulman *et al.* (2010)
*Tuber maculatum**	Angiosperms	Europe	New Zealand Australia Argentina	Morphological	Trappe & Cázares (2000)
Tuber indicum (B)*	Angiosperm & gymnosperm	Asia	Italy	Molecular	Murat *et al.* (2008)
			Oregon	Molecular	This study
Tuber melanosporum	Angiosperms	Europe	Virginia	Molecular (cultivated)	
Tuber rapeaodorum	Angiosperms	Europe	North America	Molecular	This study
			New Zealand		Bulman *et al.* (2010)
Tuber menseri nom prov	Angiosperms & gymnospoerms	North America	Europe	Molecular	This study & Bulman *et al.* (2010)
Tuber rufum	Angiosperms	Europe	New Zealand	Morphological	
Tuber separans	Angiosperms	North America	New Zealand	Morphological	
Tuber sp.19	Angiosperms & gymnosperms	Northern Hemisphere	New Zealand	Molecular	This study
					Bulman *et al.* (2010)
Tuber sp.57	Angiosperm	North America	New Zealand	Molecular	This study & Bulman *et al.* 2010

*Tablulated by Vellinga *et al.* (2009).

Host preferences for Tuber species

Host specificity at the genus and family level occurs in a minority of ectomycorrhizal fungi (Molina & Trappe 1982; Molina *et al.* 1992). However, some ectomycorrhizal clades may be restricted to particular plant phyla, such as the association between Suillineae (Boletales) and Pinaceae hosts (Taylor & Bruns 1997). In other cases, it appears that individual ectomycorrhizal taxa or clades have preferential associations with certain host groups (Murat *et al.* 2004; Ishida *et al.* 2007; Tedersoo *et al.* 2008; Smith *et al.* 2009). From this meta-analysis, which includes sequence data from over 70 molecular-based studies of ectomycorrhizal communities, we are able to document 24 *Tuber* species associated with multiple host species and genera. Clearly more data is needed to ascertain the range of host preference and specificity in *Tuber*, but from the data currently available, some speculation can be made. The Gibbosum clade appears to be the only *Tuber* lineage with strong preference for gymnosperms (Bonito *et al.* 2010). In contrast, species in the Rufum, Excavatum and Maculatum clades may be capable of forming ectomycorrhizal associations with gymnosperms, but show a strong preference towards angiosperm hosts in nature (Montecchi & Sarasini 2000; Halász *et al.* 2005; Frank *et al.* 2006b). Species in the Puberulum clade appear to associate with either gymnosperm or angiosperm hosts, and in some cases with both. Species in the Aestivum and Melanosporum clades are typically associated with angiosperm hosts, although *T. aestivum* and *T. melanosporum* have been occasionally reported with gymnosperms, whereas *T. indicum* (A & B) commonly fruits under Pinaceae hosts (Trappe 1971; Ceruti *et al.* 2003; Hall *et al.* 2007). Mycorrhizal associations with terrestrial orchid species were documented for thirteen *Tuber* species that belong to the Excavatum, Aestivum, Rufum, Maculatum and Puberulum clades (Bidartondo *et al.* 2004; Selosse *et al.*

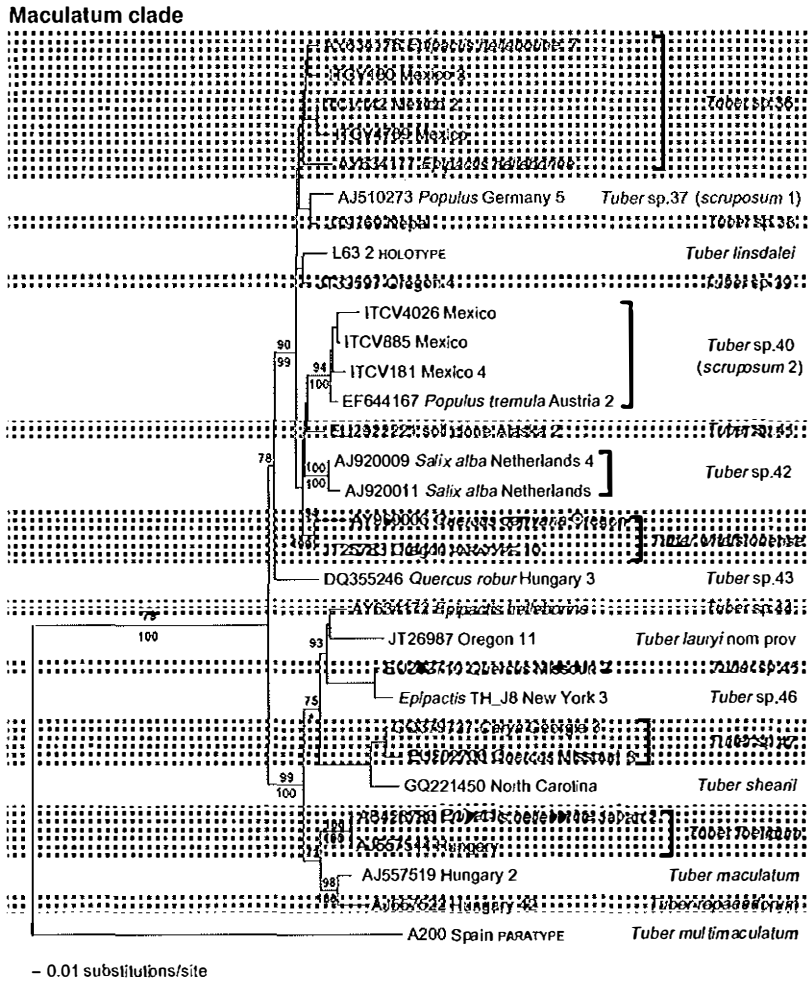

Fig. 3 Placement of insufficiently identified *Tuber* collections and sequences belonging in the *Maculatum* clade: most likely tree for *Maculatum* clade. The analysis is based on ITS nuclear rDNA (574 included characters) and a GTR+G submodel of nucleotide substitution with four substitution rate classes. Maximum likelihood (ML) bootstrap values are shown above branches and posterior probabilities based on Bayesian inference (BI) below. Values of >70 for ML and ≥99 for BI are considered significant. Asterisks (*) denote support values of 100, when space was limited. Sequences are labelled with Latin binomials, GenBank accession or collection number and geographical origin. Species are marked by alternating white and grey bands. *Tuber multimaculatum* was chosen as an outgroup.

2004; Bidartondo & Read 2008; Ogura-Tsujita & Yukawa 2008). In six of these instances, unique *Tuber* phylotypes were found only in association with *Epipactis* orchids.

Phylogenetic placement of insufficiently identified Tuber taxa

In this meta-analysis of over 2000 *Tuber* ITS sequences representing 76 studies from around the world, a total of 123 phylotypes were distinguished, 39 of which are reported from multiple studies. In some cases, *Tuber* species were dominant members of the ectomycorrhizal community (Walker *et al.* 2005; Smith *et al.* 2007a; Morris *et al.* 2009; Jumpponen *et al.* 2010), whereas in others they were less frequently detected (Hrynkiewicz *et al.* 2008; Krpata *et al.* 2008; Leski *et al.* 2008). Well over 99% of the insufficiently identified *Tuber* taxa (including sporocarp and pyrosequence data) grouped within three less studied *Tuber* clades: Puberulum, Maculatum and Rufum. To the best of our knowledge, 64 undescribed species are represented by these data and 36 are known only from DNA sequences (Table S3, supporting information). This raises questions concerning their taxonomy, as species are traditionally described by fruitbody characters (Hibbett *et al.* 2009). In a step short of sequence-based species descriptions to address taxonomic issues, we have assigned temporary numbers to undescribed species (up to *Tuber* sp.73) following the convention of Jeandroz *et al.* (2008).

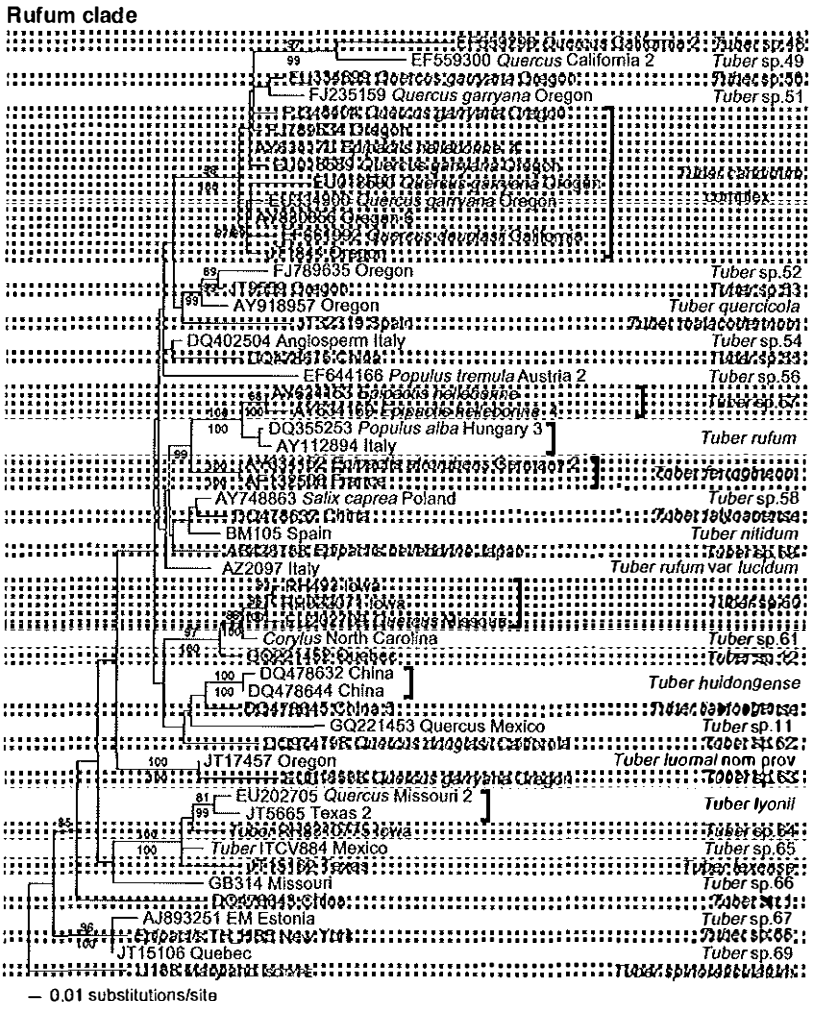

Fig. 4 Placement of insufficiently identified *Tuber* collections and sequences belonging in the *Rufum* clade: most likely tree for *Rufum* clade. The analysis is based on ITS nuclear rDNA (461 included characters) and a GTR+G+I model of nucleotide substitution with four substitution rate classes. Maximum likelihood (ML) bootstrap values are shown above branches and posterior probabilities based on Bayesian inference (BI) are below branches. Values of >70 for ML and ≥99 for BI are considered significant. An asterisk (*) was used to denote support values of 100, when space was limited. Sequences are labelled with their Latin binomials, GenBank accession or collection number and geographical origin. Species are marked by alternating white and grey bands. *Tuber spinoreticulatum* was chosen as an outgroup.

Herbaria contain a large number of unsampled taxa and their role in increasing the fungal biodiversity represented in GenBank has been addressed (Brock *et al.* 2009). However, there are known issues with the nomenclature of identified GenBank accessions (Vilgalys 2003; Trappe 2004) and it is estimated that ~20% of the entries have been incorrectly identified (Nilsson *et al.* 2006). Therefore, a phylogenetic framework is preferable for identifying unknowns, and discretion should be used when taxonomic calls are based solely on BLAST results. Our analyses indicate that GenBank accessions for *T. rufum*, *T. borchii*, *T. californicum*, *T. scruposum*, *T. maculatum* and *T. excavatum* are composed of multiple phylogenetic species. It is still unclear at this time which (if any) of these accessions are representative of type collections of these species.

Global estimations of *Tuber* biodiversity

Estimating worldwide species diversity for *Tuber* has been difficult because of numerous synonyms and misidentifications. Although Index Fungorum lists 256 described species of *Tuber* (including synonyms and varieties) and the Dictionary of the Fungi states there are 86 species (Kirk *et al.* 2008), only 70–75 species are believed to be valid (Ceruti *et al.* 2003; Jeandroz *et al.*

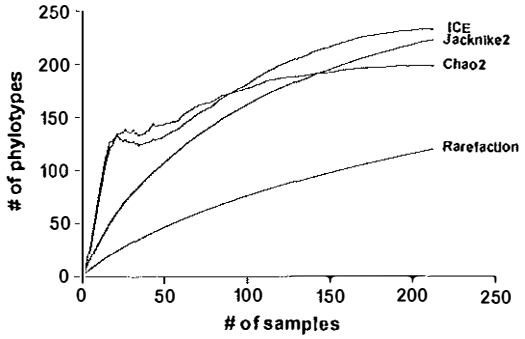

Fig. 5 Rarefaction and global species richness estimates for *Tuber*.

2008). Our meta-analysis of global ITS rDNA diversity for *Tuber* phylotypes (defined as those sharing 96% ITS rDNA sequence similarity) distinguishes 123 phylotypes, although only ~70% of the accepted species were represented in the analyses. Projections of global *Tuber* species richness from these data predict a minimum of 180–230 species worldwide, depending on the estimator used (Fig. 5). To assess the accuracy of these estimators in predicting species richness when a 'true' value is known, Petersen *et al.* (2003) compared riclmess estimators using herbarium collections for Asilidae, a group of conspicuous and well-sampled beetles. They found that the estimators were internally consistent but consistently underestimated the 'true' diversity. This did not seem to be particularly sensitive to sample size or subsampling strategies. While the true number of *Tuber* species may never be known, it is clear that considerable *Tuber* diversity awaits discovery. Novel taxa continue to be encountered, even in relatively well-sampled regions (e.g. western Europe, Pacific Northwestern USA) and even more so in less studied regions of high plant endemism including central Asia, Japan and Mexico.

In summary, working within a phylogenetic framework we demonstrate that *Tuber* is more diverse than previously realized and that most of the diversity resides within non-economical and less studied clades (i.e. Rufum, Puberulum, and Maculatum). Most *Tuber* clades show strong phylum-level preference to either angiosperm or gymnosperm hosts. We also infer from these data that *Tuber* species in Europe, Asia and North America are endemic to their respective continents and are generally not shared between continents except in cases of human-mediated long-distance dispersal.

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Supporting information

Additional supporting information may be found in the online version of this article.

Table S1 Citations for reference sequences used and insufficiently identified sequences of *Tuber* retrieved by *emerencia*. GenBank number of *Tuber* sequences are presented at the end of each citation

Table S2 *Tuber* groupings based on 98% ITS rDNA sequence similarity

Table S3 Thirty-six *Tuber* species known only from sequence data

Table S4 Most likely tree of the Excavatum clade based on ITS nuclear rDNA and a GTR+G+I model of nucleotide substitution with four substitution rate classes. Three phylogenetic species of *'T. excavatum'* are resolved. Maximum likelihood (ML) bootstrap values are shown above branches and posterior probabilities based on Bayesian inference (BI) are below branches. Values of >70 for ML and ≥99 for BI are considered significant

Table S5 Collection information and GenBank accession numbers for sequences generated during this study

Table S6 Phylogenetic placement of 1548 *Tuber* sequence data from *Quercus* sp. ectomycorrhizae in Kansas, USA produced through pyrosequencing (Jumpponen *et al.* 2010). This most likely tree includes taxa from the Maculatum and Rufum clades and is based on ITS nuclear rDNA and a GTR+G+I model of nucleotide substitution. The eleven phylotypes represented by these sequences are shown in a larger font and their abundance based on binning at 96% is presented on the taxon labels. Three phylotypes belonging to the Rufum clade were unique to this study and are labelled with and asterisk (*). Maximum likelihood (ML) bootstrap values are shown on top of the branches. Values of >70 for ML are considered significant

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