

Variability of nuclear and mitochondrial ribosomal DNA of a truffle species (*Tuber aestivum*)

THOMAS GUILLEMAUD^{1,2,4}, MICHEL RAYMOND², GABRIEL CALLOT³,
JEAN-CLAUDE CLEYET-MAREL¹ AND DIANA FERNANDEZ⁴

¹ Laboratoire de Recherche sur les Symbiotes des Racines, INRA, place Viala, 34060 Montpellier, France

² Institut des Sciences de l'Evolution (URA 327), Laboratoire Génétique et Environnement, Université de Montpellier II, CC065, 34095 Montpellier cedex 05, France

³ Laboratoire de Sciences du Sol, INRA, place Viala, 34060 Montpellier, France

⁴ Laboratoire de Phytopathologie Tropicale, ORSTOM, B.P. 5045, 34032 Montpellier cedex 01, France

The intraspecific genetic variability of *Tuber aestivum* was studied using molecular markers at various geographical scales. We used the polymerase chain reaction (PCR) coupled with restriction fragment length polymorphism (RFLP) analysis to examine the variation of the nuclear and mitochondrial ribosomal DNA (rDNA). RFLPs were found in the nuclear internal transcribed spacer (ITS) and three alleles were detected in the six populations analysed. No variability was found in mitochondrial rDNA. We found, in a very few cases, that truffles sharing different ITS genotypes could be present within a single symbiotic tree.

Tuber contains a number of economically important edible species of ectomycorrhizal fungi commonly known as 'truffles'. There is a limited amount of data available concerning the population biology of the *Tuber* species. In particular, the ecology and population dynamics of truffles have not been studied in detail. The mating system and the mode of spore dispersal are still unknown, although some mammals or insects are suspected to act as potential vectors. This general lack of ecological knowledge may account for the difficulty in artificially inoculating trees with the black truffle, *Tuber melanosporum* (Chevalier, Giraud & Bardet, 1982).

The spatial distribution of genetic variation in populations has started to be analysed in truffle species. Using protein electrophoresis, Pacioni & Pompini (1989, 1991) and Pacioni *et al.* (1993) found genetic homogeneity within *Tuber aestivum* populations and little variability between populations. Henrion, Chevalier & Martin (1994) used molecular markers (rDNA spacers) and found little variation among 11 strains of *T. melanosporum*. However, as these results are based on a small sample size no general conclusions can be drawn.

The present study was undertaken to examine the genetic variability in one truffle species, *T. aestivum*. This species was particularly well suited for this work as it is not the focus of artificial introduction and thus natural populations are probably not affected by human activities. We used nuclear and mitochondrial ribosomal DNA (rDNA) polymorphism to analyse the genetic variability in natural populations at different geographical scales including the brûlé (a 'brûlé' represents a particular surface around the symbiotic tree where plants are scarce, as if they were burnt, and where truffle ascocarps are present), the local and regional scale.

MATERIALS AND METHODS

Ascocarps from *Tuber aestivum* Vittad. were collected in three distinct regions of Southern France: Languedoc, Provence and Drôme (Fig. 1), between June 1993 and March 1994. In each region, one to four independent sites (brûlés) were sampled: St Drézéry (brûlé 1), Viols le Fort (brûlé 2), Buzygnargues (brûlé 3) and Saussines (brûlé 4) in Languedoc; Vallée du Roubion (brûlé 5) in Drôme; Eguilles (brûlé 6) in Provence. For four sites, the precise location of each truffle and its relative distance to the symbiotic tree were recorded. A total of 87 truffle ascocarps were sampled, between 4 and 34 ascocarps in each site (Table 2). The peridium was removed from the

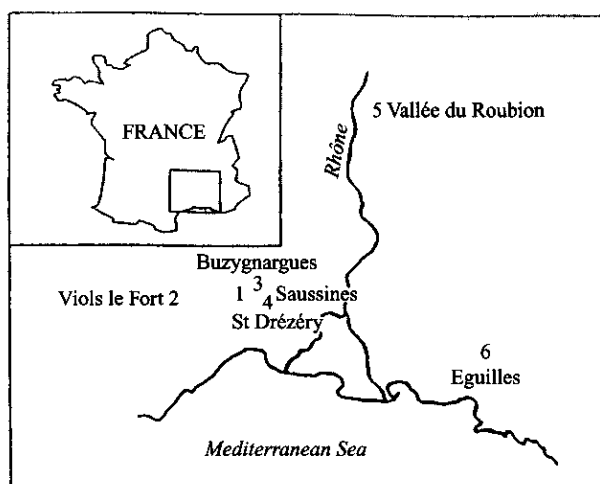


Fig. 1. Geographical location of sampled sites in Southern France.

Table 1. Pairs of primers used for PCR amplification of the three rDNA loci. Sequences are written from 5' to 3'

Primers	Locus
ITS1-F*	ITS1
ITS2†	
ITS1-F*	ITS
ITS4†	
ML5†	LSU
ML6†	
ML7†	LSU
Ctb9‡ (CGGAGGATAGGATAACTCG)	
Ms1†	SSU
Ms2†	
Mt1 (TACGTGCCAAGCAGTCGCGGTAATACG)	SSU
Mt2 (TACTCTTGAGGTGGAATGCTTACAC)	

* Gardes & Bruns (1993).

† White *et al.* (1990).

‡ Gardes, pers. comm.

ascocarps, and they were lyophilized and stored for further analysis.

DNA was extracted from individual lyophilized ascocarps using a quick CTAB miniprep protocol (M. Gardes, pers. comm.). Approximately 20 mg of fungal material were pulverized and suspended in 500 µl of 2 × CTAB extraction buffer (100 mM Tris-HCl, pH 8.0, 1.4 M NaCl, 20 mM EDTA, 2% CTAB, 0.2% mercaptoethanol) for 1 h at 65 °C. After centrifugation (15 min, 12 000 g), 300 µl of the supernatant were gently mixed with 600 µl isopropanol. DNA was then pelleted by centrifugation (10 min, 12 000 g) and washed with 300 µl of 70% ethanol and pelleted again. The air-dried pellet was resuspended in 100 µl of TE buffer (10 mM Tris-HCl, pH 7.5, 0.1 mM EDTA, pH 8.0). The quality and the quantity of extracted genomic DNA were checked by 0.8% agarose (Gibco-BRL, Eragny, France) gel electrophoresis in TAE buffer (40 mM Tris-acetate, 1 mM EDTA, pH 8.0). Gels stained with ethidium bromide (0.5 µg ml⁻¹) were photographed on a uv transilluminator. DNA samples were diluted 50-, 100- and 200-fold in distilled water for use in PCR experiments.

Six pairs of primers were used in the PCR to amplify three nuclear and mitochondrial loci (Table 1). Primers ITS2, ITS4, ML5, ML6, ML7, Ms1 and Ms2 were described by White *et al.* (1990); primer ITS1-F was described by Gardes & Bruns (1993) for enhanced specificity to filamentous fungi; primer Ctb9 was obtained from M. Gardes (pers. comm.). Finally, primers Mt1 and Mt2 were designed by comparing the mitochondrial rDNA sequences of 25 species belonging to seven ascomycetous genera deposited in the EMBL databank. These primers corresponded to 100% conserved regions of the SSU region for the 25 species.

PCR conditions were those of White *et al.* (1990) with slight modifications. The amplifications were conducted using 5–10 µl DNA template solution in 50 µl of reaction mixture. The final solution contained 10 µM each of dATP, dCTP, dGTP and dTTP (Amersham, Les Ulis, France), 2 µM each of

the primers, and 1 unit of thermostable DNA polymerase (Goldstar Polymerase, Eurogentec, Louvain-la-Neuve, Belgium) in 1 × Goldstar reaction buffer. A negative control was included for each reaction in order to detect the presence of DNA contamination. The amplification was performed with a DNA thermal cycler, either a Cyclone (Integra Bioscience, Eaubonne, France) or a Thermojet (Eurogentec). The cycling conditions consisted of an initial denaturation step at 95 °C for 3 min followed by 25–30 amplification cycles of 1 min at 95 °C, 1 min at 50–55 °C (depending on the primers used) and 1 min at 72 °C. Ten microlitres of the amplification products were visualized following 1.5 or 2% (w/v) agarose gel electrophoresis.

Between 5 and 15 µl of amplified DNA was digested, without further purification, for restriction fragment length polymorphism (RFLP) analysis. The final solution of 25 µl contained 10–20 units of restriction enzymes (Eurogentec), 0.1 × volume of 10 × restriction buffer supplied by the manufacturer. The solutions were incubated for 1–5 h at the optimal temperature recommended. For each PCR product, 11 arbitrarily chosen restriction enzymes were used: *Alu* I, *Msp* I, *Hae* III, *Taq* I, *Hha* I, *Rsa* I, *Bsh* 1236, *Hinf* I, *Hind* III, *Sau* 3A and *Sau* 96I. The restriction digests were then electrophoresed for 4 h on 4% (w/v) Nusieve (Tebu, Le Perray en Yvelines, France) or 4% agarose/Nusieve (1/2, w/w) gels. All amplifications and digests were performed twice for verification.

In order to confirm the nature of the products amplified with ITS1-F/ITS4 primers, 5 µl of the amplified DNA of a randomly chosen ascocarp were labelled with [³²P]dCTP and probed on a Southern blot of *Eco*R I-digested genomic DNAs of 18 truffles (three representatives of each population). Southern blot preparation, probe labelling and hybridization conditions were as described in Fernandez *et al.* (1994). After film exposure, the hybridized membrane was carefully stripped of the radioactive probe according to the manufacturer's specifications (Amersham, Les Ulis, France) and re-hybridized with the cloned-rDNA repeat unit of *Sordaria macrospora* provided by Landry and Lechevantou (Orsay, France).

RESULTS AND DISCUSSION

All DNA regions were successfully amplified in *T. aestivum*, with the exception of the mitochondrial LSU which did not yield clear amplification results with both primer pairs ML5/ML6 and ML7/Ctb9.

The mitochondrial rDNA SSU was amplified using Mt1/Mt2 primers only, and produced a monomorphic fragment for the 87 ascocarps tested. PCR experiments with primers MS1/MS2 (White *et al.*, 1990) were unsuccessful, producing multiple DNA fragments and smears. This is probably due to the small number (two) of ascomycetous species used by White *et al.* (1990) to design or test these primers.

The nuclear rDNA of ITS1 and ITS were amplified using primers ITS1-F/ITS4 and ITS1-F/ITS2, respectively. In both cases, we successfully amplified *T. aestivum* DNA indicating that the 18S, 5.8S and 28S regions were conserved enough to match with these primers. Hybridization with the heterologous

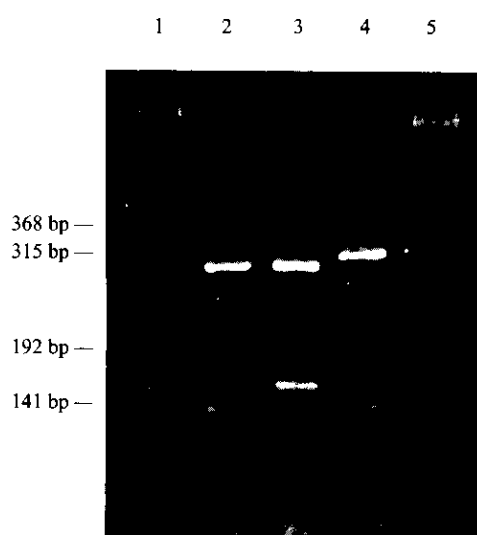


Fig. 2. RFLP analysis of the amplified nuclear rDNA ITS digested with the restriction enzyme *Msp* I. Lane 2, ascocarps from brûlé 1 (pattern A); lane 3, brûlé 6 (pattern B); lane 4, brûlé 5 (pattern C); lanes 1 and 5, fragment size markers (*Hae* II-digested pBR322 DNA and *Taq* I-digested pBR322 DNA).

nuclear rDNA probe confirmed that the amplified products contained rDNA sequences (data not shown).

Size variation and RFLPs using 11 restriction enzymes were assayed on the PCR products of mitochondrial SSU, nuclear ITS1 and ITS for the 87 *T. aestivum* ascocarps. For all ascocarps, the SSU, ITS1 and ITS produced no PCR amplification length variants, indicating that insertion/deletion polymorphisms were absent in these regions. Respectively, the sizes of the PCR products for ITS1, ITS and SSU were 330, 730 and 330 bp. For all ascocarps, none of the 11 restriction enzymes tested was able to cut the SSU fragment. This indicates that no restriction sites were present for these enzymes and that nucleotide divergence was not abundant in this region. In contrast all the restriction enzymes were able to cut ITS and RFLPs were observed with *Msp* I. Three *Msp* I-restriction patterns were obtained (Fig. 2), designated A, B and C, which differed in the size of the two highest molecular weight DNA fragments, either 310 and 140 bp (pattern A), or 310 and 150 bp (pattern B), or 320 and 140 bp (pattern C). This variation most likely corresponds to a difference of a few nucleotides rather than to a deletion-insertion event because no size variation was detected in the ITS region before or after digestion with the 10 non polymorphic restriction enzymes. To ensure a constant size (730 bp) of the non-digested ITS, the existence of a 10 bp band in profile A must be assumed. This low molecular weight band could not be detected in our electrophoresis conditions. For each ascocarp, the total length of the bands obtained by RFLP of the ITS was never greater than 730 bp. Each restriction profile thus corresponded to three distinct ITS alleles.

The amount of variability detected in the ITS is low and is compatible with results of previous work on other fungal species (Lee & Taylor, 1992; DePriest, 1993; Feibelman, Bayman & Cibula, 1994). This low variation would be negligible compared with high interspecific polymorphism

Table 2. Distribution of ITS alleles in the six 'brûlés' of the three regions sampled in France

Region	Allele			N*
	A	B	C	
Languedoc				
Brûlé 1	34	0	0	34
Brûlé 2	4	0	5	9
Brûlé 3	25	0	0	25
Brûlé 4	0	0	4	4
All	63	0	9	72
Provence				
Brûlé 5	0	0	9	9
Drôme				
Brûlé 6	0	5	1	6
All	63	5	19	87

* N refers to sample size.

when ITS is studied as a truffle species marker (Henrion *et al.*, 1994).

The populations of four 'brûlés' exhibited ITS monomorphism despite a relatively high sample size for two of them (brûlés 1 and 3 with 34 and 25 truffles, respectively) (Table 2). Two brûlés (2 and 6) displayed ITS polymorphism, indicating that multiple independent associations could take place within a single tree (Table 2). In these two sites, truffles sharing the same genotype were from the same location within a 'brûlé' and were spatially distant from truffles of the other genotype (maps not shown).

Several studies on fungi have shown high variability in the nuclear rDNA spacers, the mtDNA SSU and the mtDNA LSU when comparing different species (White *et al.*, 1990; Gardes *et al.*, 1991; Henrion, Le Tacon & Martin, 1992; Simon, Bousquet & Lévesque, 1993; Feibelman, Bayman & Cibula, 1994). However, very few studies have used these loci to analyse intraspecific polymorphism (Gardes *et al.*, 1990; Gardes, Wong & Fortin, 1990; Lee & Taylor, 1992; DePriest, 1993; Feibelman, Bayman & Cibula, 1994) and hence no population studies have been carried out using rDNA spacers or mitochondrial ribosomal DNA. We have shown here that ITS presents intraspecific polymorphism in the Tuberales. The genetic variation among individuals is low but sufficient to distinguish genotypes within a 'brûlé'.

Within a 'brûlé', multiple and independent colonizations of a tree are thus possible. Additional polymorphic loci are required to study the genetic structure of truffle populations, to allow the analysis of population differentiation and mating system of *T. aestivum*.

We thank M. Gardes (University of California, Berkeley, U.S.A.) for kindly providing most of the primers tested and for helpful comments on PCR experimental design, F. Rousset (Institut des Sciences de l'Evolution, Montpellier, France) for help in the design of the Mt1 and Mt2 primers, and M. Quinten (ORSTOM, Montpellier, France) for technical assistance. The authors are grateful to J. P. Braye, A. Jourdan, L. Brugière and G. Serane for their assistance in harvesting of ascocarps. This work was funded by the Chambre d'Agriculture

de l'Hérault, France and by the INRA (AIP INRA, 4757-1 4770). This is contribution 96.006 of the ISEM (URA 327).

REFERENCES

- Chevalier, G., Giraud, M. & Bardet, M. C. (1982). [Interaction between *Tuber melanosporum* mycorrhizae and other ectomycorrhizal fungi in soils favourable to truffle.] *Les Colloques de l'INRA* **13**, 313–321. [In French.]
- DePriest, P. T. (1993). Small subunit rDNA variation in a population of lichen fungi due to optional group-I introns. *Gene* **134**, 67–74.
- Feibelman, T., Bayman, P. & Cibula, W. G. (1994). Length variation in the internal transcribed spacer of ribosomal DNA in chanterelles. *Mycological Research* **98**, 614–618.
- Fernandez, D., Assigbetse, K., Dubois, M. P. & Geiger, J. P. (1994). Molecular characterization of races and vegetative compatibility groups in *Fusarium oxysporum* f. sp. *vasinfectum*. *Applied and Environmental Microbiology* **60**, 4039–4046.
- Gardes, M. & Bruns, T. (1993). ITS primers enhanced specificity for basidiomycete – application to the identification of mycorrhizae and rusts. *Molecular Ecology* **2**, 113–118.
- Gardes, M., Fortin, J. A., Mueller, G. M. & Kropp, B. R. (1990). Restriction fragment length polymorphism in the nuclear ribosomal DNA of four *Laccaria* spp.: *L. bicolor*, *L. laccata*, *L. proxima*, and *L. amethystina*. *Phytopathology* **80**, 1312–1317.
- Gardes, M., Mueller, G. M., Fortin, J. A. & Kropp, B. R. (1991). Mitochondrial DNA polymorphisms in *Laccaria bicolor*, *L. laccata*, *L. proxima* and *L. amethystina*. *Mycological Research* **95**, 206–216.
- Gardes, M., Wong, K. & Fortin, J. A. (1990). Interactions between monokaryotic and dikaryotic isolates of *Laccaria bicolor* on roots of *Pinus banksiana*. *Symbiosis* **8**, 233–250.
- Henrion, B., Chevalier, G. & Martin, F. (1994). Typing truffle species by PCR amplification of the ribosomal DNA spacers. *Mycological Research* **98**, 37–43.
- Henrion, B., Le Tacon, F. & Martin, F. (1992). Rapid identification of genetic variation of ectomycorrhizal fungi by amplification of ribosomal RNA genes. *New Phytologist* **122**, 289–298.
- Lee, S. B. & Taylor, J. W. (1992). Phylogeny of five fungus-like protocistan *Phytophthora* species, inferred from the internal transcribed spacers of ribosomal DNA. *Molecular Biology and Evolution* **9**, 636–653.
- Pacioni, G., Frizzi, G., Miranda, M. & Visca, C. (1993). Genetics of a *Tuber aestivum* population. *Mycotaxon* **7**, 93–100.
- Pacioni, G. & Pomponi, G. (1989). Chemotaxonomy of some Italian species of *Tuber*. *Micologia e Vegetazione Mediterranea* **4**, 63–72.
- Pacioni, G. & Pomponi, G. (1991). Genotypic patterns of some Italian populations of the *Tuber aestivum*–*mesentericum* complex. *Mycotaxon* **42**, 171–179.
- Simon, L., Bousquet, J. & Lévesque, R. C. (1993). Origin and diversification of endomycorrhizal fungi and coincidence with vascular land plants. *Nature* **363**, 67–69.
- White, T. J., Bruns, T. D., Lee, S. & Taylor, J. W. (1990). Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In *PCR Protocols: A Guide to Methods and Applications* (ed. M. A. Innis, D. H. Gelfand, J. S. Sninsky & T. J. White), pp. 315–322. Academic Press: San Diego, California.

(Accepted 16 October 1995)