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Primer Note

First identification of polymorphic microsatellite markers in the Burgundy truffle, *Tuber aestivum* (Tuberaceae)[1]

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- *Premise of the study: Tuber aestivum*, the most common truffle in Europe, plays an important role in the commercial truffle market. For the first time, microsatellite primers were developed to investigate polymorphism within this species.
- *Methods and Results:* Using direct shotgun pyrosequencing, 15 polymorphic microsatellites were identified out of the 7784 perfect microsatellites present in the 534 620 reads obtained. Tested on 75 samples, these microsatellites were highly polymorphic. The number of alleles varied from four to 15, and the expected heterozygosity ranged from 0.266 to 0.620. A multilocus analysis allowed the identification of 63 genotypes over the 75 samples analyzed.
- *Conclusions:* Direct shotgun pyrosequencing is a fast and relatively low-cost technique allowing identification of microsatellites in nonmodel species. The microsatellites developed in this study will be useful in population genetic studies to infer the evolutionary history of this species.

Key words: direct shotgun pyrosequencing; polymorphism; truffle; *Tuber aestivum*; Tuberaceae.

Truffles are edible ectomycorrhizal fungi belonging to the genus *Tuber* P. Micheli ex F. H. Wigg. and living in symbiosis with different host trees. More than 100 *Tuber* species exist in the world but only a few, such as *T. magnatum* Picco, *T. melanosporum* Vittad., and *T. aestivum* Vittad., have interesting organoleptic qualities. The first two species have a natural distribution restricted to limited European countries, but *T. aestivum* has a natural distribution across Europe. Previous studies utilizing random-amplified polymorphic DNA (RAPD) or inter-simple 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). However, the absence of codominant molecular markers, such as microsatellites or simple sequence repeats (SSRs), precluded the investigation of *T. aestivum* population genetics.

Recently, next-generation sequencing techniques have provided new prospects to develop microsatellite markers in nonmodel species, quickly and for relatively low costs (Abdelkrim et al., 2009; Csencsics et al., 2010; Perry and Rowe, 2011). 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.*

METHODS AND RESULTS

Total genomic DNA was isolated from an ascocarp of *T. aestivum* harvested in Haute-Saône (France) (MD4) (Appendix 1, group A). DNA was extracted from frozen tissues with a cetyltrimethylammonium bromide (CTAB) protocol available at the MYCORWEB website (http://mycor.nancy.inra.fr/). A total of 150 μg of genomic DNA was sent to Genoscope (Evry, France). Direct shotgun sequencing occurred in a 1/2 plate optimized run of 454 GS-FLX Titanium and produced 534 620 reads. The fasta and quality files generated were trimmed using the program "TrimSeq.pl" to trim raw sequencing reads at both ends based on moving averages of quality scores (phred = 20) and to remove reads smaller than 300 bp. After trimming and editing, 411 374 reads corresponding to 195 Mbp were retained for microsatellite identification. MISA (http://pgrc.ipk-gatersleben.de/misa/download/misa.pl) was used to identify di-, tri-, tetra-, penta-, and hexanucleotide simple sequence repeat markers. Using this software, a total of 7784 perfect microsatellites were identified (2519 di-, 3568 tri-, 1248 tetra-, 324 penta-, and 125 hexanucleotide repeat motifs). Using the MISA output files, only microsatellites beginning between 100 and 300 bp from the 5′ ends of the reads were selected. After this step, 181 microsatellites with more than 10 repeats were obtained. WebSat (Martins et al., 2009) and Primer3

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Table 1. Characteristics of the primers developed in *Tuber aestivum* for the 15 selected microsatellites.

Locus	5′ end-labeled dye	Primer sequences (5′–3′)	Repeat motif	Size range (bp)	T_a (°C)	EMBL accession no.
aest1	FAM	F: AAATAACGCTCCAGATCCCTTT R: TAGAGAGGTTCTAGCCGTCAGG	$(CCACTC)_{10}$	236–296	60	HE793313
aest6	FAM	F: CGTTTATTGTTCGCCTTTTCTC R: TAAGCTACTGAGCGACAGGTTG	$(AGTAAT)_6$	212–236	60	HE793314
aest7	FAM	F: GAGAACGTGATGTGTGATGGTT R: ATGAGATGCAAGCACTGTAGGA	$(ACAGC)_6$	256–286	60	HE793315
aest10	VIC	F: AATAAAGCACCAGATCACCACC R: GACTAGCGAAAGGGGATGAGTA	$(AGTAC)_6$	280–310	60	HE793316
aest15	VIC	F: GACTTTTCCGTCCACTTGTTTC R: GATCCATTCATCCATCCATACC	$(GGATG)_6$	297–322	60	HE793317
aest18	FAM	F: TATTCCTATCCCACACGACCAC R: AACATTGCTGTTTACGGCTCTT	$(ACTG)_{11}$	136–156	60	HE793318
aest24	VIC	F: TGAAAGTGTTAGAGATCGGCAA R: GTACTTCGCCCAGACAGAGAGT	$(TCA)_{18}$	292–319	60	HE793319
aest25	FAM	F: AGTTGTTTGTATGATGATGCCG R: ACTGGTATGACACGCTCCAAAT	$(ATT)_{10}$	122–140	60	HE793320
aest26	FAM	F: CAAGACAGAGACGCTAATCCCT R: GAGTATGCAATAATGGGTCCGT	$(TAT)_{11}$	124–178	60	HE793321
aest27	VIC	F: AAGTGTTAGAGATCGGCAAAGC R: CCTCACTCCAGACTTATCCCAC	$(TCA)_{18}$	314–341	60	HE793322
aest28	VIC	F: ACCTTCTTTTCCTGTGGCAAT R: AAATAGTCTGAGGGGAGTCGTG	$(TTA)_{18}$	344–452	60	HE793323
aest29	FAM	F: GAGTGGGGAGAATCACGTCTAC R: TATCCAAATGTTCCTGCACTTC	$(AGTAC)_6$	186–226	60	HE793324
aest31	VIC	F: TAGATACAGCACCGTAGCACCA R: TTTCGTAATTGTCTGTCGGTTG	$(CCTAC)_6$	285–325	60	HE793325
aest35	FAM	F: GTATGCGGTAGGTGGGTTTACT R: CTTGTGCTCGTACTCCACTTGT	$(TTTC)_{10}$	124–160	60	HE793326
aest36	VIC	F: ACAATACTCACCGACCTTTGCT R: CATGATACAACAGCATCCCG	$(GACT)_{13}$	310–350	60	HE793327

Note: EMBL = European Molecular Biology Laboratory; T_a = annealing temperature.

(Rozen and Skaletsky, 2000) were respectively used to check repeats and to design primers for 40 microsatellites randomly selected among the 181 microsatellites previously obtained. The PCR feasibility was checked using OligoAnalyzer 3.1 (http://eu.idtdna.com/analyzer/Applications/OligoAnalyzer/Default.aspx). Primers were tested in silico using AmplifX software (http://crn2m.univ-mrs.fr/pub/amplifx-dist).

To evaluate the microsatellite primers in a PCR reaction, two *T. aestivum* ascocarps were used. DNA was extracted with a DNeasy Plant Mini Kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions. All PCR amplifications were performed using 30 ng of template DNA in a final volume of 20 µL containing 10× PCR buffer (Invitrogen, Carlsbad, California, USA), 1 U of *Taq* DNA polymerase (Invitrogen), 0.125 mM each of dNTPs, 0.5 µM each of forward and reverse primers, and sterile water to adjust to the final volume. PCRs were conducted in a Mastercycler Gradient S PCR system (Eppendorf, Hamburg, Germany) after an initial denaturation of 3 min at 94°C, 30 cycles at 94°C for 25 s, 58°C for 25 s, and 72°C for 40 s, followed by 72°C for 15 min. PCR products were checked on a 1.5% agarose gel. According to Paolocci et al. (2006), the gleba of *Tuber* ascocarps is mainly composed of haploid maternal tissue and the DNA extracted from ascocarps therefore corresponds to a haploid tissue. For this reason, only the microsatellites producing a single band signal were selected for future investigations. Twenty-five primer pairs (62.5%) resulted in good amplification (i.e., one band for both ascocarps).

To identify polymorphic microsatellites, the 25 retained primer pairs were tested with 14 *T. aestivum* samples from different European countries (Appendix 1, group B). Genomic DNA from the 14 ascocarps was isolated from 20 mg of dried gleba as described above. PCR conditions were the same as those described above. PCR products were separated on a 4% agarose gel. Fifteen out of the 25 microsatellites (60%) showed size polymorphisms on 4% agarose gel. To assess more precisely the level of polymorphism, 15 microsatellites were used to perform a genotyping analysis using 5′ end-labeled primers (FAM or VIC) (Table 1). Seventy-five samples from seven European populations (Appendix 1, group C) were chosen to perform preliminary genotyping. PCR products were assayed, using 500 LIZ as a size standard, on an ABI 3730XL sequencer (Applied Biosystems, Foster City, California, USA) at the "Plateforme de Génotypage GENTYANE" (Clermont-Ferrand, France). All amplifications were carried out individually. Allele sizes were analyzed with GeneMapper (Applied Biosystems). Expected heterozygosity (H_e) was calculated using GenAlEx (Peakall and Smouse, 2006) (Table 2). As expected, due to the haploid nature of the ascocarp (Paolocci et al., 2006), each locus gave only one peak for all 75 samples. The number of different alleles per locus ranged from four to 15, and H_e ranged from 0.266 to 0.620. Finally, 63 different multilocus genotypes were obtained from the 75 *T. aestivum* isolates.

CONCLUSIONS

A DSP approach on a single *T. aestivum* ascocarp allowed for the identification of 7784 perfect microsatellites for 195 Mbp (nearly 40 microsatellites per Mbp). To our knowledge, our study is the first report of DSP to identify nuclear polymorphic microsatellite markers in an ectomycorrhizal fungal species. Indeed, until now, the DSP approach has only been performed on organisms belonging to the Animalia and Plantae kingdoms. This method is very quick and relatively inexpensive compared to the enriched library methods commonly used to develop polymorphic microsatellites. Indeed, with only 1/2 plate of a pyrosequencing run, we were able to identify several thousand microsatellites. DSP is therefore a useful approach to characterize polymorphic markers for nonmodel species. The markers developed in this study will enable future population genetic studies for this truffle species. Moreover, from a commercial point of view, population genetic studies could be useful to label some remarkable ecotypes showing particular alleles. Indeed, because the price of this truffle continues to increase and varies depending on geographical origin, a certificate of "true geographical origin" could be used to avoid fraud in the commercial truffle market.

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Table 2. Genotyping of the 15 microsatellites for 75 truffle samples belonging to seven *Tuber aestivum* populations.*

Locus	"Daix" (n = 23)			"Andal" (n = 9)			"Bedf" (n = 9)			"Drome" (n = 9)			"Tarsul" (n = 11)			"Sweden" (n = 6)			"Valsuz" (n = 8)		
	A	A_e	H_e	A	A_e	H_e	A	A_e	H_e	A	A_e	H_e	A	A_e	H_e	A	A_e	H_e	A	A_e	H_e
aest1	5	3.599	0.722	3	1.976	0.494	4	2.613	0.617	3	2.314	0.568	4	3.667	0.727	2	1.385	0.278	4	2.286	0.563
aest6	5	3.058	0.673	2	1.528	0.346	3	1.588	0.370	2	1.528	0.346	2	1.198	0.165	2	1.800	0.444	2	1.280	0.219
aest7	5	3.245	0.692	2	1.246	0.198	3	2.314	0.568	3	1.976	0.494	3	2.373	0.579	2	1.385	0.278	3	2.133	0.531
aest10	5	3.503	0.715	2	1.246	0.198	4	3.000	0.667	2	1.246	0.198	4	3.270	0.694	2	1.800	0.444	3	2.667	0.625
aest15	3	1.924	0.480	2	1.246	0.198	2	1.246	0.198	2	1.800	0.444	2	1.198	0.165	1	1.000	0.000	2	1.600	0.375
aest18	3	2.232	0.552	2	1.246	0.198	2	1.528	0.346	1	1.000	0.000	3	1.754	0.430	3	2.571	0.611	3	2.667	0.625
aest24	5	2.658	0.624	4	2.077	0.519	5	3.522	0.716	2	1.246	0.198	5	4.172	0.760	2	1.800	0.444	4	3.556	0.719
aest25	4	3.023	0.669	3	1.976	0.494	3	2.455	0.593	1	1.000	0.000	3	1.754	0.430	2	2.000	0.500	4	2.286	0.563
aest26	6	3.206	0.688	3	1.588	0.370	4	3.240	0.691	2	1.246	0.198	5	3.457	0.711	2	1.800	0.444	5	4.000	0.750
aest27	5	2.606	0.616	4	2.077	0.519	5	3.522	0.716	2	1.246	0.198	4	3.457	0.711	2	1.800	0.444	3	2.909	0.656
aest28	8	5.136	0.805	2	1.246	0.198	7	5.400	0.815	3	1.976	0.494	4	3.667	0.727	3	2.571	0.611	4	3.200	0.688
aest29	4	2.989	0.665	3	2.455	0.593	4	3.000	0.667	2	1.246	0.198	3	1.754	0.430	3	2.000	0.500	4	2.286	0.563
aest31	3	2.108	0.526	3	1.588	0.370	5	4.263	0.765	3	2.314	0.568	2	1.658	0.397	3	2.571	0.611	2	1.882	0.469
aest35	4	2.372	0.578	2	1.246	0.198	3	1.588	0.370	2	1.246	0.198	2	1.198	0.165	2	1.385	0.278	2	1.600	0.375
aest36	3	1.426	0.299	3	1.976	0.494	4	2.613	0.617	3	2.455	0.593	3	1.458	0.314	1	1.000	0.000	4	2.909	0.656

Note: A = number of alleles; A_e = number of effective alleles; H_e = expected heterozygosity; n = sample size.
* See Appendix 1 for locality information.

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Appendix 1. Locality information of the different samples and populations of *Tuber aestivum* used in this study.

Group	Sample/Population ID	Country, District*	Latitude*	Longitude*
Group A	MD4	France, Haute-Saône	47°55′12″N	6°4′48.9″E
Group B	E38	Hungary, Northern Great Plain (Debrecen)	47°31′47.906″N	21°38′21.6852″E
	E43	Luxembourg (Rumelange)	49°28′00″N	6°02′00″E
	E58	Switzerland (Lauzanne)	46°31′11.862″N	6°38′0.948″E
	E60	Turkey	ND	ND
	E62	China (Beijing)	39°54′15.1704″N	116°24′26.6″E
	E70	United Kingdom, Bedfordshire	52°8′9.5208″N	0°27′59.943″W
	E98	Romania (Bucharest)	44°26′15.7596″N	26°5′50.520″E
	E104	Sweden, Gotland (Roma)	57°30′28.944″N	18°27′1.116″E
	F1	France, Corsica (Santa Lucia di Moriani)	42°23′11.04″N	9°31′49.08″E
	F23	France, Drôme (Montjoyer)	44°28′38″N	4°51′10″E
	F38	France, Lozère (Montbrun)	44°20′17.02″N	3°30′15.98″E
	F68	France, Var (Ampus)	43°36′26.193″N	6°22′59.7″E
	F166	France, Aube (Vailly)	48°22′9.976″N	4°7′45.637″E
	UMF1	United Kingdom, Norfolk (Watton)	52°34′15.0522″N	0°49′40.882″E
Group C	"Daix"	France, Côte d'Or (Daix)	47°21′6.192″N	4°59′57.4542″
	"Andal"	Spain, Andalusia (Grenada)	37°10′35.3532″N	3°35′52.544″W
	"Bedf"	United Kingdom, Bedfordshire (Bedford)	52°8′9.5208″N	0°27′59.943″W
	"Drome"	France, Drôme (Montjoyer)	44°28′38″N	4°51′10″E
	"Tarsul"	France, Côte d'Or	47°31′57″N	4°59′3.4506″E
	"Sweden"	Sweden, Gotland (Roma)	57°30′28.944″N	18°27′1.116″E
	"Valsuz"	France, Côte d'Or (Val Suzon)	47°24′29″N	4°53′39″E

Note: ND = GPS coordinates not determined.

* Due to the confidentiality between authors and truffle providers, only GPS coordinates of the closest town or village are indicated instead of the precise location.

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