Chapter 9
Truffle Genomics: Investigating an Early Diverging Lineage of *Pezizomycotina*

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9.1 Introduction

In the 1980s, the term genomics came into common use to describe the emerging scientific field of sequencing and analyzing genomes, but genomics started in the second half of the twentieth century, thanks to numerous discoveries, such as the first sequencing of the alanine tRNA of *Saccharomyces cerevisiae* Meyen ex E.C. Hansen (Holley et al. 1965), the sequencing of the bacteriophage MS21 (Fiers et al. 1976), and the development of the Sanger DNA sequencing method (Sanger et al. 1977). The Sanger sequencing method was used until the mid-2000s and allowed the sequencing of the first bacterial and eukaryotic genomes, *Haemophilus influenzae* (Fleischmann et al. 1995) and *S. cerevisiae* (Goffeau et al. 1996), respectively.

In 2003, the first filamentous fungal genome was sequenced (*Neurospora crassa* Shear and B. O. Dodge; Galagan et al. 2003), and afterward the number of sequenced fungal genomes began to increase rapidly (Galagan et al. 2005). Fungal genomics benefits from different large-scale programs, such as the Fungal Genome Initiative (FGI), a consortium that aims to sequence fungal genomes, initiated in the year 2000 at the Broad Institute (http://www.broadinstitute.org/science/projects/fungal-genome-initiative/fungal-genome-initiative). The FGI aimed to sequence the fungal species that are important in medicine, agriculture, and industry. In the last decade, large-scale projects that aimed to sequence the genomes of fungi from environmental settings were launched at the Joint Genome Institute (JGI), the sequencing center of the US Department of Energy (http://jgi.doe.gov/).

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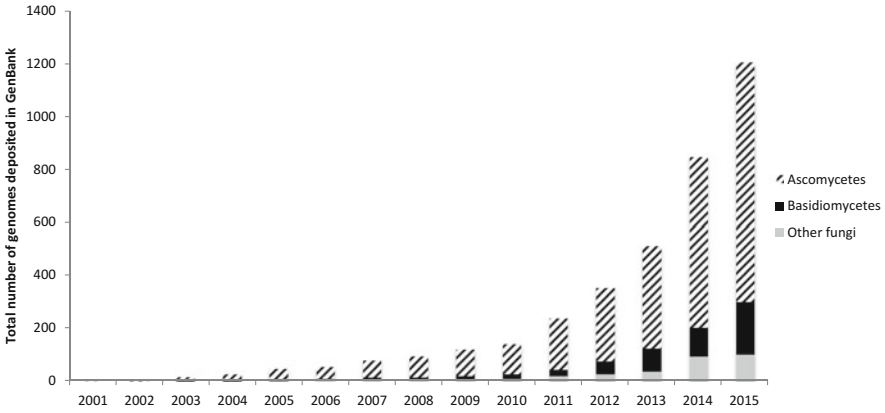

Fig. 9.1 Number of partial or complete fungal genomes deposited in GenBank from 2001 to December 10, 2015 (http://www.ncbi.nlm.nih.gov/genome/browse/)

The 1000 Fungal Genomes project (http://genome.jgi-psf.org/programs/fungi/1000fungalgenomes.jsf), which aimed to sequence at least two reference genomes from the more than 500 recognized families of fungi, started to fill in the gaps in the Fungal Tree of Life (Spatafora 2011). Additional projects, such as the Mycorrhizal Genomics Initiative (MGI; http://genome.jgi.doe.gov/Mycorrhizal_fungi/Mycorrhizal_fungi.info.html), which focuses on sequencing specific ecologically relevant groups of fungi, participated and increased the number of released fungal genomes (van der Heijden et al. 2015). All these projects rely on second-generation DNA sequencing (SGS) technologies. Indeed, until the mid-2000s, most sequencing projects were limited to one or a few genomes due to the cost of Sanger sequencing. In 2005, a revolution in genome sequencing started with SGS (Margulies et al. 2005), which dramatically dropped the cost of sequencing (Metzker 2010). To date, 1204 fungal genomes have been deposited in the GenBank database, belonging to the Ascomycetes (903), the Basidiomycetes (200), and early diverging fungi (101) (Fig. 9.1). The activity of the international consortia mentioned above explains the exponential increase in the number of fungal genomes deposited in the GenBank in the last few years (Fig. 9.1).

9.2 The Evolution of Mycorrhizal Symbiosis Unraveled by Comparative Genomics

Almost all vascular plants interact with mycorrhizal fungi, but woody shrub and tree species in particular rely on symbiotic associations with ectomycorrhizal (ECM) fungi to generate large amounts of biomass and store carbon (Smith and

Read 2010). The ability to establish ECM symbioses is a widespread characteristic of various soil Ascomycetes and Basidiomycetes; interactions with these soil symbionts are essential for efficient acquisition of growth-limiting nutrients, such as phosphorus and nitrogen. The ECM species have great potential as a versatile ecological model due to their involvement in symbioses with economically important conifer and hardwood tree species worldwide. Several ECM species have been disseminated globally via exotic eucalypt, poplar, and pine plantations. Reforestation and large-scale plantations on marginal soils introduced ECM symbionts to be successful; as a consequence, this mutualistic partnership is being exploited as a strategy to bolster biomass production for next-generation biofuels in the USA, Europe, Brazil, and Australasia. In addition, the fruiting bodies of ~400 ECM fungi are appreciated worldwide as edible mushrooms, e.g., boletes, chanterelles, and truffles, and they represent a global market evaluated at more than US$23 billion (Boa 2004).

According to Hibbett and colleagues (2000), ECM symbiosis arose several times in the evolution from saprotrophic species. In phylogenetic analyses, however, it is impossible to know if all ECM fungi use the same "tool kit" to establish symbiosis or if multiple ECM species developed their own tool kits. To gain information on mycorrhizal evolution, we sequenced the genome of the ECM species *Laccaria bicolor* (Maire) P. D. Orton (Martin et al. 2008) and *Tuber melanosporum* Vittad. (Martin et al. 2010). In the frame of the MGI project (see above), an additional 40 mycorrhizal genomes were sequenced, among them ECM, arbuscular (AMF), orchids (ORC), and ericoid (ERM) mycorrhizal species (http://genome.jgi.doe.gov/Mycorrhizal_fungi/Mycorrhizal_fungi.info.html). The questions addressed in the frame of the MGI project are:

1. Do all ECM lineages arise from similar saprotrophic ancestors?
2. Are there a common set of mycorrhiza-related genes that interact with the host plants and differentiate the symbiotic structures: a putative *symbiosis tool kit*?
3. Do the different types of mycorrhizal symbioses (ECM, AMF, ORC, ERM) use similar gene networks to differentiate the mutualistic interaction?

The first large-scale comparative analysis of the genomes from mycorrhizal species included 11 ECM (10 Basidiomycetes and one Ascomycete), 2 ORC, and 1 ERM. These genomes were compared to those of 33 white and brown rots, soil/litter decayers, and pathogenic fungi (Kohler et al. 2015). The first conclusion is that ECM symbiosis arose several times during the *Agaricomycotina* evolution from ancestors of either white-rot, brown-rot, or litter decayers. On average, ECM lineages have a reduced complement of genes encoding plant cell-wall degrading enzymes (PCWDEs), compared to ancestral white-rot wood decayers, suggesting convergent genome erosion. This decrease in PCWDE can be explained by two hypotheses: (1) being fed by their host plant, ECM fungi do not need to maintain PCWDEs in their genome to degrade the soil's organic matter or (2) their reduced number of PCWDEs allows them to avoid eliciting the host plant's defenses as a result of the oligosaccharide elicitors. Transcriptomic data allowed the identification, for each basidiomycete ECM species, of a specific set of

mycorrhizal-induced small secreted proteins (MiSSP; Kohler et al. 2015). One of these proteins, MiSSP7, is an effector protein that contributes to the dialogue between *L. bicolor* and the plant (Plett et al. 2011, 2014); most of these MiSSPs are species specific (Kohler et al. 2015). Finally, a different picture was observed for ORC and ERM, since the genomes of these fungi are very rich in PCWDEs and some PCWDEs are expressed during symbiosis (Kohler et al. 2015). In conclusion, the major types of mycorrhizal symbioses (ECM, ORC, and ERM) evolved different molecular mechanisms to interact with their host plants.

In this large-scale comparative genomic analysis of mycorrhizal genomes, one *Ascomycete* species (i.e., *T. melanosporum*) was included; can one generalize the conclusions reached with basidiomycete ECM fungi to *Ascomycete* ECM fungi?

9.3 Pezizomycetes Pan-Genome Project

True truffles belong to the genus *Tuber* and form ECM symbiosis with trees and shrubs. The *Tuber* genus evolved in the last 140 Mya in 11 phylogenetic groups (Bonito et al. 2013) and 180 species (Bonito et al. 2010; see Chap. 1). *Tuber* belongs to the Pezizomycetes class, which constitutes an early diverging lineage in *Pezizomycotina* (Spatafora et al. 2006). The Pezizomycetes class is composed of 200 genera and 1683 described species (Kirk et al. 2008) that are saprophytic, mycorrhizal, or pathogenic. The formation of apothecia that contain operculate asci with forcible spore discharge (Laessøe and Hansen 2007) characterizes Pezizomycetes, although other forms exist, such as hypogeous fungi. Fungi with subterranean fruiting bodies are called truffles, and they comprise many taxonomically unrelated species (i.e., Ascomycetes and Basidiomycetes) that show remarkable features of phenotypic convergent evolution as a result of adaptation to this specialized habitat (i.e., fruiting belowground). Truffle ascomata contain asci with passive spore dispersal, which have multiple evolutionary origins in Pezizomycetes (O'Donnell et al. 1997; Laessøe and Hansen 2007). They probably derive from epigeous fruiting bodies which evolved as an adaptation to animal grazing or water stress (Thiers 1984; Bruns et al. 1989). Although truffle lifestyles have evolved in nearly every major group of fleshy fungi and over 100 independent instances within the Ascomycota, Basidiomycota, and Mucoromycotina (Tedersoo et al. 2010), the majority of transitions to a truffle form occur in ECM fungal lineages (Trappe et al. 2009). This pattern suggests that the symbiotic association with plants may be an important driver in the evolution of the truffle fruiting body phenotype. Although protected from desiccation and other environmental stresses, like grazing, by their belowground location, truffle ascomata grow slower than those of most epigeous mushrooms, e.g., *T. melanosporum* ascomata growth during the 6–9 months underground during summer and fall (Olivier et al. 2012). It is tempting to speculate that this slow hypogeous differentiation of the truffle ascomata is only possible, thanks to the constant flux of carbohydrates from the host plant (Le Tacon et al. 2013).

Comparing *Tuber* spp. with other Pezizomycetes, therefore, allowed the investigation of the different fungal life strategies in Ascomycetes (mycorrhizal vs. saprotroph), the development of hypogeous versus epigeous ascocarps, and the evolution of the particular organoleptic qualities of some species (e.g., truffles and morels). To date, the *T. melanosporum* genome is the only truffle genome that has been published (Martin et al. 2010). Several projects to sequence additional truffle genomes are ongoing (Payen et al. 2014), and the genomes of fourteen additional *Tuber* species will be released in the next few years (Table 9.1). These genomes are sequenced in the frame of the TuberEvol project (Comparative Genomics of Truffle Species and the Evolution Ectomycorrhizal Symbiotic Genomes), involving scientists in France, Italy, the USA, and both the JGI and Genoscope sequencing centers (Payen et al. 2014). The haploid genomes of *Tuber aestivum* Vittad. and *Tuber magnatum* Pico have been sequenced and assembled, and gene annotations are now completed. The genomes of *Tuber brumale* Vittad., *Tuber indicum* Cooke and Massee, and *Tuber lyonii* Butters have been sequenced and assembled, and the gene annotation is underway. For *Tuber borchii* Vittad., *Tuber canaliculatum* Gilkey, *Tuber dryophilum* Tul. and C. Tul., *Tuber excavatum* Vittad., *Tuber gibbosum* Harkn., *Tuber macrosporum* Vittad., *Tuber maculatum* Vittad., *Tuber oregonense* Trappe, Bonito, and P. Rawl., and *Tuber rufum* Pico, genome sequencing will be carried out in 2016–2017. These 14 species belong to 8 out of the 11 clades identified in the *Tuber* genus (Table 9.1, Bonito et al. 2013); indeed, only species of the multimaculatum, japonicum, and gennadii clades are not yet represented in this genomic project.

Additional Pezizomycetes genomes are now available (http://genome.jgi.doe.gov/pezizomycetes/pezizomycetes.info.html), including:

1. The saprotrophic species: *Pyronema confluens* Tu. and C. Tul. (Traeger et al. 2013), *Ascobolus immersus* Pers., *Ascodesmis nigricans* Tiegh., *Morchella conica* Pers., *Morchella importuna* M. Kuo, O'Donnell, and T. J. Volk, and *Sarcoscypha coccinea* (Gray) Boud
2. The symbiotic species: *Choiromyces venosus* (Fr.) Th. Fr., *Terfezia boudieri* Chatin, and *Wilcoxina mikolae* (Chin S. Yang and H. E. Wilcox) Chin S. Yang and Korf

A first characteristic of truffle genomes is their large size (>125 Mbp) and their high repeat sequence content (Table 9.1). The Tuberaceae member *C. venosus* has a genome that shares features with *Tuber* species (i.e., a large size with a high repeat sequence content), in agreement with some phylogenetic studies (Percudani et al. 1999), although recent phylogenetic analyses clearly separated both genera (Bonito et al. 2013). Moreover, *W. mikolae* (Pyronemataceae) also has a large genome (111 Mbp), similar to other ECM Pezizomycetes (i.e., *Tuber* spp. and *Choiromyces*), confirming that the genomes of ECM species are generally larger than those of saprotrophic species (Payen et al. 2014). The comparative analysis of these Pezizomycetes genomes is currently underway and should provide new insights into truffle evolution, but also into ECM evolution by comparing Basidiomycetes ECM and Pezizomycetes ECM fungi.

Table 9.1 List of *Tuber* species for which the genome is sequenced or being sequenced

Species	Clade[a]	Genome size (Mb)	% of repeated sequences	Material	Statut of the project	Natural habitat	Project
T. aestivum	Aestivum	145	50	Ascomata	Complete	North Africa, Europe	T. Evolve (Génoscope-INRA)
T. magnatum	Aestivum	192	60	Ascomata	Complete	Italy, South-Central Europe	INRA-UNITO
T. excavatum	Excavatum	na	na	Mycelium	Start in 2016	Europe	1K Fungal Genome (JGI-DOE)
T. gibbosum	Gibbosum	na	na	Mycelium	Start in 2016	North America, Europe	1K Fungal Genome (JGI-DOE)
T. oregonense	Gibbosum	na	na	Ascomata	Start in 2016	North America	1K Fungal Genome (JGI-DOE)
T. canaliculatum	Macrosporum	na	na	Mycelium	Start in 2016	North America	1K Fungal Genome (JGI-DOE)
T. macrosporum	Macrosporum	na	na	Mycelium	Start in 2016	Europe	1K Fungal Genome (JGI-DOE)
T. maculatum	Maculatum	na	na	Mycelium	Start in 2016	Europe	1K Fungal Genome (JGI-DOE)
T. brumale	Melanosporum	180[b]	na	Ascomata	Annotation pending	Europe	INRA
T. indicum	Melanosporum	147[b]	na	Ascomata	Annotation pending	China	INRA-University Kunming
T. melanosporum	Melanosporum	125	58	Mycelium	Published	South-West Europe	Martin et al. (2010)
T. borchii	Puberulum	na	na	Mycelium	Pending	Europe	Metatranscriptomic of soil forest (JGI-DOE)

T. dryophilum	Puberulum	na	na	Mycelium	Start in 2016	Europe	1K Fungal Genome (JGI-DOE)
T. lyonii	Rufum	na	na	Mycelium	Pending	North America	Duke University
T. rufum	Rufum	na	na	Mycelium	Start in 2016	Europe	1K Fungal Genome (JGI-DOE)

[a]Clade in the *Tuber* phylogeny according to Bonito et al. (2013)
[b]Genome size estimation obtained by Kmer approach in AllPaths-LG genome assembler

9.4 Population Genomic: Resequencing of Geographic Accessions

The increase of genomic resources in the last few decades provided new genetic tools for population geneticists. For example, using whole genome sequences, it was possible to characterize highly polymorphic microsatellite markers for investigating the genetic structures of *T. aestivum* (Molinier et al. 2013) and *T. melanosporum* (Murat et al. 2011) populations (see Chaps. 2 and 3). Traditional population genetic studies, however, use a limited set of molecular markers (typically a dozen or less), impeding the identification of genomic evolution and adaptation signatures. Thanks to the decrease in DNA sequencing costs (see above), it is now possible to move from population genetics to the population genomics of mycorrhizal symbionts by resequencing the genome of several geographic accessions (Branco et al. 2015; Payen et al. 2015). In the latter studies, single nucleotide polymorphisms (SNP) were characterized; SNPs are more informative than microsatellites due to their distribution throughout the genome, bi-allelic nature, and high potential for automation (Brumfield et al. 2003). SNP locations in coding sequences increase the probability of identifying the signatures of adaptation to environmental cues. Such an approach was used, for example, to identify the genomic signature of adaptation to temperature in *N. crassa* (Ellison et al. 2011). Recently, Branco and colleagues (2015) re-sequenced 28 individual strains of the ECM basidiomycete *Suillus brevipes* (Peck) Kuntze from coastal and montane sites in California. Reduced nucleotide diversity was observed among coastal individuals for the *Nha1-like* gene, a membrane Na^+/H^+ exchanger known to enhance salt tolerance in plants and yeast, suggesting an adaptation to saline soils by *S. brevipes*.

The first population genomics analysis of *T. melanosporum* was recently published (Payen et al. 2015). More than 440,000 SNPs were identified by comparing seven genomes. These SNPs were used to detect genomic regions putatively under selection (see Chap. 2). Among these SNPs, 60,507 present in the intergenic regions free of selective pressure were selected to reconstruct a phylogeny with the seven geographic accessions. Interestingly, the phylogenetic tree clustered samples according to their geographical origin, with a cluster comprising the northern France samples, another with those of southeastern France and Italy, and a last one with the Spanish samples (Fig. S1B in Payen et al. 2015). The use of SNPs is now facilitated by SGS and high-throughput SNP arrays (Davey et al. 2011). Medium- to high-throughput technologies, such as the competitive allele-specific PCR (KASPar) assay from KBiosciences (Hertfordshire, UK; http://www.kbioscience.co.uk) or the Affymetrix Axiom SNP microarrays, are now available. The KASPar assay is commonly used for genotyping up to 1000–2000 SNPs, whereas the Axiom SNP microarrays allow genotyping of 1500 to several million SNPs. We are now developing an array based on the 60,507 SNPs for analyzing the population genetic structure throughout the natural regions of *T. melanosporum* production.

9.5 Conclusions

Truffle genomics began with the sequencing of the *T. melanosporum* genome (Martin et al. 2010). The genomes of 14 additional truffle species (*T. aestivum*, *T. borchii*, *T. brumale*, *T. canaliculatum*, *T. dryophilum*, *T. excavatum*, *T. gibbosum*, *T. indicum*, *T. lyonii*, *T. macrosporum*, *T. maculatum*, *T. magnatum*, *T. oregonense*, and *T. rufum*) should be released in the next 2 years. In the framework of the 1000 Fungal Genomes Project, we recently proposed the genome sequencing of about 20 Pezizomycetes belonging to the *Balsamia*, *Barssia*, *Discina*, *Helvella*, *Tuber*, *Underwoodia*, and *Verpa* genera.

Based on the ongoing studies of the *Tuber* and other Pezizomycetes genomes, we identified several key questions that future analyses can help resolve, presented below in the form of six currently unanswered questions, rather than an exhaustive list:

1. How did the different lifestyles (e.g., mutualism vs. saprotrophism) evolve in Ascomycetes?
2. Which are the key developmental genes explaining the shift from epigeous to hypogeous ascomata?
3. Are the sex-related pathways in *Tuber* species similar to those characterized in other Pezizomycetes, such as the genetic model *A. immersus*?
4. Which enzymatic pathways are at the origin of the particular organoleptic volatiles of truffle species and are these pathways species or genus specific?
5. Are truffles able to adapt to environmental stresses, such as drought or frost?
6. Is it possible to genotype the geographic origin(s) of truffles?

In summary, our knowledge of the truffle life cycle, evolution, and population dynamics have increased, thanks to the availability of genomic resources. In addition to answering fundamental questions, genomic resources could also help us respond to truffle industry requests, since the truffle industry, for several decades, has sought innovative tools to identify the geographic origin of the truffles, mainly to valorize local territories. Protected designation of origin certification was developed for boletes, i.e., "Fungo di Borgotaro" (http://www.fungodiborgotaro.com/ita/igp.jsp), although molecular markers allowing us to certify bolete origins do not exist yet. In a population genomic study, using SNPs, the seven geographic accessions are clustered according to their geographic origin (Payen et al. 2015). Using SNPs to identify the harvesting region could have many applications for the truffle industry regarding local geographic certification.

The development of truffle genome sequencing will therefore provide scientists and the truffle industry new innovative tools or molecular markers to investigate not only population genetics but also taxonomy. Indeed, the recent discovery and characterization of mating-type genes for *T. indicum* confirmed that such functional markers could also be used for taxonomic purposes (Belfiori et al. 2013; see Chap. 2).

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