

Soil Biology

Alessandra Zambonelli  
Mirco Iotti  
Claude Murat *Editors*

# True Truffle (*Tuber* spp.) in the World

Soil Ecology, Systematics and  
Biochemistry

 Springer

# **Soil Biology**

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Claude Murat  
Editors

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# Preface

As we were writing this preface, the COP21 international conference on climate change was being held in Paris, highlighting the importance of all initiatives to protect the future of the planet. Forests, and more generally trees, play a key role in carbon sequestration and greenhouse gas mitigation. Many trees live in strict symbiosis with ectomycorrhizal fungi that are important for ecosystems' functioning. Some ectomycorrhizal species, such as boletes and truffles, are also famous because they form edible fructifications, and truffles belonging to the *Tuber* genus, the so-called "true truffles," are gourmet delicacies worldwide. The genus *Tuber* includes around 180 species, most of which are naturally distributed in the northern hemisphere. Some *Tuber* species, such as *Tuber magnatum* (the Italian white truffle), *T. melanosporum* (the Perigord black truffle), *T. aestivum* (the Burgundy truffle), and *T. borchii* (the bianchetto truffle), are the most economically important fungi, but other *Tuber* species are edible and locally appreciated as well. Besides their economic and culinary importance, many truffle species play a key role in forest ecosystems, including disturbed forests, where they are often common ectomycorrhizal symbionts. Moreover, the cultivation of some truffle species such as *T. melanosporum* and *T. aestivum* has spread worldwide in the last two decades and has diversified crops and incomes for local farmers. In this context, many books have been written on truffles, but most of them in French and Italian, or they are focused on a few species or specific aspects.

In this book, we decided to cover much of the taxonomic diversity of the genus *Tuber*, in addition to economically important species, and include information generated from more recent technological innovations (e.g., second-generation DNA sequencing). The book is divided into five parts and comprises chapters written by experienced and internationally recognized scientists. The aim is to provide an inventory of the knowledge on truffle systematics, interactions with abiotic and biotic environments, strategies for spore dispersal, and biochemistry. Such multidisciplinary approach provides a unique insight and a better understanding of the truffle ecology and the role these fungi play in natural and managed ecosystems.

We are grateful to the many scientists who generously assisted us in writing and reviewing the content of this book. It would be too long to cite all the contributors, but we would like to highlight all the corresponding authors of the chapters: Antonella Amicucci, Elena Barbieri, Niccolò Benucci, Gregory Bonito, Gilberto Bragato, Zoltan Bratek, Milan Gryndler, Benoit Jaillard, Chen Juan, Enrico Lancellotti, François Le Tacon, Francis Martin, Cristina Menta, Virginie Molinier, Giovanni Pacioni, Francesco Paolocci, Xavier Parladé, Federica Piattoni, Claudio Ratti, Christophe Robin, Matthew Smith, Richard Splivallo, and Alexander Urban.

Peer review by contributors to this volume and by external internationally recognized scientists helped to maintain the rigor and high quality of material presented. We would like to thank especially all the colleagues who helped us in reviewing the chapters: Antonella Amicucci, Niccolò Benucci, Gilberto Bragato, Aurélie Deveau, Lorenzo Gardin, Milan Gryndler, Ian Hall, Benoit Jaillard, Annegret Kohler, Virginie Molinier, Giovanni Pacioni, Francesco Paolocci, Xavier Parladé, Federica Piattoni, Maria Agnese Sabatini, Elena Salerni, Massimo Turina, Giuliano Vitali, and Yun Wang. We are also grateful to Joey Spatafora who kindly revised this Preface.

We would like also to thank Ajit Varma, series editor, who gave us this great opportunity, Jutta Linderborn, Editor Life Science of Springer, and Sumathy Thanigaivelu, for their help and patience in responding to all the queries regarding the preparation of the book and for giving us the opportunity to include the color pictures provided.

We hope this book will serve as a primary research reference for researchers and research managers interested in mycology, ecology, and soil sciences. Our aim was also to provide a reference book for farmers and foresters who are interested in truffle cultivation worldwide. We are convinced that truffles deserve to be preserved in the context of climate change in order to maintain biodiversity and ecosystem functioning but also to allow future generations to appreciate these unique natural resources.

Bologna, Italy  
L'Aquila, Italy  
Champenoux, France  
December 2015

Alessandra Zambonelli  
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# Abbreviation List

1D-SDS-PAGE	One-dimensional sodium dodecyl sulfate polyacrylamide gel electrophoresis
2-DE	Two-dimensional electrophoresis
2D-PAGE	Two-dimensional polyacrylamide gel electrophoresis
AbEV1	<i>Agaricus bisporus</i> endornavirus 1
ABV1	<i>Agaricus bisporus virus 1</i>
AFLP	Amplified fragment length polymorphism
AFM	Atomic force microscope
AIDS	Acquired immune deficiency syndrome
AMF	Arbuscular mycorrhizal fungi
AMSL	Above mean sea level
AMT	Ammonium transporter
AT	Aminotransferase
ATP	Adenosine triphosphate
BACI	Before-after-control-impact
BCMV	Brown cap mushroom virus
BLAST	Basic local alignment search tool
Cazymes	Carbohydrate active enzymes
CBS	Centraalbureau voor schimmelcultures fungal biodiversity center
cDNA	Complementary deoxyribonucleic acid
CE	Capillary electrophoresis
CEC	Cation exchange capacity
CFB	Cytophaga-Flexibacter-Bacteroides
Cfu	Colony-forming unit
CP	Coat protein
CTAB	Cetyltrimethylammonium bromide
DGGE	Denaturing gradient gel electrophoresis
DNA	Deoxyribonucleic acid
dsRNA	Double-stranded RNA
DTPA	Diethylene triamine pentaacetic acid
ECM	Ectomycorrhiza(l)

EF1	Elongation factor 1
Eno	Enolase
ERM	Ericoid mycorrhiza
ESI	Electrospray ionization
EST	Expressed sequence tags
FF	Fungicolous fungi
FGI	Fungal Genome Initiative
FvBV	Flammulina velutipes browning virus
GAP	GTPase-activating protein
GC	Gas chromatography
GC-MS	Gas chromatography—mass spectrometry
GC-o	Gas chromatography—olfactometry
GDH	Glutamate dehydrogenase
Gdis	GDP-dissociation inhibitor
GDP	Guanosine diphosphate
GEF	Guanine nucleotide exchange factor
GRF1V-M	Glomus sp. strain RF1 virus-like medium dsRNA
GS	Glutamine synthetase
GTP	Guanosine triphosphate
HB	Hydric balance
HPLC	High-performance liquid chromatography
HS-SPME	Head space-solid phase micro-extraction
HS-SPME- GC-MS	Head space-solid phase micro-extraction gas chromatography– mass spectrometry
HXK	Hexokinase
HXT	Hexose transporter
INRA	Institut National de la Recherche Agronomique
ISO	International Standard Organisation
ISRIC	International Soil Reference and Information Centre
ISSR	Inter simple sequence repeat
ITS	Internal transcribed spacer
JGI	Joint Genome Institute
kDa	Kilo dalton
LC	Liquid chromatography
LeSV	Lentinula edodes spherical virus
LeV	<i>Lentinula edodes</i> mycovirus
LIV	La France isometric virus
LSU	Large subunit
MALDI	Matrix-assisted laser desorption ionization
MAT	Mating type
MBV	Mushroom bacilliform virus
ME	Emden-Meyerhof
MGI	Mycorrhizal Genome Initiative
MHB	Mycorrhiza helper bacteria

MiSSP	Mycorrhizal induced small secreted protein
MMN	Modified Melin-Norkrans
MRCA	Most recent common ancestor
mRNA	Messenger ribonucleic acid
MS	Mass spectrometry
MTL	Methanethiol
MVOC	Volatile organic compounds produced by microbe
MVX	Mushroom virus X
NADP	Nicotinamide adenine dinucleotide phosphate
NCBI	National Center for Biotechnology Information
NGS	Next-generation sequencing
NIR	Near-infrared spectrometry
NiR	Nitrite reductase
NMR	Nuclear magnetic resonance spectroscopy
NR	Nitrate reductase
Nrt	Nitrate transporter
OM	Organic matter
OMIV	Oyster mushroom isometric virus
OMSV	Oyster mushroom spherical virus
ORC	Orchid mycorrhizas
ORF	Open reading frame
OTU	Operational taxonomic unit
PCWDE	Plant cell wall degrading enzymes
PDA	Potato dextrose agar
pers comm	Personal communication
PKC	Protein kinase C
PoV1	<i>Pleurotus ostreatus virus 1</i>
PoV-SN	<i>Pleurotus</i> strain Shin-Nong
PP	Pentose phosphate
qPCR	Quantitative polymerase chain reaction
RAPD	Random amplification of polymorphic DNA
rDNA	Ribosomal deoxyribonucleic acid
RdRP	RNA-dependent RNA polymerase
RFLP	Restriction fragment length polymorphism
RNA	Ribonucleic acid
RPB1	RNA polymerase II large subunit
RPB2	RNA polymerase II second largest subunit
RPLC	Reversed-phased liquid chromatography
RPP2	Acidic ribosomal protein P2
rRNA	Ribosomal ribonucleic acid
RT-qPCR	Retrotranscription quantitative polymerase chain reaction
s.l.	Sensu lato
s.s.	Sensu stricto
SCAR	Sequence characterized amplified region

SCIF	Sporocarp-inhabiting fungi
SCL	Sandy clay loam
SD	Standard deviation
SEM	Scanning electron microscope
SGS	Second-generation DNA sequencing
SiCL	Silty clay loam
SiL	Silt loam
SL	Sandy loam
SNP	Single nucleotide polymorphism
SPME-GC-MS	Solid phase micro-extraction-gas chromatography-mass spectrometry
SRP	Signal recognition particle
ssDNA	Single-stranded DNA
SSR	Simple sequence repeat
ssRNA	Single-stranded RNA
TaEV	Tuber aestivum endornavirus
TaMV	<i>Tuber aestivum</i> mitovirus
TaV1	<i>Tuber aestivum virus 1</i>
TE	Transposable elements
TEF1	Translation elongation factor 1 $\alpha$
TeMV	Tuber excavatum mitovirus
TIF	Truffle-inhabiting fungi
tRNA	Transfer ribonucleic acid
TTGE	Temporal temperature gradient gel electrophoresis
TUNEL	Terminal deoxynucleotidyl transferase dUTP nick end labeling
UPLC	Ultra-performance liquid chromatography
USDA	United States Department of Agriculture
VOC	Volatile organic compound

# **Part I**

## **Phylogeny**



# Chapter 1

## General Systematic Position of the Truffles: Evolutionary Theories

Gregory M. Bonito and Matthew E. Smith

### 1.1 Introduction

When the “truffle” concept is evoked, what comes to mind may vary greatly between people and cultural groups. As you read this book, your own concept of what a truffle is may change, as ours has while discovering and learning about these exquisite fungi!

In the very broadest sense, truffles are fungi that sequester their spores within differentiated fruiting structures that are produced below the soil or leaf litter. These fungi have also been referred to in the past as sequestrate fungi or hypogeous fungi, depending on the author and the usage. Hypogeous fungi that belong to the phylum Basidiomycota are sometimes referred to as “false truffles,” a name historically used to distinguish these truffles from those in the Ascomycota. We regard truffles as fungi that produce these sequestrate, hypogeous fruiting bodies regardless of their taxonomic or phylogenetic relationships. However, for the purpose of this book, we will use the term truffle in reference to the “true truffles” that belong to the genus *Tuber* (e.g., *Tuber melanosporum* Vittad., *Tuber magnatum* Pico, and related species). Truffles typically fruit on the forest floor just below the leaf litter or sometimes within the mineral horizon. As you will read within this book, we know a lot about the biology and ecology of these organisms, and yet there are still many questions about truffles that remain unanswered.

Truffles often fruit within the rooting zone of forest plants and exhibit a range of variable macroscopic characteristics such as color, shape, size, texture, and aroma.

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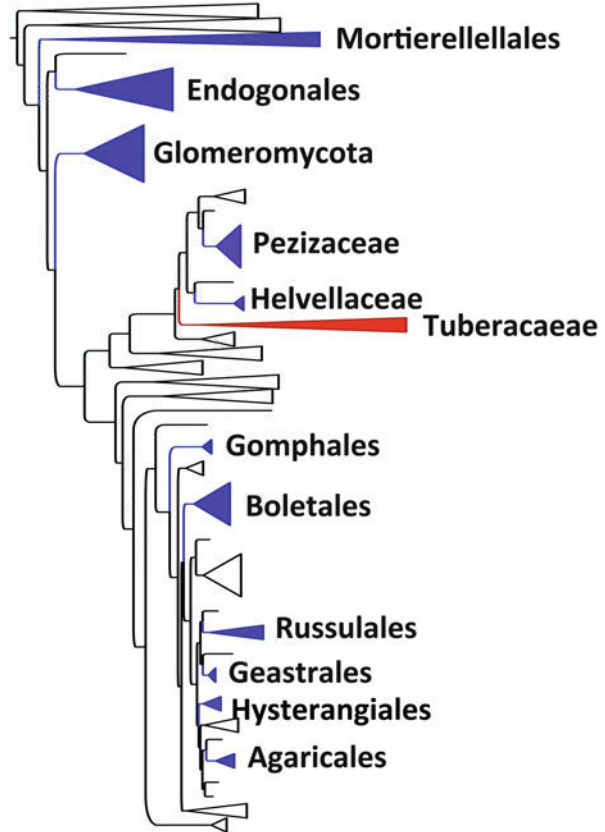
Microscopic, genomic, and developmental characters also vary widely between truffle species and truffle lineages. Details regarding the natural ecology of the majority of truffle species are still missing. Available evidence suggests that truffles co-diversified with plants and animals and the evolution and distribution of these fungal, plant, and animal symbionts forms a web that overlaps in time and space. Truffle speciation and function in ecosystems are tightly linked to their ectomycorrhizal (ECM) ecology and putative co-diversification with major plant families including Pineaceae (pines), Fagaceae (oak/beech), Myrtaceae (eucalyptus), and Salicaceae (willows/poplar) and also to adaptations for animal dispersal in the Northern and Southern Hemispheres. Because most truffles form ECMs and therefore actively exchange limiting nutrients with plants (truffles usually provide nitrogen and/or phosphorous whereas plants supply carbohydrates), truffle fungi play major roles in the functioning of forest soils and ecosystems as well as the maintenance of Earth's climate and food webs. On the other hand, human-induced climate change appears to be having effects on the distribution and fruiting of truffles and other fungi in Europe and across the globe (Kausrud et al. 2010).

Fungi that form truffle fruiting bodies have evolved independently in at least 13 orders that represent phylogenetically distant fungal lineages (Fig. 1.1) (Smith and Bonito 2013). While there may be some commonalities among these fungi, they are quite diverse in their morphology and ecology, and few generalities can be made about "truffles" at such a coarse level. We do find it interesting that most truffle fungi appear in lineages considered to be plant root-associated mutualists, such as ECM fungi that form a mantel covering the external surface of the root tip and a Hartig net forming laterally between the cortical cells of the root. These structures can be visualized under a microscope or sometimes even with the aid of a hand lens. Some truffle fungi form less evident orchid and ectendomycorrhizal structures, which are only apparent upon staining and visualization under a light microscope.

### ***1.1.1 Loss of Active Spore Discharge in Truffle Fungi***

Strong selection for active spore dispersal in fungi has led the evolution of bio-physical innovations in forcible spore discharge across the Kingdom. Particularly noteworthy, spores discharged from ascomycetes such as *Podospora curvicolla* (Winter) Niessl can shoot nearly half a meter, and those of *Gibberella zeae* (Schwein.) Petch can reach initial accelerations of  $8.5 \times 10^6 \text{ m s}^{-1}$  during spore discharge (Yafetto et al. 2008). Such intense force results from a buildup and release of turgor pressure stored in and released from fungal cells, which are critical to dispersal and in maintaining gene flow between populations. However, in truffle fungi that sequester their spores and fruit belowground the ability to actively discharge spores has been lost. This would seem to be detrimental to truffles, yet these fungi are extremely diverse and some species are dominant ECM partners in some ecosystems (Bonito et al. 2011; Smith et al. 2007). Although "self-powered"

**Fig. 1.1** Phylogram showing the distribution of truffle-forming fungi throughout the fungal tree of life. Major fungal orders (and families in the Pezizales) that include truffle taxa are color-coded *blue*. *Tuberaceae*, the taxonomic family which are the focus of this book, are shown in *red*



active spore discharge has been lost in truffles, novel “passive” mechanisms for spore dispersal have arisen in many truffle lineages. In this scenario the fungi have evolved mechanisms to attract animals through the production of olfactory or visual attractants (Beever and Lebel 2014), coaxing them into the consumption, release, and dispersal of truffle spores. There are a great many instances of coevolution of truffles with mammals in the Northern Hemisphere and with marsupials in the Southern Hemisphere (Claridge et al. 2014). There is also evidence that some birds act as truffle dispersers, such as *Paurocotylis* in New Zealand (Beever and Lebel 2014) and that some insect species could also serve to spread truffle spores (Fogel and Peck 1975).

### 1.1.2 *Enigmatic Truffles and Remaining Mysteries*

With their strong aromas, culinary and economic interest, high diversity, and importance to plant and animal nutrition, *Tuber* is a truffle genus that has attracted

much interest. However, a number of mysteries still remain concerning its evolution, ecology, and fundamental biology. Mature fruiting bodies of *Tuber* are unusual in their distinctive spore morphology, large spore size (relative to many other truffle fungi), and variable number of spores per ascus. The variable number of spores per ascus is particularly notable since this feature is atypical among Pezizales and varies between *Tuber* species and because the mechanisms for packaging post-meiotic nuclei and nuclear contents into spores are not well understood.

Recently, the sexual nature of *T. melanosporum* and *T. magnatum* was demonstrated using multiple molecular markers and population genetic approaches (Riccioni et al. 2008; Paolocci et al. 2006). Genome sequencing and subsequent studies support the existence of a bipolar sexual mating system in *Tuber* (Martin et al. 2010). In this mating system, there are two idiomorphs, mating loci characterized by large regions of nonhomologous DNA. However, at least some species of *Tuber* also produce mitotically produced (asexual) spores that are hypothesized to function in reproduction or root colonization (Urban et al. 2004; Healy et al. 2013). At least one (currently undescribed) species of *Tuber* belonging to the *Puberulum* clade is only known from ECMs and masses of these asexual spores (Healy et al. 2013). Although it is possible that these asexually derived spores function as conidia to colonize roots and establish new colonies, these spores are small and abundant and have very thin cell walls, suggesting that instead they may function as spermatia for sexual outcrossing. Improved understanding of the cues and regulation of sexual reproduction and asexual spore production in *Tuber*, aided by population genomic tools, could enable the development of controlled fertilization processes and selective breeding programs for truffles that have so far not been possible.

Truffles evolved from epigeous (mushroom) ancestors, but the specific environments and selective forces leading to truffle evolution in the genus *Tuber* are not clear. The belowground fruiting habit is believed to be adaptive for root-associated fungi, since the spores are produced in closer proximity to roots, but fruiting belowground helps to buffer against environmental fluctuations while the fruiting bodies develop. Further, because the majority of a truffle fruiting body is composed of spore mass, truffle fungi presumably shunt a greater proportion of energy into sexual spore production than do mushroom-producing fungi, which must partition reproduction resources toward the development of sterile cap and stem tissues. Several authors have suggested that sequesterate taxa evolve continually due to chance events, but that sequesterate lineages are selected for when both abiotic environmental conditions (e.g., drought, frequent fires) and biotic interactions (e.g., presence of dispersal agents) are favorable (Albee-Scott 2007; Thiers 1984).

Ancestral biogeographic reconstructions show that *Tuber* most likely had an origin in Eurasia (Bonito et al. 2013; Jeandroz et al. 2008). The most complete phylogenetic treatment of this group indicates that *Tuber* evolved from a lineage of epigeous, cup fungi and then diversified in the Northern Hemisphere throughout the Jurassic and Cretaceous periods (Bonito et al. 2013). What triggered the radiation and high level of *Tuber* diversity is still not completely clear.

In the past, most differences between truffles were considered to be species-specific and strongly influenced by the maturity of the truffle. While maturity is definitely an important factor, there is wide inter- and intraspecific variation in truffle fruiting body shape, size, and aroma and also in the community of bacteria that constitute the truffle microbiome. Evidence from recent studies suggests that the endobiotic bacterial community may be a highly influential and previously underappreciated factor that influences truffle odors and therefore interactions with other organisms (Splivallo et al. 2015; Splivallo and Ebeler 2015). An understanding of how the genomes of endobacteria interact with their fungal hosts and respond to their local environment is one of the grand challenges of truffle ecology, newly invigorated by advances in high-throughput sequencing technologies. Such knowledge will certainly lead to improved strategies for resource management, agricultural production, truffle breeding, and strain development for *Tuber* species.

## 1.2 Truffle Phylogeny: The Tuberaceae

The family Tuberaceae currently consists of six genera: *Tuber*, *Choiromyces*, *Reddellomyces*, *Labyrinthomyces*, *Dingleya*, and Southern Hemisphere cup fungi *Nothojafnea*. The two Northern Hemisphere genera, *Choiromyces* and *Tuber*, produce sizable and aromatic fruiting bodies that are highly valued in some European countries. It is interesting that species of *Tuber* are incredibly diverse across the Northern Hemisphere, and yet, in contrast, the genus *Choiromyces* includes just a few relatively rare but geographically widespread species.

Cup fungi belonging to the genus *Nothojafnea* have been described from South America and Australia, but DNA sequences are only available for the South American species, *Nothojafnea thaxteri* (E. K. Cash) Gamundí. *N. thaxteri* fruits directly on soil with species of *Nothofagus* and is presumed to form ECMs. Molecular analyses indicate that *N. thaxteri* is phylogenetically nested among Australasian truffles in the genera of *Reddellomyces*, *Labyrinthomyces*, and *Dingleya*. Species in these genera are presumed to be ECM on Australasian Myrtaceae such as *Eucalyptus*, *Corymbia*, *Melaleuca*, and *Leptospermum* as well as with species of *Acacia*, *Nothofagus*, or perhaps other woody plants. These truffle genera are broadly distributed and species rich in Australia, but none of the species are known to have any economic or gastronomic value to humans. Bonito et al. (2010a) found that at least 10 *Tuber* species are also present in Australia and New Zealand, but molecular evidence indicates that these taxa were introduced by humans. Bonito et al. (2010a) also provided molecular data to show that *Tuber clarei* Gilkey is an invalid name erroneously applied to the cosmopolitan and “pioneer” European truffle, *Tuber rapaeodorum* Tul. and Tul. More recently, another *Tuber* collection collected in Australia and deposited in the Melbourne herbarium (MEL2063143) as *Tuber hiromichii* (Imai) Trappe was shown to be *Tuber rapaeodorum* (Bonito unpublished data, GenBank accession KP311464).

## 1.2.1 Diversity, Ecology, and Distribution of the Genus *Tuber*

Recently, Bonito et al. (2013) reassessed the diversity, ecology, and historical biogeography of the genus *Tuber* using four genetic loci for inferences (RPP2, TEF1, and ITS and 28S rDNA). They distinguished 11 major clades within the genus *Tuber*. Recent estimates on the number of *Tuber* species are between 180 and 220 species, some of which are known only from “environmental” DNA sequences derived from rhizosphere soil. Characteristics of each of the major *Tuber* clades and exemplars of each are noted below.

### 1.2.1.1 Aestivum Clade

The Aestivum clade consists of some of the most morphologically diverse *Tuber* species. *Tuber aestivum* Vittad., the type species of the genus *Tuber*, is one of the most widespread and cultivated truffle species and is characterized by a dark warty peridium and aveolate-reticulated ascospores. *Tuber sinoaestivum* Zhang and Liu is an Asian species that is morphologically similar to *T. aestivum*, but *T. sinoaestivum* has ascospores that are more globose and have a shallower reticulum ornamentation (Zhang et al. 2012). In contrast, *Tuber panniferum* Tul. and Tul. is morphologically distinct and characterized by a woolly peridium and very spiny ascospores. *Tuber mesentericum* Vittad. is a species complex composed of at least two species of European truffles (see Chap. 5). Interestingly, the famous and pungent white truffle species *T. magnatum*, with its pale-colored and smooth peridium, also appears to belong within the Aestivum clade despite the fact that it is morphologically quite distinct. Species in this clade form mycorrhizal associations with diverse hosts include angiosperms (e.g., Fagaceae, Betulaceae), gymnosperms (i.e., Pinaceae), and even orchids. The evolutionary history of this clade may be deep and complex.

### 1.2.1.2 Excavatum Clade

*Tuber* species belonging in the Excavatum clade are distinguished by having a cavity within the base of their fruiting bodies. Species in this group tend to have a thick and hard peridium and generally have 3–5 coarsely reticulated ascospores per ascus. They are symbionts of angiosperms and are distributed in both Europe and Asia, but this group has never been documented in North America. Often found in association with hardwood tree hosts, several species in this group have also been found as symbionts of orchids (Illyés et al. 2010). The described species that belong to this clade include *Tuber excavatum* Vittad., *Tuber fulgens* Qué! from Europe, and *Tuber sinoexcavatum* Fan and Lee from Asia. This group has not been studied as extensively as some *Tuber* clades, yet a number of unique phylogenetic species were detected by Bonito et al. (2010a) indicating the presence of morphologically

cryptic species. Truffles in the Excavatum clade can have favorable aromas, but are not typically consumed by humans, likely because of their thick and hard peridium.

### 1.2.1.3 Gennadii Clade

An early diverging clade within *Tuber* species in the Gennadii group are only thus far known from Europe. Recently, Alvarado et al. (2012) identified two species in this clade [*Tuber gennadii* (Chatin) Pat. and *Tuber lacunosum* Mattir.]. The truffles in this group have only been found in association with the genus *Tuberaria* (Cistaceae) suggesting that these species may have very specific host requirements. Due to the fact that they are relatively rare and restricted in distribution, truffles in the Gennadii clade are generally not consumed by humans.

### 1.2.1.4 Gibbosum Clade

Endemic to *Pseudotsuga* forests of the Pacific Northwest of the USA, truffles in the *Gibbosum* clade have a light-colored peridium characterized by microscopic beaded hyphae emanating from the surface (Bonito et al. 2010b). The four known species in this group appear to associate exclusively with Pinaceae hosts, particularly *Pseudotsuga* but also occasionally with *Pinus*. Because of their economic value, *Tuber gibbosum* Harkn. and *Tuber oregonense* Trappe, Bonito, and Rawl. are two of the most important species in the *Gibbosum* clade. These truffles have not yet been cultivated but in the Pacific Northwest of the USA they are wild harvested during winter and spring (Lefevre 2013).

### 1.2.1.5 Japonicum Clade

Kinoshita et al. (2011) recently discovered a new clade of *Tuber* in Japan. Although species in this group have not yet been officially described, Kinoshita et al. (2011) noted that these species have some unique morphological traits, including pale yellow globose ascospores and fewer spores per ascus than most other *Tuber* species (often only 1 spore per ascus). Internal vein patterning within the gleba of mature truffles in the Japonicum clade tends to be more faint and less conspicuous than in other *Tuber* clades giving them the appearance of unripe truffles. This group is well supported as a monophyletic lineage, but there is still uncertainty regarding the closest relatives of this group (Bonito et al. 2013). We found no information on truffles in the Japonicum clade being consumed by humans.

### 1.2.1.6 Macrosporium Clade

Truffles in the Macrosporium clade are characterized by the presence of small warts on the outside surface of the peridium and one-, two-, or three-spored asci with relatively large (often  $>60\ \mu\text{m}$  in length) alveolate-reticulate spores. This group occurs in Asia, Europe, and North America. *Tuber glabrum* Fan and Feng and *Tuber sinomonosporum* Cao and Fan are two new species in the Macrosporium clade that were recently described from China (Fan et al. 2014). Species in this clade tend to be associated with either angiosperm or Pinaceae species. The geographical origin and ancestral host of this clade were not well resolved by Bonito et al. (2013). Paradoxically, truffles in the genus *Paradoxa* actually belong in the Macrosporium clade of *Tuber*. These truffles contain single large ascospores within their asci and had previously been difficult to place phylogenetically without DNA sequence data. Two species in the Macrosporium clade have commercial value. *Tuber macrosporium* Vittad. is found across Italy and eastern Europe and has recently been cultivated in Austria and Hungary (Benucci et al. 2012, 2014). *Tuber canaliculatum* Gilkey is one of the larger and more pungent of the North American *Tuber* species, and this species has a wide distribution in the Eastern USA from the mid-Atlantic states (e.g., North Carolina, Maryland, Virginia) to the upper Midwest (e.g., Michigan) and into Canada. Mycorrhizal synthesis and cultivation trials with *T. canaliculatum* are underway (Benucci et al. 2013).

### 1.2.1.7 Maculatum Clade

The Maculatum clade produces truffles that have a light-colored peridium with a smooth to cracked texture. The elliptical ascospores of species in this clade tend to have alveolate-reticulate ornamentation. Many species in the Maculatum lineage that have been described from North America and Asia over the past few years and several additional species remain undescribed (Guevara et al. 2013; Su et al. 2013). Truffles in the Maculatum clade are generally not as aromatic as other *Tuber* species. Aside from New Zealand, where *Tuber maculatum* Vittad. has been marketed, species in this clade are not typically consumed by humans but instead are considered undesirable “contaminants” (Amicucci et al. 2000).

### 1.2.1.8 Melanosporium Clade

Most species belonging to the Melanosporium clade are characterized by a warty outer peridium and spiny ascospore ornamentation, although a few species have spiny-reticulated spores and at least one species (*Tuber pseudoexcavatum* Wang, Moreno, Rioussset, Manjón, and Rioussset) has spores with alveolate reticulation. Many of the truffle species in this group have pigmented ascospores, giving their gleba a dark color when the spores become mature. There is one currently



undescribed species in the *Melanosporum* clade that has a light-colored outer peridium and gleba (Gregory Bonito, personal observation), putatively ancestral traits that have been fixed in this species. The black truffle *T. melanosporum* is perhaps the most cultivated truffle species internationally, and this species is economically important on several continents. Asian black truffles in the *Tuber indicum* Cooke and Masee complex are also harvested from forests on a massive scale for human consumption, and cultivation trials with this species are underway in China (Wang 2013).

### 1.2.1.9 Multimaculatum Clade

*Tuber multimaculatum* Parladé, Trappe, and Alvarez is known only from a few collections in Spain (Alvarez et al. 1992) and is the only species belonging to the Multimaculatum clade. Possibly due to its long branch on the phylogeny, its exact placement within the genus *Tuber* is still not resolved. *Tuber multimaculatum* is characterized by large ellipsoid ascospores with finely meshed alveolate reticulations. Ascospores are produced in one-spored or two-spored asci that have notable apical thickenings in the ascus walls. Because of the rarity of this species, its biology and ecology are not well known.

### 1.2.1.10 Puberulum Clade

Current data indicate the Puberulum clade has the widest geographic distribution and the most species of any *Tuber* clade. Species in this group are distributed across Europe, Asia, North America, and northern Africa in association with Pinaceae, angiosperms, or both. One species in the Puberulum clade has also been found on the roots of native *Salix humboldtiana* Willd. in South America, suggesting that this may be the only lineage of *Tuber* that has naturally spread to South America with Northern Hemisphere host trees (Bonito et al. 2013). Truffles in the Puberulum clade tend to produce light-colored truffles that have a smooth to cracked peridium, and some species in this clade are known to produce prolific mats of mitospores on soil (Healy et al. 2013). Ascospores of truffles in the Puberulum clade are generally globose to subglobose and are ornamented with alveolate reticulation. Some species in this clade appear to be pioneer ECM species that have been unintentionally introduced into locations in the Southern Hemisphere where they previously did not exist (Guerin-Laguette et al. 2013). Such species could be considered as “weedy” ECM associates. *Tuber borchii* Vittad. is the most important edible truffle species in the Puberulum group and has been shown to produce both ECMs with pine and hardwood species and arbutoid mycorrhizas with *Arbutus unedo* L. (Lancellotti et al. 2014). The list of new species in the Puberulum lineage described from Asia continues to grow, suggesting that there may be many more undescribed taxa in this group (Fan et al. 2012a, b, c). While most species in the Puberulum clade are considered to be undesirable for consumption, one recently described species,

*Tuber panzhihuanense* Deng and Wang, is reported to have favorable aromatic attributes and commercial potential (Deng et al. 2013).

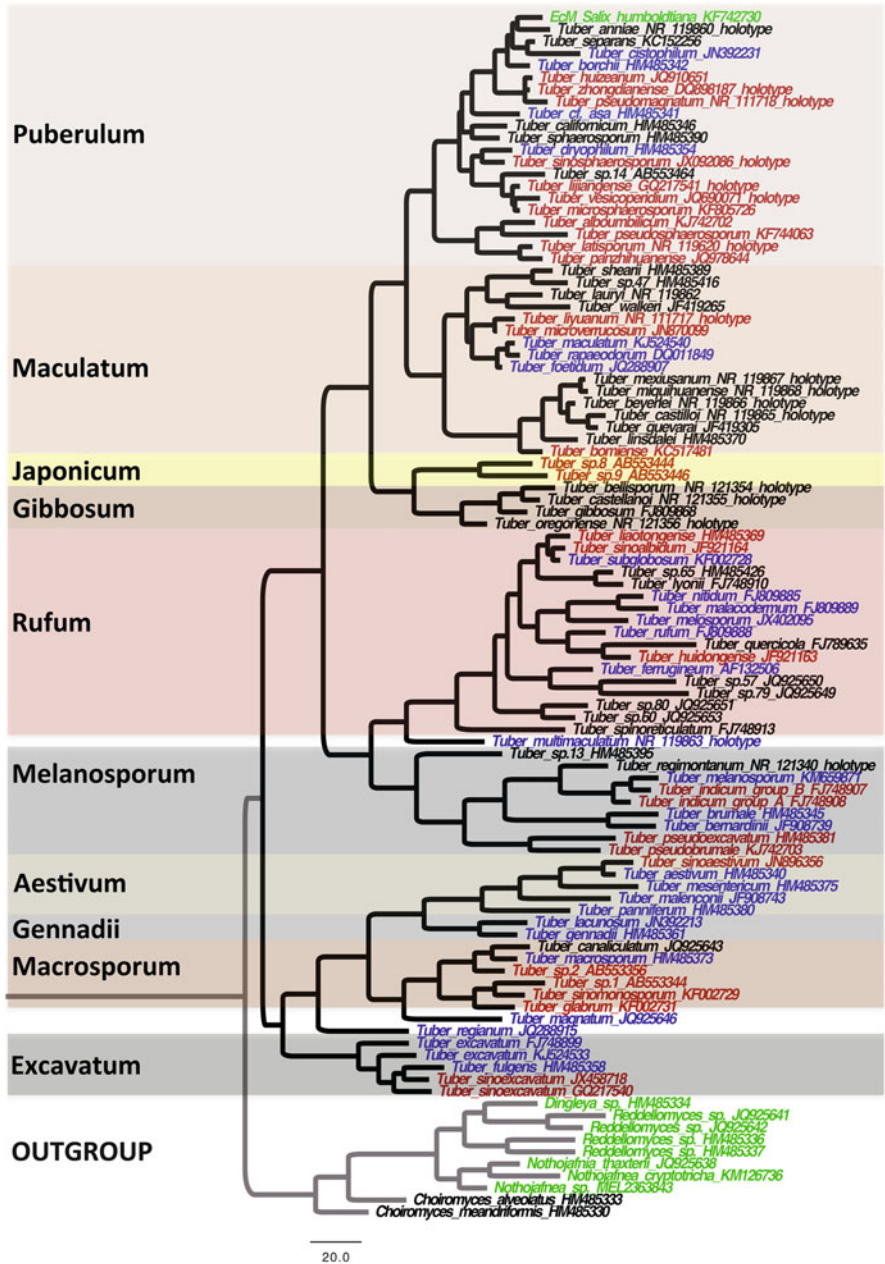
#### 1.2.1.11 Rufum Clade

The Rufum clade forms a sister group to the Melanosporum clade, and they share some morphological characteristics. For instance, most species in the Rufum clade also have spiny ascospore ornamentation. Some species have a range of spiny-alveolate reticulation, and *Tuber melosporum* (Moreno, Díez, and Manjón) Alvarado, Moreno, Manjón, and Díez in the Rufum clade is the only *Tuber* species known to have smooth ascospores (Alvarado et al. 2012). The Rufum clade is one of the most species-diverse clade in the genus *Tuber*. The peridium of truffles belonging to Rufum clade varies widely; some species may have a verrucose outer peridium covered with small warts, whereas others may have a smooth or cracked peridium. Most species in the Rufum clade have either a faint, unpleasant, or even noxious aroma and are therefore undesirable for human consumption (Iotti et al. 2007). One exception is the North American species *Tuber lyonii* Butters, which is sometimes referred to as the “pecan truffle” because it is commonly found with pecan trees (Bonito et al. 2011). This species has a pleasant aroma and is occasionally harvested and sold in the southeastern USA. Efforts are now underway to cultivate *T. lyonii* and to better understand its biology and ecology (see Chap. 8).

### 1.3 Biogeography of the Tuberaceae

There is much interest in elucidating the biogeographic origin and evolutionary history of the Tuberaceae. As shown in Fig. 1.2, most of the genera within the Tuberaceae are distributed either in the Southern Hemisphere (*Labyrinthomyces*, *Reddellomyces*, *Dingleya*, *Nothojafnea*) or the Northern Hemisphere (*Choiromyces*, *Tuber*—with the exception of the Puberulum clade), suggesting an ancient phylogeographic split within the family. Based on the work of Bonito et al. (2013), Southern Hemisphere lineages of *Tuber* are more recently diverged than Northern Hemisphere clades, although the Tuberaceae of Australasia have not been thoroughly studied. Based on the large number of undescribed species and blurred generic boundaries, a full systematic revision of the Australasian taxa will be essential to resolve some of these issues (Bonito, Kovacs, Trappe, unpublished).

The geographic origin of *Tuber* has been predicted to be either Europe or Asia. However, even global and multigene datasets assembled by Jeandroz et al. (2008) and by Bonito et al. (2013) were insufficient in reconstructing the geographic center of origin for *Tuber*. Rather than supporting each other, alternate loci mostly gave incongruent results or poorly resolved the branching order among the different lineages. This is especially problematic because several lineages are thus far restricted to only one region (e.g., Japonicum in Asia, Gibbosum in North



**Fig. 1.2** Phylogeny of the Tuberales based on ITS rDNA and including all sequence vouchered species and distinct phlotypes. Major clades are distinguished by color and are named on the left of the label. Taxon labels are color coded to represent geographic origin of the species: blue for Europe, red for China, black for North America, and green for Southern Hemisphere

America), and it is likely that a significant portion of species remains unsampled, particularly in Asia (Bonito et al. 2013). With an increasing number of *Tuber* genomes being sequenced (see Chap. 9), and new species being found and described, phylogenomic network reconstructions using these data should help to more clearly define the center of origin, genomic history, and diversification of *Tuber*.

#### 1.4 Coevolution and Co-diversification of *Tuber* with Plant Hosts and Spore Dispersers

Species in the Tuberaceae are hypothesized to be nutritionally dependent on living ECM plants in order to complete their lifecycles. Thus far, all known species of Tuberaceae form ECMs, although some species (e.g., *T. aestivum*, *T. excavatum*, *T. melanosporum*) may also form mycorrhizal associations with orchids. However, the factors involved in its establishment and persistence of mycorrhizas by *Tuber* are not completely understood. By having an ectotrophic mode of nutrition, species of *Tuber* obtain the majority of carbon for maintaining cellular processes and growth from fixed labile carbon from the living host plant. For instance, <sup>13</sup>C tracer studies indicate that even after deciduous host plants have dropped their leaves, *Tuber* mycorrhizas obtain plant carbon and transport these sugars through mycelium conduits to developing fruiting bodies (Le Tacon et al. 2013). While the carbon in truffles may be recently derived, nutrients and other minerals mined out of soil particles by these fungi can be quite old. For example, most of the soil nitrogen obtained by *T. gibbosum* comes from older (10–100 years) organic and recalcitrant fractions (Hobbie and Hogberg 2012).

The strong nutritional dependency of *Tuber* species on their plant hosts has led to coevolution between particular plant and *Tuber* lineages. Adaptive “host-generalist” or “host-specific” strategies could arise within *Tuber*, but most species tend to be host generalists that can associate with multiple genera of plants. Some taxa can associate with species of angiosperms and Pinaceae, but most species appear to be either angiosperm associated or Pineaceae associated. We hypothesize that *Tuber* and plant host populations co-migrate across landscapes in a mosaic-like fashion. Following this model, *Tuber* lineages have putatively migrated with their host symbionts across and throughout the Northern Hemisphere, but even into South America with native species of *Salix* and/or *Alnus* (Bonito et al. 2013). Similar coevolution scenarios have been proposed for other interactions, such as pollination and seed dispersal syndromes. This interplay leads to development of intricate food webs and fascinating complexity in nature (Maser et al. 1978).

## 1.5 Emergence of *Tuber* Phylogenomics and Molecular Ecology

As the revolution in DNA sequencing technologies continues, with continued interest in truffles, *Tuber* is becoming a model genus for studies of genomics, species diversity, population structure, symbiosis, and evolution at an increasing high resolution (Martin et al. 2010; Rubini et al. 2011; Bonito et al. 2013). There are now genome sequences finished for three *Tuber* species (*T. aestivum*, *T. magnatum*, and *T. melanosporum*), and genomes of four additional *Tuber* species (*T. borchii*, *Tuber brumale* Vittad., *T. indicum*, and *T. lyonii*) are currently being assembled. In total, these taxa represent four of the 11 clades within the genus (Payen et al. 2014; see Chap. 9). These genomic data will help to resolve questions pertaining to truffle growth and development, ecological adaptability, center of origin, and evolutionary history. Molecular tools and understanding arising from these genomic resources will empower a new generation of truffle growers and researchers to tackle age-old questions that have made truffles so perplexing for so long. We expect that genomic approaches will provide streamlined and sensitive protocols for detecting contamination, diseases, genetic diversity, and geographic origin of target truffle strains.

In addition, genomes of eight other fungi in the class Pezizomycetes have been sequenced and are available on the JGI Mycocosm web portal: <http://genome.jgi-psf.org/programs/fungi>. These data are already providing new perspectives on the evolution of Pezizomycetes and helping to clarify the genomic consequences of different trophic modes in fungi.

## 1.6 Conclusions

The Tuberales continues to be an important family within the Ascomycota for understanding ECM symbiosis, truffle evolution, and fruiting body production. As is evident in the chapters that follow, truffle science has entered into the phylogenomic era. We expect that genomic approaches will bring novel insights and knowledge on truffle development, symbiosis-related genes, molecular crosstalk between fungus and host, genome organization and evolution, and consequences of bacteria on fungal growth, function, and development. It is an exciting time to be studying the biology of *Tuber* and other truffle fungi.

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