Disposition

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## Topic

“Genetic structure and long-term genetic diversity assessment of Burgundy truffle populations in central Europe”

## Research question(s) and hypotheses

Prelim.: What is the population structure of the truffles? Are MLGs perennial? Differences between or within populations? Interactions…

## Research aims

The Burgundy truffle is an abundant ectomycorrhizal symbiont that occurs in a wide range of temperate climates. Despite its prized underground ascocarps, its complex lifecycle and possible response to anthropogenic climate change are largely known. A citizen science monitoring of Burgundy truffle ascocarp production was run with a 3-week resolution in 23 natural populations at the center of its European distribution over up to 11 years. More than 3000 truffles were genotyped using microsatellite markers to assess the genetic structure and diversity of these populations in space and time. The aims of this research project are to assess the population structure, compare intra- and inter populations and assess the gene flow and perenniality of individuals.

## A review of the current state of research

### Truffles in general

True truffles are ectomycorrhizal fungi belonging to the genus Tuber in Ascomycetes. This genus, which exclusively forms hypogeous fruiting bodies (i.e. ascocarps or sporocarps), known as truffles, comprises more than 200 species all over the world ((G. M. Bonito et al. 2010), (G. Bonito et al. 2013), (Guevara et al. 2013), (Reyna and Garcia-Barreda 2014a), (Berch and Bonito 2016)), of which at least 30 naturally occur in Europe (Ceruti et al., n.d.). Some of these species are well known for their organoleptic properties and their high economic value, such as *Tuber magnatum* (Alba white truffle), *Tuber melanosporum* (Périgord black truffle), and *Tuber aestivum* Vittad. (Burgundy and summer truffles, depending on the harvesting period) (Boa and Nations 2004); (G. M. Bonito et al. 2010); (Chevalier et al., n.d.). Truffle species include the most valuable fungi on earth due to their use in haute cuisine (Reyna and Garcia-Barreda 2014b).

As ectomycorrhizal fungi, truffles form symbiotic associations with roots of a wide range of ectomycorrhizal tree species (Benucci et al. 2011), (Stobbe et al. 2012) and grant their hosts access to water and nutrients, protect roots from pest and disease, and interact with and sometimes kill understory, non-host plants (Gryndler et al. 2014); (Streiblová, Gryndlerová, and Gryndler 2012); (Taschen et al. 2020)).

In contrast to other high-value forest mushrooms, such as Boletus spp., Cantharellus spp. and Laccaria spp., which can develop their fruitbodies during a few days (Moore et al. 2008); (Halbwachs, Simmel, and Bässler 2016)), hypogenous truffle fruitbodies are expected to grow more slowly (Pacioni et al. 2015a). However, it is still unknown how much time truffles need to develop and how many generations of fruitbodies can occur within one ‘growing’ season between the formation of primordia and the realisation of a fully mature and ripe gleba (Büntgen et al. 2017). The species’ subterranean life cycle dynamics, together with complex host interactions and the lack of non-destructive investigation techniques (Luoma et al., 1991), are the main reasons for the yet little understood biotic and abiotic drivers of truffle growth (Pacioni et al. 2015b). Localising truffles in situ is only possible with the help of well-trained sniffing dogs or, very rarely, with domestic trufflepigs (or occasionally also by searching and flowing truffle-specific flies, such as Suillia tuberiperda), which all have the olfactory sense capable of detecting the volatile aroma of fruitbodies from a distance of up to 50 m (Splivallo and Culleré 2016a). Consequently, our knowledge about the truffles’ hidden belowground mode of life is restricted to only those fruitbodies that were detected (Trappe and Claridge 2010). In fact, we simply do not know how selective a harvest is and how well it represents the actual abundance of fruitbodies since the absence of evidence is no evidence of absence. Non-systematic and nondestructive harvests are therefore likely dominated by specific aroma concentrations, fruitbody dimensions and locations, as well as hunter/dog ability. The aroma profile of truffles seems to be influenced by individual genotypes Virginie Molinier et al. (2015), and not by the geographical origin or maturity level Niimi, Deveau, and Splivallo (2021). Since the development of aromas most likely depends on the presence of specific bacteria Splivallo and Culleré (2016b), no correlation between gleba colour and aroma intensity has been reported. Depending on the percentage of fully pigmented spores, and thus the level of maturity and ripening Virgine Molinier et al. (2016a), the gleba of Burgundy truffles can vary from white-ochre to dark brown. Differences in the colour and texture of fruitbodies have been proofed useful visual criteria for species identification and distinction Chatin (1887), which may otherwise be considered genetically identical, such as Tuber. aestivum and Tuber uncinatum Virginie Molinier et al. (2013a). (Büntgen et al. (2021)).

### *Tuber aestivum*

*T. aestivum* is indigenous to many European countries and, unlike the other two species, has a widespread distribution range from Sweden to Spain (Stobbe et al. 2013). *T. aestivum* has also been found outside Europe in North Africa (Jeandroz et al. 2008). Since the 1970s, *T. aestivum* as well as some other truffle species have been successfully cultivated in truffle orchards (Chevalier et al. 1973).

To date, most studies have focused on the species’ taxonomic status (Mello et al. 2002;Molinier et al. 2013b;Paolocci etal. 2004;896 Mycorrhiza (2016) 26:895–907 Weden et al. 2005), aroma profiles (Cullere et al. 2010;Diaz et al. 2009; Splivallo et al. 2012) and genetic diversity (Molinier et al. 2015b) including potential links to aroma (Splivallo et al., 2012;Molinier etal. 2015a). The cultivation potential and requirements of these species (Benucci et al. 2012; Shamekh et al. 2014; Stobbe et al. 2013a;Stobbe et al. 2013b), including soil conditions (Benucci et al. 2011; Gryndler et al. 2011; Salerni et al. 2014), have also been investigated previously.

*– more recent papers? LB*

The small scale genetic structure and the distribution of the mating-type strains of *T. aestivum* remain largely unknown. The genome sequencing of *T. melanosporum* in 2010 (Martin et al. 2010), as well as numerous other studies of *T. melanosporum* (Murat et al., 2013; Riccioni et al. 2008; Rubini et al. 2005, 2011a, b), *T. magnatum* (Paolocci et al. 2006;Riccioni et al. 2008;Rubinietal. 2005, 2011a,b) and *T. indicum* (Belfiori et al. 2013) have revealed that these species are heterothallic, as has lately been confirmed for *T. aestivum* (Payen et al. 2014). While homothallic species present both mating types (MAT) in a single genome, rendering them self-fertile, heterothallic species bear only one ofthe two idiomorphs in the MAT locus (Billiard et al. 2012). The same haploid mycelium can produce male (antheridia) and female (ascogonia) organs, although these structures have hardly been observed in Tuber species (Le Tacon et al. 2015). To achieve its life cycle, an ascogonium has to be fertilized by a strain of the opposite mating type. In truffles, a fruiting body is composed of peridium, gleba and ascospores. The gleba is a haploid tissue consisting ofthe maternal parent genotype, which is usually also found as mycelia in the surrounding soil and on nearby ECM root tips (Murat et al. 2013; Rubini et al. 2011a). In contrast, ascospores originate from the recombination of the maternal and paternal genotypes (Le Tacon et al. 2015).

The popularity of truffles as an exclusive ingredient of elite gastronomy has risen tremendously in recent years (Hall et al., 2003). Not only the highly prized Alba white truffle(Tuber magnatum), and the Périgord black truffle(Tuber melanosporum), but also the more common Burgundy truffle(Tuber aestivum) are coveted. In contrast to T. magnatum and T. melanosporum, which are native to Mediterranean environments (Chevalier and Sourzat, 2012; Vasquez et al., 2014), T. aestivum is distributed across large parts of temperate Europe (Chevalier, 2010; Stobbe et al., 2012). According to Hall et al. (2003, 2007) this truffle species is the most common edible fungus in Europe. Based on inventories of hypogeous fungi in the region of Basel, Switzerland, it has even been estimated to be among the most abundant mushroom species in mixed beech forests on calcareous soils (Schw€arzel, 1967). Nevertheless, T. aestivum has only been sparsely harvested in many countries, and is even legally protected from harvest in Germany for its alleged rarity (Stobbe et al., 2013a). This discrepancy in the estimation of its abundance is due to the fragmentary knowledge about the species’ occurrence and distribution, which, in turn, is related to its hidden life belowground and the difficulty ofdetecting its hypogeous fruit bodies. The great demand for T. aestivum has increased cultivation attempts of this symbiotic ascomycete fungus (Murat, 2015). Although truffle plantations are now established over much of the species’ natural distribution range (Stobbe et al., 2013a), setting up new plantations is costly and investments have to be made on a long-term horizon since growth of truffle mycelium in soil is slow (Molinier et al., 2016) and harvests can begin no earlier than 5 y after the initial inoculation of host seedlings, reaching their maximum about a decade after planting (Callot, 1999). Like many other truffle species, T. aestivum grows predominantly in soils with a high content of limestone or exchangeable calcium (Chevalier and Sourzat, 2012) and forms ectomycorrhizas with a wide range of tree and shrub species (Stobbe et al., 2012, 2013a).

Moser et al. (2017)

### Results from WSL

Previous studies utilizing random-amplified polymorphic DNA (RAPD) or intersimple sequence repeat (ISSR) markers and sequencing of a few genes suggested the existence of genetic diversity between *T. aestivum* populations (Gandeboeuf et al., 1997 ; Mello et al., 2002 ; Weden et al., 2004 ). Direct shotgun pyrosequencing (DSP) has been successfully used for the identification of microsatellites in animals or plants but, to our knowledge, this approach has never been used for ectomycorrhizal fungi. Recently the sequencing of the black truffle genome revealed a substantial richness in repeated sequences such as microsatellites ( Murat et al., 2011 ). This result suggested that a DSP approach could be used for identifying microsatellites in Tuber species. The aim of our study was therefore to use DSP to identify, for the first time, polymorphic microsatellites in the economically important truffle *T. aestivum* (Virginie Molinier et al. (2013b)).

Among Tuber species, *Tuber aestivum* Vittad. has a wide distributional range being found naturally all over Europe. A study by Virgine Molinier et al. (2016b) performed large-scale population genetic analyses in *T. aestivum* to (i) investigate its genetic diversity at the European scale, (ii) characterize its genetic structure and test for the presence of ecotypes and (iii) shed light into its demographic history. To reach these goals, 230 ascocarps from different populations were genotyped using 15 polymorphic simple sequence repeat markers. They identified 181 multilocus genotypes and four genetic groups which did not show a clear geographical separation; although, one of them was present exclusively in Southeast France, Italy and Spain. Fixation index values between pairs ofgenetic groups were generally high and ranged from 0.29 to 0.45. A significant deficit of heterozygosity indicated a population expansion instead of a recent population bottleneck, suggesting that *T. aestivum* is not endangered in Europe, not even in Mediterranean regions. This study based on a large-scale population genetic analysis suggests that genetically distinct populations and likely ecotypes within *T. aestivum* are present.

Büntgen et al. (2021) introduced a novel eco-archaeological approach to study in situ aspects of subterranean life cycle dynamics and genetic structures of the Burgundy truffle in three different natural habitats in Baden-Württemberg, southern Germany. They applied a suite of fine-scale excavation techniques to reveal high-resolution, spatiotemporal snapshots of the composition, size and developmental stage of a wide-range of individual truffles. Our approach not only exposes fruitbodies in the investigated soil layers, but also relates each of them to its edaphic environment, and further describes the obtained genotype composition.

1 presented 8 years (2011–2018) of citizen science monitoring data on the fruitbody production of naturally occurring T. aestivum populations. Using machine learning, they modeled how truffle productivity is influenced by soil physicochemical, fungal meta-community, climate, and host tree phenology. Additionally, they characterized the broad species climatic envelope of European summer truffles. By comparing the sensitivity of summer truffle fruitbody productivity to interannual climate trends, they tested whether these center-of-range, keystone populations are locally sensitized to climate change.

Virginie Molinier et al. (2016) determined mating type and simple sequence repeat (SSR) maternal genotypes of mapped fruiting bodies to assess their genetic structure within two naturally colonized forest sites in southern Germany. Forty-one genotypes were identified from 112 fruiting bodies. According to their mating types, the maternal genotypes were aggregated only in one population. Genotypic diversity of individuals that mostly were small and occurred in 1 out of 2 years of sampling was high. Although these results suggested a ruderal colonization strategy, some genets spread several hundred meters. This result indicates that, besides sexual spore dispersal, vegetative growth or spreading by mycelial propagules contributes to dissemination. In one site, fewer individuals with a tendency to expand genets belonging to only one genetic group were observed. In the second site, numerous small individuals were found and were grouped into two clearly differentiated genetic groups that were spatially intermingled. Forest characteristics and disturbances are possible reasons for the observed genetic patterns.

Moser et al. (2017) aimed at estimating the potential of using vegetation composition as an additional parameter to detect and describe *T. aestivum* habitats, and subsequently use this information to define suitable cultivation sites. We, therefore, assess the abundance of individual plant species at 16 sites with known T. aestivum occurrences in southern Germany and Switzerland. We compare the floristic composition of these sites with that of a systematic sample of 232 Swiss F. sylvatica, Carpinus betulus and Ostrya carpinifolia (FCO) forest sites with a climate that is, according to current knowledge, favourable for T. aestivum.

## Identification of gaps in the field of research, motivation for the research and justification of the added scientific value for the field of study

Much remains unknown about the life history of these iconic species due to a lack of available data from natural truffle populations.

However, many aspects of the subterranean life cycle of the genus, such as the formation, duration, maturation and deterioration of their fruitbodies, remain enigmatic and require innovative scientific endeavours (Büntgen and Egli, 2014; Büntgen et al., 2017). Interdisciplinary and international research projects are now indicating that Burgundy truffles (Tuber aestivum) are more widely distributed in ecologically suitable habitats across central Europe than previously recognised (Stobbe et al., 2012; Molinier et al., 2016c; Cejka et al., 2020; Puliga et al., 2021).

Sequencing of the T. aestivum genome is currently being completed (Payen et al., 2014), and the first sequences have facilitated the development of specific simple sequence repeat markers (SSR) (Molinier et al. 2013a). A first assessment of the species’ genetic structure at the European-scale revealed the existence ofwell-differentiated sympatric genetic groups, indicating different ecotypes (defined as genetically distinct varieties within a species that are adapted to specific environmental conditions) with reduced gene flow, although this previous study provided no clues about how these ecotypes are spatially distributed (Molinier et al. 2015b). At much smaller scales, a recent study that focused on the link between genetic structure and aroma in an orchard naturally colonized by T. aestivum indicated that genet sizes (up to 92 m) are larger than those of T. melanosporum (Molinier et al. 2015a;Murat et al. 2013). However, the limited sample size did not allow any deep genetic structure analyses, and the mating-type genotype distribution was not considered. Virginie Molinier et al. (2016)

## Materials and Methods

Data collection

We selected a total of 20 natural *T. aestivum* populations from within the center of its known natural geographic distribution to monitor truffle fruitbody production (Figure 2a). These populations were defined as spatially restricted locations of known truffle occurrences ranging in size from 20 to 1000 m2, depending on the contiguous area under compatible host trees where trained truffle dogs found *T. aestivum* fruitbodies. Monitoring occurred every 3 weeks throughout the whole year by citizen scientists using dogs trained to scent summer truffles.. At each site, only one and the same citizen-dog team performed the monitoring throughout the whole project. For all belowground fruitbodies detected by the truffle dogs, the soil was roughly removed and the species identity, mass (g), and degree of maturity were assessed. The degrees of maturity were divided into the following qualitative categories:

1. unripe: white, hard, no or indistinct odor
2. semi ripe: light beige-light brown, hard, indistinct (nutty) odor
3. ripe: brown to dark brown, hard, intense odor

* Molecular preparation

Genomic DNA of the fruiting bodies was isolated from gleba of each sample using DNeasy® Plant Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer’s instructions. Extracts were eluted in 50 μl ofBuffer AE supplied as part of the kit. To identify the mating type of each fruiting body, specific primer pairs were designed for each MAT idiomorph based on T. aestivum genome sequences (Accession numbers: LT593973 TuaestMAT1-1 gene (European Nucleotide Archive); JB402662.1 TuaestMAT1-2 gene (Genbank)); aest-MAT1-1f (5′ CTACATTCTGGTGG GCGATT 3′)/aest-MAT1-1r (5′ TCCCGATTTGTCCA ACGTAT 3′) and aest-MAT1-2f (5′ ATCGTCGGGACTCA TCTCAC 3′)/aest-MAT1-2r (5′ CGGATATTGGGATT TGATGG 3′). For mating-type gene amplification, multiplex PCRs were performed in a total volume of25 μl consisting of 2.5 μl of 10× REDTaq PCR Buffer (11 mM MgCl2;Sigma: B5926), 5 μl dNTP, (1mM, Life Technologies), 1 μl multiplex solution containing the four primers (10 μMeach), 1.25 μl RED Taq Polymerase, (1 U/μl, Sigma: D4309), 13.25 μlof sterile water and 2 μl template DNA diluted 10 times. The PCR reactions were performed in a Veriti® thermal cycler (Applied Biosystems, Foster City, CA, USA) with the following conditions: 2 min at 94 °C followed by 28 cycles of denaturation at 94 °C for 30 s, annealing at 57 °C for 30 s, extension at 72 °C for 1 min, and a final extension at 72 °C for 7 min. PCR products were run on 1.5 % agarose gel and visualized with a UV transilluminator after ethidium bromide staining.

The extracted DNA from fruiting bodies was amplified using a set of 14 SSR loci (aest01, aest06, aest07, aest10, aest15, aest18, aest24, aest25, aest26, aest28, aest29, aest31, aest35 and aest36) developed previously by (Virginie Molinier et al. 2013a). Polymerase chain reactions were carried out using the Qiagen Multiplex PCR kit (Qiagen, Germany) following the manufacturer’s instructions. PCRs were performed in a total volume of 7 μl, consisting of 3.5 μlof Qiagen Multiplex Buffer (2×), 1.8 μl of sterile water, 0.7 μlprimer premix (2 μM) and 1 μl template DNA diluted 10 times. Two different primer premixes were used according to the expected allelic sizes to avoid overlapping. Both multiplex mixtures had the same PCR conditions: samples were denatured at 95 °C for 15 min, followed by 28 cycles consisting ofa denaturing step at 94 °C for 30 s, an annealing step at 60 °C for 1 min and an extension step at 72 °C for 1 min. A final extension step at 60 °C for 30 min was added after 28 cycles. For the subsequent genotyping step on ABI-3130 (Applied Biosystems, USA), PCR products were diluted in pure water (1/4) and then mixed with HiDi Formamide. As an internal size standard, GeneScan™ 500 LIZ™ dye Size Standard (ThermoFisher Scientific, USA) was used. Virginie Molinier et al. (2016)

* Genetic analyses

Identification of repeated multilocus genotypes

A multilocus genotype (MLG) was assigned to each fruiting body. To distinguish true clones from samples carrying the same MLG by chance, the probability that copies of a MLG arose from sexual reproduction (Psex) was calculated using MLGSIM (Stenberg et al. 2003). Psex values were generated by 10,000 simulations using a Monte Carlo simulation method. A significant Psex value suggests that multiple copies of the same MLG arose from asexual reproduction and growth (true clones). In contrast, a non-significant Psex value suggests that the number of occurrences observed for a repeated MLG resulted from sexual reproduction (by chance). In the latter case, the occurrences were treated as different individuals.

The number of MLGs and genotypic diversity, as well as versus the number of loci, were calculated for one member ofeach MLG using Multilocus 1.3 (Agapow and Burt 2001). Given the clonality observed, subsequent analyses were conducted on a global data set including all individuals (ramet level) and on a clone-corrected data set including one member of each MLG (simple clone-corrected dataset).

For some spatial analyses (i.e. aggregation index, autocorrelation analyses), we assigned up to four centred location points per MLG for large MLGs (>15 m) in the clone-corrected data set (partial clone-corrected dataset) (Fig. S2). Virginie Molinier et al. (2016)

Persistence of individuals

Comparison of genetic diversity (rarefied)

PCAs

STRUCTURE

## Expected results

Since Tuber spp. are hypogeous, their propagation mainly depends on insects and larger animals as spore vectors (Trappe and Claridge 2005). Hypogeous species are thought to have a shorter distance dispersion potential than epigeous species, which have air-dispersed spores, with related effects on the gene flow and genetic structure. The consequence of a short dispersal distance is greater population differentiation and isolation by distance at smaller spatial scales for hypogeous fungi such as Rhizopogon occidentalis, R. vulgaris and Tuber melanosporum (Murat et al. 2013;Grubisha etal. 2007). A fine-scale population structure analysis carried out in two T. melanosporum plantations using fruiting bodies and mycorrhizas demonstrated a profound isolation by distance in the first 5 m and found that belowground genets had a maximum size ofa fewmeters (Murat et al. 2013). The observed patterns were assumed to be a combination of vegetative mycelial growth of a few genets that persisted for several years and an annual recruitment of new genets via the ascospores. Intriguingly, genotypes were not spatially randomly distributed in T. melanosporum plantations and natural fields but rather occurred in clusters according to their MAT idiomorph (Murat et al. 2013;Rubini etal. 2011a). Such a spatial segregation ofmating types would decrease the probability that compatible cells meet, which could be beneficial under the assumption that sex is costly (Selosse et al. 2013). This segregation ofthe mating-type strains raises the question ofwhere paternal individuals come from and what structures they might consist of (Le Tacon et al., 2015). Virginie Molinier et al. (2016)

Virginie Molinier et al. (2016) determined mating type and simple sequence repeat (SSR) maternal genotypes of mapped fruiting bodies to assess their genetic structure within two naturally colonized forest sites in southern Germany. Forty-one genotypes were identified from 112 fruiting bodies. According to their mating types, the maternal genotypes were aggregated only in one population. Genotypic diversity of individuals that mostly were small and occurred in 1 out of 2 years of sampling was high. Although these results suggested a ruderal colonization strategy, some genets spread several hundred meters. This result indicates that, besides sexual spore dispersal, vegetative growth or spreading by mycelial propagules contributes to dissemination. In one site, fewer individuals with a tendency to expand genets belonging to only one genetic group were observed. In the second site, numerous small individuals were found and were grouped into two clearly differentiated genetic groups that were spatially intermingled. Forest characteristics and disturbances are possible reasons for the observed genetic patterns.

## Risk assessment

Errors in sampling: risk low. Errors in sample preparation (PCRs): moderate. Errors in attribution of markers in GeneMapper: moderate to high.

## Project schedule

* Data collection almost finished
* Data analysis: splitting into different parts

## Budget plan

* Costs: personnel, analyses

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