Fine-scale genetic structure of natural *Tuber aestivum* sites in southern Germany

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Abstract Although the Burgundy truffle (*Tuber aestivum*) is an ectomycorrhizal fungus of important economic value, its subterranean life cycle and population biology are still poorly understood. Here, we determine 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. Our findings contribute to a better understanding of the biology of one of the most widespread and commercially important truffle species. This knowledge is critical for establishing and maintaining sustainable long-term truffle cultivations.

Keywords Burgundy truffle · Genets · Mating-type genes · Population genetics · Propagation strategy · SSR markers

Introduction

True truffles are ectomycorrhizal fungi belonging to the genus *Tuber* in Ascomycetes. This genus, which exclusively forms hypogeous fruiting bodies, comprises more than 200 species all over the world (Bonito et al. 2013; Bonito et al. 2010), of which at least 30 naturally occur in Europe (Ceruti et al. 2003). 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). This last species is indigenous to many European countries and, unlike the other two species, has a widespread distribution range from Sweden to Spain (Stobbe et al. 2013a). *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 et al. 2004;

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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 et al. 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. However, 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; Rubini et al. 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 of the 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 of the 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).

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 et al. 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 of a few meters (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 et al. 2011a). Such a spatial segregation of mating 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 of the mating-type strains raises the question of where paternal individuals come from and what structures they might consist of (Le Tacon et al. 2015).

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 of well-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.

Here, we analysed the spatial and temporal genetic structure of two natural *T. aestivum* populations in southern Germany. Mating-type genes and SSR markers on maternal tissue from fruiting bodies were used to address (1) how the mating types are distributed, (2) how the genets are spatially structured on a small-scale, and (3) how the persistence of genotypes evolves over time. We compare our results with previous findings about the small-scale genetic structure of *T. melanosporum* and thereby provide new insight into the life cycle of *T. aestivum*.

Materials and methods

Sites description and sampling of fruiting bodies

T. aestivum fruiting bodies were collected in two forest sites (Ueberlingen (UL) and Bohlingen (BB)) located about 3 km apart from each other in southwest Germany near Lake Constance. Due to the risk of illegal truffle harvesting on these long-term monitoring sites, the exact locations are kept confidential but a schematic map is provided in Fig. S1.

Bohlingen is a single-storied, even aged (>100 years) and conventionally managed beech forest with little understory vegetation, whereas UL is a multi-storied, uneven aged and poorly managed mixed beech-oak forest with abundant understory vegetation. On both sites, the tree layer is dominated by

Fagus sylvatica, but the second-most dominant species is *Quercus robur* on UL and *Acer campestre* on BB. Further tree species are *Carpinus betulus* and *Robinia pseudoacacia* on UL, and *Fraxinus excelsior* on BB. UL was selected in 2011 due to the consistent presence of *T. aestivum* fruiting bodies over several years. This forest stand is spatially restricted to about 5000 m² by surrounding agricultural fields and is located at least 250 m away from the neighbouring forests. In 2013, the BB experimental site was selected due to its high *T. aestivum* fruiting body productivity and is part of a forest with an area of approximately 40,000 m².

To obtain more detailed information about the site characteristics (e.g. host- and non-host trees) and potential environmental differences between them, we performed a vegetation survey within a circular plot with an area of 200 m² in each study site. In each of these plots, we assessed the composition and coverage of herbs, shrubs (woody plants with a height between 0.5 m and 5.0 m) and tree species. From these data, the Shannon index and the Landolt indicator values were calculated. Indicator values refer to the ecology and biology of individual plant species and aim at characterising species' niches in natural plant communities (Landolt 2010). Average Landolt indicator values of a plant community mirror local site conditions, e.g. with respect to temperature, soil moisture or nutrients. Physico-chemical soil characteristics of each of the two truffle sites were studied by collecting 16 soil cores (0–15 cm in depth), which were pooled at the site level and then analysed by SADEF company (Aspach-France).

Fruiting body sampling was carried out in a 1-day harvest in October 2011 at site UL and in a 2-day harvest in October 2013 at both sites with four trained truffle dogs. All fruiting bodies were retrieved from the soil and precisely mapped using GPS technology. The fresh weight of each fruiting body was measured, and its maturity stage was determined according to a protocol based on Zeppa et al. (2004). Under a microscope (×400 magnification), asci (sexual spore-bearing cells produced in ascomycete fungi) were counted and sorted into three different categories: (1) asci with mature spores (yellow-brown colour with a fully formed ornamentation), (2) immature asci with immature spores (white colour and without or incompletely formed ornamentation) and (3) empty asci or absence of asci. The degree of maturity of the fruiting bodies (percent mature) was calculated as follows: the number of mature asci (category 1) divided by the total number of asci (categories 1, 2 and 3). For each fruiting body, observation and calculation were performed five times, each with a different microscopic slide. Samples of fruiting bodies were kept at −20 °C until further analyses.

DNA extraction, mating-type genes and SSR amplifications

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 of Buffer 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 TuaeMAT1-1 gene (European Nucleotide Archive); JB402662.1 TuaeMAT1-2 gene (Genbank)); aest-MAT1-1f (5' CTACATTCTGGTGG GCGATT 3')/aest-MAT1-1r (5' TCCCGATTGTCCA 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 of 25 µl consisting of 2.5 µl of 10× REDTaq PCR Buffer (11 mM MgCl₂; Sigma: B5926), 5 µl dNTP, (1 mM, Life Technologies), 1 µl multiplex solution containing the four primers (10 µM each), 1.25 µl RED Taq Polymerase, (1 U/µl, Sigma: D4309), 13.25 µl of 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 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 µl of Qiagen Multiplex Buffer (2×), 1.8 µl of sterile water, 0.7 µl primer 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 of a 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.

Data analyses

To investigate the sexual behaviour of *T. aestivum*, we first used the mating-type genes alone. Genetic and clonal

structures were investigated using 14 SSR markers combined with the mating-type gene.

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 (P_{sex}) was calculated using MLG_{SIM} (Stenberg et al. 2003). P_{sex} values were generated by 10,000 simulations using a Monte Carlo simulation method. A significant P_{sex} value suggests that multiple copies of the same MLG arose from asexual reproduction and growth (true clones). In contrast, a non-significant P_{sex} 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 of each 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).

Mating-type distribution

We tested whether the proportion of *MAT1-1* and *MAT1-2* isolates were equal within each population. For assessing the significance of deviation from a 1:1 mating-type ratio (null hypothesis) in a population, we used the exact binomial test at the ramet and genet levels. In a random distribution context, this ratio should be equal to one. On each site, we calculated the aggregation of mating types using Genclone software (Arnaud-Haond and Belkhir 2007) to study the potential clustering of the individuals harbouring the same mating type. For this analysis, two different levels were performed: at the ramet level and, also, we used the partial clone-corrected dataset with several centred location points for large MLGs. For each analysis, only the *MAT* gene information was used.

Clonal diversity and fine-scale spatial genetic structure analyses

Within each population (BB, UL 2011, UL 2013 and UL 2011/2013), according to Arnaud-Haond et al. (2007), we used Genclone software to calculate several indices thought to be the most parsimonious set of non-redundant indices of clonal diversity (Arnaud-Haond and Belkhir 2007): (1) the

genotypic diversity ($R = (\text{number of MLG} - 1) / (\text{number of ramets} - 1)$), which ranges from 0 to 1 where 0 means the entire population is one clone (with the same genotype) and 1 means every sample (here fruiting bodies) is one unique clone (each sample possesses a different genotype); (2) the adapted Simpson index for genotypic diversity (D^*), which ranges from 0 to 1 where 1 represents maximum diversity; (3) the corresponding evenness index (ED^*), which ranges from 0 to 1 where 1 means that all MLG are equal in abundance; and (4) the complement of the slope of the Pareto distribution of clonal membership (c Pareto). The Pareto coefficient increases as the diversity and evenness in the sample increase.

In order to describe the spatial component of clonal growth and the intermingling of repeated MLGs, we used Genclone software (Arnaud-Haond and Belkhir 2007) to estimate the spatial aggregation index (Ac) and the edge effect (Ee), as described by Arnaud-Haond et al. (2007). The Ac index ranges from 0, where the probability that nearest neighbours have the same MLG does not differ from the average probability across all occurrences, to 1, where all nearest neighbours preferentially share the same MLG. The statistical significance of the aggregation index was tested against the null hypothesis of a spatially random distribution of isolates using a re-sampling approach based on 1000 permutations. The edge effect (Ee) estimates the effect of sampling on the estimates of genotypic diversity, especially potential overestimation due to the presence of large clones sampled only once or a few times at the edge of the sampling area. Finally, the clonal subrange (maximum distance between two samples with same MLG) was calculated and used to represent the size of the largest genet.

A spatial autocorrelation approach was used to investigate the spatial genetic structure within each truffle site. We used the kinship coefficient (F_{ij}) (Loiselle et al. 1995) as a means of measuring the relatedness between each pair of fruiting bodies. We defined 17 distance classes and tested whether the observed values of F_{ij} within each distance class differed significantly from 0 (random structure) by using 10,000 permutations of spatial locations in the program SPAGED1 1.3 (Hardy and Vekemans 2002). Spatial genetic structure was quantified with the Sp statistic, estimated as $-b / (1 - F_1)$, where b is the slope of the regression of F_{ij} on the logarithm of the geographical distance separating the pairs of fruiting bodies, and F_1 is the mean kinship coefficient of the first distance class (Hardy 2003). We performed these analyses at the ramet level, which addresses clonal, vegetative growth, and with the partial clone-corrected dataset, which focuses on sexual spore dispersal.

Population subdivision

We investigated population subdivision using the entire dataset and the Bayesian method implemented in STRUCTURE 2.3.4 (Pritchard et al. 2000). STRUCTURE infers the best number of genetic groups (K) or sub-populations. The admixture model

with correlated allele frequencies was selected as an appropriate option for the analysis. The burn-in period and Markov Chain Monte Carlo value (MCMC) were set to 500,000 and 750,000 iterations, respectively, and 20 replicates per K were run. As recommended, successive K values (number of populations) from 1 to 10 were used to obtain the distinct genetic group and to estimate the number of subpopulations. The final posterior probability of K , $\ln P(K)$ and Delta K (ΔK), defined as the rate of change of $\ln P(K)$ between successive K values (Evanno et al. 2005), was calculated using Structure Harvester (Earl and vonHoldt 2012) to determine the most likely number of genetic groups. Genetic group pairwise F_{st} calculations, pairwise kinship coefficients (F_{ij}) and intra-genetic group kinship coefficients (G_{ij}) were calculated using SPAGeDi software (Hardy and Vekemans 2002). When subpopulations were observed, we calculated the average fruiting body maturity and weight, as well as the correlation between these two parameters within each genetic group (Pearson's correlation), and tested if genetic groups differ in these parameters (ANOVA by permutation). When subpopulations were observed within the site, we performed spatial aggregation analyses as described above.

Control for sampling biases

To infer a possible sampling bias due to differences in sampling area surface, clonal parameters were calculated for several sampling scenarios. In 2013, truffles were harvested from a larger area on site BB than on site UL, but the same sampling area (80×45 m) was used for calculations for each site (Fig. S3, case 1). Because the sampling area on UL was smaller in 2011 than in 2013, the calculations were done with a subset of the samples collected in 2013 (Fig. S3, case 2). To investigate if the localization of the sampling area within an individual site plays a role in the genetic results, calculations were performed for three rectangular sampling areas. The rectangles, each with a size of 95×35 m, were placed randomly on BB (Fig. S3, case 3).

Mapping

To map mating types and MLG, and to calculate distance values for the genetic group analysis, we used the program ArcGIS 10.2 (ESRI, Redlands, CA, USA).

Results

Truffles harvests

Vegetation and soil characteristics for BB and UL are summarized in Table S1 and Fig. S4. In total, 112 truffle fruiting bodies were harvested. On BB, 42 fruiting bodies were collected within 9540 m^2 in 2013. In UL, 31 fruiting bodies were

collected in an area of approximately 1000 m^2 in 2011, and 39 fruiting bodies within 2350 m^2 in 2013. No significant differences in maturity or weight were found between fruiting bodies collected in the two different sites. Details about weight, maturity and correlation calculations for each population are given in Table S2.

Mating-type genes and their spatial distribution within the truffle sites

For fruiting body gleba (i.e. maternal tissue), due to the haploid nature of this tissue (Paolocci et al., 2006), only one *MAT* locus was detected. When looking at all sampled fruiting bodies (ramet level), the ratio *MAT1-1*:*MAT1-2* maternal tissue was 0.90 in BB and did not deviate significantly from a 1:1 mating-type ratio (not significant; Table 1). In UL, however, significantly more fruiting body maternal tissue presented the *MAT1-1* locus, with *MAT1-1*:*MAT1-2* ratios of 3.11 (p value < 0.01 , UL samples from 2011 and 2013 pooled (UL 2011/2013)), 2.1 (not significant, samples from 2011) and 4.57 (p value < 0.01 , samples from 2013; Table 1). When considering all fruiting bodies, the mating-type distribution visually seemed rather homogenous in the site BB, whereas a more clustered distribution of mating types seemed to be present in UL (Fig. 1). This was confirmed by aggregation analyses, which showed weak aggregation indexes in BB (0.30, not significant) and significant, high aggregation indices in UL (UL 2011/2013: 0.77, p value = 0; UL 2011: 0.93, p value = 0 and UL 2013: 0.75, p value = 0; Table 1). Since this reflects clonal growth rather than clustering of different genets bearing the same mating type, we performed these analyses at the genet level using the clone-corrected dataset including several centred locations for large genets (Fig. S2). Here, we found no indication of mating-type aggregation in the BB site but still a significant aggregation value in UL 2011/2013 (0.55; p value = 0.03) and UL 2013 (0.57; p value = 0.03). With this dataset, a *MAT1-1*:*MAT1-2* ratio of 0.90 (not significant) was found in BB, a ratio of 2 was found for UL 2011/2013 (p value = 0.03), and a ratio of 5 was found for UL 2011 (p value = 0.03). For UL 2013, the ratio *MAT1-1*:*MAT1-2* was 1.75 and did not deviate significantly from a 1:1 mating-type ratio.

MLGs analyses

The 112 fruiting bodies of *T. aestivum* were successfully genotyped using the 14 SSR markers. All SSRs were polymorphic except one, the locus *aest15* (Table S3a). SSR markers presented 1 to 4 alleles (Table S3a). One and seven SSRs were monomorphic for BB and UL, respectively (Table S3b). By combining the mating-type and SSR profiles, a total of 41 MLGs were identified, 26 for BB and 15 for UL (Table 1). When plotting the genotypic diversity and the number of

Table 1 Genetic characteristics and clonal diversity parameters of the two *T. aestivum* populations

Site	Year	Number of fruiting bodies (N)	Number of genotypes (G)	Genotypic diversity ((G-1)/(n-1))	Edge effect (p value)	Simpson's diversity index (D)	Evenness of D	c Pareto	Aggregation index (p value)	Clonal subrange (in meters)	Ratio MAT1-1: MAT1-2 (p value) of fruiting bodies	Aggregation index (p value) considering fruiting bodies	Ratio MAT1-1: MAT1-2 (p value) of genotypes	Aggregation index (p value) considering genotypes
BB	2013	42	26	0.61	0.10 (ns)	0.96	0.82	1.96	0.26 (0)	155	0.90 (ns)	0.30 (ns)	0.90 (ns)	-0.06 (ns)
UL	2011, 2013	70	15	0.20	0.22 (ns)	0.88	0.90	1.31	0.70 (0)	55	3.11 (<0.01)	0.77 (0)	2.83 (0.03)	0.55 (0.03)
UL	2011	31	9	0.27	-0.23 (ns)	0.86	0.87	1.53	0.92 (0)	5	2.10 (ns)	0.93 (0)	5 (0.03)	0.57 (0.03)
UL	2013	39	11	0.26	0.33 (ns)	0.84	0.81	1.41	0.54 (0)	45	4.57 (<0.01)	0.75 (0)	1.75 (ns)	0.45 (ns)

ns not significant (p value > 0.05)

MLG against the number of loci, no plateau was observed; there was a genotypic diversity of 0.949 for 15 loci (i.e. with mating gene) and increasing numbers of MLGs (Fig. S5). Since no plateau was reached, it cannot be excluded that additional markers would increase the genotypic diversity.

No MLG was shared between the two sites (Table S4). In BB, seven MLG (27 %) had several ramets (i.e. several fruiting bodies), and a significant P_{sex} value indicated that they belonged to the same genet (Table S4). In the UL site, nine MLG (60 %) had several ramets and, with the exception of MLG 21 and MLG 35, all MLG can be considered as genets (Table S4). For MLG21 and MLG35, the two fruiting bodies were each treated as different MLGs (e.g., 21a, 21b). Two thirds of MLGs found in UL were present in only one of the two seasons, whereas five MLGs were found in both 2011 and 2013 (Table S4). Although collected at the same time, many fruiting bodies formed by the same MLG showed different weights and maturity stages from unripe to fully ripe ones in both sites (e.g. MLG 17, MLG 36; Table S2).

Clonal diversity and spatial genetic structure

In BB, the genotypic diversity (R) was high (0.61). Lower R values were obtained for UL in each year and in 2011 and 2013 combined (0.20–0.27; Table 1). No significant edge effects (E_e) were detected in any analyses, indicating that there was no sampling bias and that the sampling scheme was adequate to estimate the genotypic diversity. High values were found for both the Simpson's diversity index (0.96 in BB and 0.84 to 0.88 in UL) and the evenness index (0.82 in BB and from 0.81 to 0.90 in UL) (Table 1). The c Pareto values ranged from 1.31 to 1.96 (Table 1). These three indices were quite similar for both truffle sites and indicate that the populations show a high diversity, with relatively equal abundances of different MLGs.

The estimated aggregation index (Ac) was 0.26 (p value < 0.001) for BB and 0.70 (p value < 0.001) for UL (2011 and 2013 combined) (Table 1). This indicates that on BB, only 26 % of the nearest neighbours share the same MLG, whereas the majority (70 %) of the nearest neighbours on UL share the same MLG. The largest genet size was found in BB, stretching 155 m, whereas in UL the maximum sizes were 5, 45 and 55 m for 2011, 2013 and 2011/2013 combined, respectively (Table 1, Fig. 1). However, genet sizes were generally small in both sites (Table S4). In BB, 70 % of the genets were only found once, whereas a maximum of 40 % of the genets were unique in UL for single years and for 2011 and 2013 combined (Table S4). Clonal parameters seem to be unbiased by the different sampling areas except for the maximum genet size observed (Fig. S2, Table S5).

Spatial autocorrelation of the kinship coefficient (F_{ij}) revealed a significant positive correlation for distances up to 4 m in BB and up to 6.5 m in UL when all sampled fruiting bodies were considered (ramet level; Fig. 2). Sp, which

Fig. 1 Schematic representation of BB and UL sites. Mating type and genetic group memberships of each sample are given in (a) and genotype mapping is given in (b). In a, mating-type strains (Mat 1 and Mat 2) are indicated in *dashed* (Mat 1) or *solid* (Mat 2) lines. The three different genetic groups are indicated in colour (1 = blue, 2 = pink and 3 = black).

In b, *crosses* represent samples showing a unique multi-loci genotype, whereas shared multi-loci genotypes are represented by triangles (samples from 2011) or *dots* (samples from 2013). Sizes of genets are represented by the sizes of *colored ovals*

Fig. 2 Spatial autocorrelogram of the kinship coefficient (F_{ij}) as a function of the log of the spatial distance. Panels (a), (b), (c) and (d) represent the autocorrelograms for which all ramets were used for BB and UL, UL in 2011 and UL in 2013, respectively. The dashed lines correspond to the 95 % confidence interval for the null hypothesis of complete spatial randomness of genotypes, constructed by 10,000 permutations of genotypes across individual positions. The slope of the

regression of kinship with $\log(\text{dist})$ is indicated as b for each correlogram. The statistic (Sp), defined as the ratio $-b/(1-F_1)$, where b is the regression slope of the autocorrelogram and F_1 is the mean F_{ij} between the individuals belonging to the first distance class that includes all pairs of neighbours (Vekemans & Hardy, 2004), is indicated for each autocorrelogram. An asterisk indicates a b -log with a p value < 0.05

reflects the rate of decrease in pairwise kinship over distance, was higher in UL for single and combined years (0.59, 0.68 and 0.25 for UL 2011/2013, UL2011 and UL2013, respectively) than in BB (0.17), indicating a stronger spatial genetic structure (SGS) in the first site when ramets are considered (Fig. 2). These values increased strongly if autocorrelation was analysed within 5 m only (Fig. 2). At the genet level, we still found a positive spatial autocorrelation of kinship in both sites, although this relationship was less clear, and similar slopes and Sp values were found for both sites when all distance ranges were considered. However, within the first 5 m, a much more pronounced SGS was observed for BB than for UL, with the highest significant Sp value (1.43) of all datasets (Fig. S6).

STRUCTURE analysis revealed three well-differentiated genetic groups, two in BB (genetic group 1 and genetic group 2) and one in UL (genetic group 3; Fig. 1). The fixation indices (F_{st}) between all genetic groups were high (0.49–0.65; Table 2), indicating a clear differentiation between genetic groups even when present within the same site. The genetic relatedness of samples within a genetic group was high, while lower and negative kinship values were observed between genetic groups (Table 2). The two genetic groups present in the BB site seemed to be spatially intermingled because no significant aggregation was found according to the genetic

group membership (0.39; not significant). Almost twice as many genets were found in genetic group 2 compared to genetic group 1, the first harbouring larger genets and the last only showing unique genets (Table S4). The average maturity of fruiting bodies from these two genetic groups did not differ (Table S2). We added the dataset from this particular study into the European dataset published in 2015 (Molinier et al. 2015b) and reran the STRUCTURE analysis. It revealed that all genotypes of the dataset presented here belong to the European genetic group 4.

Table 2 Matrix of genetic differentiation between pairs of genetic clusters. Genetic distance (F_{st}) values are indicated in the lower half of the matrix. Kinship coefficients (F_{ij}) between pairs of genetic groups and within genetic groups (G_{ij}) are indicated in the upper half of the matrix and in the diagonal line (in italics), respectively

ALL LOCI	Genetic group 1	Genetic group 2	Genetic group 3
Genetic group 1	0.47	−0.15	−0.08
Genetic group 2	0.52	0.50	−0.38
Genetic group 2	0.49	0.65	0.56

Discussion

Sex and mating-type distribution of *T. aestivum*

Taking advantage of the genomic resources recently generated (Payen et al., 2014), we confirm in the present study that *T. aestivum*, like many other *Tuber* spp., has a dominant haploid life cycle and a heterothallic sexual reproduction mode requiring two compatible mating-type strains. Similar to studies on *T. melanosporum* (Murat et al. 2013; Rubini et al. 2011a), there is some indication of maternal mating-type aggregation in our study site UL but not in BB. To be able to compare mating-type aggregation indices obtained in the current study with *T. melanosporum* data on fruiting bodies in the plantation of Rollainville (Murat et al. 2013), we calculated these indices for data presented by Murat et al. (2013) and found that aggregation was considerably higher in the *T. melanosporum* plantation (0.83 and 0.84 at the ramet and the genet level, respectively). We also observed significant aggregation of mating types in both sites when all fruiting bodies (ramets) were considered, and in the UL site when only genets were considered. However, the first analyses based on ramets reflect clonal growth rather than mating-type clustering, whereas the second analysis might be biased in the UL site because *MAT1-1* genets were much more abundant than those bearing *MAT1-2*. Therefore, more studies are needed to learn whether or not mating-type clustering occurs in *T. aestivum*. Our data indicates that such a tendency is less pronounced in *T. aestivum* than in *T. melanosporum*, which suggests that not all *Tuber* species behave in the same way. However, if aggregation is present, it might be explained by the vegetative incompatibility system, which is responsible for rejection of non-compatible strains during vegetative growth (Leslie and Zeller, 1996; Glass et al. 2000). The mating-type locus is one of the loci controlling vegetative incompatibility (Glass et al. 2000). These mechanisms, which block the formation of heterokaryotic mycelia, also exist in *Tuber* spp. (Sbrana et al. 2007). Selosse et al. (2013) proposed that one possible explanation from an evolutionary point of view justifying the link between mating-type genes and vegetative incompatibility is that the resulting spatial segregation decreases the probability that compatible cells meet. A lower probability of mating is beneficial under the assumption that sex is costly (Selosse et al. 2013).

Interestingly, unequal abundances of mating types with significantly more *MAT1-1* samples were previously observed on tree roots in Australian *T. melanosporum* populations (Linde and Selmes 2012). The authors hypothesized that, if not due simply to insufficient sample sizes, such a skew could arise from competition on the host trees, and the dominant mating type could be linked to a gene under selection, providing a competitive advantage. However such a bias was not found in some other studies of *T. melanosporum* (Murat

et al. 2013; Rubini et al. 2011a); clearly, this possibility needs to be investigated further, both in the field with large sample sizes of fruiting bodies and mycorrhizal root tips and in genomic studies.

Propagation strategy of *T. aestivum*

The propagation of ECM fungi is intimately related to the host-plant roots since they are obligatory symbionts (Smith and Read 2008). To maintain and extend a population, fungi need to colonize the root system either by mycelial spread from existing individuals or via the recruitment of new genets through spore germination. The trade-off between asexual growth and sexual reproduction can be species specific (r- or K-strategy) or correlated with some extrinsic environmental factors, such as disturbance or forest age (Douhan et al. 2011). While it is clear that *T. aestivum* is an ECM fungus adapted to a wide range of host trees and climatic conditions (Molinier et al. 2016; Stobbe et al. 2013a), not much is known about its propagation strategy and its reaction to disturbances within a population. The present results show that *T. aestivum* forms many rather small genets with mostly only one fruiting body per genet and with a high genet turnover, suggesting a ruderal colonization strategy (r-strategy). This finding was more obvious on site BB than on UL. R-strategists spread and colonize by sexual spores rather than by vegetative growth (Dahlberg and Stenlid 1994; Deacon 1992). The same pattern was described for *T. melanosporum* (Murat et al. 2013) and in naturally colonized *T. aestivum* plantations (Molinier et al. 2015a). The high genetic diversity found here is in agreement with previous results from small-scale (Molinier et al. 2015a) and large-scale studies of *T. aestivum* (Gandeboeuf et al. 1997; Mello et al. 2002; Molinier et al. 2013a; Molinier et al. 2015b; Pacioni and Pomponi 1991; Paolocci et al. 2004; Weden et al. 2004). This high diversity is probably linked to the ubiquitous presence of *T. aestivum* throughout Europe and its adaptation to different climate and site conditions.

Although mostly small genets were observed in our study, some individuals spanned more than 100 m, which is significantly larger than the observed maximum size of 4.7 m for *T. melanosporum* in artificial plantations (Murat et al. 2013). Our results are in agreement with the maximum size of 92 m recently published in a study on naturally colonized *T. aestivum* sites (Molinier et al. 2015a). If the observed genet sizes originated from mycelial growth, *T. aestivum* would be one of the ECM fungal species forming the largest genets (Douhan et al. 2011). Maximum genet sizes of 110 m have been observed for the basidiomycete *Xerocomus chrysenteron* (Fiore-Donno and Martin 2001), followed by the *Tricholoma sculpturatum* complex (Carriconde et al. 2008) and *Suillus pungens* (Bonello et al. 1998), both with a maximum size of 40 m. Besides the species-specific colonization strategy and growth capacity, environmental parameters such as forest age

and degree of disturbance can influence vegetative mycelial growth (review in Douhan et al. 2011). The large genet sizes observed in our study can be explained by the fact that our study took place in natural, only sporadically managed forests including trees more than 100 years old. However, *T. aestivum* has an in vitro growth of 2–4 mm per week, which is slow compared to other mycorrhizal fungi (Iotti et al. 2002 and our own unpublished observations). Gryndler et al. (2015) observed similar growth rates in a field survey (3 mm per week). To reach the size observed in our study of 155 m would therefore take almost 1000 years. Although unlikely, this would be possible if our site was continuously forested for this long period. Unfortunately, no long-term data about forest history are available. However, such a large genet size might alternatively have emerged from mycelial fragments sticking to and thus being distributed by animals or humans. In addition, the genets could spread via mitospores, the formation of which has been shown for other *Tuber* species (Healy et al. 2013).

Our kinship analyses indicate that *T. aestivum* shows significant spatial genetic structure (SGS) in natural sites within the first 5 m. Such a high SGS is typical for species that disseminate spores by animals rather than wind (Vekemans and Hardy 2004) and our observations are consistent with those for *T. melanosporum* (Murat et al. 2013). This finding indicates that offspring preferentially stays close to the parents, maybe through the decay of fruiting bodies not harvested by animals. Such a pattern might also arise by the active vegetative incompatibility system mentioned above, in which kin and non-kin individuals are differentiated and the local distribution of genets is shaped by the sharing of space among kin and the protection from resource access by non-kin.

Even if *T. aestivum* appears to have the same life strategy as other truffles, the season and the timing of sexual encounters and fruiting body development is still unclear. *Tuber magnatum* and *T. melanosporum* show a relatively short season in which fruiting bodies can be found (from September to the end of December and from December to March, respectively). In contrast, fully mature *T. aestivum* fruiting bodies can be harvested over a much longer season (Stobbe et al. 2013a). In the present study, we found no clear correlation between maturity and weight of fruiting bodies from a single harvest. Further, the same maternal mycelium was found in fruiting bodies of all maturity stages. Given the fact that a period of 5 months has been suggested for completion of maturation for *T. melanosporum* (Pacioni et al. 2014), we can assume that the same maternal tissue of *T. aestivum* can fructify more than once per year. Therefore, the life cycle of *T. aestivum* is likely to have several short reproductive periods and can be more complex than that of other studied truffle species.

In one of the present study sites, we found two well-differentiated genetic groups, indicating limited gene flow between them, although they were spatially not clearly

separated. Another type of barrier might exist and explain their sympatric presence. Interestingly, the two genetic groups seem to differ in their colonization strategy: whereas one consists solely of unique and small genets, the other one harbours individuals that had formed several fruiting bodies and which had spread vegetatively to form larger genets. These different strategies might explain the high genetic differentiation between the two genetic groups, and a genetic determinant might exist that enables competitive vegetative spread.

Differences in the genetic structure of the *T. aestivum* populations within the two natural sites

Although the sites BB and UL are located only 3 km apart and show similar physico-chemical soil characteristics, no gene flow seems to occur. Such a high genetic differentiation has already been shown for the hypogeous species *Rhizopogon occidentalis*, where a high F_{st} value (0.26) was found between populations separated by 8.5 km (Grubisha et al. 2007). The authors attributed this finding to geographic dispersal barriers, a limited number of mammal dispersers in the area, and a large *Rhizopogon* spore bank. In our study, the site UL is isolated by agricultural fields, and thus geographic barriers, at least for vegetative dispersion, could explain the limited gene flow. In addition, small mammals like rodents are efficient short-distance dispersers (Urban 2016), with dispersal limits below 50 m (Frank et al. 2009), which might not be far enough to cross these agricultural fields. Larger mammals such as boars can also be effective vectors of *T. aestivum* (Piattoni 2012; Piattoni et al. 2014). However, even if present in this area, such animals apparently do not lead to a genetic exchange of *T. aestivum* between the two studied sites, and further studies are required to estimate how efficient they are as truffle dispersers.

Besides the lack of gene flow, we also observed some differences in genetic diversity and spatial genetic structure between the two sites. On site UL, we observed fewer genetic individuals belonging to one genetic group, which tend to show expanded genet sizes, than on BB. In addition, the genotypic diversity was higher and genets tended to have more restricted sizes, stronger spatial genetic structuring, and clearer grouping into two well-differentiated genetic groups on site UL compared to site BB. On BB, we observed a genetic pattern very similar to that found for *T. melanosporum* (Murat et al. 2013), except for maternal mating-type genotype aggregation. The most obvious environmental difference between the two sites is the fact that agricultural fields surround UL, whereas BB is a large and interconnected forest. Therefore, UL is an isolated site and has a limited effective population size, whereas in BB, spore dispersion by animals from larger areas is more probable and may allow incoming spores to establish small genets. A more intense genetic turnover in the connected forest compared to in the isolated one may have led to the observed higher genotypic diversity on site BB.

Interestingly, mycelial growth seems to be more favoured on site UL, which might be a consequence of fewer disturbances in an unmanaged forest with limited human activity. Over the longer term, the composition of tree species (potential *T. aestivum* hosts) and of shrubs and herbs in the understory layer could influence the genetic structure of *T. aestivum* if genets have different host preferences, as shown previously for arbuscular mycorrhizal fungi (Croll et al. 2008).

To conclude, *T. aestivum* growing in natural forest stands is genetically highly diverse, mainly forms small genets (although very large genets do occur occasionally) and presents a high spatial genetic structure over only a few meters. *T. aestivum* can have different colonization strategies depending on site conditions. This new knowledge on the life history traits of this truffle species is highly important for maintaining sustainable long-term truffle production, both in artificial plantations and in natural habitats.

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