oubliée au profit d'une autre analyse biochimique plus simple : l'analyse des isoenzymes.

Analyse des Isoenzymes

Les isoenzymes sont des enzymes ayant un substrat commun, mais qui diffèrent par leur mobilité électrophorétique. En fait, elles peuvent correspondre à l'expression de plusieurs gènes situés sur plusieurs loci, aux produits d'une même protéine ayant subi des transformations ou des molécules synthétisées par un même gène ou un groupe de gènes et ayant subi des modifications post-traductionnelles. Elles reflètent donc l'expression des gènes. Les isoenzymes sont utilisées pour identifier les espèces de truffes mais aussi pour étudier la variabilité génétique, nous reviendrons dans le § 1.4.3 sur ces études.

C'est en 1988 au Congrès International sur les Truffes que Palenzona et al. présentent, pour la première fois, l'utilisation des isoenzymes pour identifier les espèces de truffes. Pacioni et Pomponi (1989, 1991) et Gandeboeuf et al. (1994) ont montré qu'il est possible de séparer chaque espèce de *Tuber* par son profil enzymatique et, entre autres, *T. aestivum* et *T. mesentericum*, confirmant ainsi les données morphologiques (Pacioni et Pomponi, 1991). Parmi les enzymes utilisées, les profils enzymatiques obtenus par le système SOD sont homogènes au sein d'une même espèce et hétérogènes entre espèces différentes (Gandeboeuf et al., 1994 ; Chevalier et al., 2000). Gandeboeuf et al. (1994) ont réussi à différencier *T. brumale* de *T. moschatum*.

Superoxide dismutase : SOD
Malate deshydrogenase : Mdh
Isocitrate deshydrogenase : Idh
6-phosphogluconate dehydrogenase : 6-Pgdh
Glutamate deshydrogenase : Gdh
Menadione reductase : Mr
Asparate aminotransferase : Aat
ß-glucosidase : ß-Glu
Leucine aminopeptidase : Lap
Peptidase : Pep
Mannose phosphate isomérase : Mpi
Glucose phosphate isomérase : Gpi
Phosphoglucomutase : Pgm

Urbanelli et al. (1998a) ont réalisé l'analyse de 18 loci enzymatiques (Mdh-1, Mdh-2, Idh-1 6-Pgdh, Gdh, Mr-1, Sod-1, Sod-2, Sod-3, Aat-1, Aat-2, ß-Glu, Lap, PepC, PepD, Mpi, Gpi et Pgm), sur 835 ascocarpes récoltés dans 26 localités espagnoles, françaises et italiennes. Ces auteurs identifient 20 types électrophorétiques qui se regroupent en 7 groupes correspondant à 7 espèces de truffes (Figure 19).

En revanche, ils n'ont pas pu différencier T. aestivum de T. uncinatum, confirmant ainsi les résultats de Pacioni et Pomponi (1991) et de Pacioni et al. (1993).

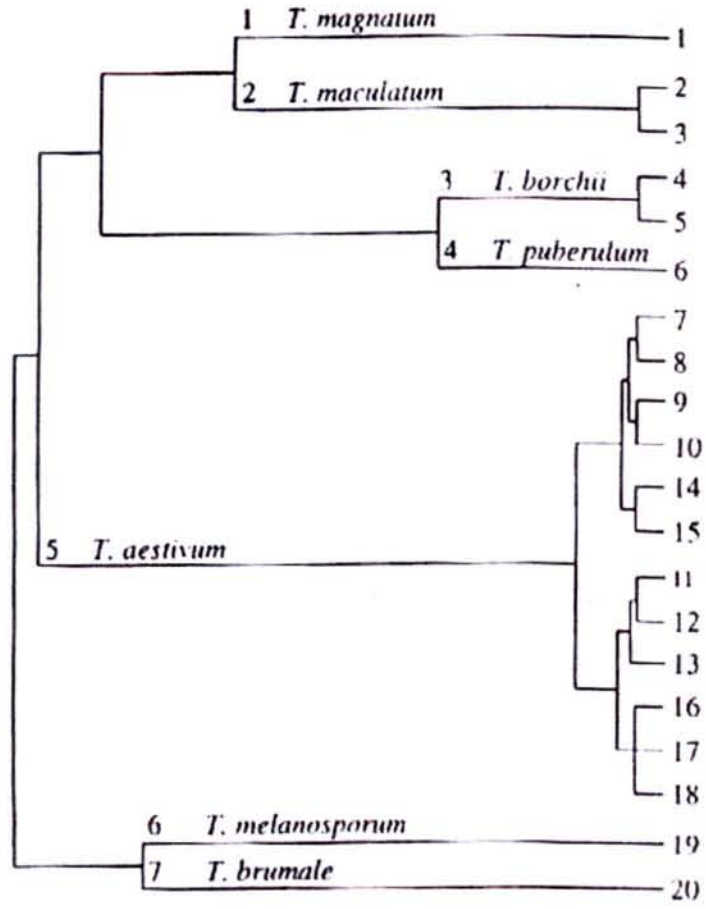


Figure 19 Dendogramme UPGMA, généré avec les distances génétiques de Nei, montrant les relations entre les 20 types enzymatiques et permettant de séparer 7 taxons de *Tuber* (d'après Urbanelli et al., 1998a)

De même, Dupré (1997) n'a pas pu séparer ces deux taxa en analysant 5 systèmes enzymatiques (Acp, Gdh, Gpi, Mdh et Sod), or ils avaient pu être séparés par l'analyse des protéines totales (Mouchès et al, 1981 ; Dupré et al., 1985). Urbanelli *et al.* (1998a) ont aussi rencontré des difficultés pour séparer *T. brumale* et *T. moschatum* alors que Gandeboeuf et al. (1994) et Dupré (1997) sont arrivés à séparer ces deux taxa qui ne peuvent être différenciés que par l'odeur des corps fructifères (Riousset et al., 2001).

Contrairement à l'analyse des protéines totales, les 5 systèmes enzymatiques étudiés par Dupré (2000) montrent une parfaite correspondance entre les cultures mycéliennes et les ascocarpes.

D'autre part, les isoenzymes peuvent être utilisés au niveau des mycorhizes. Urbanelli et al. (1998b) ont analysé quatre systèmes enzymatiques (NADP-Gdh, ß-Glu, Gpi et Pgm-1) permettant ainsi l'identification des mycorhizes de *T. melanosporum*, *T. aestivum* et *T.*

magnatum. De même, Dupré (1997) montre qu'il est possible d'analyser la NADP-Gdh au niveau des mycorhizes, mais le nombre d'apex nécessaire pour avoir une activité enzymatique suffisante s'est révélé important, ce qui rend difficile l'utilisation de cette technique.

Les méthodes d'analyse biochimique ont permis, pour la première fois, de vérifier la clé de classification morphologique des Tuber. De plus, elles sont applicables sur des cultures pures de mycélium, voire sur des apex racinaires. Cependant, malgré cela elles présentent des contraintes expérimentales limitantes: elles nécessitent de grosses quantités de matériel et ce dernier ne doit pas avoir subi de modifications. De plus, elles n'ont pas permis de régler certaines ambiguïtés taxonomiques comme cela est le cas pour T. uncinatum et T. aestivum et elles ne sont pas très efficaces pour identifier les mycorhizes. Il a donc fallu se pencher sur de nouvelles techniques plus performantes comme l'analyse moléculaire du génome.

1.4.2.2 Méthodes moléculaires

Méthodes d'amplification aléatoire

C'est Lanfranco et al, qui, en 1993, commencent à utiliser les techniques de biologie moléculaire pour la caractérisation des *Tuber*. En effet, ces auteurs ont mis en évidence un haut degré de diversité interspécifique par amplification RAPD (la similarité des profils interspécifiques est comprise entre 2,5% et 17,4%) pour six espèces de *Tuber* : *T. magnatum*, *T. melanosporum*, *T. albidum*, *T. aestivum*, *T. macrosporum* et *T. rufum*. En revanche, le niveau de diversité intraspécifique pour *T. magnatum* est beaucoup plus faible (similarité entre les profils de 80 à 90%). Cette même technique a été utilisée par Potenza et al. (1994) pour l'identification de *T. magnatum*, *T. maculatum* et *T. borchii*. Ces auteurs ont réussi à identifier un fragment spécifique de *T. magnatum*. Dans un second temps, ce fragment a été utilisé comme sonde pour l'identification des ascocarpes par hybridation.

Comme nous l'avons vu, la RAPD peut être utilisée pour différencier plusieurs espèces de *Tuber*, surtout celles ayant un niveau de diversité intraspécifique faible comme *T. magnatum*, *T. melanosporum*, *T. brumale* et *T. moschatum* (Gandeboeuf et al., 1997 ; Chevalier et al., 2000). Cependant il existe des problèmes avec les espèces ayant un haut niveau de diversité intraspécifique comme, par exemple, *T. aestivum, T. uncinatum*, *T. borchii*, *T. rufum*, et *T. excavatum* (Gandeboeuf et al., 1997 ; Dupré, 1997). D'autre part, la RAPD présente plusieurs désavantages. Tout d'abord, elle ne peut pas être utilisée au niveau des mycorhizes car

lors de la PCR, l'ADN de la plante est aussi amplifié. Il existe aussi des problèmes de contamination des profils par l'ADN d'autres organismes (bactéries, champignons, levures) comme cela a été montré par Rabouam et al. (1999) et Murat (2001). Enfin, il peut aussi y avoir des problèmes de reproductibilité des profils. Ceci a conduit Longato et Bonfante (1997) à utiliser une autre technique dérivée de la RAPD, la RAMS (Tableau 8). Au lieu d'utiliser une amorce arbitraire, ces auteurs utilisent une amorce microsatellitaire $(GTG)_5$ qui a permis de différencier les 11 espèces de truffes étudiées. Cette même amorce a été utilisée par Murat (2001) pour identifier *T. melanosporum*, car elle permet de mettre en évidence d'importantes variations intrerspécifiques, alors que les profils obtenus pour cette espèce (= intraspécifique) sont très homogènes.

Enfin, Mello et al. (2002) ont réussi à distinguer T. uncinatum de T. aestivum en utilisant les amorces $(GTG)_5$, $(GAC)_5$, $(AAG)_8$ et $(AAC)_8$ (Figure 20).

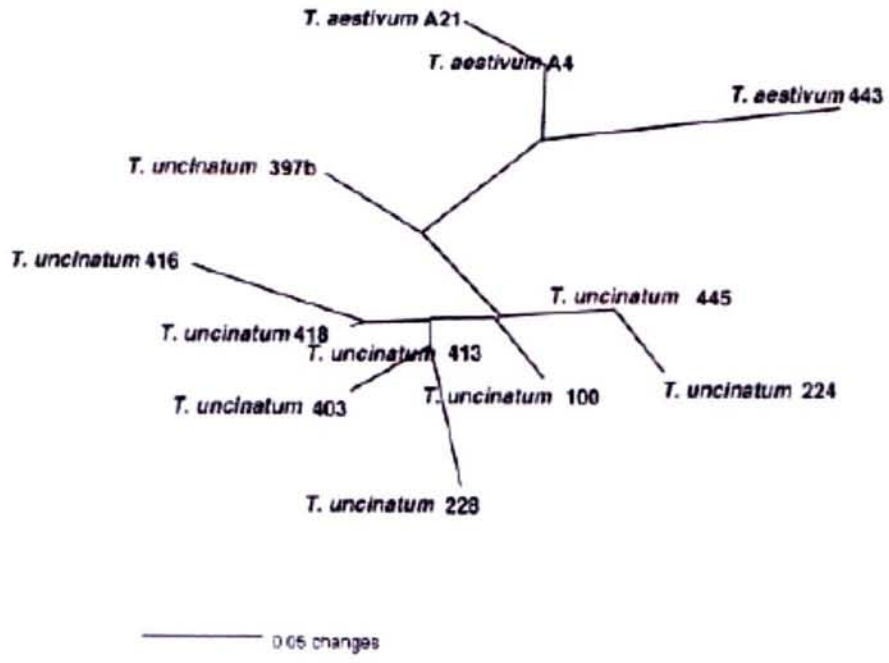


Figure 20. Arbre Neighbour-Joining obtenu avec les amorces microsatellites sur 12 ascocarpes *de T. uncinatum* et *T. aestivum* (Mello et al., 2002).

Les techniques d'amplification aléatoire permettent d'identifier un certain nombre d'espèces morphologiques, mais l'existence d'importantes variations intraspécifiques pour certaines d'entre elles rendent ces méthodes inadéquates. De plus, leur utilisation afin de génotyper les tissus fongiques sur les apex ectomycorrhizés est impossible compte tenu de la présence de l'ADN de la plante. D'autres techniques de génotypage utilisant l'amplification PCR d'un locus bien défini du génome fongique se sont rapidement développées.

Analyse moléculaire par PCR dirigée

L'utilisation de la PCR dirigée a comme principal avantage d'être utilisable sur toutes les phases du cycle biologique des truffes (ascocarpes, mycélium et ectomycorhizes), nous verrons aussi qu'elle peut être utilisée sur des aliments pour éviter les fraudes. L'ADN ribosomique et, entre autre, l'*Internal transcribed spacer* (ITS) est la région la plus utilisée. Plusieurs raisons conduisent à choisir ce locus: (i) il existe toute une série d'amorces universelles pour l'amplifier (White et al., 1990 ; Gardes et Bruns, 1993) ; (ii) il s'agit de régions répétées dans le génome ce qui facilite son amplification (Cassidy et al., 1984) ; (iii) généralement, il existe un grand polymorphisme interspécifique, alors que le polymorphisme intraspécifique est beaucoup plus limité (Selosse, 1998).

Le polymorphisme de l'ITS des *Tuber* a été mis en évidence par restriction enzymatique (RFLP) (Henrion et al., 1994 ; Amicucci et al, 1996 ; Paolocci et al., 1999 ; Roux et al., 1999), par hybridation avec une sonde *(southern blot)* (Paolocci et al., 1995), puis par séquençage direct (Roux et al., 1999 ; Mabru et al., 2001 ; Rubini et al., 2001 ; Mello et al., 2001 ; Mello et al., 2002 ; Paolocci et al., 2004).

A l'exception de *T. brumale*, qui a un ITS d'environ 1000 pb, toutes les autres espèces présentent une bande à 600-680 pb (Henrion et al., 1994 ; Roux et al., 1999). Henrion et al. (1994) ont pu différencier par RFLP de l'ITS avec *Alu1 / Hinf1* et *Rsa1 / Mbo1* plusieurs espèces : *T. borchii*, *T. brumale*, *T. excavatum*, *T. ferrugineum*, *T. magnatum*, *T. melanosporum* et *T. rufum*. Ces auteurs ont aussi utilisé *l'Intergenic spacer* (IGS) pour différencier les mêmes espèces. Ces résultats ont été confirmés par séquençage de l'ITS (Roux et al., 1999).

A l'exception de Mello et al. (2002), qui ont distingué *T. uncinatum* de *T. aestivum* par séquençage de l'ITS, les autres études basées sur ITS/RFLP et IGS / RFLP (Henrion et al., 1994), le séquençage de l'ITS, de la ß-tubuline et du facteur d'élongation 1∝ (Paolocci et al., 2004) n'ont pas réussi à les distinguer.

En fait, la majorité des analyses se sont focalisées sur les espèces les plus intéressantes économiquement (*T. magnatum* et *T. melanosporum*). En effet, le développement des techniques de trufficulture nécessite des outils pour vérifier l'identité du champignon colonisant les apex racinaires des plants inoculés expérimentalement. Ces études ont conduit à la mise au point d'amorces spécifiques définies dans l'ITS ou bien correspondant à des régions amplifiées lors de la RAPD qui ont été clonées et séquencées (SCAR : *Sequence Characterized Amplified Random*) (Tableau 9).

Tableau 9. Amorces spécifiques de quelques espèces de *Tuber*, cette liste n'est pas exhaustive.

Espèce	Locus	Nom des amorces	Référence
T. uncinatum*-*T. aestivum	ITS	TulTS1-TulTS4	Luis, 2000
	ITS	UncI-UncII	Mello et al. (2002)
	SCAR	Tu1400f-Tu1400r	Luis, 2000
	SCAR	Tu1800f-Tu1800r	Luis, 2000
T. melanosporum	ITS	ITSML-ITS4LNB	Paolocci et al. (1999)
	ITS	MELF-MELR	Douet et al. (2004)
T. brumale	ITS	ITSB-ITS4LNB	Paolocci et al. (1999)
	ITS	SYLV1-SYLV2	Douet et al. (2004)
T. indicum	ITS	ITSCHCH-ITS4LNB	Paolocci et al. (1999)
	ITS	IndF1-IndR	Mabru et al. (2001)
	ITS	IndF2-IndR	Mabru et al. (2001)
T. magnatum	ITS	ITSMAGN-ITSBACK3	Rubini et al. (2001)
	ITS	TmagI-TmagII	Amicucci et al. (1998)
	ITS	P7-M3	Mello et al. (2001)
	SCAR	TAR I-TAR II	Amicucci et al. (1996)
T. puberulum	ITS	TpuI-TpuII	Amicucci et al. (1998)
T. dryophilum	ITS	TdryI-TdryII	Amicucci et al. (1998)
T. maculatum	ITS	TmacI-TmacII	Amicucci et al. (1998)
T. borchii	ITS	TbolI-TbolII	Amicucci et al. (1998)
	ITS	TBA-TBB	Mello et al. (2000)

Le développement de ces techniques de diagnostic moléculaire permet également de vérifier quelle espèce de truffe est utilisée dans les aliments en conserve. En effet, il existe dans cette filière agroalimentaire un risque de fraude, par exemple, *T. brumale* ou *T. indicum* sont employées à la place de *T. melanosporum*. Douet et al. (2004) ont testé des amorces PCR spécifiques définies dans l'ITS (Tableau 9) pour *T. melanosporum*, *T. brumale* et *T. indicum*. Après optimisation de l'extraction d'ADN et de la réaction PCR, ces auteurs ont réussi à amplifier ces trois espèces sur des ascocarpes qui avaient été stérilisés 1h30 à 115°C. Dans un travail similaire, Mabru et al. (2004) ont optimisé l'extraction de l'ADN ribosomique mitochondrial et dessiné les amorces (ADL1-ADL3) amplifiant neuf espèces de *Tuber*. Cette technique permet de détecter jusqu'à 5% de truffes contaminant une préparation cuisinée de *T. melanosporum*.

Existe-t-il encore des espèces de truffes qui soient difficiles à séparer ?

Nous pouvons conclure en disant que ces méthodes de PCR dirigée permettent d'identifier la majorité des espèces de truffes dans leurs différentes phases du cycle biologique. Il est même maintenant possible de détecter des contaminations par d'autres espèces dans les aliments. Toutefois, il persiste encore des doutes sur l'existence de certaines espèces morphologiques et principalement entre *T. uncinatum* et *T. aestivum*. (Encadré 10). Comme nous l'avons vu, Mouchès et al. (1978 et 1981), par l'analyse des protéines totales, et Mello et al. (2002), par une analyse avec des amorces microsatellites et le séquençage de l'ITS, sont parvenus à séparer ces deux taxa.

En revanche, l'analyse des isoenzymes (Pacioni et Pomponi, 1991 ; Pacioni et al., 1993 ; Dupré, 1997), de l'ITS/RFLP (Henrion et al., 1994) et du séquençage de l'ITS, de la ß-tubuline et du facteur d'élongation 1-∝ (Rubini et al., 2004) ne sont pas parvenus à les distinguer.

Parmi les autres taxa posant problème (cf §1.1.3), *T. brumale* et *T. moschatum* sont séparés par les isoenzymes (Gandeboeuf et al., 1994), alors qu'ils ne le sont pas par RAPD (Gandeboeuf et al., 1997). En fait, ces deux taxa sont très proches et sont considérés comme deux variétés de la même espèce par Riousset et al. (2001).

T. hiemalbum et *T. melanosporum*, ne sont pas différenciées par l'analyse moléculaire (Riousset et al., 2001). Mais *T. hiemalbum* n'est pas un taxon très étudié car il est peu fréquent. Même si l'existence de ce taxon est très controversée, Riousset et al. (2001) le considèrent comme valide de par ses caractéristiques morphologiques.

Encadré 10 *T. aestivum* Vittad. et *T. uncinatum* Chatin deux taxa très discutés (d'après Chevalier et al., 1994).

Les truffes du groupe *aestivum* sont l'objet d'une controverse dont l'origine remonte à 1887, année où le mycologue français Chatin a décidé de scinder l'espèce *T. aestivum*, définie plus tôt par Vittadini (1831), en deux taxons distincts : *T. aestivum* Vittad. et *T. uncinatum* Chat. Chatin différenciait les deux espèces par les critères suivants :

- Taille et morphologie des verrues du péridium (moins grosses et non striées transversalement chez *T. uncinatum*)
- Ornementation des spores (présence de papilles crochues chez *T. uncinatum*)
- Couleur de la glèba (plus foncée à maturité chez *T. uncinatum*)
- Odeur (plus forte et agréable à maturité chez *T. uncinatum*)
- Date de maturité (mai à juillet pour *T. aestivum* ; octobre à décembre pour *T. uncinatum*)

Les avis des auteurs postérieurs à Chatin sur le bien-fondé du nouveau taxon ont profondément divergé. Si Fischer (1897), Hollos (in Szemere, 1965), Knapp (1950-1951), Lange (1956) considèrent *T. uncinatum* soit comme une espèce à part entière, soit comme une variété de *T. aestivum*, Hawker (1954), Ceruti (1960) et Szemere (1965) assimilent tout simplement *T. aestivum.* à *T. uncinatum.*

D'après Chevalier et al. (1978) les seules différences entre les deux taxons sont : la couleur de la gléba qui est généralement plus foncée à maturité dans le type *uncinatum* et le réseau qui orne les ascocarpes est plus développé (jusqu'à 8 µm de hauteur) ; les alvéoles des spores sont également plus grandes, mieux fermées et plus régulières. Ils en concluent que le type *T. aestivum* (plus méridional) se caractérise par des ornementations sporales hautes en moyenne de 2 µm (« forme 2 µm »), alors que l'autre type, *T. uncinatum* (plus septentrional), se caractérise par des ornementations de 4 µm (« forme 4 µm »).

Comme nous l'avons vu dans le paragraphe sur les outils d'identification des *Tuber*, les analyses moléculaires sont contradictoires, certains auteurs sont capables de séparer les deux taxa alors que d'autres ne le sont pas. Mais comment expliquer ces résultats divergents ? Il n'existe donc pas encore de réponse et le mystère *T. uncinatum* / *T. aestivum* reste entier...

1.4.3 Que sait-on sur la diversité intraspécifique et sur la structure génétique des *Tuber* ?

En fait, peu d'études se sont focalisées sur l'analyse de la diversité génétique intraspécifique et la différenciation génétique entre populations de truffes. Toutefois, les analyses de diversité génétique interspécifique, présentées dans le paragraphe précédent, permettent d'avoir une idée sur le niveau de diversité génétique intraspécifique des *Tuber*.

En effet, l'analyse RAPD de plusieurs espèces a permis de révéler deux groupes chez les *Tuber* (Gandeboeuf et al., 1997). Le premier est composé d'espèces présentant un haut niveau de diversité génétique parmi lesquelles : *T. uncinatum, T. aestivum, T. mesentericum, T. excavatum, T. borchii* et *T. macrosporum* (Figure 21). En revanche, le second groupe comprend des espèces ayant un plus bas niveau de diversité génétique comme *T. melanosporum, T. magnatum, T. brumale* et *T. moschatum* (Figure 21).

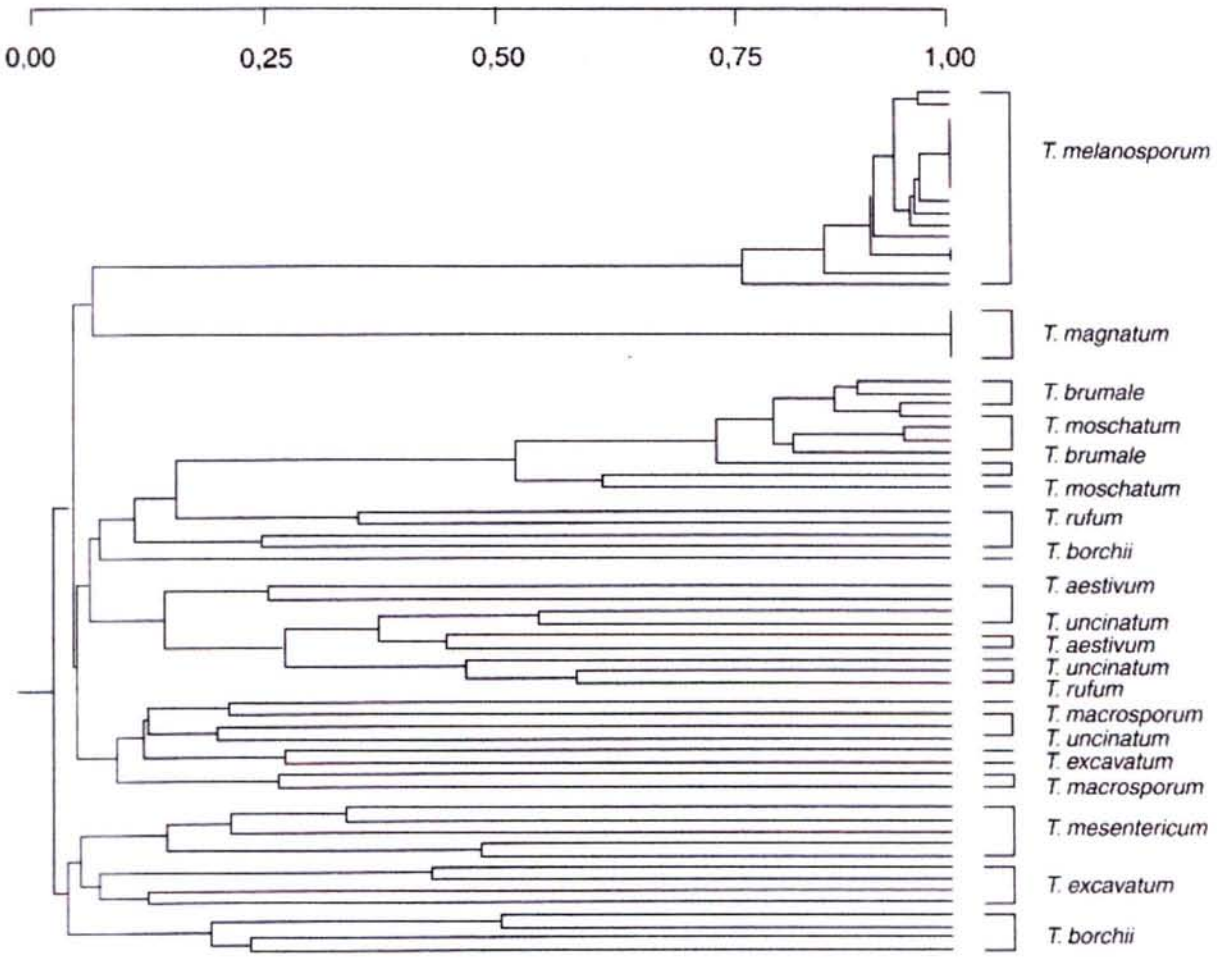


Figure 21. Dendogramme UPGMA réalisé sur 11 taxons de *Tuber* à l'aide de la RAPD (indice de similarité de Jaccard) (Gandeboeuf et al., 1997).

Le bas niveau de polymorphisme de *T. magnatum* et *T. melanosporum* a été confirmé par les isoenzymes (Cameleyre et Olivier, 1993 ; Frizzi et al., 2001), par RAPD (Lanfranco et al., 1993 ; Bertault et al., 1998 ; Murat, 2001) et par le séquençage de régions génomiques SCAR (Murat, 2001). Toutefois, si lors de mon DEA j'ai trouvé une faible diversité génétique pour la plupart des échantillons de *T. melanosporum* étudiés par RAPD, j'ai aussi identifié un polymorphisme plus élevé pour certains ascocarpes provenant principalement de Bourgogne (Figure 22 ; Murat, 2001). De même, Rubini et al. (2004) ont récemment publié 8 microsatellites polymorphes de *T. magnatum* dont un présente jusqu'à 18 allèles.

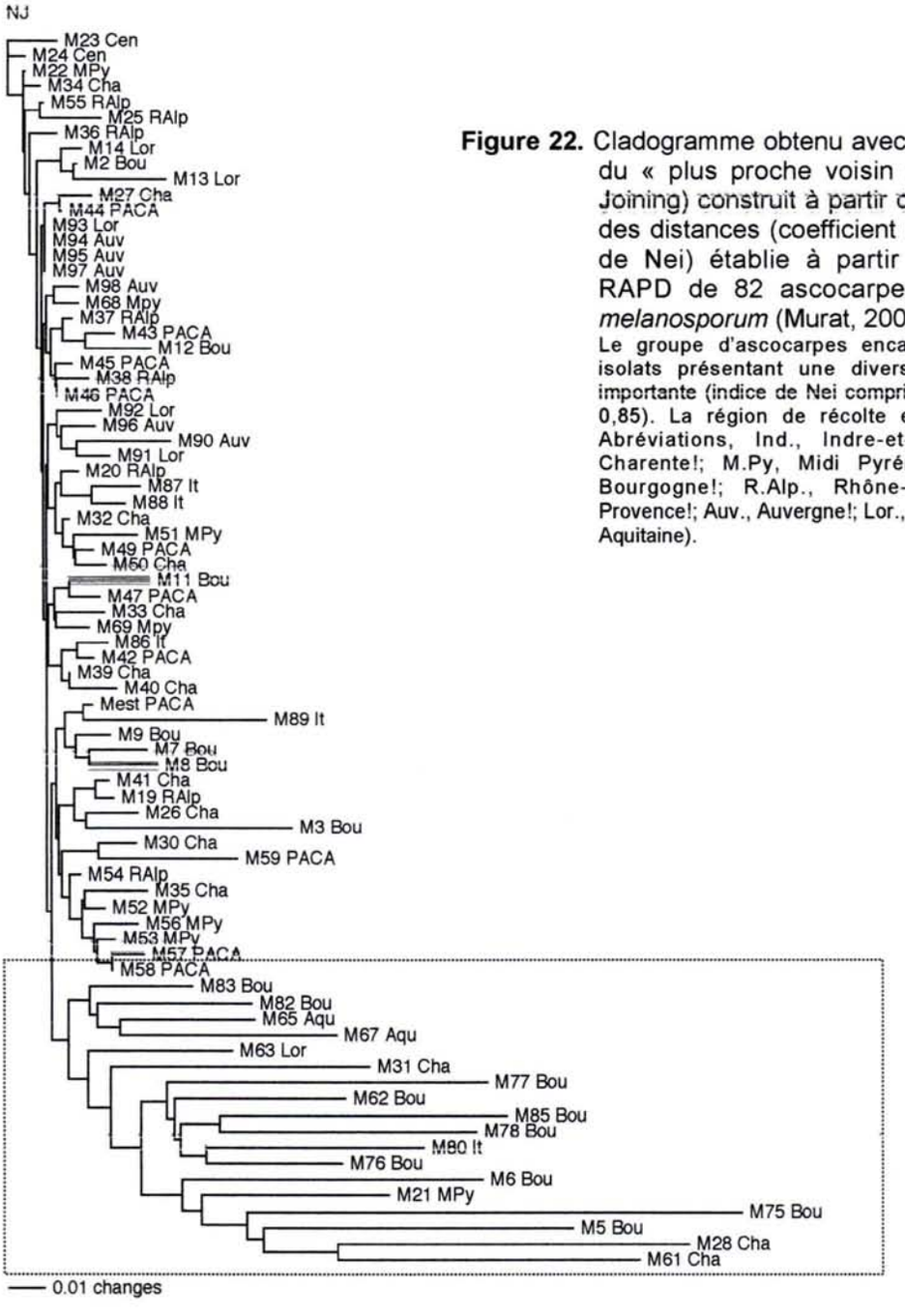


Figure 22. Cladogramme obtenu avec l'algorithme du « plus proche voisin » (Neighbor Joining) construit à partir de la matrice des distances (coefficient de similarité de Nei) établie à partir des profils RAPD de 82 ascocarpes de *Tuber melanosporum* (Murat, 2001).
Le groupe d'ascocarpes encadré inclut les isolats présentant une diversité génétique importante (indice de Nei compris entre 0,55 et 0,85). La région de récolte est indiquée (Abréviations, Ind., Indre-et-Loire; Cha., Charente!; M.Py, Midi Pyrénées ; Bou., Bourgogne!; R.Alp., Rhône-Alpes!; Pro., Provence!; Auv., Auvergne!; Lor., Lorraine! Aqu., Aquitaine).

Donc, même si le niveau de diversité génétique est plus faible pour ces deux espèces, il existe des régions génomiques qui présentent du polymorphisme et qui peuvent être utilisé pour des analyses de génétique des populations.

Existe-t-il une différenciation génétique entre les populations de truffes ?

Il n'existe que très peu de données sur la structure génétique des espèces de truffes. Longato et Bonfante (1997) rapportent une différenciation génétique pour *T. maculatum* puisqu'ils ont séparé avec l'amorce $(GTG)_5$ les échantillons provenant du Piémont de ceux de la Sardaigne. Mais leur objectif étant l'identification des espèces, ils n'ont pas poussé plus loin l'analyse de la différenciation génétique.

Frizzi et al. (2001) ont quant à eux étudié 13 populations italiennes de *T. magnatum* par les isoenzymes. Ces auteurs trouvent deux enzymes polymorphes : la malate deshydrogènase (*Mdh-2*) et l'enzyme malique (*Me-2*) pour un total de 6 types enzymatiques se distribuant dans les différentes populations. Les types A et B sont les plus fréquents, alors que le type D n'a été retrouvé que dans une population (Pianoro, Italie). Même s'il est difficile de tirer des conclusions sur la structure génétique de cette espèce, la distribution non homogène des types enzymatiques semble indiquer une différenciation génétique. Comme je l'ai déjà dit précédemment, Rubini et al. (2004) ont publié 8 microsatellites polymorphes qu'ils ont analysés sur 370 ascocarpes récoltés dans 28 populations. Malheureusement, ils n'ont pas encore publié les résultats de cette analyse et nous ne savons pas quelle est la distribution géographique de ces allèles dans les populations.

Donc, pour le moment nous ne savons presque rien sur la structure génétique de T. magnatum.

Les deux taxons *T. uncinatum* et *T. aestivum* n'ont pas été étudiés seulement du point de vue taxonomique. En effet, lors de son DEA, Patricia Luis a analysé la diversité génétique de ces truffes par RAPD et RAMS (Luis, 2000). Elle a mis en évidence une importante différenciation génétique entre communes (*Fst* = 0,398) et régions (*Fst* = 0,112). De même, Weden et al. (2004) ont aussi montré une différenciation génétique par RAPD du complexe *T. uncinatum* / *T. aestivum* récoltées sur l'île de Gotland en Suède. Malheureusement, ces études sont réalisées avec des marqueurs RAPD qui sont dominants, il faudrait donc reprendre ces analyses avec des marqueurs codominants. Mello et al. (2002) sont parvenus à séparer ces deux taxa par l'analyse moléculaire (RAMS et séquençage de l'ITS). Mais en regardant de plus près leurs résultats, il est

possible que cette distinction repose sur une différenciation génétique entre populations. Comme le montre la figure 23, le dendrogramme réalisé avec la RAMS sépare non seulement les *T. uncinatum* des *T. aestivum* mais aussi les échantillons de par leur origine géographique. Enfin, Paolocci et al. (2004) ont montré que l'ITS mais aussi la ß-tubuline (4 allèles) et le facteur d'élongation 1-∝ (8 allèles) sont polymorphes pour ces taxa, il s'agit donc de bons marqueurs pouvant être utilisés pour des études de phylogéographie chez ces truffes.

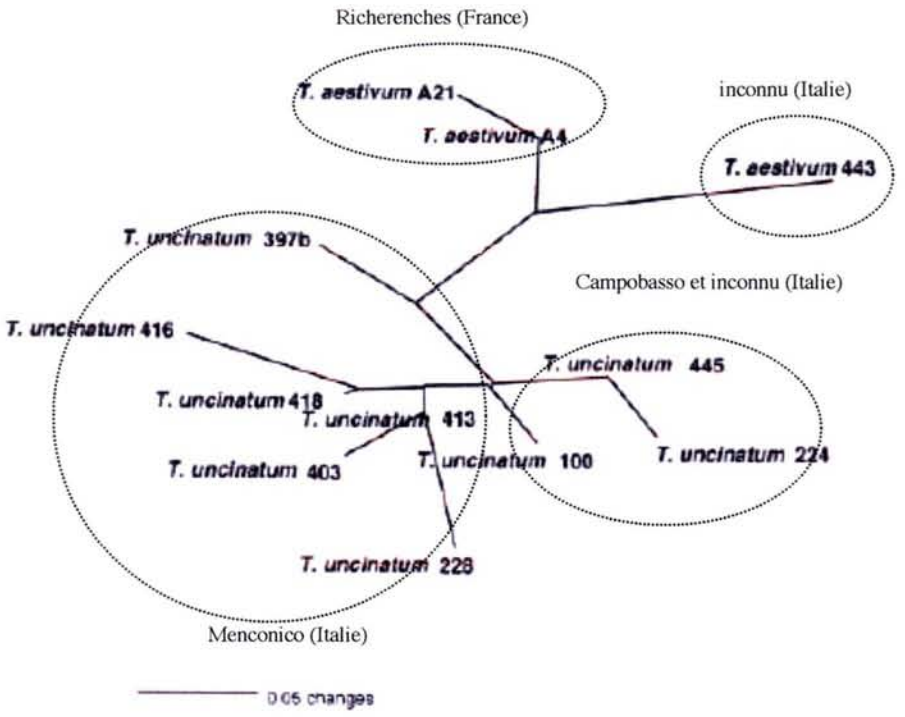


Figure 25. Cladogramme obtenu avec l'algorithme du « plus proche voisin » (Neighbor Joining) avec des amorces microsatellites sur 12 ascocarpes *de T. uncinatum* et *T. aestivum,* les origines géographiques des échantillons sont indiquées (Mello et al., 2002).

Les travaux de Luis (2000), Mello et al. (2002) et Weden et al. (2004) suggèrent une différenciation génétique relativement importante pour les taxa T. uncinatum et T. aestivum. Mais, pour le moment, il n'existe pas encore d'étude à grande échelle, sur toute l'aire de distribution (Tableau 4) de ces truffes, avec des marqueurs codominants.

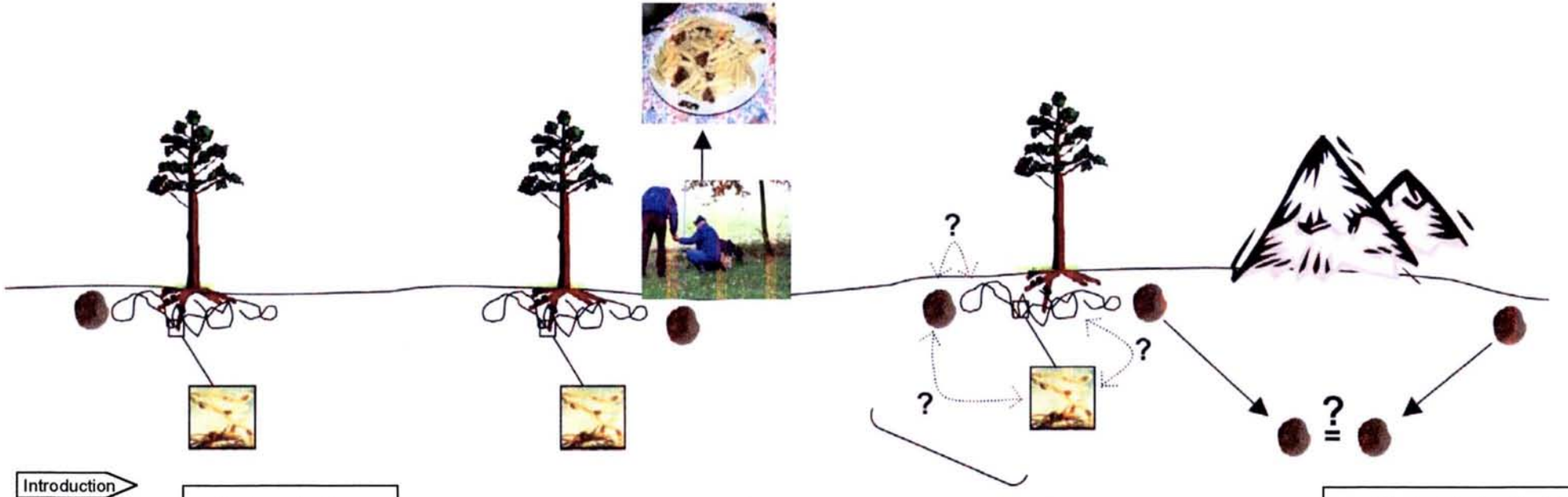
T. melanosporum est l'espèce la plus étudiée du point de vue de la structure génétique. Bertault et al. (1998, 2001) ont analysé des échantillons provenant d'Italie, d'Espagne et du sud de la France par RAPD, RAMS et SSR (Tableau 8). Ces travaux confirment le bas niveau de

polymorphisme de cette truffe. D'autre part, le test de Mantel n'a pas révélé de corrélation entre la diversité génétique et la distance géographique. Ces auteurs concluent qu'il n'existe pas de différenciation génétique entre populations de *T. melanosporum*, suggèrant que les différences organoleptiques entre truffes seraient liées au terroir et non à la génétique.

Le faible niveau de diversité génétique de T. melanosporum est expliqué par un effet « bottleneck » lors de la dernière glaciation, suivi d'une recolonisation rapide expliquant l'absence de structure génétique (Bertault et al., 1998).

Contrairement à Bertault et al. (1998), l'analyse que j'ai réalisée lors de mon DEA, sur un échantillonnage comprenant des populations du Nord-Est de la France (Bourgogne et Lorraine), a mis en évidence une importante différenciation génétique avec un équivalent du *Fst* calculé par l'indice de Shannon de 0,5 (Encadré 7) (Murat, 2001). Tout comme Bertault et al. (1998, 2001) je n'ai pas identifié de corrélation entre les distances génétiques et les distances géographiques, rejetant ainsi l'isolement par la distance pour expliquer la différenciation génétique observée. Mais il est possible que d'autres facteurs, comme la fragmentation ancestrale ou la recolonisation rapide (Figure 18), soient à l'origine de cette différenciation génétique. Sachant que les données RAPD sont sujettes à caution (Tableau 8), ces résultats doivent être confirmés avec d'autres marqueurs génétiques, comme la séquence de l'ITS ou l'analyse de microsatellites (SSR).

Comme nous venons de le voir, la structure génétique des différentes espèces de *Tuber* est en partie inconnue, seule celle de *T. melanosporum* a été réellement étudiée. Mais pour cette espèce les résultats sont encore contradictoires. En effet, ils dépendent de l'échantillonnage, des marqueurs moléculaires utilisés et des analyses statistiques effectuées. Donc, si après les travaux de Bertault et al. (1998) on pensait que deux truffes récoltées dans deux régions différentes étaient génétiquement identiques, les résultats de mon DEA relancent le débat (Murat, 2001).



Introduction

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Présentation générale § 1.1

— Les truffes sont des champignons Ascomycètes ectomycorhiziens.

— Les truffes appartiennent au genre *Tuber* qui fait partie de la famille des *Tuberaceae* classées dans l'ordre des Pezizales.

— La taxonomie des truffes est étudiée depuis plusieurs siècles; mais des doutes subsistent pour certaines espèces!

Les truffes et les hommes § 1.2

— Les truffes présentent plusieurs intérêts: humain, écologique, culturel et développement rural.

— Pour le moment la culture des truffes fonctionne avec *T. melanosporum*, *T. uncinatum* et *T. borchii*.

— Chaque truffe a ses propres exigences mais la plupart poussent dans des terrains calcaires, de plus elles interagissent avec des bactéries, des champignons et des levures..

Biologie des truffes § 1.3

— Le cycle biologique des truffes est composé d'une phase saprotrophique, une phase symbiotique et une phase reproductive.

— Le mode de reproduction des truffes est encore inconnu, mais elles sont probablement homothalliques

— Les truffes seraient autonomes tôt dans leur développement.

Variabilité génétique et génétique des populations § 1.4

— Maintenant, la biologie moléculaire permet d'identifier les truffes dans les aliments, mais il est encore impossible de distinguer certains taxa : *T. uncinatum*/*T. aestivum*

— La différenciation génétique des espèces de truffes est inconnue, on ne peut donc pas encore différencier deux truffes récoltées dans deux régions différentes!

— *T. melanosporum* aurait subit une forte diminution démographique lors de la dernière glaciation

Figure 24. Schéma de conclusion de l'introduction générale dans lequel nous avons répondu aux questions que nous nous posions à la Figure 1.

Les objectifs de l'étude

Comme nous l'avons vu dans ce premier chapitre, la plupart des études portant sur les truffes concernent l'identification des espèces. Leur diversité génétique ainsi que leur structure génétique sont encore en grande partie inconnues. Ce travail de thèse a donc pour objectifs :

1- L'étude de la variabilité génétique et de la structure génétique de la truffe noire du Périgord (*Tuber melanosporum*) par le séquençage direct de l'ITS sur un échantillonnage provenant de toutes les régions productives françaises (échelle macro-géographique). Nous espérons ainsi avoir une idée de la différenciation génétique entre populations et de la phylogéographie de cette espèce.

2- L'étude de la diversité génétique de la truffe blanche du Piémont (*T. magnatum*) surtout à l'échelle micro-géographique dans une truffière naturelle. Lors de cette étude nous avions aussi quelques truffes récoltées dans d'autres régions. Ce travail pourrait donner une idée sur la dynamique et l'évolution d'une truffière naturelle.

3- Enfin, dans la même truffière de *T. magnatum*, nous avons récolté des apex racinaires afin d'avoir une idée sur la distribution spatiale et temporelle de cette truffe dans le sous-sol, mais aussi pour identifier les champignons ectomycorhiziens se trouvant dans cet écosystème truffier.

Références de l'introduction générale

Agostini D., Polidori E., Palma F., Ceccaroli P., Saltarelli R., Tonelli D., Stocchi V., 2001. Cloning, expression, and characterization of the *hxk-1* gene from the white truffle *Tuber borchii* Vittad.; a first step toward understanding sugar metabolism. Fungal Genetic and Biology, 33(1), 15-23.

Ambra R., Grimaldi B., Zamboni S., Filetici P., Macino G., Ballario P., 2004. Photomorphogenesis in the hypogeous fungus *Tuber borchii*: isolation and characterization of Tbwc-1, the homologue of the blue-light photoreceptor of *Neurospora crassa*. Fungal Genetics and Biology 41 (7), 688-697

Amicucci A., Rossi I., Potenza L., Zambonelli A., Agostini D., Palma F., Stocchi V., 1996. Identification of ectomycorrhizae from *Tuber* species by RFLP analysis of the ITS region. Biotechnology Letters, 18, 821-826

Anderson I.C., Chambers S.M., Cairney J.W.G., 1998. Use of molecular methods to estimate the size and distribution of mycelial individuals of the ectomycorrhizal basidiomycete *Pisolithus tinctorius*. Mycological Research, 102, 295-30

Avise J.C., 1998. The history and purview of phylogeography: a personal reflection. Molecular Ecology, 7 (4), 371-379

Balestrini, R., Mainieri D., Soragni E., Gamero L., Rollino S., Viotti A., Ottonello S., Bonfante P., 2000. Differential expression of Chitin Synthase III and IV mRNAs in Ascomata of *Tuber borchii* Vittad. Fungal Genetics and Biology. 31, 219-232.

Barbieri, E., J. Bertaux, P. Frey-Klett, J. Garbaye, A. Hartmann, M. Schmid, and V. Stocchi, 2002a. New evidence for *in situ* detection of a *Cytophaga-Flexibacter-Bacteroides* bacterium associated to the ectomycorrhizal *Tuber borchii* Vittad. mycelium. 6th European Conference on Fungal Genetics. Pisa, Italy.

Barbieri E, Riccioni G, Pisano A, Sisti D, Zeppa S, Agostini D, Stocchi V., 2002b. Competitive PCR for quantitation of a *Cytophaga-Flexibacter-Bacteroides* phylum bacterium associated with the *Tuber borchii* Vittad. mycelium. AEM 68 (12): 6421-6424

Barbieri E., Potenza L., Rossi I., Sisti D., Giomaro G., Rossetti S., Beimfohr C., Stocchi V. 2000. Phylogenetic characterization and in situ detection of *Cytophaga-Flexibacter-Bacteroides* phylogroup bacterium in *Tuber borchii* Vittad. ectomycorrhizal mycelium. Applied Environemental Microbiology, 66 (11), 5035-5042.

Barry D., Staunton S., Callot G, 1994. Mode of the absorption of water and nutrients by ascocarps of *Tuber melanosporum* and *Tuber aestivum*: a radioactive tracer technique. Canadian Journal of Botany 72 (3), 317-322

Belloli S, Bologna F., Gregori G., Zambonelli A., 1999. Il tartufo di Fragno (*Tuber uncinatum* Ch.) : ecologia e coltivazione. Proceedings of the Fifth International Congress on truffle, Aix en Provence, France, 4-6 march 1999. Fédération nationale des trufficulteurs eds., Paris, 367-371

Bergero R., Perotto S., Girlanda M., Vidano G., Luppi A.M., 2000. Ericoid mycorrhizal fungi are common root associates of a Mediterranean ectomycorrhizal plant (*Quercus ilex*). Molecular Ecology 9, 1639-1649

Bertault G., Raymond M., Berthomieu A., Callot G., Fernandez D., 1998. Trifling variation in truffles. Nature 394, 734.

Bertault G., Rousset F., Fernandez D., Berthomieu A. Hochberg, M. E., Callot G., Raymond M., 2001. Population genetics and dynamics of the black truffle in a man-made truffle field. Heredity 86, 451-458.

Bond U., Schlesinger M.J., 1985. Ubiquitin is a heat shock protein in chiken embryo fibroblasts. Mol. Cell. Biol. 5, 949-956.

Bonello P., Bruns T.D., Gardes M., 1998. Genetic structure of a natural population of the ectomycorrhizal fungus *Suillus pungens*. New Phytologist, 138, 533-542

Bonfante P., Balestrini R., Martino E., Perotto S., Plassard C., Mousain D., 1998. Morphological analysis of early contacts between pine roots and two ectomycorrhizal *Suilius* strains. Mycorrhiza. 8, 1-10.

Booth M.G., 2004. Mycorrhizal networks mediate overstorey-understorey competition in a temperate forest. Ecology Letters 7 (7), 538-546

Botton B., Chalot M., 1999. Nitrogen assimilation: enzymology in ectomycorrhizas. In Mycorrhiza. Structure, function, molecular biology and biotechnology. Varma, A., and Hock, B. (eds.) Heidelebrg: Springer-Verlag, 333-372

Buscot F., Munch J.C., Charcosset J.Y., Gardes M., Nelhs U., Hampp R., 2000. Recent advances in exploring physiology and biodiversity of ectomycorrhizas highlight the functioning of these symbioses in ecosystems. FEMS Microbiol. Reviews 24, 601-614

Callot G. 1999. La Truffe, la Terre, la Vie. . INRA, Paris, 210 p.

Cameleyre I., Olivier J.M., 1993. Evidence for intraspecific isoenzyme variations among French isolates of *Tuber melanosporum* (Vitt.). FEMS Microbiology Letters, 110 : 159-162

Carbone I., Kohn L.M., 2001. Multilocus nested haplotype networks extended with DNA fingerprints show common origin and fine-scale, ongoing genetic divergence in a wild microbial metapopulation. Molecular Ecology, 10 (10), 2409-2422

Cassidy J.R., Moore D., Lu B.C., Pukkila P.J., 1984. Unusual organization and lack of recombination in the ribosomal RNA genes of *Coprinus cinereus*. Current Genetics, 8, 607-613

Ceccaroli P., Saltarelli R., Buffalini M., Piccoli G. and Stocchi V., 1999. Three different forms of hexokinase are identified during *Tuber Borchii* mycelium growth. Mol. Cell. Biol. 194 (1-2): 71-77.

Ceccaroli P., Saltarelli R., Cesari P., Pierleoni R., Sacconi C., Vallorani L., Rubini P., Stocchi V., Martin F., 2003. Carbohydrate and amino acid metabolism in *Tuber borchii* mycelium during glucose utilization: a C-13 NMR study. Fungal Genetics and Biology, 39 (2), 168-175

Ceccaroli P., Saltarelli R., Cesari P., Zambonelli A. and Stocchi V. 2001. Effects of different carboyhydrate sources on the growth of *Tuber borchii* Vittad. mycelium strains in pure culture. In *Molecular and Cellular Biochemistry*. 218(1-2):65-70.

Ceruti A., 1960. Elaphomycetales e Tuberales. In : J. Bresadola, Iconographia Micologica, 28, suppl. 2, Tridenti, Trento, 100 p.

Ceruti,A., Fontana,A., Nosenzo C., 2003. Le Specie Europee del Genere *Tuber*. Museo Regionale,.560 p.

Cesalpino A., 1583 De plantis libri XVI, Florence.

Chatin A.D., 1869. La truffe. Ed. Bouchard-Huzard, Paris, 202 p.

Chatin A.D., 1887. Une nouvelle espèce de truffe : *Tuber uncinatum*. C.R. Acad. Sci., Paris, 104, 1132.

Chevalier G ;, 1973. Synthèse axénique des mycorhizes de tuber brumale Vitt. à partir de cultures pures du champignon. Annals of Phytopathology, 5 (2), 163-182.

Chevalier G. & Grente J., 1979. Application pratique de la symbiose ectomycorhizienne : production à grande échelle de plants mycorhizés par la truffe. Mushr. Sci. 10 (2), 483-505.

Chevalier G., Frochot H. 1989. Ecology and possibility of culture in Europe of the Burgundy truffle (*Tuber uncinatum* Ch.). Agric. Ecosystems Environ., 28, 1/4, p. 71-73.

Chevalier G., Frochot H. 1990. Ecologie et possibiltés de culture en Europe de la truffe de Bourgogne (*Tuber uncinatum* Ch). C. R. 2e Congr. Int. Sur la truffe, Spoleto, Italie, 24-27 nov. 1988, p. 323-330.

Chevalier G., Frochot H., 1997. *La truffe de Bourgogne*, Ed. Pétrarque, 257p

Chevalier G., Gandeboeuf D., Masclaux F., Dupré C. 2000. Application des méthodes biochimiques et moléculaires à la taxonomie et la systématique des *Tuber*. Bull. Famm. N. S.,

18, 57-
Chevalier G., Riousset L. & G., Dupré C., Gandeboeuf D. 1994. *Tuber uncinatum* Chat. et *T. aestivum* Vitt., espèces différentes ou simples variétés de la même espèce ? Doc. Mycol., 24 (95), 17-20
Chevalier, G., G. Riousset & C. Dupré, 1985. Taxonomie des truffes européennes. *In* Les Mycorhizes. Proceedings of the 1st European Symposium on Mycorrhizae (édité par V. Gianinazzi-Person & S. Gianinazzi). INRA, Paris, p. 631-653
Chevalier, G.; Gregori, G.; Frochot, H.; Zambonelli, A., 2002. The cultivation of the Burgundy truffle. Edible mycorrhizal mushrooms and their cultivation. Proceedings of the Second International Conference on Edible Mycorrhizal Mushrooms, Ed. Hall, I.; Yun Wang; Danell, E.; Zambonelli, A., Christchurch, New Zealand, 3-6 July, 2001
Citterio B., Malatesta M., Battistelli S., Marcheggiani F., Bajone W., Saltarelli R., Stocchi V., 2001. Possible involvement of *Pseudomonas fluorescens* and Bacillaceae in structural modification of *Tuber* borchii fruit bodies. Canadian Journal of Microbiology, 47, 264-268.
Citterio B., P. Cardoni, L. Potenza, A. Amicucci, V. Stocchi, G. Gola & M. Nuti, 1995. Isolation of bacteria from sporocarps of *Tuber magnatum* Pico, *Tuber Borchii* Vitt. And *Tuber maculatum* Vit. *In* Biotechnology of Ectomycorhizae (Edited by Vilberto Stocchi *et al.*) Plenum Press, New York.
Courvoisier M., 1992. Bilan de la filière truffe en France, bio markets research. Ed. Ctifl, Bio Markets, Paris.
Dahlberg A., 1997. Population ecology of *Suillus variegatus* in old Swedish Scots pine forests. Mycological Research, 101, 47-54
Dahlberg A., 2001. Community ecology of ectomycorrhizal fungi: an advancing interdisciplinary field. New Phytologist, 150, 555-562
Dahlberg A., Jonsson L., Nylund J.E., 1997. Species diversity and distribution of biomass above and below ground among ectomycorrhizal fungi in an old-growth Norway spruce forest in south Sweden. Canadian Journal of Botany, 75, 1323-1335
Dahlberg A., Stenlid J., 1994. Size, distribution and biomass of genets in populations of *Suillus bovinus* (L.: Fr.) Roussel revealed by somatic incompatibility. New Phytol., 128, 225-234
Dahlberg A., Stenlid J., 1995. Spatiotemporal patterns in ectomycorrhizal populations. Canadian Journal of Botany, 73, S1222-S1230
De Bellis R., Agostini D., Piccoli G., Vallorani L., Potenza L.., Sisti D, Amoresano A., Pucci P., Arpaia G., Macino G., Balestrini R., Bonfante P., Stocchi V., 1998. The tbf-1 gene from the white truffle *Tuber borchii* codes for a structural cell wall protein specifically expressed in fruitbody. Fungal Genetics and Biology 25 (2), 87-99
De Miguel A., De Roman M., Etayo M.L., 2002. Mycorrhizal fungi competing with *Tuber melanosporum* Vitt. In cultivated truffle beds in north-eastern Spain. Edible mycorrhizal mushrooms and their cultivation. Proceedings of the Second International Conference on Edible Mycorrhizal Mushrooms, Ed. Hall, I.; Yun Wang; Danell, E.; Zambonelli, A., Christchurch, New Zealand, 3-6 July, 2001
De Vries G. A., 1971. De fungi van Nederland 3 : Hypogaea, Wetenschap. Meded. Konink. Nedwerlandske Natuurhist. Verenig., 88, p. 1-64
De Vries G. A., 1977. Contribution à la connaissance des champignons hypogés de la Belgique. Lejeunia, 86, n.s.
Dennis R.W.G. 1981. British Ascomycetes. J. Cramer, Vaduz. Developmental Biology. 1:177-184.
Dickie I.A., Xu B., Koide R.T., 2002. Vertical niche differentiation of ectomycorrhizal hyphae in soil as shown by T-RFLP analysis. New phyt. 156, 527-537
Diez J., Anta B., Manjon J.L., Honrubia M., 2001. Genetic variability of *Pisolithus* isolates associated with native hosts and exotic *eucalyptus* in the western Mediterranean region. New Phytologist, 149, 577-587
Donadini J.C., Riousset L.J., Chevalier G., 1978. *Tuber malençonii*. Bull. Soc. Mycol. Fr., 94, 351-358.
Douet J. P., Castroviejo M., Mabru D., Chevalier G., Dupre C., Bergougnoux F., Ricard J. M., Medina B., 2004. Rapid molecular typing of *Tuber melanosporum*, *T. brumale* and *T. indicum* from tree seedlings and canned truffles. Analytical and Bioanalytical Chemistry 379 (4), 668-673
Dupré C., 1997. De nouveaux outils au service de la trufficulture. ENESAD, mémoire en vue de l'obtention du Diplôme d'Ingénieur délivré par l'état. 84 p.
Dupré C., Chevalier G., Branlard G., 1985. Caractérisation des mycorhizes de différents *Tuber* par l'étude du polymorphisme enzymatique. C.R. 1er Coll. Natl. Sur les technies de purification des protéines, Paris, 1-3 octobre 1984, DIPC-INPL public, 465-467
Emerson B.C., Paradis E., Thébaud C., 2001. Revealing the demographic histories of species using DNA sequences. Trends in Ecology and Evolution, 16 (12), 707-716
Ewase J.O., Moore D., Stewart G.R., 1978. Coordinate regulation of enzymes involved in ornithine metabolism and its relation to sporophore morphogenesis in *Coprinus cinereus*. J. Gen. Microbiol. 107, 343-357.
Fassi B., De Vecchi E., 1962. Ricerche sulle micorrize ectotrofiche del Pino strobo in vivaio. I. Descrizione di alcune forme più diffuse in Piemonte. Allionia, 8, 133-152
Fassi B., Fontana A., 1969 . Sintesi micorrizica tra *Pinus strobus* e *Tuber maculatum*. II. Sviluppo dei semenzali trapianti e produzione di ascocarpi. Allionia, 15, 115-120
Fiore-Donno A.M., Martin F., 2001. Populations of ectomycorrhizal *Laccaria amethystina* and *Xerocomus* spp. show contrasting colonization patterns in a mixed forest. New Phytologist, 152, 533-542
Fischer E., 1897. Tuberaceen und Hemiasceen. In : Die Pilze Deutschlands, Osterreich und der Schweiz. V. Ascomyceten. L. Rabenhorst's Kryptogamenflora von Deutschland, Osterreich und der Schweiz, Bd 1, Abt. 5 : Ascomyceten, 2e éd., Reprint J. Cramer, Weinheim, Leipzig, 37-61
Frank, B., 1885. Über die auf Wurzelsymbiose beruhende Ernährung gewisser Baume durch unterirdische Pilze. Berichte der Deutschen Botanischen Gesellschaft, Berlin, v. 3, 128-145.
Frankland J.C., 1998. Fungal succession - unravelling the unpredictable. Mycological Research, 102, 1-15
Frey P., Frey-Klett P., Garbaye J., Berge O., Heulin T., 1997. Metabolic and genotypic fingerprinting of fluorescent pseudomonads associated with the Douglas fir-Laccaria bicolor mycorrhizosphère. Applied and Environmental Microbiology, 63, 1852-1860
Fries N., Serck-Hanssen K., Häll Dimberg L., Theander O., 1987. Abietic acid an activator of basidiospore germination in ectomycorrhizal species of the genus *Suillus* (Boletaceae). Exp Mycol 11: 360-363
Frizzi G., Lalli G., Miranda M., Pacioni G., 2001. Intraspecific isoenzyme variability in Italian population of the white truffle *Tuber magnatum*. Mycological Research, 105 (3), 365-369.
Gandeboeuf D., 1997. Caractérisation et identification moléculaire de différentes espèces de genre *Tuber*. Thèse de doctorat de l'université Blaise Pascal, Clermont-Ferrand. France. 65 p.
Gandeboeuf D., Dupré C., Chevalier G, 1994. Différenciation des truffes européennes d'intérêt commercial par analyse des isoenzymes. Acta botanica Gallica, 141 (4), 455-463
Gandeboeuf D., Dupré C., Roeckel-Drevet P., Nicolas P., Chevalier G., 1997. Typing *Tuber* ectomycorrhizae by polymerase chain amplification of the internal transcribed spacer of rDNA and the sequence characterized amplified region markers. Canadian Journal of Microbiology 43, 723-728
Garbaye J., 1991. Utilisation des mycorhizes en sylviculture. In : Strullu D.G. ed Les mycorhizes des arbres et plantes cultivées. Lavoisier, Paris, 197-248.
Gardes M., Bruns T.D., 1993. ITS primers with enhanced specificity for basidiomycetes: application to the identification of mycorrhizae and rusts. Mol. Ecol., 2, 113-118
Garnero L., Lazzari B., Manieri D., Viotti A., Bonfante P., 2000. TMchs4, a class IV chitin synthase gene from the ectomycorrhizal *Tuber magnatum*. Mycological Research 104 (6), 703-707.
Gherbi H., Delaruelle C., Selosse M.A., Martin F., 1999. High genetic diversity in a population of the ectomycorrhizal basidiomycete *Laccaria amethystina* in a 150-year-old beach forest. Molecular Ecology, 8, 2003-2013
Gianinazzi-Pearson V., Brechenmacher L., Tamasloukht M. Bécard G., La Camera S., Gianinazzi S., Franken P., 2002.

Functional genomics of fungal-plant communication in the arbuscolar mycorrhizal symbiosis. Int. Mycol. Congre. 7, 11-17.

Giomaro G., Sisti D., Zambonelli A., Amicucci A., Cecchini M., Comandini O., Stocchi V., 2002. Comparative study and molecular characterization of ectomycorrhizas in *Tilia americana* and *Quercus pubescens* with *Tuber brumale*. FEMS Microbiol. Lett. 216 (1): 9-14.

Glass N.L. and Staben C. 1990. Genetic control of Mating in *Neurospora crassa*. Seminars in Developmental Biology, 1, 177-184

Glass N.L., Donaldson G.C., 1995. Development of primer sets designed for use with the PCR to amplify conserved genes from filamentous Ascomycetes. Appl Environ Microbiol 61 (4), 1323-1330.

Glass N.L., Kuldau G.A. 1992. Matin Type and negative incompatibility in filamentous Ascomycetes. Annual Review of Phythopatology. 30:201-224.

Gregori L., 2002. Problems and expectations with the cultivation of *Tuber magnatum*. Edible mycorrhizal mushrooms and their cultivation. Proceedings of the Second International Conference on Edible Mycorrhizal Mushrooms, Ed. Hall, I.; Yun Wang; Danell, E.; Zambonelli, A., Christchurch, New Zealand, 3-6 July, 2001

Grime J. P., Mackey J. M. L., Hillier S. H., Read D. J., 1987. Floristic diversity in a model system using experimental microcosms. Nature, 328, 420-422.

Gross G., 1987. Schlüssel zu den europäischen Sippen der Gattung *Tuber*. In : Atlas der Pilze des Saarlandes. Eds. H.E. Derbsch und J.A. Schmitt, Teil 2, Delattinia, Saarbrücken, 79-100.

Gryta H., Debaud J.C., Effosse A., Gay G., Marmeisse R., 1997. Fine-scale structure of populations of the ectomycorrhizal fungus *Hebeloma cylindrosporum* in coastal sand dune forest ecosystems. Molecular Ecology 6, 353-364

Guelh J.M., Garbaye J., Wartinger A., 1992. The effects of ectomycorrhial status on plant-water relations and sensitivity of leaf gas exchange to soil drought in Douglas fir (*Pseudotsuga menziesii*) seedlings. In Mycorrhizas ecosystems (édité par D.J. Read, D.H. Lewis, A.H. Fitter et I.J. Alexander). CAB International, Wallingford, p. 323-332

Guescini M., Pierleoni R., Palma F., Zeppa S., Vallorani L., Potenza E., Sacconi G., Giomaro G., Stocchi V., 2003. Characterization of the *Tuber borchii* nitrate reductase gene and its role in ectomycorrhizae. Molecular Genetic and Genomics, 269, 807-816.

Guidi C., Zeppa S., Barbieri E., Zambonelli A., Polidori E., Potenza L. and Stocchi V. 2003. A putative mitochondrial fission gene from the ectomycorrhizal ascomycete *Tuber borchii* Vittad.: cloning, characterization and phylogeny. Current Genetics 44(3), 148-154.

Guidot A., Debaud J.C., Effosse A., Marmeisse R., 2004. Below-ground distribution and persistence of an ectomycorrhizal fungus. New Phytologist, 161, 539-547

Guidot A., Debaud J.C., Marmeisse R., 2001. Correspondence between genet diversity and spatial distribution of above- and below-ground populations of the ectomycorrhizal fungus *Hebeloma cylindrosporum*. Molecular Ecology, 10, 1121-1131

Guidot A., Debaud J.C., Marmeisse R., 2002. Spatial distribution of the below-ground mycelia of an ectomycorrhizal fungus inferred from specific quantification of its DNA in soil samples. FEMS Microbiology Ecology, 42, 477-486

Gutiérrez A., Morte A., Honrubia M., 2003. Morphological characterization of the mycorrhiza formed by *Helianthemum almeriense* Pau with *Terfezia claveryi* Chatin and *Picoa lefebvrei* (Pat.) Maire. Mycorrhiza. 14(1), 26-37.

Hall I.R., Yun W., Amicucci A. 2003. Cultivation of edible ectomycorrhizal mushrooms. Trends Biotechnol. 21 (10): 433-438

Hare M.P., 2001. Prospects for nuclear gene phylogeography. Trends in Ecology and Evolution. 16 (12), 700-706

Hartl D. 1994. Génétique des populations. Ed. Flammarion, 305 p.

Hawker L.E., 1954. Britisch Hypogeous fungi. Phil. Trans. Roy. Soc. London, ser. B., Biol. Sci., 237, 429-546.

He Q.Y., Cheng P., Yang Y.H., Wang L.X., Gardner K.H., Liu Y., 2002. White collar-1, a DNA binding transcription factor and a light sensor. Science 297 (5582), 840-843

Helgason T., Daniell T.J., Husband R., Fitter A.H., Young J.P.W., 1998. Ploughing up the wood-wide web? Nature 394, 431

Henrion B., G. Chevalier & F. Martin, 1994. Typing truffle species by PCR amplification of the ribosomal DNA spacers. Mycologia 98, 37-43

Hey J., Machado C.A., 2003. The study of structured populations - new hope for a difficult and divided science. Nature Reviews Genetics 4 : 535-543

Hoekstra R.F., 1996. Horizontal transmission in fungal populations. In Fungal genetics, principle and Practices. Edited by Cees J. Bos

Horton T.R., Bruns T.D., 2001. The molecular revolution in ectomycorrhizal ecology: peeking into the black-box. Molecular Ecology, 10, 1855-1871

Iotti M., Amicucci A., Stocchi V. and Zambonelli A. 2002. Morphological and molecular characterization of mycelia of some *Tuber* species in pure culture. New Phytologist 155, 499-505

Jang J.C., Sheen J.. 1997. Sugar sensing in higher plants. Trends in Plant Science, 2: 208-214

Jany J.L., Garbaye J., Martin F., 2002. Cenococcum geophilum populations show a high degree of genetic diversity in beech forests. New Phytologist, 154, 651-659

Javelle A., Morel M., Rodriguez-Pastrana B.R., Botton B., André B., Marini A.M., Brun A, Chalot, M., 2003. Molecular characterization, function and regulation of ammonium transporters (Amt) and ammonium-metabolizing enzymes (GS, NADP-GDH) in the ectomycorrhizal fungus *Hebeloma cylindrosporum*. Molecular Microbiology 47, 411-430

Javelle A., Rodriguez-Pastrana B.R., Jacob C., Botton B., Brun A., André B., Marini A.M., Chalot, M., 2001. Molecular characterization of two ammonium transporters from the ectomycorrhizal fungus *Hebeloma cylindrosporum*. FEBS Letters 505, 393-398

Jerome C.A., Ford B.A., 2002. Comparative population structure and genetic diversity of Arceuthobium americanum (Viscaceae) and its Pinus host species: insight into host-parasite evolution in parasitic angiosperms. Molecular Ecology 11 (3), 407-420

Johannesson H. 2000. Ecology of Daldinia spp. With special emplasis on *Daldinia loculata*. Doctoral thesis, University of Uppsala, 43 p.

Jonsson L., Dahlberg A., Nilsson M.C., Zackrisson O., Karen O., 1999. Ectomycorrhizal fungal communities in late-successional Swedish boreal forests, and their composition following wildfire. Molecular Ecology 8, 205-215

Kagan-Zur V., Freeman S., Luzzati Y., Roth-Bejerano N., Shabi E. 2001. Survival of introduced Tuber melanosporum at two sites in Israel as measured by occurrence of mycorrhizas. Plant and Soil 229, 159-166

Kennedy P.G., Izzo A.D., Bruns T.D., 2003. There is high potential for the formation of common mycorrhizal networks between understorey and canopy trees in a mixed evergreen forest. Journal of Ecology 91 (6), 1071-1080

Knapp A., 1950. Die europäischen Hypogaeen-Gattungen und ihre Gattungstypen. Schweiz. Zeitschr. f. Pilzkd., 28 (10), 175-179

Knapp A., 1951. Die europäischen Hypogaeen-Gattungen und ihre Gattungstypen. Schweiz. Zeitschr. f. Pilzkd., 29 (4), 84-87

Knowles L.L., Maddison W.P., 2002. Statistical phylogeography. Molecular Ecology 11: 2623-2635.

Koide R.T., 2000. Functional complementary in the arbuscular mycorrhizal symbiosis. New Phytologist, 147, 233-235

Koide R.T., Dickie I.A., 2002. Effects of mycorrhizal fungi on plant populations.Plant and Soil, 244 (1-2): 307-317

Lacourt I., Duplessis S., Abba S., Bonfante P. and Martin F. 2002. Isolation and characterization of differentially expressed genes in the mycelium and fruit body of *Tuber borchii*. Applied and Environmental Microbiology 68(9), 4574-82.

Landeweert R., Leeflang P., Kuyper T.W., Hoffland E., Rosling A., Wernars K., Smit E., 2003. Molecular identification of ectomycorrhizal mycelium in soil horizons. Applied and Environmental Microbiology. 69, 327-333

Lanfranco L., Arlorio M., Matteucci A., Bonfante P. 1995. Truffles:

their life cycle and molecularcharacterization. In Biotecnology of ectomycorrhizae. Molecular approaches (Stocchi V., Bonfante P., Nuti M.). Plenum Press. New York and London.

Lanfranco L., Wyss P., Marzachi C., Bonfante P. 1993. DNA probes for identification of the ectomycorrhizal fungus *Tuber magnatum* Pico. FEMS Microbiology Letters, 114, 245-25

Lange M., 1956. Danish hypogeous macromycetes. Dansk. Bot. Arkiv. 16, 1-84.

Largent D.L., Sugihara N., Brinitzer A., 1980a. *Amanita gemmata*, a non-host specific mycorhizal fungus af *Arctostaphylos manzanita*. Mycologia 72, 435-439

Largent D.L., Sugihara N., Wishner G., 1980b. Occurrence of mycorrhizae on ericaceous and pyrolaceous plants in northern California. Canadian Journal of Botany 58, 2274-2279

Le Tacon F., J. Garbaye, D. Bouchard, G. Chevalier, J. M. Olivier, J. Guimberteau, N. Poitou & H. frochot, 1988. Field result from ectomycorrhizal inoculation in France. *In* Canadian workshop on Mycorrhizal in Forestry (edited by Lalonde, Pichey) : 51-74

Le Tacon F., Selosse M.A. 1994. La place des symbioses mycorhiziennes dans l'évolution et la colonisation des continents pour la vie. Acta botanica Gallica, 141, 405-419

LePage B.A., Currah R.S., Stockey R.A., Rothwell G.W. 1997. Fossil ectomycorrhizae from the middle Eocene. American Journal of Botany, 84, 410-412

Lewis D.H., 1986. Inter-relations between carbon nutrition and morphogenesis in mycorrhizas. In Mycorrhizas : their physiology and genetics. Prodeeding of the First European Symposium on Mycorrhizas (ed. V. Gianinazzi-Pearson & S. Gianinazzi, INRA, Paris, 85-100.

Leyval C., Turnau K., Haselwandter K., 1997.Effect of heavy metal pollution on mycorrhizal colonization and function: physiological, ecological and applied aspects. Mycorrhiza, 7 (3), 139-153

Longato S., Bonfante P., 1997. Molecular identification of mycorrhizal fungi by direct amplification of microsatellite regions. Mycological Research, 101, 425-43

Lowe A.J., Gillies A.C.M., Wilson J., Dawson I.K., 2000. Conservation genetics of bush mango from central/west Africa: implications from random amplified polymorphic DNA analysis. Molecular Ecology 9 (7), 831-841

Luis P., 2000. Caractérisation de la diversité génétique des populations chez *Tuber uncinatum* et *Tuber melanosporum*. . DEA de l'université Claude Bernard, Lyon I-France. 47 p.

Luis P., Walther G., Kellner H., Martin F., Buscot F., 2004. Diversity of laccase genes from basidiomycetes in a forest soil. Soil Biology and Biochemistry, 36, 1025-1036

Luppi-Mosca A.M., 1973. La microflora della rizosfera nelle tartufaie. Allionia, 19, 29-32

Mabru D., Douet J.P., Mouton A., Dupre C., Ricard J.M., Medina B., Castroviejo M., Chevalier G., 2004. PCR-RFLP using a SNP on the mitochondrial Lsu-rDNA as an easy method to differentiate *Tuber melanosporum* (Perigord truffle) and other truffle species in cans.International Journal of Food Microbiology, 94 (1), 33-42

Mabru D., Dupré C., Douet J. P., Leroy P., Ravel C., Ricard J. M., Medina B., Castroviejo M., Chevalier G., 2001. Rapid molecular typing method for the reliable detection of Asiatic black truffle (*Tuber indicum*) in commercialized products : fruiting bodies and mycorrhizal seedlings. Mycorrhiza 11 : 89-94

Malloch D.W., Pirozynski K.A., Raven P.H., 1980. Ecological and evolutionary significance of mycorrhizal symbioses in vascular plants (a review) Proceedings of the National Academy of Sciences USA, 77, 2113-2118

Mamoun M. & J. M. Olivier, 1991.Influence du substrat carboné et de la forme d'azote minéral sur la croissance de *Tuber melanosporum* Vitt. en culture pure. Application à la production de biomasse mycélienne. Agronomie, 11, 521-527

Mamoun M. & J. M. Olivier, 1993. Competition between *Tuber melanopsorum* and other ectomycorrhizal fungi under two irrigation regimes. Plant and Soil 149 : 211-218

Mantel, N. 1967. The detection of disease clustering and a generalized regression. Cancer Research, 27, 209-220

Martin F., Duplessis S., Ditengou F., Lagrange H., Voiblet C., Lapeyrie F., 2001. Developmental cross talking in the ectomycorrhizal symbiosis: signals and communication genes. New Phytologist 151, 145-154

Martin F., Tagu D., 1999. Developmental biology of a plant-fungus symbiosis : the ectomycorrhiza. In Mycorrhiza (eds Varma A. and Hock B.), second edition, Springer-Verlag, Berlin, Heidelberg, Nedw-York

Melin E., Nilsson H., 1950. Transfer of radioactive phosphorus to pine seedlings by means of mycorrhizal hyphae. Physiologia Plantarium 3, 88-92

Mello A., Cantisani A., Vizzini A., Bonfante, P., 2002. Genetic variation of *Tuber uncinatum* and its relatedness to other black truffles. Environmental Microbiology, 4 (10), 584-594

Mello, A., Fontana, A., Meotto, F., Comandini, O., Bonfante P., 2001. Molecular and morphological characterization of *T. magnatum* mycorrhizas in a long-term survey. Microbiol Res 155: 279-284

Menotta M., Amicucci A., Sisti D., Gioacchini A.M., Stocchi V., 2004a. Differential gene expression during pre-symbiotic interaction between *Tuber borchii* Vittad. and *Tilia americana* L. Current Genetics. 46 (3), 158-165

Menotta M., Gioacchini A.M., Amicucci A., Buffalini M., Sisti D., Stocchi V., 2004b. Headspace solid-phase microextraction with gas chromatography and mass spectrometry in the investigation of volatile organic compounds in an ectomycorrhizae synthesis system. Rapid Communications in Mass spectrometry 18 (2), 206-210.

Miozzi (2003) La transizione dalla fase vegetativa a quella simbiotica in *Tuber borchii* Vittad.: unianalisi cellulare e molecolare. PhD thesis, università di Torino (Italy), 190 p.

Molina R.H., Massicotte H., Trappe J., 1992. Specificity phenomena in mycorrhizal symbioses : community-ecological consequences and practical implications. In Mycorrhizal functioning- an integrated plant-fungal process (édité par M.F. Allen). Chapman and Hall, New York, 357-417

Montanini B., 2003. Risposte molecolari alla deprivazione di azoto in un fungo filamentoso simbiotico. PhD thesis, Università di Parma, 159 p.

Montanini B., Betti M., Marquez A.J., Balestrini R., Bonfante P., Ottonello, S., 2003. Distinctive properties and expression profiles of glutamine synthetase from a plant symbiotic fungus. Biochemichal Journal, 373, 357-368.

Montanini B., Moretto N., Soragni E., Percudani R., Ottonello, S., 2002. A high-affinity ammonium transporter from the mycorrhizal ascomycete *Tuber borchii*. Fungal and Genetic Biology, 36, 22-34.

Montecchi A., Lazzari G. 1987. Un nuovo tartufo di montagna : *Tuber regianum* sp. Nov. Riv. Micol. (AMB), 30 (1-2), 3-11.

Montecchi A., Lazzari G. 1993. Atlante fotografico di Funghi Ipogei. Ed. Associazione Micologica Bresadola, Centro Studi Micologici, Trente, 490 p.

Moore D., 1998. Fungal morphogenesis. Cambridge University Press, Cambridge, England.

Mouches C., Duthil P., Bove J.M., Valjalo J, Delmas J., 1978. Caractérisation des champignons supérieurs par électrophorèse de leurs protéines. Mushr. Sci., 10 (1), 491-503

Mouches C., Duthil P., Poitou N, Delmas J., Bove J.M., 1981. Caractérisation des espèces truffières par analyse de leurs protéines en gels de polyacrylamide et application de ces techniques à la taxonomie des champignons. Mushr. Sci., 11, 819-831

Murat C., 2001. Etude de la diversité génétique de la Truffe noire du Périgord (*Tuber melanosporum* Vittad.). Diplôme d'Etude Approfondie, Université de Nancy

Nelson M.A., 1996. Mating systems in ascomycetes: a romp in the sac. Trends in Genetics ,12, 69-74

O'Donnell K., Cigelnik E., Weber N.S. and Trappe J.M. 1997. Philogenetic relationships among ascomycetous truffles and the true and false morels inferred from 18S and 28S ribosomal DNA sequence analysis. Mycologia, 89 (1): 48-65

Pacioni G. 1988. Impatto di nuove metodologie sulla tassonomia. C. R. 2e Congr. Int. Sur la truffe, Spoleto, Italie, 24-27 novembre 1988, 51-52

Pacioni G., Fantini P. 1997. *Tuber bellonae* un tartufo mediterraneo del complesso *Tuber aestivum-Tuber mesentericum*. Micol. E Veg. Mediterr., 12 (1), 15-20.

Pacioni G., Frizzi G., Miranda M., Visca C., 1993. Genetics of a

Tuber aestivum population (Ascomycotina, Tuberales). Mycotaxon, XL, 93-100

Pacioni G., Pomponi G. 1989. Chemotaxonomy of some Italian species of *Tuber*. Micol. E Veg. Mediterr., 4 (1), 63-72.

Pacioni G., Pomponi G. 1991. Genotypic patterns of some Italian species of *Tuber aestivum-Tuber mesentericum* complex. Mycotaxon, 42, 171-179.

Palenzona M., 1969. Sintesi micorrizica tra Tuber aestivum Vitt., Tuber brumale Vitt., Tuber melanosporum Vitt. e semenzali di Coryllus avelana. Allionia, 15, 121-131

Palenzona M., Chevalier G., Fontana A. 1972. Sintesi micorrizica tra i miceli in coltura di *Tuber brumale, T. melanosporum, T. rufum* e semenzali di conifere e latifoglie. Allionia, 18, 41-52

Paolocci F., Angelini P., Cristofari E., Granetti B., Arcioni S., 1995. Identification of *Tuber* spp and corresponding ectomycorrhizae through molecular markers. J. Sci. Food Agric., 69, 511-517

Paolocci F., Rubini A., Granetti B., Arcioni S., 1999. Rapid molecular approach for a reliable identification of *Tuber* spp. Ectomycorrhizae. *FEMS Microbiology Ecology* 28 : 23-30.

Paolocci F., Rubini A., Riccioni C., Topini F., Arcioni S., 2004. *Tuber aestivum* and *Tuber uncinatum*: two morphotypes or two species? FEMS Microbiology Letters 235 (1), 109-115

Pegler D.N., Spooner B.M., Young T.W.K. 1993. *British Truffles : a revision of British Hypogeous Fungi*. Royal Botanic Gardens, Kew, 216 p.

Percudani R., Trevisi A., Zambonelli A. and Ottonello S. 1999. Molecular Phylogeny of Truffles (Pezizales: Terfeziaceae, Tuberaceae) Derived from Nuclear rDNA Sequenze Analysis. Molecular Phylogenetic Evolution, 13 (1), 169-180.

Piazza A., 2003. Genetica di popolazione. In: Dizionario di Biologia. A Fasolo Ed. UTET Torino

Pierleoni R., Buffalini M., Vallorani L., Guidi C., Zeppa S., Sacconi C., Pucci P., Amoresano A., Casbarra A., Stocchi V., 2004. *Tuber borchii* fruit body: 2-dimensional profile and protein identification. Phytochemistry, 65 (7), 813-820

Pierleoni R., Vallorani L., Sacconi C., Sisti D., Giomaro G., Stocchi V., 2001. Evaluation of the enzymes involved in primary nitrogen metabolism in *Tilia platyphyllos Tuber borchii* ectomycorrhizae. In Plant Physiol. Biochem. 39 (12), 1111-1114

Pöggeler S., 2001. Mating-type genes for classical strain improvements of ascomycetes. Applied Microbiology Biotechnology, 56, 589-601

Polidori E., Agostini D., Zeppa S., Potenza L., Palma F., Sisti D., Stocchi V., 2002. Identification of differentially expressed cDNA clones in *Tilia platyphyllos-Tuber borchii* ectomycorrhizae using a differential screening approch. Mol. Genet. Genomics. 266, 858-864.

Polidori E., Saltarelli R., Ceccaroli P., Buffalini M., Pierleoni R., Palma F., Bonfante P., Stocchi V., 2004. Enolase from the ectomycorrhizal fungus *Tuber borchii* Vittad.: biochemical characterization, molecular cloning, and localization. Fungal Genetics and Biology 41 (2), 157-167

Poma A., Venora G., Miranda M., Pacioni G. The karyotypes of three *Tuber* species (Pezizales, Ascomycota). Caryologia 55 (4), 307-313

Pons O., Petit R.J., 1996. Measuring and testing genetic differentiation with ordered versus unordered alleles. *Genetics*, 144, 1237-1245.

Posada D., Crandall K.A., 2001. Intraspecific gene genealogies : trees grafting into networks. Trends in Ecology and Evolution, 16, 37-45

Potenza L., Amicucci A., Rossi I., Palma F., De Bellis R., Cardoni P., Stocchi V., 1994. Identification of *Tuber magnatum* pico DNA markers by RAPD analysis. In "Biotechnology Techniques", Vol. 8, 93-98

Prospero S., Rigling D., Holdenrieder O., 2003. Population structure of Armillaria species in managed Norway spruce stands in the Apls. New Phytologist, 137, 357-369

Rabouam C.,Comes A. M., Bretagnolle V., Humbert J.-F., Periquet G., BigotY., 1999. Features of DNA fragments obtained by random amplified polymorphic DNA (RAPD) assays. Molecular Ecology 8, 493-503.

Read D., 1997. Mycorrhizal fungi. The ties that bind. Nature 388, 517-51

Redecker D., Szaro T.M., Bowman R.J., Bruns T.D., 2001. Small genets of Lactarius xanthogalactus, Russula cremoricolor and Amanita francheti in late-stage ectomycorrhizal successions. Molecular Ecology, 10, 1025-1034

Reyna, S.; Rodriguez Barreal, J. A.; Folch, L.; Perez-Badia, R., Garcia S., Jimenez E., 2002. Truffle silviculture in Mediterranean forests. Edible mycorrhizal mushrooms and their cultivation. Proceedings of the Second International Conference on Edible Mycorrhizal Mushrooms, Ed. Hall, I.; Yun Wang; Danell, E.; Zambonelli, A., Christchurch, New Zealand, 3-6 July, 2001

Ridley M., 1996. Evolution (seconde édition). Blackwell Science, Cambrigde, Massachussetts (USA), 719p.

Riousset L., Riousset G. 1988. *Tuber requieni* Tulasne L.R. & C. une espèce méconnue. Bull Soc Et Sci Nat Vaucluse, n.s., 58e année, 73-80

Riousset L., Riousset G. 1990. *Tuber oligospermum* (L. & Ch. Tulasne) J.M. Trappe, espèce mal connue. Il Fungo (suppl ; n°4), 12-13

Riousset L., Riousset G. 1993. *Tuber oligospermum* (Tulasne & Tulasne) Trappe, une espèce mal connue. Bull Fed Assoc Mycol Mediterr, n.s., 36-39

Riousset L., Riousset G., Chevalier G., Bardet M.C. 2001. Truffes d'Europe et de Chine. Ed. INRA, 181 p.

Robert L.M. 1995. The sexual cycle in *Neurospora*. In Biotecnology of ectomycorrhizae. Molecular approaches (Stocchi V., Bonfante P., Nuti M.). Plenum Press. New York and London

Robinson D, Fitter A., 1999. The magnitude and control of carbon transfer between plants linked by a common mycorrhizal network. Journal of Experimental Botany, 50 (330), 9-13

Rossi I., Bertini L ;, Amicucci A., Cecchini M., Gregori L., Sacchi A., Zambonelli A., Stocchi V., 2002. Study of a natural *Tuber magnatum* Pico truffière in central Italy. Edible mycorrhizal mushrooms and their cultivation. Proceedings of the Second International Conference on Edible Mycorrhizal Mushrooms, Ed. Hall, I.; Yun Wang; Danell, E.; Zambonelli, A., Christchurch, New Zealand, 3-6 July, 2001

Roux, C., Séjalon-Delmas N., Martins M., Parguey-Leduc A., Dargent R., Bécard G., 1999. Phylogenetic relationships between european and chinese truffles based on parsimony and distance analysis of ITS sequences. FEMS Microbiology Letters 180 : 147-155.

Rubini A, Paolocci F, Granetti B, Arcioni S (2001) Morphological characterization of molecular-typed *Tuber magnatum* ectomycorrhizae. Mycorrhiza 11: 179-185.

Rubini A., Topini F., Riccioni C., PaolocciF., Arcioni, S., 2004. Isolation and characterization of polymorphic microsatellite loci in white truffle (*Tuber magnatum*). Molecular Ecology Notes 4, 116-118

Saltarelli R., Ceccaroli P., Cesari P., Zeppa S., Potenza L. and Stocchi V., 1999. Strain differences in the mycelium of the ectomycorrhizal *Tuber borchii*. Mycological Research 103, 1524-1528

Saltarelli R., Ceccaroli P., Vallorani L., Zambonelli A., Citterio B., Malatesta M., Stocchi V. 1998. Biochemical and morphological modifications during the growth of *Tuber borchii* mycelium. Mycol. Res. 102, 403-409

Sbrana C., Agnolucci M., Bedini S., Lepera A., Toffanin A., Giovannetti M., Nuti M.P., 2002. Diversity of culturable bacterial populations associated to *Tuber borchii* ectomycorrhizas and their activity on T-borchii mycelial growth. FEMS Microbiol. Lett. 211 (2), 195-201

Sbrana C., Bagnoli G., Bedini S., Filippi C., Giovannetti M., Nuti M.P., 2000. Adhesion to hyphal matrix and antifungal activity of Pseudomonas strains isolated from Tuber borchii ascocarps. Can. J. Microbiol. 46 (3), 259-268

Schardl C.L., Leuchtmann A., Chung K.R., Penny D., Siegel M.R., 1997. Coevolution by common descent of fungal symbionts (Epichloe spp) and grass hosts. Molecular Biology and Evolution 14 (2), 133-143

Schärzel C. 1967. Beitrag zum Hypogäenflora des Kantons Basel-Stadt und seiner näheren Umgebung. Schweiz. Zeiteschr. F. Pilzkd. , 45 (9), 129-141

Selosse M.A., 1998. Etude de populations de champignons basidiomycétes ectomycorhiziens du genre Laccaria,

introduits ou spontanés sous Douglas (Pseudotsuga menziesii. Thèse de l'université Paris XI, Orsay

Selosse M.A., Jacquot D., Bouchard D., Martin F., Le Tacon F., 1998a. Temporal persistence and spatial distribution of an American inoculant strain of the ectomycorrhizal basidiomycete *Laccaria bicolor* in a French forest plantation. Molecular Ecology 7, 561-573

Selosse M.A., Martin F., Le Tacon F., 1998b. Survival of an introduced ectomycorrhizal Laccaria bicolor strain in a European forest plantation monitored by mitochondrial ribosomal DNA analysis. New Phytologist, 140, 753-761

Selosse M.A., Faccio A., Scappaticci G., Bonfante P., 2004.Chlorophyllous and achlorophyllous specimens of Epipactis microphylla (Neottieae, Orchidaceae) are associated with ectomycorrhizal septomycetes, including truffles. Microbiology Ecology, 47 (4), 416-42

Simard S. W., Perry D. A., Jones M. D., Myrold D. D., Durall D. M., Molina R., 1997. Net transfer of carbon between ectomycorrhizal tree species in the field. Nature, 388, 579-582

Singer R., 1977. Die gruppe der Laccaria laccata (Agaricales). Plant Systematic and Evolution. 126, 347-370

Sisti D., Giomaro G., Cecchini M., Faccio A., Novero M., Bonfante P., 2003. Two genetically related strains of *Tuber borchii* produce *Tilia* mycorrhizas with different morphological traits. Mycorrhiza 13(2), 107-15

Sisti D., Zambonelli A., Giomaro G., Rossi I., Ceccaroli P., Citterio B., Benedetti P.A., Stocchi V., 1998. *In vitro* mycorrhizal synthesis of micropropagated *Tilia platyphyllos* Scop. plantlets with *Tuber borchii* Vittad. mycelium in pure culture. Acta Horticul. 457, 379-387

Smit E., Leeflang P., Glandorf B., Van Elsas J.D., Wernars K., 1999. Analysis of fungal diversity in the wheat rhizosphere by sequencing of cloned PCR-amplified genes encoding 18S rRNA and temperature gradient gel electrophoresis. Applied and Environmental Microbiology 65, 2614-26

Smith S.E., Read D.J., 1997. Mycorrhizal symbiosis (second edition). Academic press, London, UK. 605 p.

Soragni E., Bolchi A., Balestrini R., Gambaretto C., Percudani R., Bonfante P., Ottonello S., 2001. A nutrient-regulated, dual localization phospholipase A2 in the symbaiotic fungus *Tuber borchii*. In EMBO J. 20(18), 5079-5090.

Sourzat P., 1994. Guide de trufficulture. L.P.A. Le Montat eds, Cahors, 96 p.

Szmere L. 1965. *Die unterirdischen Pilze des Karpatenbeckens*. Académia Kiado, Budapest, 319 p.

Templeton A.R., 1998. Nested clade analyses of phylogenetic data: testing hypothesis about gene flow and population history. Molecular Ecology 7: 381-397

Templeton A.R., Routman E., Phillips C., 1995. Separating population structure from population history: a cladistic analysis of the geographical distribution of mitochondrial DNA haplotypes in the tiger salamander, *Ambystoma tigrinum,* Genetics, 140, 767-782.

Thõen D. 1988. Catalogue des champignons de Belgique et du Grand-Duché de Luxembourg. Dumortiera, 41, 4-18.

Thompson J.N., Cunningham B.M., 2002. Geographic structure and dynamics of coevolutionary selection. Nature 417:735-738

Thompson J.N., Nuismer S.L., Gomulkiewicz R., 2002. Coevolution and maladaptation Integrative and Comparative Biology 42 (2), 381-387

Thompson J.N.., 1989. Concepts of coevolution. Trend Ecol Evol 4:179–183

Thorn R.G., Reddy C.A., Harris D., Paul E.A., 1996. Isolation of saprophytic basidiomycetes from soil. Applied and Environemental Microbiology 62, 4288-4292

Trappe, J.M., 1979. The orders, families and genera of hypogeous Ascomycotina (truffles and their relatives). Mycotaxon 9 : 297-340.

Urban A., Neuner-Plattner I., Krisai-Greilhuber I., Haselwandter K., 2004. Molecular studies on terricolous microfungi reveal novel anamorphs of two Tuber species. Mycological Research, 108 (7), 749-58.

Urbanelli S., Sallicandro P., De Vito E., Bullini L., Biocca E., 1998a. Biochemical systematics of some species in the genus *Tuber*. Mycologia, 90, 537-546

Urbanelli S., Sallicandro P., De Vito E., Bullini L., Palenzona M., Ferrara AM 1998b. Identification of *Tuber* mycorrhizae using multilocus electrophoresis. Mycologia 90 (3): 389-395.

Vallorani L., Bernardini F., Sacconi C., Pierleoni R., Pieretti B., Piccoli G., Buffalini M., Stocchi V., 2000. Identification of Tuber borchii Vittad. mycelium proteins separated by two-dimensional polyacrylamide gel electrophoresis using amino acid analysis and sequence tagging. Electrophoresis, 21 (17), 3710-3716

Vallorani L., Polidori E., Sacconi C., Agostini D., Pierleoni R., Piccoli G., Zeppa S., Stocchi V., 2002. Biochemical and molecular characterization of NADP glutamate dehydrogenase from the ectomycorrhizal fungus *Tuber borchii*. New Phytol. 154 (3), 779-790

Villarreal-Ruiz L., Anderson I.C., Alexander I., 2004. Interactions between an isolate from the *Hymenoscyphus ericaceae* aggregate and roots of *Pinus* and *Vaccinium*. New Phytologist 164, 183-192

Vralstad T., 2004. Are ericoid and ectomycorrhizal fungi part of a common guild ? New Phytologist 164, 7-10

Wang T., Moreno G., Riousset L.J., Manjon J.L., Riouset G., Fourre G., Di Massimo G., Garcia-Montero L.G., Diez J., 1998. *Tuber pseudoexcavatum* sp nov, a new species from China commercialised in Spain, France and Italy, with additional comments on Chinese truffles. Cryptogam. Mycol. 19 (1-2), 113-120.

Weden C., Danell E., Camacho F.J., Backlund A., 2004. The population of the hypogeous fungus *Tuber aestivum* syn. *T-uncinatum* on the island of Gotland. Mycorrhiza, 14 (1), 19-23

Weig M., Haynes .K, Rogers T.R., Kurzai O., Frosch M., Muhlschlegel F.A., 2001. A GAS-like gene family in the pathogenic fungus Candida glabrata. Microbiology-SGM 147 (8): 2007-2019

Weiss M., Mikolajewski S., Peipp H., Schmitt U., Schmitt J., Wray V., Strack D., 1997. Tissue-specific and developpment-dependent accumulation of phenylpropanoids in larch mycorrhizas. Plant physiology, 114, 15-27

White T.J., Bruns T.D., Lee S.B. Taylor J.W., 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In Innis M.A., Gelfand D.H., Sninshy J.J., White T.D. (Eds.), PCR Protocols : a Guide to Methods and Applications. Academic Press, London, 315-322

Wright S., 1978. Evolution and the Genetics of Populations, Vol. 4. Variability Within and Among Natural Populations. University of Chicago Press, Chicago

Wu B., Nara K., Hogetsu T., 1999. Competition between ectomycorrhizal fungi colonizing *Pinus densiflora*. Mycorrhiza 9, 151-159

Zacchi L., Vaughan-Martini A., Angelini P., 2003. Yeast distribution in a truffle-field ecosystem. Annals of Microbiology, 53 (3), 275-282

Zambonelli A, Branzanti M.B., 1989. Mycorrhizal synthesis of *Tuber albidum* Pico with *Castanea sativa* Mill. And *Alnus cordata* Loisel. Agricultural Ecosystems, Environmental, 563-567

Zambonelli A, Branzanti M.B., 1990. Competizione fra *Tuber albidum* e alcuni basidiomiceti nella formazione di ectomicorrize su semenzali di Pinus pinea. Atti del 2° Congresso internazionale sul tartufo, Spoleto 24 Novembre 1988, 443-449

Zambonelli A., Iotti M., Giomaro G., Hall I., Stocchi V., 2002. T. borchii cultivation : an interesting perspective. Edible mycorrhizal mushrooms and their cultivation. Proceedings of the Second International Conference on Edible Mycorrhizal Mushrooms, Ed. Hall, I.; Yun Wang; Danell, E.; Zambonelli, A., Christchurch, New Zealand, 3-6 July, 2001

Zeppa S., Gioacchini A.M., Guidi C., Guescini M., Pierleoni R., Zambonelli A., Stocchi V., 2004. Determination of specific volatile organic compounds synthesised during Tuber borchii fruit body development by solid-phase microextraction and gas chromatography mass spectrometry Rapid Communications in Mass Spectrometry, 18 (2), 199-205

Zeppa S., Guidi C., Zambonelli A., Potenza L., Vallorani L., Pierleoni R., Sacconi C., Stocchi V., 2002. Identification of putative genes involved in the development of *Tuber borchii* fruit body by mRNA differential display in agarose gel.

Current Genetics 42 (3), 161-168
Zeppa S., Potenza L., Polidori E., Guescini M., Agostini D., Giomaro G. and Stocchi V. 2001. Cloning and characterization of a polyubiquitin gene from the ectomycorrhizal fungus *Tuber borchii* Vittad. In Curr. Genet. 40(1), 49-53
Zeppa S., Vallorani L., Potenza L., Bernardini F., Pieretti F., Guescini M., Giomaro G. and Stocchi V., 2000. Estimation of fungal biomass and transcript levels in *Tilia platyphyllos-Tuber borchii* ectomycorrhizae. FEMS Microbiol. Lett. 188, 119-124
Zeringue H.J., McCormick S.P., 1989. J. Am. Chem. Soc., 66, 581
Zhang D.X., Hewitt G.M., 2003. Nuclear DNA analyses in genetic studies of populations: practice, problems and prospects. *Molecular Ecology*, 12, 563-584
Zhou Z., Hogetsu T., 2002 . Subterranean community structure of ectomycorrhizal fungi under *Suillus grevillei* sporocarps in a Larix kaempferi forest. New Phytologist, 154, 529-539
Zhou Z., Miwa M., Matsuda Y., Hogetsu T., 2001. Spatial distribution of the subterranean mycelia and ectomycorrhizae of Suillus grevillei genets. Journal of Plant Research, 114, 179-185

2. RESULTATS

2.1 Etude de la variabilité génétique de la truffe noire du Périgord, *Tuber melanosporum* Vittad.

Dans cette partie, je me suis intéressé à la diversité génétique de *T. melanosporum*. Lors de mon DEA j'avais étudié sept régions génomiques (SCAR) par séquençage direct sur un échantillonnage d'ascocarpes récolté dans plusieurs régions. Malheureusement ces loci n'ont pas montré de polymorphisme. En revanche, j'avais trouvé plusieurs haplotypes pour l'ITS.

Nous avons donc décidé d'utiliser ce marqueur pour étudier la variabilité génétique de cette truffe. Pour cela, nous avons échantillonné 188 ascocarpes dans toutes les régions productives françaises. À la différence de Bertault et al. (1998), nous avons élargi notre analyse aux régions du nord de la France (Bourgogne et Lorraine) représentant la limite de production de *T. melanosporum*. Le séquençage direct de l'ITS pour ces ascocarpes a permis l'identification de 10 haplotypes. L'étude de leur distribution géographique dans les populations a montré une importante différenciation génétique confirmée par les valeurs de Fst (0,20), Nst (0,22) et Gst (0,22). Cette différenciation génétique était d'autant plus grande si l'on comparait les régions de l'Est et de l'Ouest du Massif Central. Une analyse phylogéographique (*Nested Clade Analysis*) a montré une association entre les haplotypes et les régions et a permis de suggérer que l'actuelle structure génétique de *T. melanosporum* serait due à une fragmentation ancestrale correspondant à deux lignées séparées par le Massif Central.

L'analyse des données disponibles sur la structure génétique des populations de chênes, nous a permis de suggérer que la truffe aurait pris refuge dans le sud de l'Italie associée à ses hôtes. Nous émettons l'hypothèse que ces deux lignées correspondent à deux voies de recolonisation post-glaciaire à partir d'une population du sud de l'Italie. À la fin de la glaciation, les truffes auraient suivi leurs hôtes et auraient recolonisé la France par deux voies : la voie de la vallée du Rhône et la voie de l'Atlantique.

Ces résultats ouvrent la voie à l'identification d'écotypes et de flux de gènes entre populations de *T. melanosporum*. Ces études sont fondamentales pour la réalisation d'Appellations d'Origine Contrôlée de cette truffe qui a un important impact économique (cf. § 1.2.1.2).

Polymorphism at the ribosomal DNA ITS and its relation to postglacial re-colonization routes of the Perigord truffle *Tuber melanosporum*

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Summary

- Glaciations and postglacial migrations are major factors responsible for the present patterns of genetic variation we see in natural populations in Europe. For ectomycorrhizal fungi, escape from refugia can only follow range expansion by their specific hosts.
- To infer phylogeographic relationships within *Tuber melanosporum*, sequences of internal transcribed spacers (ITS) and the 5.8S coding region of the ribosomal DNA repeat were obtained for 188 individuals sampled over the entire distribution of this species in France, and in north-western Italy and north-eastern Spain.
- Ten distinct ITS haplotypes were distinguished, mapped and treated using *F*- and N_{ST}-statistics and nested clade (NCA) analyses. They showed a significant genetic differentiation between regional populations. NCA revealed a geographical association of ITS haplotypes, an old fragmentation into two major groups of populations, which likely colonized regions on different sides of the French Central Massif.
- This re-colonization pattern is reminiscent of the one observed for host trees of the Perigord truffle, such as oaks and hazelnut trees. This suggests that host postglacial expansion was one of the major factors that shaped the mycobiont population structure.

Key words: ectomycorrhizal fungi, genetic differentiation, nested clade analysis, phylogeography, ribosomal DNA, truffle.

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Introduction

The Quaternary climatic fluctuations dramatically influenced the distribution of the flora and fauna in Europe (Hewitt, 1999). Repeated climate changes and the advance of glaciers forced animal and plants to retreat to southern refugia. The biota expanded after the last glaciations when the climate became warmer (Hewitt, 1996). Extensive studies on the postglacial expansion of animals and plants (Taberlet *et al.*, 2001), including forest trees (Petit *et al.*, 2003), contrast with the lack of knowledge on the populations of rhizospheric microorganisms. Ectomycorrhizal fungi are root-associated mutualistic symbionts of trees in boreal and temperate forests (Smith & Read, 1997) and their populations have probably been shaped by the reduction and expansion of the forests.

The Perigord truffle (*Tuber melanosporum* Vittad.) is an ascomycete endemic to calcareous soils in southern Europe and is found in symbiotic association with roots of deciduous trees, mostly oaks (*Quercus* spp.) and hazelnut trees (*Corylus avellana*) (Delmas, 1978). The fruiting body of *T. melanosporum*

is an edible truffle (= hypogeous ascocarp), which is highly appreciated for its delicate organoleptic properties (i.e. taste and perfumes) (Hall *et al.*, 2003). The high prize of the Perigord truffle has prompted the development of its culture through man-made inoculation of seedlings (Chevalier & Grente, 1978; Chevalier & Dupré, 1990). It can be assumed that the natural distribution and genetic structure of populations of this black truffle species have been structured by at least five major factors: first the distribution of its host plant species (i.e. ectomycorrhizal deciduous trees); second the spore dispersal by mycophagous animals; third limiting ecological factors (calcareous soils and a temperate climate); fourth geographical barriers (i.e. Mediterranean Sea, which limits its expansion towards North Africa), and fifth historical events (i.e. northward re-colonization routes from glacial refugia in southern Europe). The genetic diversity of *T. melanosporum* is strikingly low (Gandeboeuf *et al.*, 1997) and a population bottleneck probably occurred during the last, and coldest, glaciation (*c.* 10 000 yr ago), when the broadleaved forest of Europe was considerably reduced and restricted mainly to the Mediterranean coastal zone (Bertault *et al.*, 1998, 2001). To our knowledge, the analysis of genetic differences among southern populations of *T. melanosporum* and those occurring at the northern limits of this truffle species expansion (i.e. north-eastern France) has not yet been analyzed. Information on the genetic differentiation among these populations may shed light on postglacial phylogeography of this edible fungus.

We assessed the genetic variability of *T. melanosporum* isolates by sequencing the rDNA ITS and five sequenced characterized amplified regions (SCAR). Methods based on *F*- and N_{ST}-statistics showed a genetic differentiation between populations. As these methods did not detect historical events (e.g. fragmentation or range expansion) that affected the populations, we implemented our genetic study with nested clade analysis (NCA) (Templeton *et al.*, 1995; Templeton, 1998, 2004) to shed light on the possible migration processes after the last glaciation. These analyses strengthened the 'glaciation hypothesis' (Bertault *et al.*, 1998) with a rapid colonization of Western Europe from relict Italian populations of *T. melanosporum*. The identified migration routes in France are similar to those observed for oaks species (e.g. *Quercus pubescens*) and other hardwood tree species (Petit *et al.*, 2002a, 2003) which can host *Tuber* species.

Materials and Methods

Sampling sites

The study organism, *Tuber melanosporum* Vittad. (Ascomycota, Pezizomycotina, Pezizomycetes, Pezizales, Tuberaceae), is endemic to France and certain areas of Southern Europe in Italy and Spain, and occurs only in calcareous soils in association with deciduous trees. This black truffle does not tolerate the cold temperatures of high mountains or northern Europe. These ecological requirements cause a fragmented distribution of *T. melanosporum* in France (Callot *et al.*, 1999). For instance, this fungus is absent from the acid and coldest regions of the Central Massif and the Alps, and the acidic sandy soils of Landes (south-western France). Our sampling strategy aimed to cover the natural geographical range of *T. melanosporum* in France to investigate whether genetic variation among regional populations occurs. Fruiting bodies of *T. melanosporum* were collected in natural truffle grounds in 17 geographical regions of France, northern Italy (Piedmont) and north-eastern Spain (Iberian Mountains) (Supplementary material Table 1). For the analysis of molecular variance (AMOVA) and nested clade analysis (NCA), the 17 sampled regional populations were pooled to have sufficient sample sizes for meaningful comparisons in seven larger geographical areas corresponding to: first Lorraine, the northernmost known limits of expansion; second Burgundy; third the calcareous Pre-Alps (Isere, Drome) and southern Jura; fourth the coastal southern France (Rhone Provence, Inner Provence, and Languedoc) and the Pyrenees (Roussillon and Ariege); and fifth the western France regions including the foothills of the Central Massif range (Tarn, Perigord-Quercy, Lower Quercy) and Charentes and Touraine (Fig. 1). Ascocarps from north-western Italy (Piedmont, region 6) and north-eastern Spain (region 7) were pooled and included in our study. Most Spanish samples were from a series of valleys along the Iberian Mountains, a calcareous mountain system that extends *c.* 400 km along the north-eastern edge of the Meseta (Central Plateau) in Spain. The Italian truffles were sampled in the Val Curone valley (Alessandria area), a sedimentary-calcareous region, which extends at the southern edge of the Po Plain. Owing to the low polymorphism of *T. melanosporum* and its selfing reproduction, a few individuals can reflect the regional populations better than extensive sampling per locality (Bertault *et al.*, 2001). We thus sampled one or two fruit bodies per truffle ground in 120 localities over a wide geographical range (Supplementary material Table 1) for ITS sequencing. A single ITS sequence was analyzed per truffle ground (except when several ITS haplotypes were found) to avoid bias linked to the repeated use of ascocarps originating from the same clonal mycelium. Fruit bodies were collected with the help of local pickers and trained dogs in natural truffle grounds during winter from December 1998 to February 2003. Precise geographical information is not provided to protect the truffle grounds from furtive harvesting. Each ascocarp was washed, its peridium peeled and its inner part (= gleba) was conserved at −80°C pending DNA extraction.

DNA manipulations

Total DNA was extracted from 20 mg of gleba with the DNeasy Plant Mini Kit (Qiagen SA, Courtaboeuf, France) following the manufacturer's instructions. Single nucleotide polymorphisms (SNP) were sought in the rDNA internal

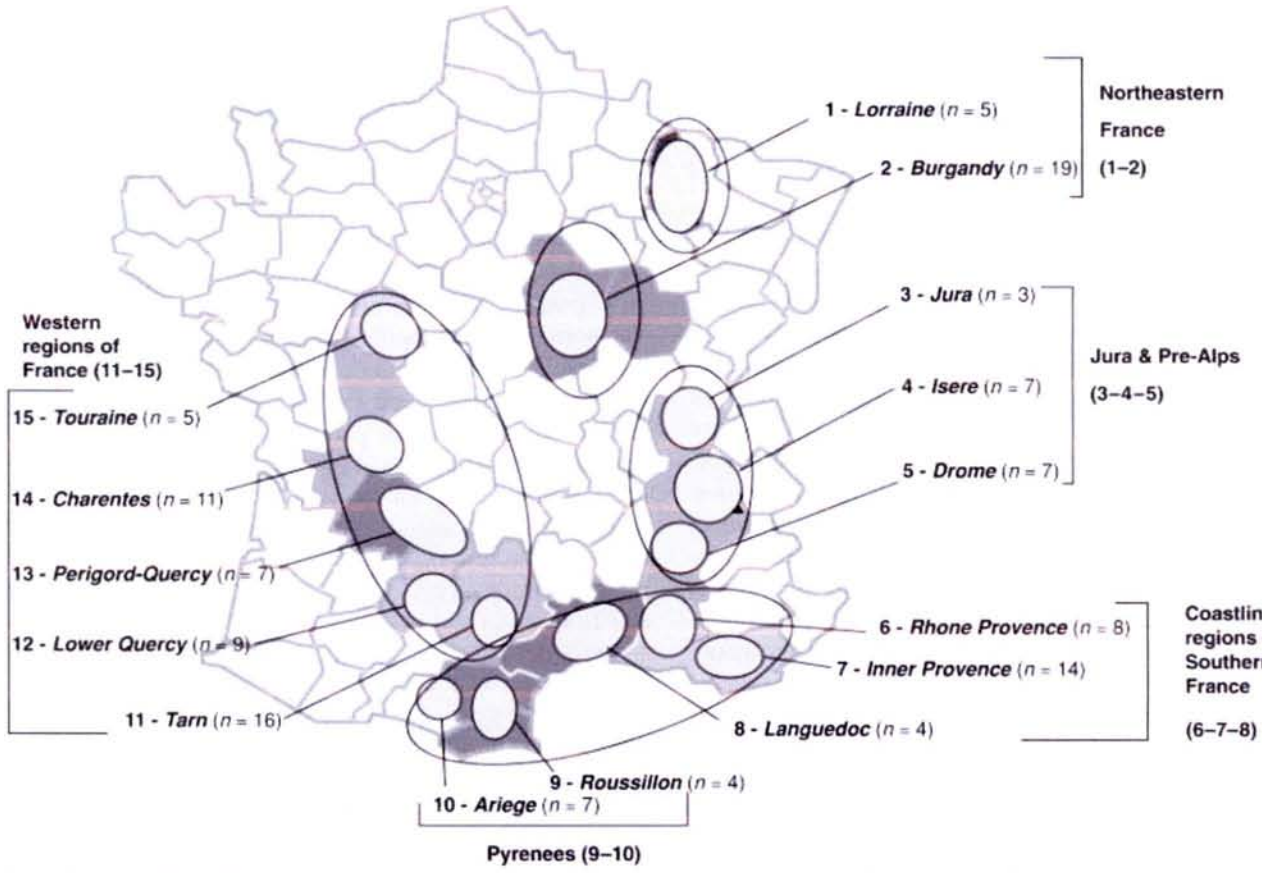

Fig. 1 Map of France showing the regions were accessions of *Tuber melanosporum* were sampled. Borders of the French administrative departments are outlined. The number of samples for each population is given in brackets (n). See Supplementary material Table 1 Data for details on sampling sites. The groupings of regional populations used in the analysis of molecular variance (AMOVA) are indicated.

transcribed sequences (ITS) and in randomly amplified genomic sequences (SCAR). Amplification and sequencing of the nuclear rDNA ITS from 188 specimens were carried out as previously described (Henrion *et al.*, 1994; Martin *et al.*, 2002). Sequencing of rare ITS haplotypes was carried out at least twice on different DNA extracts to rule out any amplification and sequencing artefacts. RAPD amplifications were carried out with primers E4, E20, and G14 (Operon Technology, Alameda, CA, USA), using the conditions described by Gandeboeuf *et al.* (1997). Five nonpolymorphic RAPD bands were cloned and sequenced from the specimen MEst collected in Bauduen (Var, France). RAPD products were ligated into a pCR4-TOPO plasmid vector of a TOPA-TA Cloning Kit (Invitrogen, Groningen, The Netherlands). Both strands of inserts were sequenced with a *Taq* Big DyeDeoxy Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA), and analyzed with an ABI 310 DNA sequencer (Applied Biosystems). Sequences were assembled using Sequencher 3.1.1 (GeneCodes, Ann Arbor, MI, USA) and SCAR primers were designed (available upon request). For a set of specimens representative of the different regional populations, we amplified the SCAR sequences using primers herein designed and PCR conditions used for ITS amplifications. The sequences of ITS and SCARs were deposited in GenBank (National Center for Biotechnology Information, NCBI). Accession numbers are provided in Supplementary material Table 1. SNPs were detected by sequence multialignments using the 'assembly contig' routine of Sequencher 3.1.1.

Data analyses

We used three approaches to investigate genetic differences among regional populations: first the index of fixation (Wright, 1978) implemented with AMOVA (Excoffier *et al.*, 1992); second N_{ST}-statistics (Pons & Petit, 1996); and third NCA (Templeton, 1998, 2004). The AMOVA analyses was carried out with ARLEQUIN 2.001 (Excoffier *et al.*, 1992), which calculated Φ_{ST} analogous to Wright's F_{ST}. We partitioned the genetic variation between three hierarchical components: among seven groups of regional populations (i.e. geographical areas), among 17 regional populations and within regional populations (Fig. 1). Fixation index was calculated for the whole geographical range, between the seven groups of regional populations, and between all region pairs (Schneider *et al.*, 2000). A Neighbor-Joining (NJ)

dendrogram was constructed using Slatkin's genetic distance calculated in ARLEQUIN, to analyze the relationship between genetic distance among regional populations with their relative geographical positions. The Neighbor-Joining analysis was carried out using PAUP4.08b (Swofford, 2002). Genetic differentiation among the seven groups of regional populations and among the 17 regional populations was also tested applying N_{ST}-statistics (Pons & Petit, 1996) using the programme PERMUT (available from http://www.pierroton.inra.fr/genetics/labo/Software/). This test compares N_{ST} with values of G_{ST}. N_{ST} estimates consider not only differences in the frequencies of haplotypes between populations, as with G_{ST}, but also genetic distances between haplotypes. In cases of correspondence between haplotype phylogenies and their geographical distribution, estimates for N_{ST} will be greater than G_{ST} values (Pons & Petit, 1996).

We used a Mantel test to analyze a potential correlation between geographical (Km) and genetic distance and to estimate the effect of isolation-by-distance (Rousset, 1997; Hutchison & Templeton, 1999). A matrix of distances among the seven groups of regional populations or the 17 regional populations was calculated with the module GEODISTANCES of the Package R. 4.0d3 (Casgrain & Legendre, 2001), which used latitudes and longitudes. Mantel statistics were calculated in ARLEQUIN on the matrices of geographical distances [Ln (distance in Km)] and genetic distance as Slatkin's linearized pairwise $\emptyset_S(\emptyset_{ST}/1 - \emptyset_{ST}$; Slatkin, 1995). The signification of the correlations was tested by 9999 random permutations.

The null hypothesis that there is no geographical association between sequence types and geographical localities (i.e. populations) was tested by permutation (10 000 replicates) using the program CHIPERM version 1.2 (Posada, 2000; available at: http://bioag.byu.edu/zoology/crandall_lab/programs.htm). We then estimated a sequence-type network using the programme TCS (Clement *et al.*, 2000), which uses statistical parsimony (Crandall, 1996) for network estimation. The maximum number of mutational steps that constitute a parsimonious connection between two sequence types was calculated with 95% confidence. The resulting network was used to construct the nested clade design with TCS (Clement *et al.*, 2000). NCA has become a common tool in intraspecific phylogeography (Templeton, 2004) and has been used to investigate phylogeographic relationships of rDNA ITS haplotypes (James *et al.*, 2001; Rodríguez-Lanetty & Hoegh-Guldberg, 2002; Wörheide *et al.*, 2002). After the nesting procedure, we employed the NCA of geographical distances (Templeton *et al.*, 1995; Templeton, 1998, 2004) using the program GEODIS version 2.0 (Posada *et al.*, 2000), with 10 000 permutations. GEODIS addresses the sampling strategy adequacy and quantifies the degree of confidence in the quantitative distance measure by testing the null hypothesis that the haplotypes (or clades) nested within a high nested clade show no geographic associations given their overall sample numbers.

The NCA of geographical distances, as implemented in the program GEODIS, has two parts. Part one looks for statistically significant geographical associations of sequence types by permutational X^2 statistics for each clade. Then part two, using different geographical information, estimates two different geographical parameters for each clade in the nested design. $D_c(x)$, the clade distance, measures the average distance of each member of a clade from its geographical centre and measures how geographically widespread are the individuals that bear haplotypes from this clade, that is $D_c(x)$ is a measure for the geographical range of clade x. $D_n(x)$, the nested clade distance, measures the average geographic distance of all members of a clade from the geographical centre of its higher-level nesting clade, which is also estimated by averaging the coordinates of all members of the higher-level nesting clade. (I–T)D_c and (I–T$_n$)D_n give the average D_c and D_n values for all the interior clades within a nesting clade minus the average D_c and D_n values for all the tip clades in the same nesting clade. All calculated distances can either be significantly large, small or nonsignificant. Significant values from the second part of the analysis are then used to work with the inference key (Templeton, 2004) (available at http://bioag.byu.edu/zoology/crandall_lab/geodis.htm) to infer patterns of population structure, population history or a combination of both. The last version of the inference key reduces the incidence of false positive to a minimum at a cost of a slight reduction in power (Templeton *et al.*, 1995; Templeton, 1998; Cruzan & Templeton, 2000; Templeton, 2004). However, one must be cautious in inferring cause and effect in NCA analyses (Knowles & Maddison, 2002). Criticisms of Knowles & Maddison (2002) and Hey & Machado (2003) regarding NCA do not, however, apply to our study as NCA performed well in the case of range expansion (Templeton, 2004). The criticism of Petit & Grivet (2002), that genetic fixation within populations can bias the outcome of NCA, should not apply to our data, as we analyzed single individuals from isolated truffle grounds.

Results

SNP analyses and haplotype frequencies in regional populations

Sequencing 50 kbp of the PCR-amplified SCAR fragments from a representative set of *T. melanosporum* fruit bodies from the different geographical areas revealed no SNP. By contrast, there were nine variable sites corresponding to 10 different haplotypes (Table 1) across all 188 ITS sequences. For the statistical analyses, only one fruiting body was considered from each truffle ground to avoid the possibility of sampling ascocarps originating from the same fungal individual mycelium. A total of 148 ITS sequences were finally analyzed. ITS haplotypes (variants) I and II were the most common haplotypes (60% and 28%, respectively), whereas haplotype

Table 1 Single nucleotide polymorphism (SNP) sites in the 10 rDNA ITS haplotypes of *Tuber melanosporum* found among 188 specimens collected throughout its natural range in 17 regions in France, north-western Italy (Piedmont) and north-eastern Spain (Iberian Mountains)

Haplotypes	Regional populations	Nucleotide position 0 1 1 2 3 4 4 5 5 / 0 4 7 1 6 2 9 0 3 / 7 3 2 5 9 9 7 4 5
I	All, except Languedoc	T C C C C C C T G
II	All, except Jura, Roussillon, Ariege & Touraine	G . . . T . . . C
III	Burgundy, Piedmont, Inner Provence & Languedoc	 C
IV	Tarn	G . . . . .
V	Ariege	. . T . . . . .
VI	Ariege	. . . . . T . . .
VII	Tarn	G . . . T . T . C
VIII	Jura	G . . . T . G . C
IX	Drome	G . . A T . . . C
X	Languedoc	. G C

The most frequent haplotype (haplotype I) was used as the reference sequence. Identical nucleotides are indicated by dots. Regions where haplotypes were sampled are indicated (see Supplementary Material Table 1 for details).

Table 2 Analysis of molecular variance (AMOVA) of ITS haplotypic diversity for 17 regional populations within France, north-western Italy and north-eastern Spain

Source of variation	d.f.	SDD	Variance components	% Total
Among groups of regional populations	6	20.490	0.1213	16.52
Among populations within groups	10	16.982	0.02480	3.38
Within populations	131	11.344	0.58816	80.10
Total	147	77.048	0.73427	100.0

The analyses were carried out using 148 collections from different truffle grounds (see sampling localities in Supplementary Material Table 1). Variance was partitioned among groups of regional populations (i.e. geographical areas), among regional populations within these areas, and within regional populations (see Fig. 1). Degrees of freedom (d.f), sums of square deviations (SSD), variance component estimates, and the percentages of the total variance (% Total) contributed by each component. F_{ST} was = 0.20, P-value < 0.001.

III was less frequent (7%) (Supplementary material Table 1). The remaining haplotypes presented a very low frequency (< 2%). These 10 haplotypes showed a differential geographical distribution (Supplementary material Table 1; Fig. 2). Haplotypes I and II were distributed widely in most sampling sites in France, Italy and Spain, whereas the other haplotypes presented a geographically restricted range. Haplotype I was more frequent in the western French Atlantic regions (e.g. 90% of ascocarps in Charentes) than in populations in eastern and north-eastern France (e.g. 20% in Burgundy). By contrast, haplotype II was more frequent in eastern France and north-western Italy (e.g. 71% in Isere and 54% in Italian Piedmont) (Fig. 2). Haplotype III presented a disjunct distribution and was found only in Burgundy (32%), Inner Provence (6%), Languedoc (25%), and Piedmont (18%). Haplotypes IV to X were distributed locally and were restricted to single truffle grounds (Supplementary material Table 1; Fig. 2). Additional surveys are, however, needed to confirm the regional ITS haplotype frequencies in the smaller populations.

Genetic differences among regional populations

A F_{ST} value of 0.20 ($P < 0.001$) indicates a significant genetic differentiation among *T. melanosporum* populations (Wright, 1978). A test of correspondence, carried out using PERMUT (Pons & Petit, 1996), confirmed this genetic differentiation ($N_{ST} = G_{ST} = 0.22$). Since the large geographical areas (eastern and western France) were further subdivided into regions occupied predominantly by a few haplotypes (Supplementary material Table 1; Fig. 2), hierarchical partitioning of variation (AMOVA) was conducted within the 17 regional populations, among regional populations, and between geographical areas (i.e. groups of regional populations). The bulk of the total haplotype diversity was found within populations (*c.* 80%). Additional variation was attributable to differences among populations within a geographical area (*c.* 3%) (Table 2). A significant variation (*c.* 17%) was attributable to the difference between geographical areas (groups of regions). The higher pairwise genetic distance value was found for populations found on the eastern and western sides of the Central Massif (i.e. Atlantic regions vs Rhone valley). A NJ dendrogram of F_{ST} genetic distances reflected the geographical distribution of the

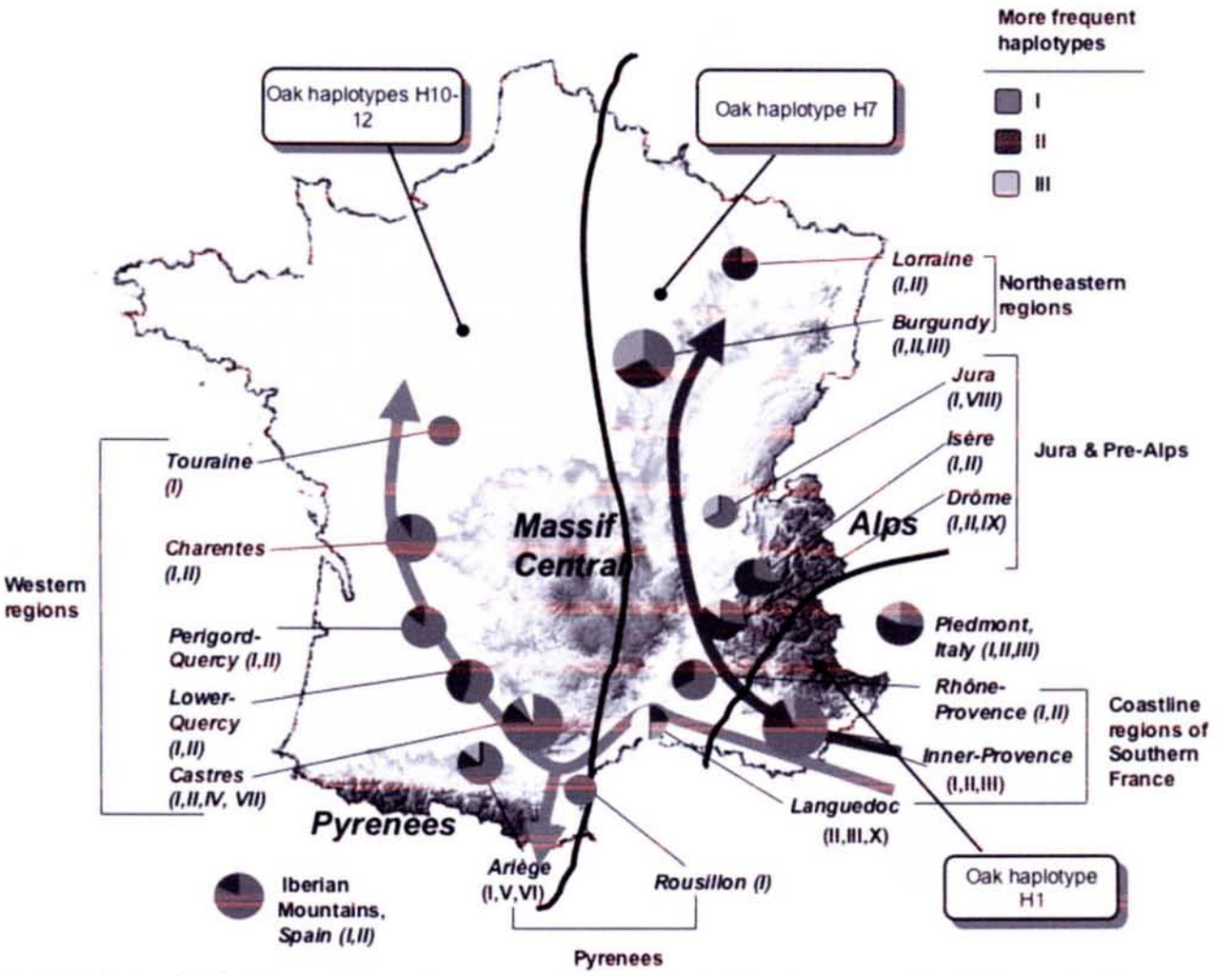

Fig. 2 Distribution of ITS haplotypes of *Tuber melanosporum* in France. The pie chart diameters are proportional to the number of ascocarps analyzed per region. Black lines delimit areas of distribution of the chlorosplastic DNA (cpDNA) haplotypes of oaks in France (Petit *et al.*, 2002a): haplotype 1 was found in the southern corner of France; haplotype-7 in the Rhone valley, and haplotypes 10–12 in western France. Arrowed lines show potential postglacial re-colonization routes for the Perigord truffle: the Atlantic route and the Rhone valley route.

haplotypes described above (Fig. 3). Two main clusters were characterised: populations of the 'western' cluster have a frequency of haplotype I higher than haplotype II and presented rare haplotypes (e.g. IV & VII in Tarn). The populations within the 'western' cluster were characterized by a low frequency of haplotype II, and included populations found in the Pyrenees, western Atlantic regions and southern France (e.g. haplotype V and VI in Ariege). Populations of the 'eastern' cluster have a higher frequency of haplotype II and were found in north-eastern France (Lorraine), and in the Alps (Drome, Isere, Piedmont). Burgundy presented similar percentages of haplotypes I and II and nested outside the main clusters. Thus, the *F*-statistics showed that the position of the regional populations related to the Central Massif range impacted the haplotype frequencies in the populations and the genetic distances among them.

According to N_{ST}-statistics ($N_{ST} = G_{ST}$), there is no or only weak overall correspondence between the haplotype phylogenies and geographical distribution (Pons & Petit, 1996). This result is presumably the result of the wide geographical codistribution of the most numerous haplotypes (I & II). However, the NJ tree based on the F_{ST} genetic distances between ITS haplotypes (Fig. 3) joined adjacent subregions. The increase in genetic differentiation with geographical distance was marginally significant (Mantel test, $P = 0.036$). However, the genetic difference drastically increased for population pairs located on different sides of the Central Massif suggesting a potential geographical isolation by this mountainous range. The AMOVA (Table 2), the N_{ST} test (data not shown), the NJ tree (Fig. 3), and the Mantel test (data not shown) were not significantly affected by the small size of several of the analyzed populations and the low frequencies of the rare alleles as shown by analyses performed using only subsamples of the entire populations (data not shown). For example, F_{ST}, N_{ST} and G_{ST} values of 0.18, 0.16 and 0.12 were found when the

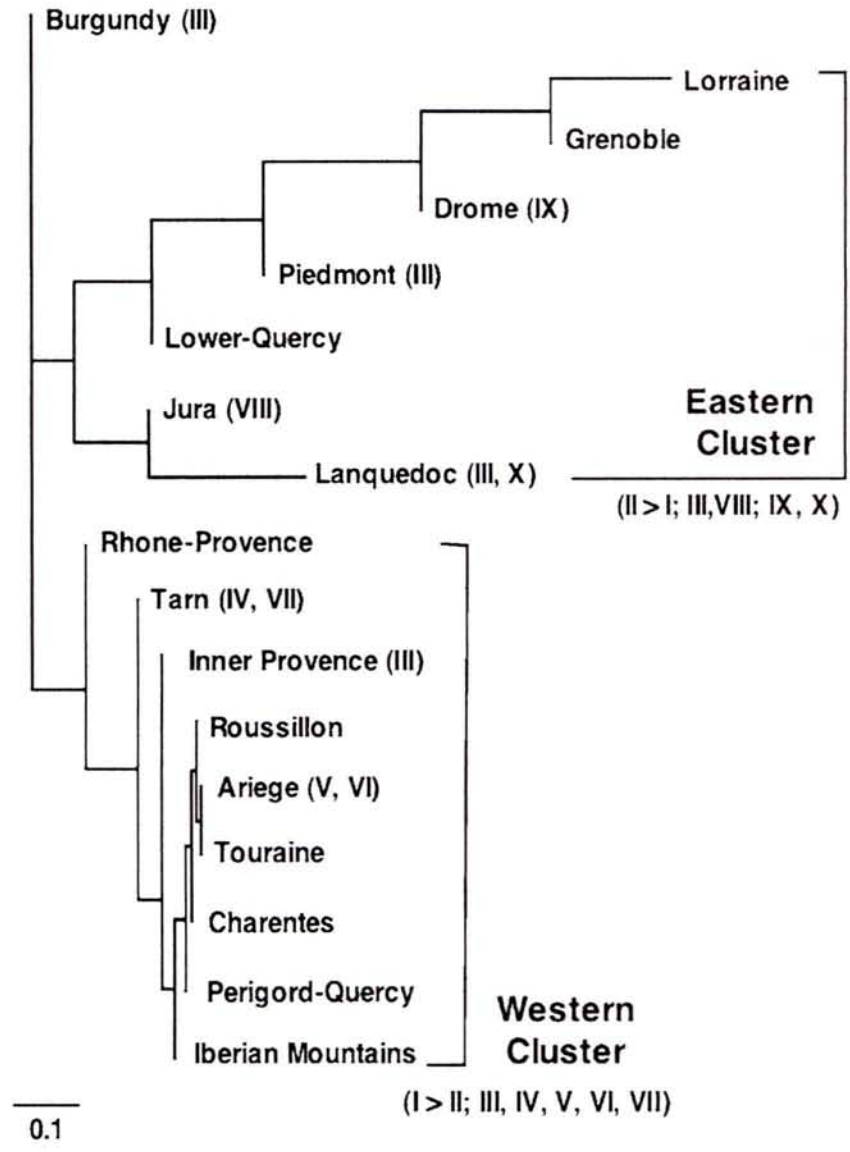

Fig. 3 Neighbour-joining tree of the 17 regional populations sampled in France, north-western Italy (Piedmont) and north-eastern Spain (Iberian Mountains) based on Slatkin's genetic distances. Frequent and rare haplotypes are indicated.

analyses were carried out on the seven larger geographical areas (Fig. 1) confirming the significant genetic differentiation among groups. Most of the results were also obtained even when the analyses were limited to the three common alleles.

Haplotype network and nested design

In this study, we applied a nested clade analysis approach (NCA; Templeton *et al.*, 1995, Templeton, 1998, 2004), to investigate the roles of contemporary ecological processes and population histories in shaping the natural populations of *T. melanosporum* in France. The utility and applicability of ITS rDNA sequences for phylogeography and NCA has been demonstrated in a study of the basidiomycetous species *Schizophyllum commune* (James *et al.*, 2001). The initial test for geographical association of haplotypes revealed a significant association between sequence types and geographical locations (Monte Carlo significance < 0.001); therefore we proceeded with the NCA. The nested cladogram comprises 11 0-step clades (haplotypes), four one-step clades (1–1, 1–2, 1–3 and 1–4) and one 2-step clade (2–1) (Fig. 4). The 95% connection limit was established at nine steps, far from the largest number of connections detected between two sequences. Ambiguities were evident in only one area of the sequence-type network, where a closed loop between four haplotypes (I, III, IV and one hypothetical one which was not observed) was found. This ambiguous loop did not affect subsequent nesting procedures. The nesting routine of the programme TCS generated three 1-step nested clades. The 1-step nested clades 1–1, 1–2 and 1–3 are tip clades, and clade 1–4 is an interior clade. Haplotypes located at the tips of the cladogram tended to have restricted geographical distributions, whereas ubiquitous, and presumably ancestral haplotypes, were on interior nodes (i.e. haplotypes I to IV) (Templeton *et al.*,

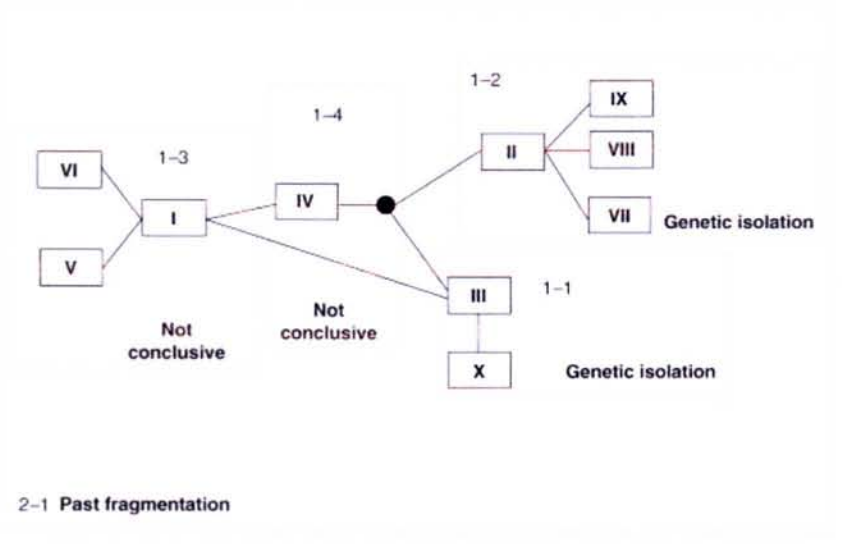

Fig. 4 Nested clade design used for nested contingency analysis of geographical associations of 10 ITS haplotypes of *Tuber melanosporum*. The black dot denotes a hypothetical (not observed) intermediate haplotype. Each line in the network represents one mutational step. Nested design was based on the sequence type network generated by the TCS programme and inferred using the rules defined by Templeton (2004). The Templeton's inference series are given at the bottom of the figure.

1995). Clade 1–1 comprised the haplotype III found only in northern Italy, and eastern and southern France (Burgundy, Languedoc, and Inner Provence) and the rare haplotype X collected in Languedoc. In clade 1–2 nested the haplotype II (highly frequent in northern Italy and eastern France) and the tip haplotypes VII (Tarn), VIII (Jura) and IX (Drome). The ancestral haplotype I, predominant in western France, nested in clade 1–3 with the rare haplotypes V and VI (Ariege). Finally, a nonsampled haplotype and haplotype IV (Tarn) nested in clade 1–4. The null hypothesis was rejected ($P < 0.0001$) for the overall tree (and for all the clades) showing that our sampling strategy was adequate for the phylogeographic analysis.

The NCA inference analysis (Templeton, 2004) for the two-step clade 2–1 (Fig. 4) indicated a past fragmentation. Such a past fragmentation could account for the different frequency of haplotypes I and II in western and eastern France (see Discussion). The inference series for the more recent outer part of the haplotype tree indicated an isolation of the populations (Fig. 4). In particular, clade 1–2 indicated a restricted gene flow with genetic isolation of populations having the rare haplotypes VII to IX. The analyses of clade 1–1 indicated as well an isolation of the populations, which restricted the dispersion of haplotype X (Languedoc) after arising from haplotype III. The series of inference for clades 1–3 and 1–4 were not conclusive (Fig. 4).

Discussion

The moderate variation of the nuclear rDNA ITS from *Tuber melanosporum* revealed a strong pattern of geographic differentiation. Within 188 isolates collected over the whole geographical distribution of the Perigord truffle, including the northern limits of expansion of this species (Burgundy and Lorraine), 10 distinct haplotypes were distinguished (Table 2; Supplementary material Table 1). One of these, haplotype I, was particularly common, predominant in every population except in eastern France where haplotype II was the most frequent. The presence of one very common haplotype in most populations confirms that this species went through a population bottleneck, after which new allelic haplotypes have originated in low frequencies (Bertault *et al.*, 1998). It appears that some of the southern populations (e.g. Languedoc) had the largest number of haplotypes, while Touraine and Roussillon had only one haplotype each (Fig. 2; Table 1; Supplementary material Table 1), although further surveys (i.e. larger sample size) may modify this distribution. Ascocarps were harvested in different truffle grounds ruling out the sampling of fruiting bodies belonging to the same mycelium.

Index of fixation approach ($F_{ST} = 0.20$), N_{ST} (0.22), and AMOVA showed a significant geographic differentiation between populations. We also found a significant association between genetic (F_{ST}) and geographical distances (Mantel test) when we clustered the distance values between populations in two groups (one with populations from western France and the other formed by the populations of the Rhone Valley). This suggests that geographical factors (e.g. the Central Massif range) may have had an important impact on the gene flow among populations. These analyses were based on a single locus that can represents, at the best, only a snapshot of the evolutionary history of *T. melanosporum* (Beerli & Felsenstein, 1999). The occurrence of significant allelic variation in ITS contrasts with the lack of sequence polymorphisms within the

SCAR sequences. It is thought that concerted evolution limits variation in the ITS and other regions of ribosomal DNA repeat (Ganley & Scott, 2002) and the current data was therefore unexpected. Additional analyses using the vegetative incompatibility gene sequence and microsatellite loci are currently underway to confirm the geographic differentiation deduced from the ITS polymorphism.

Truffle postglacial history suggested by nested clade analysis

Using NCA, it may be possible to infer the factors that generated genetic differentiation between populations and to predict migration routes (Templeton *et al.*, 1995; Templeton, 2004), although its validity and limitations are debated (Knowles & Maddison, 2002; Petit & Grivet, 2002; Templeton, 2004). NCA incorporates the geographical distribution of the haplotypes to the nested phylogenetic relationships among haplotypes to infer population events in historical order (Templeton, 1998). The geographical contingency analysis supported the differences among populations, as Monte Carlo's test for the association of haplotypes with regions was highly significant ($P < 0.001$).

If we accept the 'interior equals oldest' predictions from coalescent theory (Templeton, 1998), then we can infer that the interior haplotypes (I, II and III) of the network are the oldest ones (Fig. 4). All these haplotypes are present in northern Italy, and they likely represented the main haplotypes present in the glacial refugia. This is in agreement with the hypothesis by Bertault *et al.* (1998) of a drastic glacial bottleneck as origin of the low polymorphism of the Perigord truffle, and the reduced number of ITS haplotypes of *T. melanosporum* is consistent with the survival of small and isolated populations in southern Europe refugia. The bottleneck of *T. melanosporum* populations likely occurred during the last and the coldest glacial period (10 000–16 000 yr ago) (Bertault *et al.*, 1998). Such a population bottleneck probably fixed most of the loci, accounting for the lack of SNPs within the SCAR markers (present work). Subsequent expansion from these smaller populations would carry different combinations of ITS haplotypes to colonized areas. The presence of the three ancestral haplotypes of *T. melanosporum* in Piedmont supports the 'Italian peninsula origin' hypothesis (Bertault *et al.*, 1998) with a migration pathway through the Po plain to the western Alps and a subsequent northward expansion along the western and eastern France migration routes. Following Templeton's inference key, we found a statistically significant past fragmentation in the two step clade 2.1. This past fragmentation probably occurred during the colonisation of calcareous foothills along the Central Massif (Fig. 2). Regions having a higher level of diversity (e.g. Languedoc) may correspond to the separation point of the two colonisation routes, whereas regions at the edge of migration routes, such as Touraine and Lorraine, contain only a subset of the lineages described and therefore exhibit lower levels of allelic richness. After the initial rapid colonization, the Central Massif mountainous range, the mosaic pattern of limestone soils in France and the strict soil requirement of the Perigord truffle may have represented effective barriers limiting the gene flow between regions and maintaining the genetic differences until today. Due to the unfavorable acidic sandy soils of Landes (south-western France), a plausible route to colonize Spanish calcareous regions may have been the eastern coastline of the Pyrenean region, from which the black truffle could reach the calcareous Iberian Mountains in north-eastern Spain (Fig. 2). On the other hand, *T. melanosporum* probably survived in refugia in Spain with haplotype I and II moving out of Spain to colonize western France and types I, II and III coming out of Italy to colonize Eastern France. A more extensive data set from the Iberian Mountains and other Spanish regions will surely provide further clues to support these contrasted scenarii.

Black truffle and its hosts shared the routes of postglacial re-colonization in France

During the maximum expansion of the glaciers, the deciduous forest of Europe was restricted to the Mediterranean costal zone (Bennet *et al.*, 1991). Climatic and fossil data support the hypothesis that three regions hold the main glacial refugia for the host trees of *T. melanosporum*: the Iberian and Italian Peninsulas, and the Balkans (Comes & Kadereit, 1998; Taberlet *et al.*, 2001). Since *T. melanosporum* is an ectomycorrhizal symbiont of oaks and other temperate deciduous trees, such as *Tilia* and *Corylus* species, this symbiotic fungus was likely restricted to some of these regions. Molecular and fossil (i.e. pollen) data have revealed that the two main northwards re-colonization routes for oaks and many hardwood species (including *Tilia* and *Corylus* species) were the Rhone valley and the western plain of France (Brewer *et al.*, 2002; Petit *et al.*, 2002a; 2002b; 2003). They determined the current distribution of the haplotypes of oak species (e.g. the thermophilous *Quercus pubescens*) in France (Fig. 3 in Petit *et al.*, 2002a). In Provence, the oak haplotype 1 is the most prominent haplotype (Fig. 2). In the Rhone valley, haplotype 7 dominates, whereas the western France populations are dominated by the closely related oak haplotypes 10, 11 and 12. Although edaphic (e.g. calcareous soils) and paleoclimatic factors likely influenced *T. melanosporum* postglacial recolonization patterns, the NCA-suggested routes of expansion for the Perigord truffle in France closely resemble that of *Q. pubescens* (Petit *et al.*, 2002a). It is therefore tempting to speculate that the oak re-colonization routes have impacted on the distribution of *T. melanosporum* populations during their northward migration (Fig. 2). It has already been suggested that the northwards expansion of plant and animal species from southern refugia likely followed the establishment of deciduous woodland. For example, oak and hedgehog species followed similar

expansion patterns (Seddon *et al.*, 2001). Mycophagous animals (e.g. squirrels, wild boar) known to dispersed truffles spores probably favored this expansion. Oak species can withstand the cold conditions of northern Europe, while *T. melanosporum* is restricted to mid-latitudes. It is therefore possible that this truffle species migrated northwards later than oaks and thus oaks could have been widespread by the time *T. melanosporum* reached France. These various hypotheses will be investigated using additional methods to infer population histories, and larger fruit body surveys.

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Supplementary material

The following material is available as Supplementary material at http://www.blackwellpublishing.com/products/journals/suppmat/NPH/NPH1189/NPH1189sm.htm

Table S1 List of *Tuber melanosporum* specimens and sampling localities used in the present study.

References

Beerli P, Felsenstein J. 1999. Maximum-likelihood estimation of migration rates and effective population numbers in two populations using a coalescent approach. *Genetics* **152**: 763–773.

Bennet KD, Tzedakis PC, Willis KJ. 1991. Quaternary refugia of North European trees. *Journal of Biogeography* **18**: 103–115.

Bertault G, Raymond M, Berthomieu A, Callot G, Fernandez D. 1998. Trifling variation in truffles. *Nature* **394**: 734.

Bertault G, Rousset F, Fernandez D, Berthomieu A, Hochberg ME, Callot G, Raymond M. 2001. Population genetics and dynamics of the black truffle in a man-made truffle field. *Heredity* **88**: 451–458.

Brewer S, Cheddadi R, De Beaulieu JL, Reille M. 2002. The spread of deciduous *Quercus* throughout Europe since the last glacial period. *Forest Ecology and Managment* **156**: 27–48.

Callot G, Byé P, Raymond M, Pargney JC, Parguey-Leduc A, Janex-Favre MC, Moussa R, Pagès L. 1999. *La Truffe, la Terre, la Vie.* Paris, France: INRA Press.

Casgrain P, Legendre P. 2001. *The R package for multivariate and spatial analysis*, Version 4.0d3 – User's Manual. URL. http://www.fasumontreal.ca/BIOL/legendre/.

Chevalier G, Dupré C. 1990. Recherche et expérimentation sur la Truffe et la trufficulture en France. In: Bencivenga M, Granetti B, eds. *2nd Congresso Internazionale Sul Tartufo.* Spoleto, Italy: Comunità Montana dei Monti Martani e del Serano, 157–166.

Chevalier G, Grente J. 1978. Applications pratiques de la symbiose ectomycorhizienne: production à grande échelle de plants mycorhizés par la truffe (*Tuber melanosporum*). *Mushroom Science* X: 483–505.

Clement M, Posada D, Crandall KA. 2000. TCS: a computer program to estimate gene genealogies. *Molecular Ecology* **9**: 1657–1659.

Comes H, Kadereit JW. 1998. The effect of Quaternary climatic changes on plant distribution and evolution. *Trends in Plant Science* **3**: 432–438.

Crandall KA. 1996. Multiple interspecies transmissions of human and simian T-cell leukemia/lymphoma virus type I sequences. *Molecular Biology and Evolution* **13**: 115–131.

Cruzan MB, Templeton AR. 2000. Paeloecology and coalescence phylographic analysis of hypothesis from the fossil record. *Trends in Ecology and Evolution* **15**: 491–496.

Delmas J. 1978. *Tuber* spp. In: Chang ST, Hayes WA, eds. *The biology and cultivation of edible mushrooms*, London, UK: Academic Press, 645–681.

Excoffier L, Smouse PE, Quattro JM. 1992. Analysis of molecular variance inferred from metric distances among DNA haplotypes: application to human mitochondrial DNA restriction data. *Genetics* **131**: 479–491.

Gandeboeuf D, Dupré C, Roecket-Drevel P, Nicolas P, Chevalier G. 1997. Grouping and identification of *Tuber* species using RAPD markers. *Canadian Journal of Botany* **75**: 36–45.

Ganley ARD, Scott B. 2002. Concerted evolution in the ribosomal RNA genes of an *Epichloe* endophyte hybrid: comparison between tandemly arranged rDNA and dispersed 5S rRNA Genes. *Fungal Genetics and Biology* **35**: 39–51.

Hall IR, Yun W, Amicucci A. 2003. Cultivation of edible ectomycorrhizal mushrooms. *Trends in Biotechnology* **21**: 433–438.

Henrion B, Chevalier G, Martin F. 1994. Typing truffle species by PCR amplification of the ribosomal DNA spacers. *Mycological Research* **98**: 37–43.

Hewitt GM. 1996. Some genetic consequences of ice ages and their role in divergence and speciation. *Biology Journal of the Linnean Society* **58**: 247–276.

Hewitt GM. 1999. Post-glacial re-colonization of European biota. *Biology Journal of the Linnean Society* **68**: 87–112.

Hey J, Machado CA. 2003. The study of structured populations – new hope for a difficult and divided science. *Nature Reviews Genetics* **4**: 535–543.

Hutchison DW, Templeton AR. 1999. Correlation of pairwise genetic and geographic distance measures: inferring the relative influences of gene flow and drift on the distribution of genetic variability. *Evolution* **53**: 189–1914.

James TY, Moncalvo JM, Li S, Vilgalys R. 2001. Polymorphism at the DNA spacers and its relation to breeding structure of the widespread mushroom *Schizophyllum commune*. *Genetics* **157**: 149–161.

Knowles LL, Maddison WP. 2002. Statistical phylogeography. *Molecular Ecology* **11**: 2623–2635.

Martin F, Díez J, Dell B, Delaruelle C. 2002. Phylogeography of the ectomycorrhizal *Pisolithus* species as inferred from the nuclear ribosomal DNA ITS sequences. *New Phytologist* **153**: 345–358.

Petit RJ, Aguinagalde I, De Beaulieu JL, Bittkau C, Brewer S, Cheddadi R, Ennos R, Fineschi S, Grivet D, Lascoux M, Mohanty A, Muller-Starck G, Demesure-Musch B, Palme A, Martin JP, Rendell S, Vendramin GG. 2003. Glacial refugia: hotspots but not melting pots of genetic diversity. *Science* **300**: 1563–1565.

Petit RJ, Brewer S, Bordacs S, Burg K, Cheddadhi R, Coart E, Cottrell J, Csaikl UM, Van Dam B, Deans JD, Espinel S, Finschi S, Finkeldey R, Glaz I, Giocoechea PG, Jensen JS, Könìg AO, Lowe AJ, Madsen SF, Matyas G, Munro MC, Popescu F, Slade D, Tabbener H, de Vries SGM, Ziegenhagen B, de Beaulieu JL, Kremer A. 2002b. Identification of refugia and post-glacial colonisation routes of European white oaks based on chloroplast DNA and fossil pollen evidence. *Forest Ecology and Management* **156**: 49–74.

Petit RJ, Grivet D. 2002c. Optimal randomization strategies when testing the existence of a phylogeographic structure. *Genetics* **161**: 469–471.

Petit RJ, Latouche-Hallé C, Pemonge MH, Kremer A. 2002a. Chloroplast DNA variation of oaks in France and the influence of forest fragmentation on genetic diversity. *Forest Ecology and Management* **156**: 115–129.

Pons O, Petit RJ. 1996. Measuring and testing genetic differentiation with ordered versus unordered alleles. *Genetics* **144**: 1237–1245.

Posada D. 2000. *Testing geographic association – Chiperm, version 2.1.* Provo, UT, USA: Department of Biology, Brigham Young University.

Posada D, Crandall KA, Templeton AR. 2000. GeoDis: a program for the cladistic nested analysis of the geographical distribution of genetic haplotypes. *Molecular Ecology* **9**: 487–488.

Rodríguez-Lanetty M, Hoegh-Guldberg O. 2002. The phylogeography and connectivity of latitudinally widespread sceractinian coral *Plesiastrea versipora* in the western Pacific. *Molecular Ecology* **11**: 1177–1189.

Rousset F. 1997. Genetic differentiation and estimation of gene flow from F-statistics under isolation by distance. *Genetics* **145**: 1219–1228.

Schneider S, Roessli D, Excoffier L. 2000. *ARLEQUIN, Version 2.001: a software for population genetics data analysis.* Geneva, Switzerland: Genetics and Biometry Laboratory, University of Geneva.

Seddon JM, Santucci F, Reeve NJ, Hewitt GM. 2001. DNA footprints of European hedgehogs, *Ericaceus europaeus* and *E. Concolor*: Pleistocene refugia, postglacial expansion and colonization routes. *Molecular Ecology* **10**: 2187–2198.

Slatkin M. 1995. A measure of population subdivision based on microsatellite allele frequences. *Genetics* **139**: 457–462.

Smith SE, Read DJ. 1997. *Mycorrhizal symbiosis, 2nd edn.* New York, NY, USA: Academic Press.

Swofford DL. 2002. *PAUP: Phylogenetic analysis using parsimony (and other methods), Version 4.08b10 for Macintosh.* Sunderland, MA, USA: Sinauer Associates Inc.

Taberlet P, Fumagalli L, Wust-Saucy AG, Cosson JF. 2001. Comparative phylogeography and postglacial colonization routes in Europe. *Molecular Ecology* **7**: 475–464.

Templeton AR. 1998. Nested clade analyses of phylogenetic data: testing hypothesis about gene flow and population history. *Molecular Ecology* **7**: 381–397.

Templeton AR. 2004. Statistical phylogeography: methods of evaluating and minimizing inference errors. *Molecular Ecology* **13**: 789–809.

Templeton AR, Routman E, Phillips CA. 1995. Separating population structure from population history: a cladistic analysis of the geographical distribution of mitochondrial DNA haplotypes in the tiger salamander, *Ambystoma tigrinum. Genetics* **140**: 767–782.

Wörheide G, Hooper JN, Degnan MB. 2002. Phylogeography of western pacific *Leucetta 'chagosensis'* (Porifera: Calcarea) from ribosomal DNA sequences: implications for populations history and conservation of the Great Barrier Reef World Heritage Area (Australia). *Molecular Ecology* **11**: 1753–1768.

Wright S. 1978. *Evolution and the genetics of populations*, Vol. 4. *Variability within and among natural populations.* Chicago, IL, USA: University of Chicago Press.

2.2 Etude de la variabilité génétique de la truffe blanche du Piémont, *Tuber magnatum* Pico : exemple de la truffière naturelle de Montemagno (Asti, Italie)

Contrairement à *T. melanosporum*, nous n'avons pas étudié la diversité génétique de *T. magnatum* sur toute son aire de distribution, ceci à cause principalement des problèmes rencontrés pour avoir un bon échantillonnage. Nous nous sommes donc focalisés sur une truffière naturelle qui est étudiée au laboratoire du département de biologie végétale de l'université de Turin depuis 1997.

Pour cette analyse, nous disposions de 42 ascocarpes récoltés durant cinq saisons de 1997 à 2002. Afin d'avoir une première idée sur la structure génétique en dehors de la truffière, nous avions 20 ascocarpes récoltés dans le sud de l'Italie, le Piémont et en Croatie. Nous avons analysé par séquençage direct plusieurs régions génomiques : l'ITS, deux parties du gène de la ß-tubuline et une région SCAR (A21inf). Concernant les échantillons de la truffière de Montemagno, seulement la région A21inf c'est montré polymorphe, avec deux haplotypes. Ils ont été retrouvés dès la première saison (1997) et durant les cinq autres. Leur distribution n'est pas aléatoire puisqu'ils sont toujours présents dans les mêmes zones de la truffière.

Ceci nous a permis de dire qu'il existe au moins deux génotypes dans cette truffière naturelle.

Concernant les échantillons provenant d'autres régions, nous avons trouvé un troisième haplotype pour la région A21inf parmi les truffes du sud de l'Italie. De plus, la distribution de ces trois haplotypes n'est pas homogène : l'haplotype 1 est majoritaire dans les échantillons du Piémont alors que le 2 l'est parmi ceux de la Croatie. De même, nous avons identifié trois haplotypes ITS, dont un majoritaire. Les deux autres étant limités à une seule région, l'haplotype 2 au Piémont et le 3 à la Croatie. En revanche la ß-tubuline est monomorphe.

Même si notre échantillonnage ne nous permet pas de conclure réellement sur la structure génétique de cette truffe, la distribution des allèles de l'ITS et d'A21inf indique une différenciation génétique. Une analyse sur un plus ample échantillonnage permettrait d'avoir une idée plus précise de la structure génétique de *T. magnatum.*

L'analyse caryologique, présenté dans l'article qui suit, fait partie de la thèse de doctorat d'Alfredo Vizzini.

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Tuber magnatum Pico, a species of limited geographical distribution: its genetic diversity inside and outside a truffle ground

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Summary

The aim of this work was to clarify the genetic structure of the ectomycorrhizal fungus, *Tuber magnatum* Pico, in a natural truffle ground located in north Italy. Ascomata of this population of *T. magnatum* were collected over a period of up to 5 years. For comparative analysis, *T. magnatum* fruit bodies of different geographical origin were also considered. We used single locus markers, such as the variable region of ribosomal genes (ITS), the β-tubulin gene and sequence-characterized amplified regions (SCAR), as tools to identify single-nucleotide polymorphisms (SNPs). On the basis of the molecular results, which were indirectly supported by a karyological analysis, a self-fertilization mechanism is suggested. A SCAR region was polymorphic within the samples of the truffle ground, leading to the identification of two genotypes. In addition, both the SCAR and the ITS proved to be polymorphic among samples coming from different geographical regions, revealing a genetic differentiation in *T. magnatum*.

Introduction

Ectomycorrhizal (ECM) fungi are known to act as crucial biological determinants of temperate and boreal forest ecosystems by improving the mineral nutrition of their host plants (Smith and Read, 1997). In addition to this widely acknowledged role, some ECM fungi produce edible fruit bodies, which are a product in great demand on the food market in many countries. Among them, *Tuber magnatum* Pico, commonly known as the white truffle of Alba (http://www.truffle.org), is of special interest. It belongs to the order Pezizales and hence is one of the few ectomycorrhizal Ascomycetes. However, unlike *Tuber maculatum* Vittad., which is ubiquitous, or *T. borchii* Vittad., which is found throughout Europe (Mello *et al.*, 2000), *T. magnatum* fruit bodies have so far been collected in Italy, Croatia, Slovenia and Hungary, showing a narrow distribution range. Their unique taste and fragrance, as well as their limited availability, make *T. magnatum* fruit bodies one of the most expensive delicacies on the market (sold at 300–400 euros per 100 g during the 2003–04 season), a fact that led Pöggeler (2001) to call them 'vegetable gold'.

Although many studies on the molecular structure of ECM fungal populations have been published (Bonello *et al.*, 1998; Gherbi *et al.*, 1999; Zhou *et al.*, 2000; Fiore-Donno and Martin, 2001; Redecker *et al.*, 2001; Bergemann and Miller, 2002; Jany *et al.*, 2002), few studies have focused on the genetic variability of truffle populations (Guillemaud *et al.*, 1996; Bertault *et al.*, 1998; 2001; Mello *et al.*, 2002; Murat *et al.*, 2004). In particular, Guillemaud and colleagues (1996) found, using polymerase chain reaction (PCR) coupled with restriction fragment length polymorphism (RFLP), intraspecific polymorphism in the variable region of ribosomal genes (ITS) of six natural French populations of *Tuber aestivum*. They also showed that the genetic variation among individuals is sufficient to distinguish genotypes within a 'brûlé'. Consistent variability was found by Mello and colleagues (2002) in the ITS of a population of *Tuber uncinatum* in a natural truffle ground. On the basis of rapid amplification of polymorphic DNA (RAPD) and microsatellite analyses, Bertault and colleagues (1998; 2001) found a very low level of genetic diversity for *Tuber melanosporum*. They did not find isolation by distance in the larger scale of the European range and suggested a glaciation impact on genetic variation followed by rapid post-glaciation expansion. Murat and colleagues (2004) confirmed the low level of polymorphism of this species: the sequencing of 50 kbp of PCR-amplified sequence-charac-

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terized amplified region (SCAR) fragments from a representative set of fruit bodies did not show any variability. However, the ITS sequencing allowed them to discover genetic differentiation between regional populations of *T. melanosporum*. They hypothesized an old past fragmentation into two groups of populations, which probably colonized regions on different sides of the French Central Massif after the last glaciation period.

Data currently available on *T. magnatum* genetic variability are very limited: they rely on RAPD (Gandeboeuf *et al.*, 1997) and ITS-RFLP (Mello *et al.*, 2001). Both analyses were performed on a few samples (eight and 17 respectively) harvested in several Italian regions and revealed no genetic diversity. The study of the ITS was principally aimed at designing primers specific to *T. magnatum* to distinguish this species from other *Tuber* species, especially at the mycorrhizal level (Amicucci *et al.*, 1998; Mello *et al.*, 1999). A study on isozyme variability made on 13 Italian populations of *T. magnatum* (Frizzi *et al.*, 2001) revealed three alleles at two of the 11 enzymes investigated. Alleles were distributed among the different Italian populations, without an interpretable evolutionary occurrence correlated with geological or climatic phenomena (Frizzi *et al.*, 2001). More recently, a paper described the isolation and characterization of eight polymorphic microsatellite loci in the white truffle (Rubini *et al.*, 2004). In all these studies realized on *T. aestivum*, *T. uncinatum*, *T. melanosporum* and *T. magnatum*, heterozygosity was never found, and this led the authors to hypothesize self-reproduction for these truffle species (Bertault *et al.*, 1998; 2001; Frizzi *et al.*, 2001; Murat *et al.*, 2004; Rubini *et al.*, 2004). However, questions concerning the temporal and spatial structure of *T. magnatum* populations are still open.

A population analysis (irrespectively of how a population is defined) deals, among other aspects, with the identification of individuals. As illustrated in many papers dealing with population genetics of fungi (Bonello *et al.*, 1998; Zhou *et al.*, 1999; 2000; Redecker *et al.*, 2001), the key solution to the identification of fungal individuals is to recognize the mycelial clone, the so-called genet. The successful strategy for distinguishing genets in ECM fungi is to sample fruit bodies within an area and to look for genetic variability among them. Once genets have been determined, they can differ in the extent of the mycelial phase and their life span.

In order to tackle these questions, our specific objective was to start unravelling the genetic diversity of a *T. magnatum* population. *T. magnatum* fruit bodies were collected in a natural truffle ground for as long as 5 years. For comparative analysis, *T. magnatum* fruit bodies from different geographical origins were also considered. We used single locus markers such as ITS, the functional β-tubulin gene and SCAR to look for single-nucleotide polymorphisms (SNPs). Although ITS and the β-tubulin sequences are usually known to be highly conserved at the intraspecies level in fungi, there is evidence that these markers are polymorphic in some species (Guillemaud *et al.*, 1996; Glen *et al.*, 2001; Johannesson *et al.*, 2001; Jany *et al.*, 2002; Mello *et al.*, 2002; Murat *et al.*, 2004). With the long-term aim of correlating the genetic status of truffles (potentially self-fertile organisms) with the crucial events of their life cycle (meiosis and mitosis during spore formation), a karyological analysis was performed, and the nuclear status of the spores, at different developmental steps, was investigated.

Even if the sampling was obviously limited by the high economic value of truffles, our analysis allowed us to demonstrate a local and global genetic diversity in *T. magnatum*: (i) two haplotypes were present in the truffle ground; and (ii) additional haplotypes were present on different sampling scales.

Results

In order to clarify the local genetic variability of the white truffle, *T. magnatum*, 42 fruit bodies were harvested in a natural truffle ground, located at Montemagno, during the winters of 1997–2002 (Table 1). These fruit bodies were found exclusively under specific trees of *Quercus robur* and *Populus nigra*, leading to the identification of productive and not productive areas (Fig. 1).

The genetic variability of the white truffle population was assessed with different single locus markers. As a first step, the variable region of ribosomal genes (ITS) and the gene for β-tubulin were analysed.

ITS and β-tubulin

As ITS sequences are polymorphic in some species, including *Tuber* species, and given that only four ITS sequences from *T. magnatum* were present in GenBank at the start of our investigation, ITS regions were sequenced as a first step.

ITS from the 42 *T. magnatum* fruit bodies collected in the Montemagno truffle ground was amplified, producing a band of 640 bp, and sequenced with primers ITS1 and ITS4 (except those from A8, A12 and A24 on account of manipulation problems). Alignment of the sequences did not show any polymorphism (the accession numbers are listed in Table 1). For comparison, we also amplified and sequenced the ITS from the 20 *T. magnatum* fruit bodies coming from outside the truffle ground (the accession numbers listed in Table 1). Two SNPs were found: one in sample B6, coming from Piedmont; and the other one in GO2, IS1 and IS2, coming from Croatia. The two SNPs generated three haplotypes: haplotype 1, the most common one; haplotype 2, corresponding to B6; and haplo-

Table .1. Fruit bodies of *T. magnatum* collected since 1997, the nearest host species, accession numbers of the sequences from the genomic regions analysed and the associated haplotypes.

		ITS		β-tub1	β-tub2	A21inf	
Sample name	Nearest host species	Acc. no.	Haplotype	Acc. no.	Acc. no.	Acc. no.	Haplotype
Season 1997–98							
A2	*Populus nigra* L.	AJ586307	1	AJ586417	AJ586424	AJ578828	II
A3	*Populus nigra* L.	AJ586306	1	AJ586418	AJ586425	AJ578829	II
A8	*Quercus robur* L.					AJ578830	I
A12	*Quercus robur* L.					AJ578831	II
A21	*Quercus robur* L.	AJ586305	1			AJ578832	I
A22	*Populus nigra* L.	AJ586304	1			AJ578833	II
A23	*Populus tremula* L.	AJ586303	1			AJ578834	II
A24	*Quercus robur* L.					AJ578835	I
A27	*Quercus robur* L.	AJ586302	1			AJ578836	I
A29	*Populus nigra* L.	AJ586301	1			AJ578839	I
A30	*Quercus robur* L.	AJ586300	1			AJ578838	I
A31	*Quercus robur* L.	AJ586299	1			[a]	I
A34	*Quercus robur* L.	AJ586298	1			AJ578882	II
A35	*Populus nigra* L.	AJ586297	1			AJ578840	II
A37	*Quercus robur* L.	AJ586296	1			AJ578841	II
Season 1998–99							
C1	*Populus nigra* L.	AJ586295	1			AJ578842	I
C2	*Quercus robur* L.	AJ586294	1	AJ586419	AJ586426	AJ578843	I
C3	*Populus nigra* L.	AJ586293	1			AJ578844	II
C5	*Quercus robur* L.	AJ586292	1			AJ578845	II
C7	*Populus nigra* L.	AJ586291	1			AJ578846	I
C8	*Quercus robur* L.	AJ586290	1			AJ578847	II
C9	*Quercus robur* L.	AJ586289	1			AJ578848	I
C10	*Quercus robur* L.	AJ586288	1			AJ578849	II
C11	*Quercus robur* L.	AJ586287	1			AJ578850	I
C12	*Populus nigra* L.	AJ586286	1	AJ586420	AJ586427	AJ578851	II
C13	*Quercus robur* L.	AJ586285	1			AJ578852	I
C14	*Populus nigra* L.	AJ586284	1			AJ578853	II
C15	*Populus nigra* L.	AJ586283	1			AJ578854	II
C16	*Quercus robur* L.	AJ586282	1			AJ578855	II
C17	*Populus nigra* L.	AJ586281	1			AJ578856	II
Season 1999–2000							
Tm1	*Quercus robur* L.	AJ586280	1	AJ579836	AJ579845	AJ578857	I
Tm2	*Quercus robur* L.	AJ586279	1			AJ578858	II
Tm3	*Quercus robur* L.	AJ586278	1			AJ578859	I
Tm4	*Quercus robur* L.	AJ586308	1			AJ578860	II
Season 2000–01							
D1	*Quercus robur* L.	AJ586277	1	AJ579837	AJ579846	AJ578861	I
Season 2001–02							
F1	*Populus nigra* L.	AJ586276	1			[a]	I
F2	*Quercus robur* L.	AJ586275	1			AJ578862	I
F3	*Populus nigra* L.	AJ586274	1			AJ578863	I
F6	*Quercus robur* L.	AJ586273	1			AJ578864	I
F7	*Quercus robur* L.	AJ586272	1			[a]	II
F8	*Quercus robur* L.	AJ586270	1			AJ578865	I
F9	*Quercus robur* L.	AJ586269	1			AJ578866	I

		ITS		β-tub1	β-tub2	A21inf	
Sample name	Collection site	Acc. No.	Haplotype	Acc. no.	Acc. no.	Acc. no.	Haplotype
Samples from Piedmont (north Italy)							
B1	San Desiderio (AT)	AJ586268	1	AJ579834	AJ579843	[a]	I
B2	Zanco (AT)	AJ586267	1			AJ578869	I
B3	Ponzano (AC)	AJ586266	1			AJ578870	I
B6	San Desiderio (AT)	AJ586271	2	AJ586421	AJ586428	AJ578868	I
B8	Castellalfero (AT)	AJ586265	1			AJ578871	II
B9	Castellalfero (AT)	AJ586264	1			AJ578872	I
B19	Montemagno (AT)	AJ586263	1			AJ578867	I
B11	Montemagno (AT)	AJ586262	1			AJ578873	II
E1	Montemagno (AT)	AJ586261	1	AJ579835	AJ579844	AJ578874	I
b2	Unknown	AJ586260	1	AJ579833	AJ579842	AJ578875	I
b4	Unknown	AJ586259	1			AJ578876	I

Table .1. *cont.*

Sample name	Collection site	ITS Acc. No.	ITS Haplotype	β-tub1 Acc. no.	β-tub2 Acc. no.	A21inf Acc. no.	A21inf Haplotype
Samples from Campania and Molise (south Italy)							
Tm sal	Salerno	AJ605110	1	AJ579838		AJ578877	I
XL	Campobasso	AJ586258	1		AJ579848	AJ578837	III
Y	Campobasso	AJ586257	1	AJ579839	AJ579849	AJ578878	II
Z	Campobasso	AJ586256	1	AJ579841	AJ579847	AJ578879	III
Samples from Croatia (Istria)							
IS1	Motovun	AJ586252	3			AJ605111	I
IS2	Motovun	AJ586253	3			AJ605112	II
IS3	Motovun	AJ586254	1			AJ605113	II
GO1	Motovun-Montona	AJ586255	1	AJ586422	AJ586429	AJ578880	II
GO2	Motovun-Montona	AJ586251	3	AJ586423	AJ586430	AJ578881	II

a. A sequence not provided, in which only the kind of haplotype could be well identified.

type 3, corresponding to GO2, IS1 and IS2 (Table 1). The similarity percentage was calculated in order to compare *T. magnatum* ITS with that of other fungal species: a similarity of 99.61% (507 conserved positions out of 509 alignment length) was found within the 59 *T. magnatum* fruit bodies analysed.

Primer sets, designed by Glass and Donaldson (1995) to amplify the gene for β-tubulin, revealed the presence of introns in *Neurospora crassa*. As introns are likely to be polymorphic, these primers were used to look for variability in *T. magnatum*.

The gene for β-tubulin, from six *T. magnatum* fruit bodies sampled in the truffle ground and nine fruit bodies of different geographical origins, was amplified with the primer sets Bt1a/Bt1b and Bt2a/Bt2b. The amplification products were 537 bp long with Bt1a/Bt1b and 495 bp

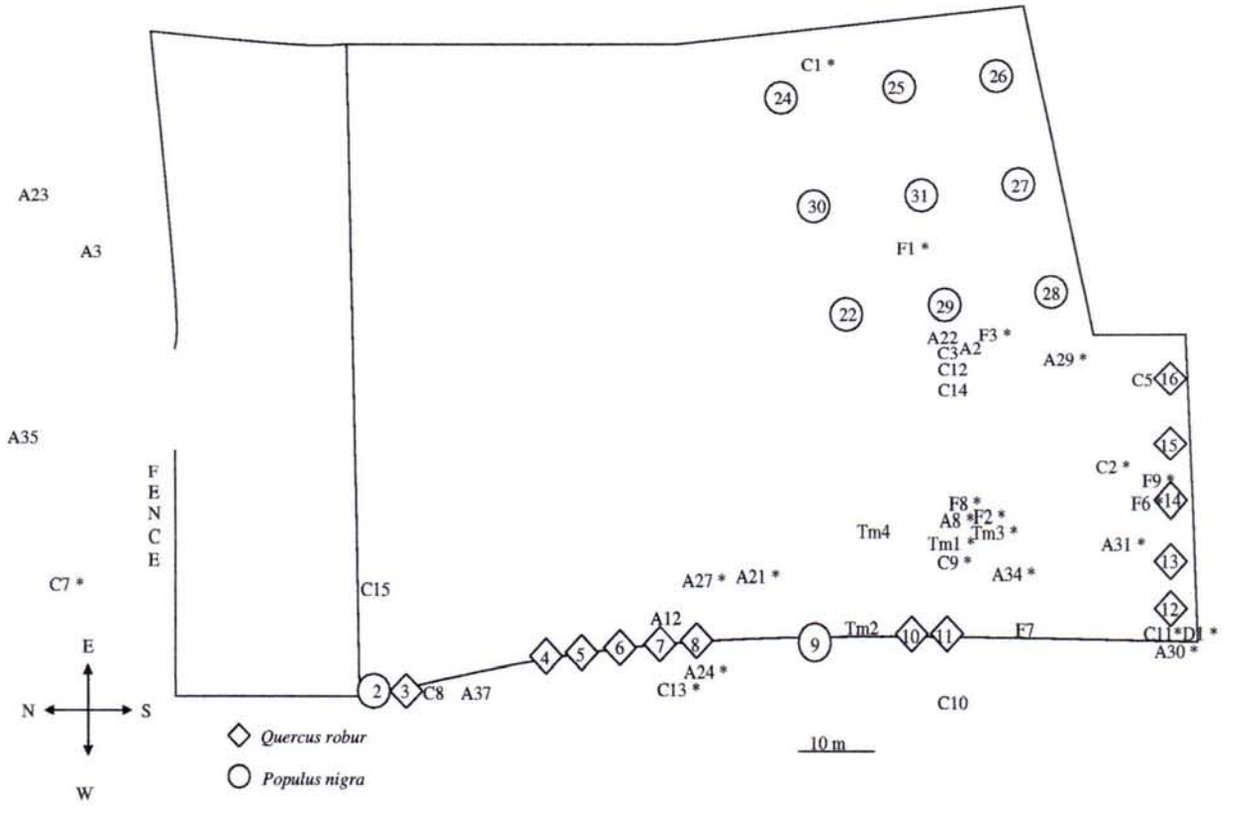

Fig. 1. Map of the truffle ground located at Montemagno (AT). Fruit bodies of *T. magnatum* are represented by letters and numbers. Letters A, C, Tm, D and F indicate fruit bodies harvested during the seasons 1997–98, 1998–99, 1999–2000, 2000–01 and 2001–02 respectively. Samples A23, A3, A35 and C7 are included in the analysis of the truffle ground because of their proximity to it and the absence of any barrier. The presence and the absence of the star indicate, respectively, the haplotypes I and II revealed by the A21inf sequencing of *T. magnatum*.

Table 2. Primer pair designed on the investigated locus and size of the amplified product.

Locus	Primer name	Sequence 5′ → 3′	Size of PCR product (bp)
SCAR A21-inf	CL1 CL2	CTTGAGCAAACTCCAATAGAG GACACGATCCAAGTCGAGAG	572

long with Bt2a/Bt2b. The sequences of the products obtained showed no polymorphism, although there was one intron in the region amplified by the Bt1 primers and three introns in that amplified by the Bt2 primers (accession numbers in Table 1).

This first set of experiments, covering samples collected during all five winters, demonstrates that ITS, as well as a functional gene such as β-tubulin, do not reveal any polymorphism in the samples of the truffle ground, whereas two SNPs at the ITS are revealed in the samples coming from outside.

SCAR characterization and SNP identification

One polymorphic RAPD fragment obtained with the primer OPA-7 from the fruit bodies collected in the truffle ground during the first 2 years was chosen for fine analysis on the 62 *T. magnatum* fruit bodies. It was cloned and sequenced, producing a SCAR named A21-inf. This sequence showed no significant similarity to known sequences in GenBank. In order to amplify and sequence the anonymous region from all the samples and to search for polymorphism, the primer pair CI1/CL2 was designed.

Amplification of the SCAR with CI1/CL2 produced a band of the expected size (Table 2) for the 42 samples from the truffle ground and the 20 of different origins (data not shown). The PCR products from SCAR A21-inf were successfully sequenced from all 62 fruit bodies. The alignment of the sequences from SCAR A21-inf showed two SNPs, which generated three haplotypes (Table 1, Fig. 2). Haplotypes I and II were distributed in the truffle ground and among the other samples, irrespective of their geographical origins (Table 1). In contrast, haplotype III was only found in two samples from south Italy (Table 1).

As it is assumed that the direct sequencing of PCR products allows the identification of the heterozygote (Johannesson *et al.*, 2002; Selosse *et al.*, 2002), the chromatograms of the sequences were also carefully inspected from this point of view. Double peaks were never found, suggesting that heterozygosity is not present.

Karyological analysis

In order to correlate the genetic status of the truffles with the crucial events in their life cycle (meiosis and mitosis

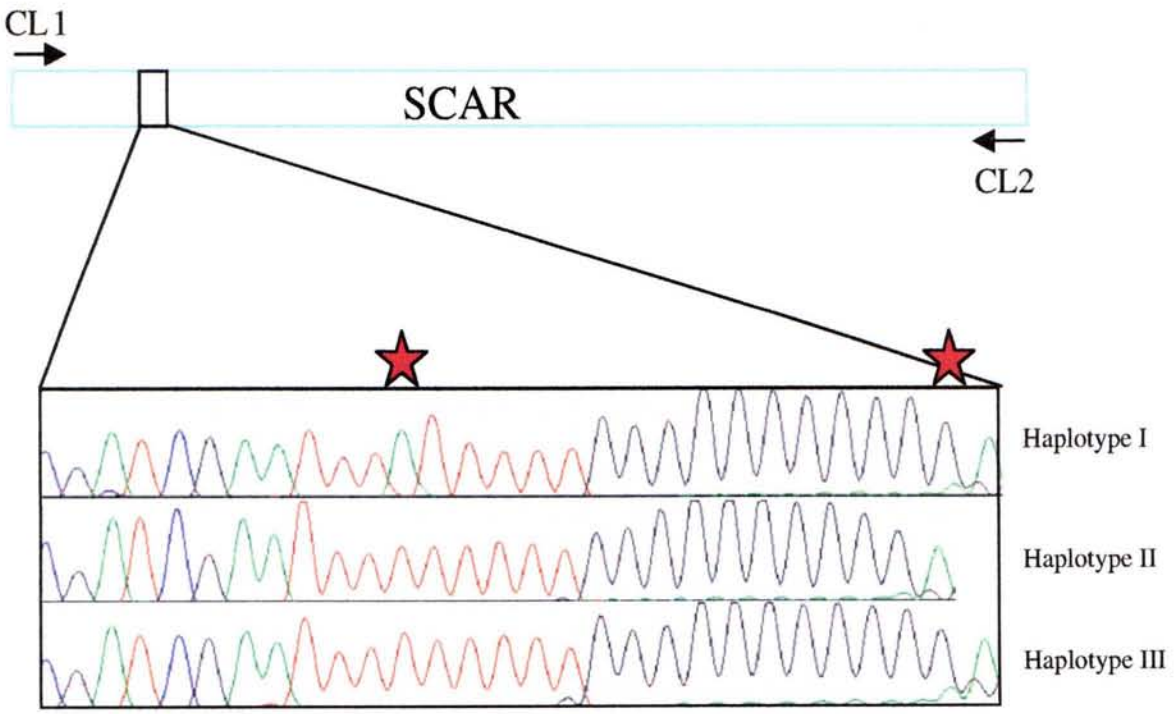

Fig. 2. Two SNPs, indicated by a star, in SCAR A21-inf, originating the haplotypes I, II and III.

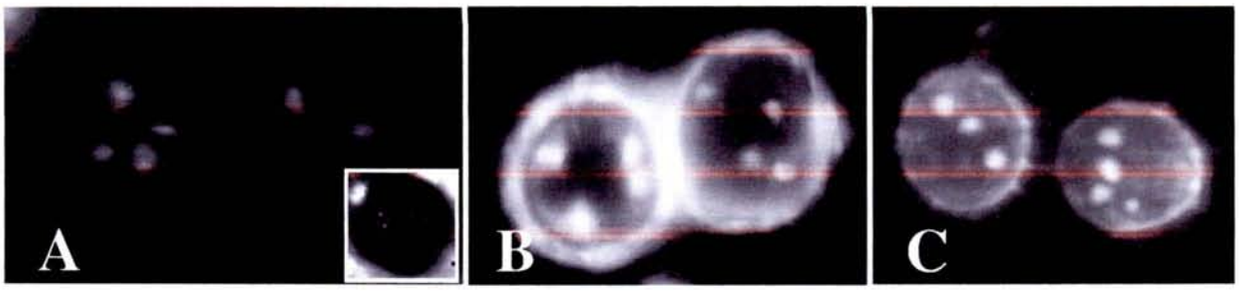

Fig. 3. *T. magnatum* immature (A) and mature (B and C) ascospores after DAPI staining (900× magnitude). The small bordered picture in (A) shows the whole ascus. In (C), the fourth nucleus in the left ascospore is not in focus.

during spore formation), the number of nuclei within the spores was counted. *T. magnatum* fruit body slices were incubated with the fluorochrome DAPI and observed under an epifluorescence microscope. Young and mature asci usually contain two or three ascospores. A total of 120 ascospores from four fruit bodies was observed. Developing ascospores quickly achieve a tetranuclear condition in most of the observed samples (Fig. 3A). Ripened ascospores (with clear ornamentations and pigmented cell walls) contained four nuclei in 63.3% of cases (Fig. 3B and C).

Discussion

Notwithstanding the drawback of having a limited number of sampled fruit bodies (42: about 60% of the whole production in the first 2 years, plus about 10% in the following years), our investigation allowed us, for the first time, to define some genetic features of a *T. magnatum* population living in a natural truffle ground. Analysis of ITS, β-tubulin and SCAR-A21inf, 640 bp, 1032 bp and 572 bp long, respectively, showed a very low genetic diversity: only two SNPs out of 2244 bp (i.e. 0.089%). The two SNPs found in SCAR-A21inf revealed the presence of two haplotypes in the Montemagno truffle ground. The identification of fungal individuals is a principal goal of population genetics. Understanding whether fruit bodies of a truffle species, found in the same area, originate from a common mycelium or from different mycelia is a challenging objective. The key solution is to recognize the mycelial clone, the so-called genet. They are defined as repeatedly sampled multilocus genotypes, which are unlikely to arise by chance in sexual reproduction (Anderson and Kohn, 1995). Even if one of our first goals was to delineate genets, which is a difficult task in a low polymorphic species, the little allelic diversity found on a local scale did not allow us to reach this goal.

As the two haplotypes found in the truffle ground are based only on one polymorphic locus, the SCAR-A21inf, we consider the two distinguished units as genotypes rather than genets.

Diversity within and outside the truffle ground

The SNPs can be broadly defined as any single base substitution/indel in the genome of an individual (Brookes, 1999). They are used as a powerful marker for mutational analysis in humans and are normally found in non-coding regions subject to less selection. They have been found in man, in passerine birds and in maize, as an average of one SNP every 100–300 bp, 150–175 bp and 27.6 bp respectively (Tenaillon *et al.*, 2001; Primmer *et al.*, 2002). Their presence in the ITS of *T. melanosporum* has allowed the identification of a genetic differentiation that reflects a regional distribution of this fungus (Murat *et al.*, 2004). *T. magnatum* ITS sequences obtained from the truffle-ground samples did not reveal any polymorphism. This result confirms the absence of polymorphism in the four ITS sequences that were present in the GenBank at the beginning of our investigation. However, the finding of two SNPs in samples outside the truffle ground revealed three alleles out of a total of 59 fruit bodies. It seems that one allele is dominant and that the other two are rare. However, because of the small number of *T. magnatum* samples investigated, the allele frequency cannot be indicated exactly without making mistakes. Of the 509 aligned positions, only two positions (0.39%) displayed SNP. In the case of *T. melanosporum*, Murat and colleagues (2004) identified 10 alleles out of a total of 188 fruit bodies. Two out of the 10 alleles were dominant, i.e. present in all the regionally sampled populations, a third allele was limited to some populations, and all the others were exclusive to single populations. Of the 539 aligned positions, only nine positions (1.67%) displayed SNP.

Mello and colleagues (2002) found 11 alleles in 12 fruit bodies of *T. uncinatum*, mostly coming from the same area. Unlike *T. magnatum* and *T. melanosporum*, of the 640 aligned positions of *T. uncinatum*, 38 (6%) displayed SNP or polymorphic insertion/deletion events (indels).

Regarding other species within the genus *Tuber*, data from the ITS sequences of *T. indicum* indicate a very high variability (13.10%) (Mabru *et al.*, 2001) that could change as soon as phylogenetic investigations for other Asian truffles, close to *T. indicum*, clarify the taxonomic position

of these truffles. From these comparisons, *T. magnatum* ITS shows a very low variability, similar to that of *T. melanosporum* ITS, whereas *T. uncinatum* and *T. indicum* ITS are more polymorphic. Several ITS-RFLP genotypes of *Cenococcum geophilum* co-exist within the same limited soil, suggesting that *C. geophilum* does not fit the model of low-numbered and exclusive genotypes that one would expect for an ECM fungus that lacks meiospore production (Jany *et al.*, 2002). The population structure of *Schizophyllum commune* has been addressed by studying the phylogeny and evolutionary dynamics of ITS and IGS1 (Timothy *et al.*, 2001). Of the 27 sequences obtained for ITS, only 31 positions (5%) were variable. In contrast, of the 195 strains sampled for IGS1, 172 (33.9%) displayed variability. Although ITS possessed less sequence variation than IGS1, phylogenetic analysis of the ITS recovered the same three evolutionarily distinct lineages within the *S. commune* global population. Therefore, ITS sequences have been applied in studying the population structure whereas, generally, ITS sequences are used to delineate relationships among fungal species within genera (e.g. Yan *et al.*, 1995; Aanen *et al.*, 2000; Høiland and Holst-Jensen, 2000), including the genus *Tuber* (Mabru *et al.*, 2001; Mello *et al.*, 2002).

Concerning the genetic variability within some *Tuber* species investigated by RAPD, Gandeboeuf and colleagues (1997) found that *T. magnatum* and *T. melanosporum* groups presented the strongest intraspecific homogeneity, with Jaccard genetic similarity indices of 1 and 0.80 respectively. For the other species such as *T. borchii*, *T. rufum*, *T. macrosporum*, *T. mesentericum* and *T. excavatum*, the genetic similarity index was very low (0.10–0.20).

The β-tubulin fragments, obtained with two different primer sets, were not polymorphic in *T. magnatum*, thus revealing that the two regions that we amplified are not a good molecular marker for studying the population genetics of this fungus. In contrast, in *Daldinia loculata*, the β-tubulin locus, amplified by the primer set bt1a/bt1b, presents multiple deletions and insertions in the intron sequence, thus being capable with other markers of revealing a genetic differentiation in Eurasian populations of this post-fire ascomycete (Johannesson *et al.*, 2001).

SCAR markers have already been used in fungi for population analysis. Within *Magnaporthe grisea* populations, they have been used as markers to monitor recombination and migration (Soubabere *et al.*, 2001) whereas, among populations of *T. melanosporum*, five SCAR markers did not reveal any polymorphism (Murat *et al.*, 2004).

In our investigation, a SCAR marker let us identify two truffle haplotypes in the truffle ground. They were always detected over several years: in the first sampling year and then for the next 5 years, except in the fourth year (Table 1 and Fig. 1). That year was characterized by dry weather in August and September, by freezing temperatures at the beginning of winter and by a very limited sampling: only one fruit body. Although only 1/10th of the fruit bodies produced in the truffle ground have been collected in the last 3 years, we found that each genotype occurred in the following seasons, often in the same zone of the truffle ground and always not far from the trees (Fig. 1).

Finally, the recent paper by Rubini and colleagues (2004) described eight polymorphic microsatellite loci in *T. magnatum*. The availability of such markers will be helpful in the assessment of the genetic structure and population dynamics of the white truffle.

An important outcome

Our investigation on the *T. magnatum* samples of different geographical origins has revealed that two genomic regions present additional alleles outside the selected truffle ground. A third allele at A21inf is present only in two samples from Campobasso, south Italy, while a second allele at the ITS region is present in one sample from Piedmont and a third in three Croatian samples (Table 1). This is the first time that molecular analysis has revealed genetic variability in *T. magnatum*, either within a population or among samples of different geographical origins. Furthermore, Frizzi and colleagues (2001) found, by isozyme analysis of populations of *T. magnatum*, three electrophoretic types in Campobasso, instead of the two commonly present in most Italian locations. Although our first aim was to study the genetic variability of a specific *T. magnatum* population, our results led us to hypothesize a genetic differentiation in *T. magnatum* over its habitat (Italy and Croatia), as observed recently in *T. melanosporum* (Murat *et al.*, 2004). This finding could have important consequences when the geographical origin of *T. magnatum* is required.

In conclusion, our long-term analysis has demonstrated that a natural truffle ground, productive since at least 1850, presents two *T. magnatum* genotypes. They do not seem to intermingle, suggesting that a self-fertilization mechanism may operate, given that environmental conditions are unchanged.

The discovery of additional polymorphic markers for *T. magnatum* will be a very useful tool for investigating *T. magnatum* genets and their temporal and spatial distribution within the Montemagno truffle ground.

Pseudohomothallism versus heterothallism: still an open question

It is generally assumed and convincingly illustrated by the paper of Guidot and colleagues (2002) that sexual reproduction must be a rather rare event when small genets are absent. Although our analysis does not identify genets, our finding of only two genotypes in an area of 7000 m^2

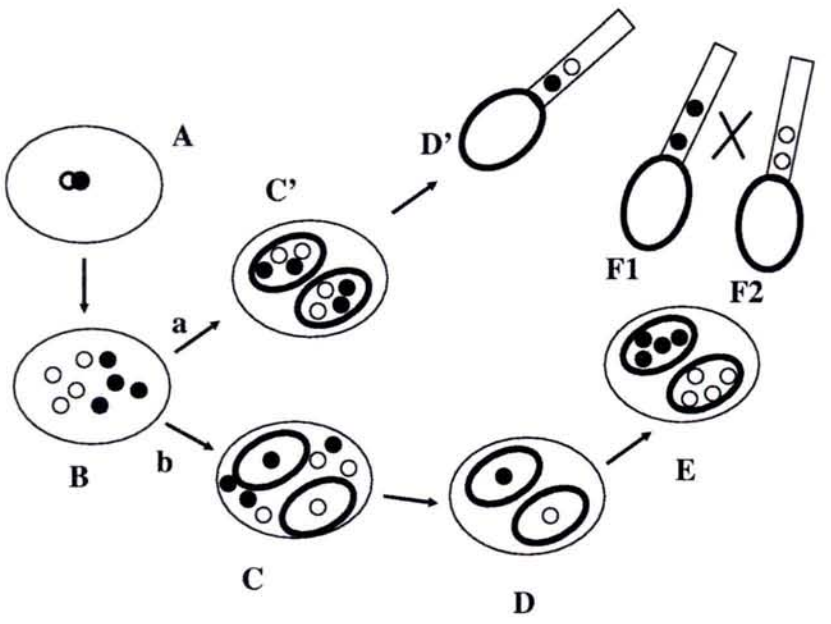

Fig. 4. Two possible mechanisms to explain the condition of plurinuclearity observed in *T. magnatum*.
A. Fusion of nuclei of opposite sexuality in the ascus.
B. Nuclei derived from meiosis and mitosis.
C. Formation of ascospores around a single nucleus.
D. The nuclei left in the ascus degenerate.
E. Mitotic divisions. (a) All the nuclei derived from mitosis are compartmentalized (A, B, C'). (b) Compartmentalization around a single nucleus that then undergoes mitotic divisions (A, B, C, D, E). D', F1 and F2, germinating ascospores and incipient promycelia.

may suggest self-reproduction, as already hypothesized by Frizzi and colleagues (2001). The absence of heterozygosity in SCAR-A21inf, as well as in eight polymorphic microsatellite loci (Rubini *et al.*, 2004), reinforces our suggestion, even if such an absence does not prove in itself that *T. magnatum* is not heterothallic in the absence of an experimental system to perform crossings.

Within Ascomycetous fungi, sexually reproducing species usually follow one of the three basic sexual reproductive strategies: homothallism, pseudohomothallism (secondary homothallism) and heterothallism (Nelson, 1996; Pöggeler, 2001). Individual haploid mycelia of homothallic and pseudohomothallic (haploid but heterokaryotic) fungi are self-fertile (Pöggeler, 2001). In a young ascus, two haploid nuclei fuse to form a diploid nucleus that quickly undergoes meiosis giving four nuclei, generally followed by mitosis with the production of eight nuclei. Around each nucleus, the haploid sexual progeny, the ascospore, is produced. The eight ascospores are then discharged from the fruiting bodies formed by most filamentous Ascomycetes.

Tuber species have plurinucleated ascospores (Donadini, 1987; Vizzini, 2001). According to our observations, each of the two or three spores of *T. magnatum* usually has four nuclei, revealing the expected condition of plurinuclearity (Fig. 3). In the case in which two spores are produced (this was the most common condition in our observation), two possible mechanisms may lead to this condition (Fig. 4). In the first one, the eight nuclei that derive from meiosis and one mitotic event inside the ascus are compartmentalized to give rise to two ascospores (A, B, C'). In the second situation, ascospore formation could occur around a single nucleus that then undergoes further mitotic divisions (A, B, C, D, E). In this case, the nuclei that remain free outside the ascospore degenerate. The nuclei inside the ascus can be of identical or opposite sexuality. In the former situation, the nuclei give rise to a self-fertile mycelium (homothallism). Whether nuclei of opposite sexuality are present in the ascus or not, two final situations can be envisaged: (i) given that nuclei of opposite sexuality are both present in the ascospores, they give rise to a self-fertile mycelium (pseudohomothallism); (ii) given that nuclei of opposite sexuality are present in separate ascospores, they give rise to a self-sterile mycelium (heterothallism). To the best of our knowledge, mating type genes, which determine heterothallism and pseudohomothallism, have never been reported for truffles, unlike for other filamentous Ascomycetes, such as *N. crassa* (Glass *et al.*, 1990; Staben and Yanofsky, 1990; Ferreira *et al.*, 1996) and *Podospora anserina* (Debuchy and Coppin, 1992; Debuchy *et al.*, 1993). Our hypotheses therefore cannot be verified experimentally. Moreover, a further limiting step results from the absence of an experimental system based on spore germination and therefore of the classical breeding of the resulting mycelia. However, the dominant presence of two ascospores per ascus, each containing four nuclei, and the apparent absence of degenerating nuclei free in the asci (Fig. 3) suggest that the first hypothesis is more probable.

In conclusion, taken together, the experimental results from our molecular analysis and the hypotheses drawn on karyological observations tend to exclude heterothallism in favour of self-fertilization. Self-fertilization can explain

the low genetic variability of *T. magnatum*, which was probably affected by glaciation, as Bertault and colleagues (1998; 2001) have already suggested for *T. melanosporum*.

Experimental procedures

Identification of the truffle ground, geographical data

The truffle ground, of about 7.000 m², is situated in a valley at Montemagno (Asti, Piedmont, north Italy, 8°19′ 35″ 4 E, 44°59′ 2″ 40 N). It is characterized by the persistence of a moist soil, even in the hot season. The area is particularly useful for research studies because it does not present signs of anthropic activities, with the exception of the enlargement of a natural stream and the seasonal mowing. In this truffle ground, there are some trees that are 50 years old, although most are younger. The following tree species are present: *Populus nigra* L., *P. alba*, *P. tremula* L., *Quercus robur* L., *Salix alba* L. and *Tilia cordata* Miller. Records on productivity claim that this truffle ground has produced truffles since at least 1850. Trees usually start to produce, depending on their age and species, when they are 10–20 years old. Figure 1 only shows trees at least 10–15 years old and therefore expected to be productive.

To clarify the local genetic variability and the dynamics of the white truffle, *T. magnatum*, 42 fruit bodies were harvested during the winters of 1997–2002 (Table 1). The agreement with the owner of the truffle ground allowed us to collect in the first 2 years about 60% and in the following years 10% of the whole *T. magnatum* fruit bodies produced. Their sampling position was mapped with respect to the nearest tree (Fig. 1). In order to perform a comparative analysis of the observed genetic diversity, additional fruit bodies of *T. magnatum* were investigated: 11 were collected from Piedmont (north Italy), four from Campania and Molise (south Italy) and five from Croatia (Table 1). All 62 fruit bodies were then analysed.

Sampling and DNA extraction

A total of 62 fruit bodies of *T. magnatum* of different geographical origins were considered for DNA analysis (Table 1). Each fruit body was washed and the peridium peeled. The gleba was cut into small pieces and stored at −80°C. Total DNA was extracted using the Dneasy plant mini kit (Qiagen) according to the manufacturer's instructions.

DNA amplification and data analysis

ITS from 62 *T. magnatum* fruit bodies was amplified with primers ITS1/ITS4 (White *et al.*, 1990) according to the protocol described by Mello and colleagues (1996). The PCR products from all the samples were sequenced, except those from A8, A12 and A24 because of problems with manipulations (accession numbers are listed in Table 1).

Two primer sets, Bt1a/Bt1b and Bt2a/Bt2b, constructed from the *N. crassa* β-tubulin gene (Glass and Donaldson, 1995), were used for DNA amplification from six *T. magnatum* fruit bodies sampled in the truffle ground and from nine of different geographical origins. The PCR products were sequenced, and the accession numbers are listed in Table 1.

RAPD amplifications were carried out with primer OPA-7 (Operon Technologies), according to the method of Lanfranco and colleagues (1998), for some samples collected in the truffle ground in the first 2 years. One polymorphic RAPD band was converted into SCAR and used to look for SNPs.

A 694 bp band from fruit body A21, named A21-inf, was isolated from agarose gel with a Qiaex II gel extraction kit (Qiagen) according to the manufacturer's instructions. The band was cloned into the plasmid pGEM-T system I vector (Promega) and transformed in the ultracompetent cells *Escherichia coli* XL-2 according to the manufacturer's instructions. Inserts were amplified with universal primers SP6 and T7 and sequenced by Genome Express. The primer pair CI1/CL2 was designed by AMPLIFY version 1.2 to amplify A21-inf from all the samples (Table 2). Amplification conditions with these primers were identical to ITS amplifications except for the annealing temperature, which was set at 55°C. PCR-amplified SCARs were purified with a PCR purification kit (Qiagen) and sequenced (accession numbers are listed in Table 1).

Sequencing was done for all the loci analysed, except when indicated otherwise, by GeneLab. Significant alignment of the SCAR was searched in GenBank using BLASTN and BLASTX on the NCBI website: http://www.ncbi.nlm.nih.gov/blast/ (Altschul *et al.*, 1997).

The sequences obtained from all ascocarps were verified visually on the chromatograms using SEQUENCHER 3.1.1 (GeneCodes). SNPs were detected by sequence multialignment using the 'assembly contig' of routine SEQUENCHER 3.1.1. Similarity of the ITS sequences was calculated with CLUSTAL W multiple alignment (at http://www.npsa-pbil.ibcp.fr/).

Karyological analysis

In order to observe nuclei within the spores, the fluorochrome DAPI (4′,6-diamidino-2-phenilindole) diluted in Tris buffer at a final saturating concentration of 5.6 μM was used. This stain specifically binds AT-rich, double-stranded DNA. Hand-made sections of the inner fertile portion of four *T. magnatum* fruit bodies were incubated in DAPI for 15 min and observed directly under an epifluorescence Zeiss microscope.

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References

Aanen, D.K., Kuyper, T.W., Boekhout, T., and Hoekstra, R.F. (2000) Phylogenetic relationships in the genus *Hebeloma*

based on ITS 1 and 2 sequences, with special emphasis on the *Hebeloma crustuliniforme* complex. *Mycologia* **92:** 269–281.

Altschul, S.F., Madden, T.L., Schaffer, A.A., Zhang, J., Zhang, Z., Miller, W., and Lipman, D.J. (1997) Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res* **25:** 3389–3402.

Amicucci, A., Zambonelli, A., Giomaro, G., Potenza, L., and Stocchi, V. (1998) Identification of ectomycorrhizal fungi of the genus *Tuber* by species-specific ITS primers. *Mol Ecol* **7:** 273–277.

Anderson, J.B., and Kohn, L.M. (1995) Clonality in soilborne, plant-pathogenic fungi. *Annu Rev Phytopathol* **33:** 369–391.

Bergemann, S.E., and Miller, S.L. (2002) The size, distribution, and persistence of genets in local populations of the late-stage ectomycorrhizal basidiomycete, *Russula brevipes*. *New Phytol* **156:** 313–320.

Bertault, G., Raymond, M., Berthomieu, A., Callot, G., and Fernandez, D. (1998) Trifling variation in truffles. *Nature* **394:** 734.

Bertault, G., Rousset, F., Fernandez, D., Berthomieu, A., Hochberg, M.E., Callots, G., and Raymond, M. (2001) Population genetics and dynamics of the black truffle in a man-made truffle field. *Heredity* **86:** 451–458.

Bonello, P., Bruns, T.D., and Gardes, M. (1998) Genetic structure of a natural population of the ectomycorrhizal fungus *Suillus pungens*. *New Phytol* **138:** 533–542.

Brookes, A. (1999) The essence of SNPs. *Gene* **234:** 177–186.

Debuchy, R., and Coppin, E. (1992) The mating types of *Podospora anserina*: functional analysis and sequence of fertilization domains. *Mol Gen Genet* **233:** 133–121.

Debuchy, R., Arnaise, S., and Lecellier, G. (1993) The *mat*-allele of *Podospora anserina* contains three regulatory genes required for the development of fertilized female organs. *Mol Gen Genet* **241:** 667–673.

Donadini, J.C. (1987) Pezizales et Tuberales (2). Le genre *Tuber* (*T. borchii* Vitt. et *T. puberulum* Berk and Broome). Cytologie des spores, paraphyses et poils par coloration microscopie électronique (*Tuber melanosporum*). *Doc Mycol* **18:** 47–60.

Ferreira, A.V.B., Saupe, S., and Glass, N.L. (1996) Transcriptional analysis of the *mt A* idiomorph of *Neurospora crassa* identifies two genes in addition to *mt A-1*. *Mol Gen Genet* **250:** 767–774.

Fiore-Donno, A.M., and Martin, F. (2001) Populations of ectomycorrhizal *Laccaria amethystina* and *Xerocomus* spp. show contrasting colonization patterns in a mixed forest. *New Phytol* **152:** 533–542.

Frizzi, G., Lalli, G., Miranda, M., and Pacioni, G. (2001) Intraspecific isoenzyme variability in Italian population of the white truffle *Tuber magnatum*. *Mycol Res* **105:** 365–369.

Gandeboeuf, D., Dupré, C., Roechel-Drevet, P., Nicolas, P., and Chevalier, G. (1997) Grouping and identification of *Tuber* species using RAPD markers. *Can J Bot* **75:** 36–45.

Gherbi, H., Delaruelle, C., Selosse, M.A., and Martin, F. (1999) High genetic diversity in a population of the ectomycorrhizal basidiomycete *Laccaria amethystina* in a 150-year-old beech forest. *Mol Ecol* **8:** 2003–2013.

Glass, N.L., and Donaldson, G.C. (1995) Development of primer sets designed for use with the PCR to amplify conserved genes from filamentous Ascomycetes. *Appl Environ Microbiol* **61:** 1323–1330.

Glass, N.L., Grotelueschen, J., and Metzenberg, R.L. (1990) *Neurospora crassa* A mating type region. *Proc Natl Acad Sci USA* **87:** 4912–4916.

Glen, M., Tommerup, I.C., Bougher, N.L., and O'Brien, P.A. (2001) Interspecific and intraspecific variation of ectomycorrhizal fungi associated with *Eucalyptus* ecosystems as revealed by ribosomal DNA PCR-RFLP. *Mycol Res* **105:** 843–858.

Guidot, A., Gryta, H., Gourbière, F., Debaud, J.C., and Marmeisse, R. (2002) Forest habitat characteristics affect balance between sexual reproduction and clonal propagation of the ectomycorrhizal mushroom *Hebeloma cylindrosporum*. *OIKOS* **99:** 25–36.

Guillemaud, T., Raymond, M., Callot, G., Cleyet-Marel, J.C., and Fernandez, D. (1996) Variability of nuclear and mitochondrial ribosomal DNA of a truffle species (*Tuber aestivum*). *Mycol Res* **100:** 547–550.

Høiland, K., and Holst-Jensen, A. (2000) *Cortinarius* phylogeny and possible taxonomic implications of ITS rDNA sequences. *Mycologia* **92:** 694–710.

Jany, J.L., Garbaye, J., and Martin, F. (2002) *Cenococcum geophilum* populations show a high degree of genetic diversity in beech forests. *New Phytol* **154:** 651–659.

Johannesson, H., Vasiliauskas, R., Dahlberg, A., Penttila, R., and Stenlid, J. (2001) Genetic differentiation in Eurasian populations of the postfire ascomycete *Daldinia loculata*. *Mol Ecol* **10:** 1665–1677.

Johannesson, H., Ihrmark, K., and Stenlid, J. (2002) Differential decay extension capability of *Daldinia* spp. in wood of *Betula pendula*, *Alnus glutinosa* and *Fraxinus excelsior*. *For Ecol Managem* **167:** 295–302.

Lanfranco, L., Perotto, S., and Bonfante, P. (1998) Applications of PCR for studying the biodiversity of mycorrhizal fungi. In *Applications of PCR in Mycology*. Bridge, P.D., Arora, D.K., Reddy, C.A., and Elander, R.P. (eds). CABI 6: 107–124.

Mabru, D., Dupré, C., Douet, J.P., Leroy, P., Ravel, C., Ricard, J.M., *et al.* (2001) Rapid molecular typing method for the reliable detection of Asiatic black truffle (*Tuber indicum*) in commercialized products: fruiting bodies and mycorrhizal seedlings. *Mycorrhiza* **11:** 89–94.

Mello, A., Nosenzo, C., Meotto, F., and Bonfante, P. (1996) Rapid typing of truffle mycorrhizal roots by PCR amplification of the ribosomal DNA spacers. *Mycorrhiza* **6:** 417–421.

Mello, A., Garnero, L., and Bonfante, P. (1999) Specific PCR-primers as a reliable tool for the detection of white truffles in mycorrhizal roots. *New Phytol* **141:** 511–516.

Mello, A., Vizzini, A., Longato, S., Rollo, F., Bonfante, P., and Trappe, J.M. (2000) *Tuber borchii* versus *T. maculatum*: neotype studies and DNA analyses. *Mycologia* **92:** 326–333.

Mello, A., Fontana, A., Meotto, F., Comandini, O., and Bonfante, P. (2001) Molecular and morphological characterization of *T. magnatum* mycorrhizas in a long-term survey. *Microbiol Res* **155:** 279–284.

Mello, A., Cantisani, A., Vizzini, A., and Bonfante, P. (2002)

Genetic variation of *Tuber uncinatum* and its relatedness to other black truffles. *Environ Microbiol* **4:** 584–594.

Murat, C., Diez, J., Luis, P., Delaruelle, C., Dupré, C., Chevalier, G., *et al.* (2004) Polymorphism at the ribosomal DNA ITS and its relation to post-glacial recolonization routes of the Perigord truffle *Tuber melanosporum*. *New Phytol* **164:** 401–411.

Nelson, M.A. (1996) Mating systems in ascomycetes: a romp in the sac. *Trends Genet* **12:** 69–74.

Pöggeler, S. (2001) Mating-type genes for classical strain improvements of ascomycetes. *Appl Microbiol Biotechnol* **56:** 589–601.

Primmer, C.R., Borge, T., Lindell, J., and Saetre, G.P. (2002) Single-nucleotide polymorphism characterization in species with limited available sequence information: high nucleotide diversity revealed in the avian genome. *Mol Ecol* **11:** 603–612.

Redecker, D., Szaro, T.M., Bowman, J.R., and Bruns, T.D. (2001) Small genets of *Lactarius xanthogalactus*, *Russula cremaricolor* and *Amanita francheti* in late stage ectomycorrhizal successions. *Mol Ecol* **10:** 1025–1034.

Rubini, A., Topini, F., Riccioni, C., Paolocci, F., and Arcioni, S. (2004) Isolation and characterization of polymorphic microsatellite loci in white truffle (*Tuber magnatum*). *Mol Ecol Notes* **4:** 116–118.

Selosse, M.A., Weiss, M., Jany, J.L., and Tillier, A. (2002) Communities and populations of sebacinoid basidiomycetes associated with the achlorophyllous orchid *Neottia nidus-avis* (L.) L.C.M. Rich. and neighbouring tree ectomycorrhizae. *Mol Ecol* **11:** 1831–1844.

Smith, S.E., and Read, D.J. (1997) *Mycorrhizal Symbiosis*, 2nd edn. London, UK: Academic Press.

Soubabere, O., Jorge, V., Notteghem, J.L., Lebrun, M.H., and Tharreau, D. (2001) Sequence characterized amplified region markers for the rice blast fungus, *Magnaporthe grisea*. *Mol Ecol Notes* **1:** 19–21.

Staben, C., and Yanofsky, C. (1990) *Neurospora crassa* a mating type region. *Proc Natl Acad Sci USA* **87:** 4917–4921.

Tenaillon, M.I., Sawkins, M.C., Long, A.D., Gaut, R.L., Doebley, J.F., and Gaut, B.S. (2001) Patterns of DNA sequence polymorphism along chromosome 1 of maize (*Zea mays* ssp. *mays* L.). *Proc Natl Acad Sci USA* **98:** 9161–9166.

Timothy, Y.J., Moncalvo, J.M., Li, S., and Vilgalys, R. (2001) Polymorphism at the ribosomal DNA spacers and its relation to breeding structure of the widespread mushroom *Schizophyllum commune*. *Genetics* **157:** 149–161.

Vizzini, A. (2001) Relazioni filogenetiche del genere *Tuber* (Tuberaceae) nell'ambito dell'ordine. Pezizales: analisi molecolari, cariologiche, macro- e micromorfologiche. Tesi Dottorato XIV ciclo. PhD Thesis, University of Turin, Italy.

White, T.J., Bruns, T., Lee, S., and Taylor, J. (1990) Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In *PCR Protocols. A Guide to Methods and Applications*. Innis, M.A., Gelfand, D.H., Sninshy, J.J. and White, T.D. (eds). London, UK: Academic Press, pp. 315–322.

Yan, Z.H., Rogers, S.O., and Wang, C.J.K. (1995) Assessment of *Phialophora* species based on ribosomal DNA internal transcribed spacers and morphology. *Mycologia* **87:** 72–83.

Zhou, Z., Miwa, M., and Hogetsu, T. (1999) Analysis of genetic structure of a *Suillus gevillei* populations in a *Larix kaempferi* stand by polymorphism of inter-simple sequence repeat (ISSR). *New Phytol* **144:** 55–63.

Zhou, Z., Miwa, M., and Hogetsu, T. (2000) Genet distribution of ectomycorrhizal fungus *Suillus gevillei* populations in two *Larix kaempferi* stands over two years. *J Plant Res* **113:** 365–374.

2.3 Identification des mycorhizes de T. *magnatum* et des champignons ectomycorhiziens dans la truffière de Montemagno (Asti, Italie)

Contrairement à *T. borchii*, nous ne disposons pas encore de mycorhizes *in vitro* de T. *magnatum*. Donc, le seul moyen d'étudier la phase symbiotique de cette truffe est l'observation *in situ*. En fait, il persiste de nombreuses questions sur l'écologie des truffes et sur leur phase symbiotique : (1) A quelle période de l'année se forment les mycorhizes ?; (2) les mycorhizes de truffes sont-elles présentes dans les zones non productives des truffières ? (3) Quels sont les champignons ectomycorhiziens vivant dans les sols truffiers ?

Pour essayer de répondre à ces interrogations, nous avons échantillonné des mycorhizes en Novembre 2001 (période productive) et en Mai 2002 (période non productive) dans la truffière de *T. magnatum* (Montemagno, Asti). Même si le trufficulteur nous a laissé prélever des racines, nous n'avons pas pu réaliser un ample échantillonnage. Nous avons réalisé, tout d'abord, une analyse morphologique des mycorhizes en les classant par morphotype. Dans un deuxième temps, les apex racinaires correspondant à chaque morphotype ont été utilisés pour une analyse moléculaire avec l'ITS.

Au total nous avons identifié 39 morphotypes différents. Parmi ceux-ci, les *Telephoraceae* ont été les plus nombreux, suivis par les *Pezizales* et les *Sebacina*. Deux morphotypes ont été identifiés comme étant *T. magnatum*, en Mai 2002. Un dans une zone productive et l'autre dans une zone non productive.

Ce travail a permis de dire que les mycorrhizes de *T. magnatum* sont très rares même dans les zones productives. De plus, il ne semble pas y avoir de liaison entre la présence de mycorhizes et la production d'ascocarpes car les mycorhizes ont été trouvées pendant une période non productive. D'autre part, les *Telephoraceae*, *Pezizales* et *Sebacina* sont les taxa les plus abondants dans l'écosystème truffier étudié.

Morphological and molecular typing of the subterranean ectomycorrhizal community in a *Tuber magnatum* Pico truffle ground

Summary

Data on *Tuber magnatum* ecology are very limited. In order to elucidate the white truffle dynamic during its symbiotic stage and to identify fungi associated with it, ectomycorrhizal root tips were harvested in a *T. magnatum* natural truffle ground. Both morphological identification of morphotypes and molecular analysis of the ITS were realized and we found that i) *T. magnatum* mycorrhizae were rare and found only in Spring 2002 in productive and non productive areas; ii) the dominant fungal species were represented by the *Telephoraceae* and *Pezizales*.

Introduction

Some truffles are products in great demand on the food market in many countries. Among them, *Tuber magnatum* Pico, commonly known as the white truffle of Alba (*internet site: www.truffle.org),* is of special interest. Its unique taste and fragrance, as well as its limited availability, make *T. magnatum* fruitbodies one of the most expensive delicacies on the market. While *T. melanosporum* Vittad. is generally sold at €30-40/100gr in France, *T. magnatum* reached €300-400/100gr in Fall 2003. A productive truffle-ground represents therefore a conspicuous economic source for rural communities.

Truffles are ascomycetous fungi that form ectomycorrhizas (ECM) with the roots of trees, such as oak, poplar, willow, hazel (Harley and Smith 1983), and shrubs, such as *Cistus* (Fontana and Giovannetti 1978, 79). As a consequence, their life cycle is usually described as a succession of vegetative, symbiotic and reproductive phases (Lanfranco *et al.*, 1995), the last one being temporally dependent on the second one. Data on truffle ectomycorrhizas ecology are very limited, previous studies being mainly focussed on truffle mycorrhizal identification with isoenzymes (Urbanelli et al., 1998) or molecular tools (Henrion et al., 1994; Amicucci et al., 1996; Paolocci et al., 1999; Mabru et al., 2001; Mello et al., 2001; Rubini et al., 2001). However, advances on *Tuber* spp. mycelium cultivation has allowed the mycorrhizal seedlings development with several truffles species. *T. melanosporum* was the first truffle produced in experimental fields using these inoculated seedlings (Chevalier & Grente, 1979). For example, in a *T. melanosporum* experimental plantation, first ascocarps were recorded after four years and 82% of transplanted seedlings had produced truffles after six years (Le Tacon et al., 1988). These cultural techniques are available for *T. uncinatum* (Chevalier & Frochot, 1997) and *T. borchii* (Zambonelli et al., 2002), however controlled production of *T. magnatum* is not yet possible.

T. magnatum ectomycorrhizas were identified only one times in wild samples using multilocus electrophoresis (Urbanelli et al., 1998). Mello et al. (2001) and Rubini et al. (2001) used specific primers, based on the *T. magnatum* Internal Transcribed Spacer (ITS) of the ribosomal DNA sequence, for a molecular characterization of mycorrhizal seedlings raised under controlled conditions. These molecular probes enabled a morphological characterization to be formulated for *T. magnatum* mycorrhizas. Furthermore, Mello et al. (2001), after screening a large number of samples, could estimate the occurrence of *T. magnatum* in the examined mycorrhizal seedlings, *T. magnatum* mycorrhizas being found in 20% of the examined samples. However, all of them were only found in controlled situations, i.e. a growth chamber or greenhouses. Failure to obtain *T. magnatum* mycorrhizas in nursery, i.e. under field conditions, was explained in term of competition with more aggressive truffles, such as *T. maculatum* and *T. borchii* (Mello et al., 2001). According to Marmeisse et al (2004), questions which are at the basis of our knowledge on the genetic diversity and dynamics of ectomycorrhizal fungi in natural ecosystems are still fully open: (i) How are abundant truffle mycorrhizas in a natural truffle ground? (ii) Does their presence match the fruitbody production? (iii) As a consequence, can we detect mycorrhizas exclusively in productive zones, or also in non-productive ones? (iv) Are they present all the year around or are temporarily limited? (v) Which other ectomycorrhizal fungi are present in a truffle ground?

Here, we aimed to elucidate the white truffle dynamic during its symbiotic stage and to identify fungi associated with *T. magnatum* in a natural truffle-ground. We took advantage on our previous work, where we mapped and genotyped *T. magnatum* fruitbodies collected in the natural truffle-ground of Montemagno (Piedmont, Italy) (Mello et al., 2004; cf. §2-2). During this survey two genotypes were found, each year for as long as five years, and productive and non productive areas were identified. As a second step, mycorrhizas samples were harvested and sorted into morphotypes by morphological criteria. This method, however, is dependent on many factors, as the age, the host tree species and environmental conditions. For this reason, morphological typing of ectomycorrhizas was supported by molecular methods using ITS. Molecular and morphotype techniques allowed ECM fungi identification on root tips, the fungal distribution analysis and provided information on the subterranean ECM fungal community. Even if the sampling was not extensive and many cautions were taken in digging the soil of the productive area under owner scrutinity, this work represents the first study on the *Tuber* symbiotic phase in a natural truffle ground.

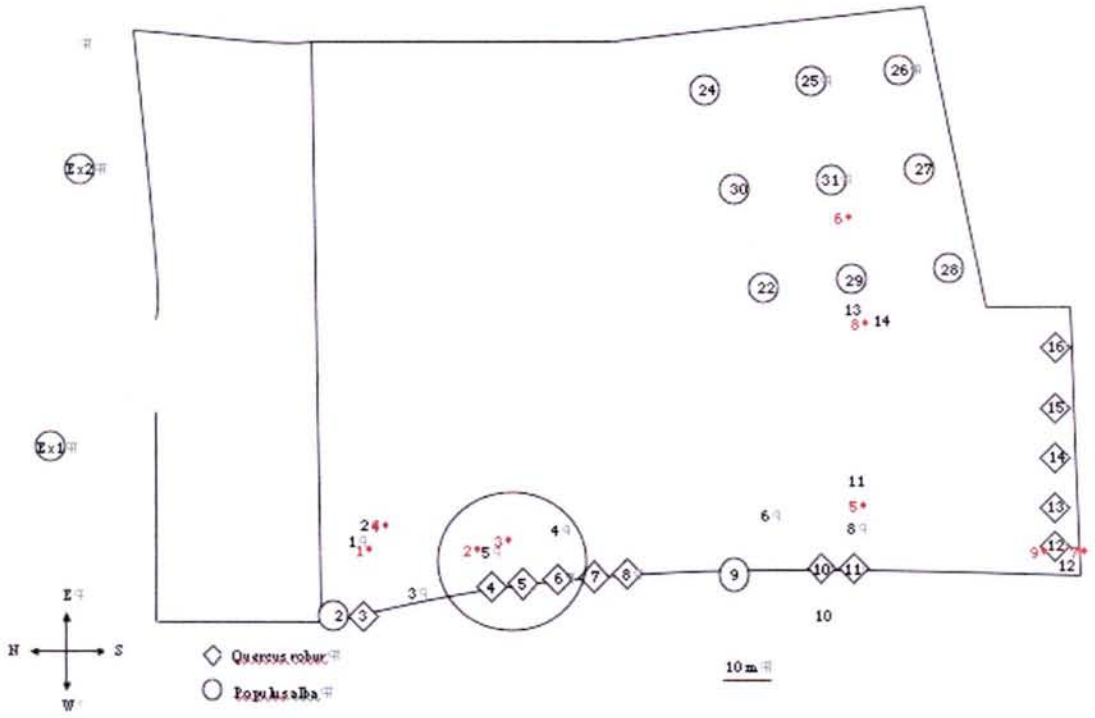

Figure 1. Map of the truffle-ground located al Montemagno (AT). Mycorrhizal samplings are indicated in black for November 2001 and in Red with asterisk for May 2002. According to Mello et al. (2004) (Fig. 1; §2.2) a non productive area has been defined and localized by black circle. In truffle ground there is grass vegetation.

Materials and Methods

Location of the truffle ground and geographical data

The truffle ground (≈ 7.000 m^2) is placed in a valley in Montemagno (Asti, Piedmont, North Italy, 8° 19' 35" 4 East, 44° 59' 2" 40 North). It is characterised by the persistence of a moist soil, even in the hot season. The area is particularly useful for research studies because it does not present signs of anthropic activities, with the exception of the enlargement of a natural stream and the seasonal mowing. In this truffle ground *Populus nigra* L., *P. alba, P. tremula* L, *Quercus robur* L., *Salix alba* L., *and Tilia cordata* Miller are present; some trees are fifty years old, while most are younger. Records on productivity claim that this truffle ground has produced truffles since, at least, 1850. Trees start to produce, usually after 10-20 years. Fig. 1 shows the localisation of trees which are expected to be productive. Each years about 30 *T. magnatum* fruitbodies are harvested. Ascocarps harvesting positions analyzed by Mello et al. (2004) are indicated in Fig. 1. Productive and non-productive areas in could be defined in this truffle ground (Fig. 1). Twenty-one root samples were collected in the winter 2001 –2002 and in the Spring 2002: 12 samples were collected in November 2001, when it is period of the harvest of fruitbodies, and 9 in May 2002, when the harvesting is over (Table 1). The sampling position

was mapped in respect to the nearest tree (Figure 1, Table 1). When possible, up to three samples were collected within a small area under the tree (Table 1) and all together are indicated by only one star in Fig. 1. As already mentioned, the limited number of samplings was due to the risk of breaking down the fungal web in the soil of this productive truffle-ground.

Morphological analysis

Root samples were collected in soil volume of 10 x 10 x 10 cm. The root system was rinsed in water and observed under a stereo dissecting microscope to look for ectomycorrhizal roots. Mycorrhizal root tips of each sample were sorted into morphotypes on the basis of colour, mantle shape and surface texture, presence of cystidia, and EM branching pattern. When necessary, cross sections were made and examined under a light microscope for the presence of the Hartig net.

Molecular analysis

Each morphotype, grouping from 1 to 10 similar mycorrhizal tips, was subjected to the molecular analysis.
Total DNA was extracted by using the Dneasy Plant Mini Kit (Qiagen SA, Courtaboeuf, France) following the manufacturer's instructions. PCR reactions were performed with ITS1f (Gardes & Bruns, 1993) and ITS4 (White *et al.*, 1990) primers. rDNA libraries were produced by isolating the amplicons from gel, ligating them into the pGEM-T vector (Promega, Madison Wis.) and transforming the plasmids into competent JM109 cells. Bacterial colonies containing plasmids with rDNA inserts were identified by PCR. To sort the clones into groups the rDNA inserts were amplified by PCR, digested individually with two DNA restriction endonuclease treatments (*Hinf* I, *Sau3A* I) and resolved on 2% agarose gels. Clones, which showed different restriction patterns, were sequenced. Similarities of the rDNA clones to sequences in the GenBank database were determined by using BLAST (NCBI). Accession numbers are listed in Table 1.

Results and Discussion

This survey is the first attempt to identify and to study the ecology of the symbiotic phase of *T. magnatum*. Notwithstanding the drawback of having a limited number of samples (21 samplings sorted in 40 morphotypes) our investigation allowed us to identify *T. magnatum* mycorrhizas in a natural truffle ground. In addition, morphotyping and ITS sequencing of these mycorrhizal samples provided novel information on the ectomycorrhizal and endophytic species living in a *T. magnatum* truffle ground (Table 1).

Table 1. Morphological and molecular typing of two root samplings in November 2001 and May 2002. Nearest tree is indicated. Samplings (S) have been realized in a productive, i.e; where at least one fruitbody has already been harvested, (Prod.) and non productive, where it has never been harvested fruitbody, (Not Prod.) area of the truffle ground.

		November 2001			May 2002		
Tree	Area	S	Morphotypes	Molecular analysis	S	Morphotypes	Molecular analysis
2	Prod.	1	unidentified		1	unidentified	*Tomentella/Telephora*
			unidentified	*Epicoccum* sp, Pezizaceae		*Tuber magnatum*	*Tuber magnatum*, *Helvella* sp (nr)
						Tuber sp	*Tuber maculatum*
	Prod.	2	*Tomentella* sp	*Tomentella* sp			
			Tuber maculatum	*Tuber maculatum*	4	*Tomentella* sp	*Tomentella* sp, *Verticillium* sp, Dothideomycetes
	Prod.	3	*Tomentella* sp	*Tomentella* sp			
			unidentified	*Helvella lacunosa*, *Tetracladium* sp, *Tuber rufum*			
4	Not prod.	5	*Scleroderma* sp	*Scleroderma* sp	2	*Tuber* sp	*Helvella* sp (nr)
			unidentified	*Strumella* sp, *Scleropezicula* sp, *Sebacina* sp		*Tomentella* sp	*Tomentella*
			unidentified	*Tomentella* sp		*Tomentella* sp	Amphisphaeriaceae, *Alternaria* sp
			unidentified	Sebacinaceae	3	unidentified	*Tuber magnatum*, *Sebacina incrustans*, Helotiales (ericoids and endophytes)
6	Not prod.	4	unidentified	*Mortierella alpina*, Fungal endophyte, Nectriaceae			
			unidentified	*Tomentella* sp			
			Tomentella sp				
9	Prod.	6	*Tuber* sp	Ericoid endophyte (Helotiales), Nectriaceae			
10	Prod.	10	unidentified	Sebacinaceae			
11	Prod.	8	*Tomentella* sp	Agaricales, *Tomentella* sp	5	No mycorrhizas	
	Prod.	11	*Tuber sp*	Agaricales			
12	Prod.	12	*Tuber* sp	Sebacinaceae	9	unidentified	Sebacinaceae
			Tomentella sp	*Tomentella* sp		*Tomentella* sp	
			Tuber sp	*Nectria*, *Tomentella* sp	7	unidentified	
29	Prod.	13	*Tomentella* sp	*Tomentella* sp	8	*Tomentella* sp	*Tomentella* sp
			unidentified	Cortinariaceae, Sebacinaceae		unidentified	*Tetracladium* sp
	Prod.	14	*Tuber* sp	*Leptospheria* sp, *Nectria* sp, *Sebacina epigea*, *Tetracladium* sp, *Pezizomycotina*			
			unidentified	Cortinariaceae, Agaricales			
			Tomentella sp	*Tomentella* sp, *Nectria* sp			
31	Not prod.				6	*Tuber* sp	*Tuber maculatum*

First glance at the subterranean ECM community

Morphological analysis allowed to distinguish 39 morphotypes, among them 16 were unidentified (Table 1). Morphotypes identities were confirmed in 14 out of 23 by the molecular analysis, while in 6 cases there were differences between morphological and molecular identification (Table 1). *Telephoraceae* was the most frequent family (13 out of 39 morphotypes, 32%), often recognized by both morphological and molecular analysis (Table 1). Except for one sample (S 1 of May 2002), this family was represented by *Tomentella*. *Tomentella* sequences alignment – which were found in our samplings - showed a broad heterogeneity suggesting the presence of different species. About 70 species of tomentelloid fungi are known, assembled into four genera, viz. *Amaurodon*, *Pseudotomentella*, *Tomentella* and *Tomentellopsis* (Larsen 1971, 1974; Stalpers 1993; Koljalg 1996). *Thelephora*, a genus closely related to tomentelloid fungi, comprises about 40 species, of which seven are found in Sweden (Corner 1968; Stalpers 1993; Koljalg 1996; Hallingbäck and Aronsson 1998). Recent studies have indicated that tomentelloid fungi may be a widespread and important component of EM communities, with the highest species richness being found in temperate coniferous and broad-leaved forests (Koljalg et al. 1996, 2000).

A second important group in the truffle ground was represented by the *Pezizales* order (11 out of 39 morphotypes). These were *Pezizaceae*, *Helvella* sp, *Strumella* sp and *Tuber* sp. (Table 1). They were mainly found in one area of the truffle ground near trees 2 and 4 in two samplings. For example, under the tree N 2 we have observed that: 1) *T. magnatum* was identified in May 2002; 2) *T. rufum* was found in November 2001; 3) *T. maculatum* was present in November, which is a fructification period, as well as in May; 4) the fungal species close to *Helvella* was present both, in November and May, always associated to *Tuber* species. In fact, *T. maculatum* ascocarps have been harvested in this truffle ground near trees 2 and 4 but we do not know if *T. rufum* ascocarps have already been produced (Gavazza, personnal communication).

Sebacina incrustans was detected under the tree N 4 where *T. magnatum* and ericoid and/or endophyte fungi belonging to *Helotiales* are present. *Sebacina* sp. were found on 9 out of 39 morphotypes. Until recently, this family was not considered in the major compilations of ectomycorrhizal taxa (Agerer 1987-98, Molina et al. 1992, Agerer et al., 1996-2001, Smith and Read 1997). However, Urban et al. (2003) identified the fungal partner of one ectomycorrhizal sample as *Sebacina incrustans* confirming the hypothesis that the ectomycorrhizal status might be common in the *Sebacinaceae* (Weib and Oberwinkler 2001). A Heterobasidiomycetous species of the *Sebacinaceae* family has been shown to form ectomycorrhizas on temperate forest trees (Selosse et al., 2002), on *Polygonum viviparum* in alpine grasslands (Sonstebo 2002) and in

Australian forests where they are common ectomycorrhizal associates of *Eucalyptus marginata* (Glen et al., 2002). Our finding of a *Sebacina* sp associated to *T. magnatum* ectomycorrhiza is in agreement with the discovery of sebacinoid ectomycorrhizas associated to ascomycetous ectomycorrhizas by Urban et al., (2003) and Selosse et al., (2002). Buscot & Kottke (1990) and Buscot (1994) reported a close association between the ectomycorrhizas of *Morchella* (Pezizales) and those of an unidentified heterobasidiomycete that, because of its dolipore ultrastructure (i.e. not perforated parenthesome), likely belong to Sebacinaceae.

Are Truffle ecosystems a particular one or there are commun characteristic with other ecosystems? In a *T. magnatum* natural truffle ground study in Italy, Rossi et al. (2002) identified several *Tuber*-like ectomycorrhizas morphotypes. However, they did not confirm morphological identification by molecular analysis and no information on other ectomycorrhizal species are provided. Other similar ecosystems have been studied by De Miguel et al. (2002), which analysed severals *T. melanosporum* cultivated beds in Spain. They have harvested sporocarps of seven truffle species (*T.aestivum* Vitt., *T. brumale* Vitt., *T. excavatum* Vitt., *T. melanosporum* Vitt., *T. mesentericum* Vitt., *T. panniferum* Tul &C. Tul) and ten other hypogeous and Gastromycetes (*Genea* sp., *Hymenogaster* sp., *Pisolithus tinctorius*, *Rhizopogon roseolus* and *Schleroderma verrucosum*). Twenty mophological morphotypes of ectomycorrhizas have been identified, among them six truffle species, *Schleroderma* type and *Tomentella subtestacea.* So, it seems that *Telephoraceae* (i.e. Tomentella) and Pezizales (i.e. *Tuber* and *Heliotiales*) are abondant in truffle ecosystem. In fact, *Telephoraceae* is among the most abundant and frequent taxon on ECM roots in conifer communities in both Europe and North America (Gardes & Bruns, 1996; Jonsson et al., 1999; Taylor & Bruns 1999). This suggest that truffle ecosystem in sud Europe is predominated by same ECM species than other community in North Europe (e.g. *Telephoraceae*).

Identification of T. magnatum mycorrhizas

Until now, *T. magnatum* mycorrhizas were reported in experimental conditions (Mello et al., 2001; Rubini et al., 2001) and only one time with wild samples (Urbanelli et al., 1998). In the present study mycorrhizal tips of *T. magnatum* were identified twice. In one case, the morphological identification was confirmed by the ITS analysis (S1 May 2002; Table 1). In the second case the morphotype was unidentified and the identification of its old mycorrhizas was limited to the molecular analysis. These results indicated that *T. magnatum* mycorrhizae are rare even in a well productive area (Mello et al., 2004), 2 out of 39 morphotypes, representing ≈ 5%. They were found both during spring 2002, e.g. in a non-productive period, and in a non-productive area (near tree 4 see Fig. 1 *In* Mello et al., 2004), suggesting that there is no a direct linkage between mycorrhizas and

fruitbodies. Moreover, we show that *T. magnatum* is present in non-productive area.

The very low occurence of *T. magnatum* mycorrhizas is not so surprising, since a low abundance of a fungal species at the mycorrhizal level has been already reported by Gardes and Bruns (1996). These authors found that *Suillus pungens* ECM root tips were rare in a *Pinus muricata* forest whereas *S. pungens* sporocarps were abundant. In the same forest *Russula amoenolens* root tips were abundant, whereas *R. amoenolens* sporocarps were rare. Dahlberg et al. (1997) highlight that macrofungi species, which accounted for 70% of the annual fruiting biomass correspond to less than 30% of the colonized root tips. According to Taylor (2002), *Cortinarius* was the most abundant genus forming 42.3% of sporocarps in a *Pinus sylvestris* stand at Riddarhhyttan; however belowground only 1.6% of the mycorrhizal tips examined could be attributed to this genus. Bonello et al. (1998) explain the condition of low mycorrhizal percentage and the spread and persistence of a large *S. pungens* genet fruiting abundantly with 3 hypothesis: (1) *S. pungens* is efficient to obtain C from its host that few mycorrhizal connections are needed for significant C transfer; (2) large genets visit the roots of more trees, pooling, consequently, more total C than smaller genets; (3) a fraction of the C is obtained saprotrophycally.

In conclusion, this study represents a first attempt to give a glimpse at the mycorrhizosphere in a *T. magnatum* natural truffle ground. We have found that *T. magnatum* ectomycorrhizas are very rare. Moreover, root tips sampling did not correspond to fructification harvesting, since we identified one T. magnatum morphotype in a non productive area of the truffle ground. However, further samplings are surely necessary to define *T. magnatum* mycelium distribution. On the other hand, most abundant morphotypes belong to *Telephoraceae*, *Pezizales* and *Sebacina*. These species are also the most abondant symbiotic taxa in other ecosystems in North Europe, America and Australia. In the future, it will be interesting to analyse fungal diversity not only at root level but directly in the soil. Such analysis would allow us to improve knowledge on truffle ecosystems and as a consequence to enhance the success of experimental truffle production.

Bibliography

Agerer R (ed.) (1987-98) Colour Atlas of Ectomycorrhiza. Einhorn Verlag, Scwäbissch Gmünd.

Agerer R , Danielson RM, Egli S, Ingleby K, Luoma, D Tre R (eds) (1996-2001) Descriptions of Ectomycorrhiza 1-7.

Amicucci A., Rossi I., Potenza L., Zambonelli A., Agostini D., Palma F., Stocchi V., 1996. Identification of ectomycorrhizae from *Tuber* species by RFLP analysis of the ITS region. Biotechnology Lett., 18, 821-826

Bonello P., Bruns T.D., Gardes M., 1998. Genetic structure of a natural population of the ectomycorrhizal fungus *Suillus pungens*. New Phytologist, 138, 533-542

Buscot F., 1994. Ectomycorrhizal types and endobacteria associated with ectomycorrhizas of Morchella elata (fr) Boudier with Picea abies (L) Karst. Mycorrhiza. 4 (5), 223-232

Buscot F., Kottke I., 1990. The association of Morchella-rotunda (Pers) Boudier with roots of Picea abies (L) Karst. New Phytologist, 116 (3), 425-430.

Chevalier G. & Grente J., 1979. Application pratique de la symbiose ectomycorhizienne : production à grande échelle de plants mycorhizés par la truffe. Mushr. Sci. 10 (2), 483-505.

Chevalier G., Frochot H., 1997. *La truffe de Bourgogne*, Ed. Pétrarque, 257p

Corner EJH (1968) A Monograph of Thelephora (Basidiomycetes). Beihefte zur Nova Hedwigia 27: 1-110.

Dahlberg A., Jonsson L., Nylund J.E., 1997. Species diversity and distribution of biomass above and below ground among ectomycorrhizal fungi in an old-growth Norway spruce forest in south Sweden. Canadian Journal of Botany, 75, 1323-1335

De Miguel A., De Roman M., Etayo M.L., 2002. Mycorrhizal fungi competing with *Tuber melanosporum* Vitt. In cultivated truffle beds in north-eastern Spain. Edible mycorrhizal mushrooms and their cultivation. Proceedings of the Second International Conference on Edible Mycorrhizal Mushrooms, Ed. Hall, I.; Yun Wang; Danell, E.; Zambonelli, A., Christchurch, New Zealand, 3-6 July, 2001

Fontana A. and G. Giovannetti - 1978-1979: *Simbiosi micorrizica fra Cistus incanus L. ssp. incanus e Tuber melanosporum Vitt.* Allionia 23: 5-11.

Gardes M and Bruns TD, 1993. ITS primers with enhanced specificity for basidiomycetes-application to the identification of mycorrhizae and rusts. Molecular Ecology 2: 113-118.

Gardes M, Bruns TD. 1996. Community structure of ecto-mycorrhizal fungi in a *Pinus muricala* forest: above- and below-ground views. Canadian Journal of Botany 74: 1572-1583.

Gehring CA, Theimer TC, Whitham TG, Keim P 1998. Ectomycorrhizal fungal community structure of pinyon pines growing in two environmental extremes. Ecology 78: 1562-1572.

Glen M, Tommerup IC, Bougher NL, O'Brien PA 2002. Are Sebacinaceae common and widespread ectomycorrhizal associates of Eucalyptus species in Australian forests? Mycorrhiza 12 (5): 243-247.

Hallingbäck T and Aronsson G (1998) Macrofungi and myxomycetes in Sweden and their ecology. 2nd revised and extended printing. Artdatabanken, SLU, Uppsala.

Harley J.L. & S.E. Smith - 1983: *Mycorrhizal Symbiosis*. Academic Press, London.

Henrion B., G. Chevalier & F. Martin, 1994. Typing truffle species by PCR amplification of the ribosomal DNA spacers. Mycologia 98, 37-43.

Jonsson T, Kokalj S, Finley R, Erland S 1999. Ectomycorrhizal community structure in a limed spruce forest. Mycol. Res. 103 (4): 501-508.

Karen O, Hogberg N, Dahlberg A, Jonsson L, Nylund JE 1997. Inter- and intraspecific variation in the ITS region of rDNA of ecto-mycorrhizal fungi in Fennoscandia as detected by endonuclease analysis. New Phytologist 136: 313-325.

Koljalg U (1996) Tomentella (Basidiomycota) and related genera in temperate Eurasia, Synopsis Fungorum 9. Fungiflora, Oslo.

Kõljalg, U. , A. Dahlberg, A. F. S. Taylor, E. Larsson, N. Hallenberg, J. Stenlid, K.-H. Larsson, P. M. Fransson, O. Kårén, L. Jonsson 2000. Diversity and abundance of resupinate thelephoroid fungi as ectomycorrhizal symbionts in Swedish boreal forests. Molecular Ecology 9 (12): 1985-1996.

Larsen MJ (1971) The genus Pseudotomentella (Basidiomycetes, Thelephoraceae s.str.) Nova Hedwigia, 22: 599-619.

Larsen MJ (1974) A contribution to the taxonomy of the genus Tomentella, Mycologia Memoirs, 4: 1-145.

Le Tacon F., J. Garbaye, D. Bouchard, G. Chevalier, J. M. Olivier, J. Guimberteau, N. Poitou & H. frochot, 1988. Field result from ectomycorrhizal inoculation in France. *In* Canadian workshop on Mycorrhizal in Forestry (edited by Lalonde, Pichey) : 51-74

Mabru D., Dupré C., Douet J. P., Leroy P., Ravel C., Ricard J. M., Medina B., Castroviejo M., Chevalier G., 2001. Rapid molecular typing method for the reliable detection of Asiatic black truffle (*Tuber indicum*) in commercialized products : fruiting bodies and mycorrhizal seedlings. *Mycorrhiza* 11 : 89-94.

Marmeisse R, Guidot A, Gay G, Lambilliotte R, Sentenac H, Combier JP, Melayah D, Fraissinet-Tachet L, Debaud JC, 2004. Hebeloma cylindrosporum - a model species to study ectomycorrhizal symbiosis from gene to ecosystem. New Phytologist 163 (3): 481-498

Mello A., Murat C., Vizzini A., Gavazza V., Bonfante P., 2004. Tuber magnatum Pico, a species of limited geographic distribution: its genetic diversity inside and outside a truffle ground. Environmental Microbiology, In press.

Mello, A., Fontana, A., Meotto, F., Comandini, O., Bonfante P., 2001. Molecular and morphological characterization of *T. magnatum* mycorrhizas in a long-term survey. Microbiol Res 155: 279-284.

Molina R, Massicotte H, Trappe JM (1992) Specificity phenomena in mycorrhizal symbiosis community – ecological consequences and pratical implications. In Mycorrhizal Functioning (M.F. Allen, ed.): 357-423. Chapman and Hall, London.

Paolocci F., Rubini A., Granetti B., Arcioni S., 1999. Rapid molecular approach for a reliable identification of *Tuber* spp. Ectomycorrhizae. *FEMS Microbiology Ecology* 28 : 23-30.

Pritsch K, Boyle H, Munch JC, Buscot F 1997. Characterization and identification of black alder mycorrhizas by PCR/RFLP analyses of the rDNA internal transcribed spacer (ITS). New Phytologist 137: 357-369.

Rossi I., Bertini L ;, Amicucci A., Cecchini M., Gregori L., Sacchi A., Zambonelli A., Stocchi V., 2002. Study of a natural *Tuber magnatum* Pico truffière in central Italy. Edible mycorrhizal mushrooms and their cultivation. Proceedings of the Second International Conference on Edible Mycorrhizal Mushrooms, Ed. Hall, I.; Yun Wang; Danell, E.; Zambonelli, A., Christchurch, New Zealand, 3-6 July, 2001

Rubini A, Paolocci F, Granetti B, Arcioni S (2001) Morphological characterization of molecular-typed Tuber magnatum ectomycorrhizae. Mycorrhiza 11: 179-185.

Selosse M-A., Bauer R., Moyersoen B. (2002) Basal hymenomycetes belonging to the Sebacinaceae are ectomycorrhizal on temperate deciduous trees. New Phytologist 155: 183-195.

Smith SE, Read DJ (1997) Mycorrhizal Symbiosis. Academic Press, New York.

Sønstebø J.E. - 2002: *Molecular Ecology of Ectomycorrhizal Fungi on Bistorta vivipara in four alpine tundra communities*. MSc thesis, University of Oslo, Norway.

Stalpers JA (1993) The Aphyllophoraceous fungi I. Keys to the species of the Thelephorales. Studies in Mycology, 35: 1-168.

Taylor AFS 2002. Fungal diversity in ectomycorrhizal communities: sampling effort and species detection. Plant and Soil 244: 19-28.

Taylor DL and Bruns TD 1999. Community structure of ectomycorrhizal fungi in a *Pinus muricata* forest: minimal overlap between the mature forest and resistant propagule communities. Mol. Ecol. 8: 1837-1850.

Urban A., Weiβ M., Bauer R. (2003) Ectomycorrhizas involving sebacinoid mycobionts. Mycol Res 107 (1): 3-14.

Urbanelli S., Sallicandro P., De Vito E., Bullini L., Palenzona M., Ferrara AM 1998 Identification of Tuber mycorrhizae using multilocus electrophoresis. Mycologia 90 (3): 389-395.

Weiß M and Oberwinkler F. (2001) Phylogenetic relationships in Auriculariales and related groups – hypotheses derived from nuclear ribosomal DNA sequences. Mycological Researh 105: 403-415

White T.J., Bruns T.D., Lee S.B. Taylor J.W., 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In Innis M.A., Gelfand D.H., Sninshy J.J., White T.D. (Eds.), PCR Protocols : a Guide to Methods and Applications. Academic Press, London, 315-322

Zambonelli A., Iotti M., Giomaro G., Hall I., Stocchi V., 2002. T. borchii cultivation : an interesting perspective. Edible mycorrhizal mushrooms and their cultivation. Proceedings of the Second International Conference on Edible Mycorrhizal Mushrooms, Ed. Hall, I.; Yun Wang; Danell, E.; Zambonelli, A., Christchurch, New Zealand, 3-6 July, 2001

3. DISCUSSION GENERALE

Les truffes sont étudiées depuis plus de 500 ans (cf. § 1.1.3), mais les études se focalisant sur leur biologie, leur taxonomie et la maîtrise de leur culture se sont multipliées les vingt dernières années. Les progrès de la biologie moléculaire ont permis d'améliorer nos connaissances sur la taxonomie de plusieurs espèces, mais aussi de mettre au point des diagnostics moléculaires utilisés par les services des fraudes. Il est maintenant possible d'identifier rapidement la plupart des truffes européennes et asiatiques à partir d'échantillons environnementaux ou d'aliments (Douet et al., 2004 ; Mabru et al., 2004). En revanche, les données concernant la diversité génétique et la structure génétique des populations de truffes étaient encore limitées au début de ce projet de thèse.

Lors de cette étude, je me suis focalisé sur l'analyse de la structure génétique de la truffe noire du Périgord (*T. melanosporum*), sur l'ensemble des régions productives françaises (échelle macro-géographique), et de celle de la truffe blanche du Piémont (*T. magnatum*). J'ai aussi étudié plus précisément une truffière de *T. magnatum* (échelle micro-géographique) au niveau des ascocarpes mais aussi des mycorhizes. Ce travail a permis d'émettre des idées nouvelles sur l'écologie de ces deux truffes. En particulier :

1-Il existe une différenciation génétique chez *T. melanosporum*, avec deux lignées, l'une à l'Est et l'autre à l'Ouest du Massif Central. Nous avons suggéré deux voies de recolonisations post-glaciaires pour cette truffe : la voie de la vallée du Rhône et la voie de l'Atlantique.

2- La variabilité génétique de *T. melanosporum* pourrait expliquer les variations phénotypiques observées, entre autres celles concernant les différences organoleptiques.

3- Nous avons trouvé des marqueurs polymorphes pour *T. magnatum* suggérant ainsi une variabilité génétique notable pour cette truffe. Les outils que nous avons mis au point pourront dans le futur être utilisés à grande échelle dans l'étude de la diversité génétique de cette truffe.

4- Au sein d'une truffière naturelle de *T. magnatum* (Montemagno, Asti), il peut exister plusieurs génotypes.

5- Nous avons identifié des mycorhizes de *T. magnatum* lors d'une période non productive. Nous avons aussi montré que ce champignon est présent dans des zones non productives de la truffière. La production des tissus symbiotiques (ectomycorhizes) peut donc être disjointe de la formation des corps fructifères. Enfin, les espèces mycorhiziennes majoritaires dans le sous-sol de la truffière de Montemagno sont les *Telephoraceae*, les *Pezizales* et les *Sebacina*.

Dans la troisième partie de cette thèse, nous discuterons de ces différents points et verrons quelles sont leurs applications possibles pour le monde de la trufficulture.

3.1 Diversité génétique et structure génétique chez *T. melanosporum* et *T. magnatum*

3.1.1 L'ITS, un locus polymorphe chez les truffes

Comme nous l'avons vu au paragraphe 1.4.3, les études RAPD (Lanfranco et al., 1993 ; Gandeboeuf et al., 1997) indiquent qu'il existe deux groupes d'espèces de truffes : (1) les espèces ayant un niveau élevé de diversité génétique (par exemple *T. uncinatum* et *T borchii*) ; (2) les espèces ayant un faible niveau de diversité génétique (par exemple *T. melanosporum* et *T. magnatum*). Le premier objectif que nous avions au début de cette thèse était donc d'identifier des régions génomiques variables pour ces deux espèces considérées comme peu variables. Dans le cas de *T. melanosporum*, nous savions que l'ITS était polymorphe (Roux et al., 1999 ; Mabru et al., 2001 ; Murat, 2001). En revanche, pour *T. magnatum*, les seules données disponibles concernaient les isoenzymes (Frizzi et al., 2001). Pour ces deux espèces, le séquençage de l'ITS a permis d'identifier plusieurs haplotypes (10 pour *T. melanosporum* et 3 pour *T. magnatum*). Étant donné que ce locus est considéré comme conservé au niveau intraspécifique, comment expliquer ce polymorphisme pour des espèces ayant par ailleurs un bas niveau de diversité génétique ?

En effet, le polymorphisme peut être observé à trois niveaux : (1) au niveau interspécifique ; (2) au niveau intraspécifique, c'est-à-dire entre les individus de la même espèce et (3) au niveau de l'individu.

Chez la plupart des Eucaryotes, le taux d'évolution concertée est suffisant pour homogénéiser les variations entre les répétitions d'ADNr au sein des individus et de l'espèce (Dover, 1982 ; Vollmer et Palumbi, 2004). Ce processus d'évolution concertée a été observé pour la première fois lors d'une étude de l'ADNr de deux espèces de Grenouilles (Brown et al., 1972). En fait, l'évolution des séquences ITS est liée à la sélection naturelle, à la dérive génétique et au mécanisme de recyclage moléculaire (*molecular turnover mechanism*) (Dover, 1982). Ces derniers comme la *gene conversion* et les *crossing-over* inégaux joueraient un rôle important dans les processus d'évolution concertée, mais leurs impacts dépendent des taux de recombinaisons et de mutations (Elder et Turner, 1995).

Toutefois, les analyses de restriction et de séquençage direct de l'ITS de plusieurs espèces fongiques, et entre autres des truffes, révèlent un niveau

de polymorphisme intraspécifique non négligeable de ce locus (Glen et al., 2001 ; Mello et al., 2002 ; Jany et al., 2002). Il semble donc que l'ITS soit plus variable, au niveau intraspécifique, que ce que l'on pensait. D'autre part, il existe des espèces pour lesquelles l'ITS est polymorphe au sein de l'individu, c'est-à-dire entre les différentes copies d'un même noyau. C'est le cas des Sauterelles (Parkin et Butlin, 2004), des coraux (Vollmer et Palumbi, 2004) et des champignons endomycorhiziens (Lanfranco et al., 1999 ; Pawlowska et Taylor, 2004). Chez les truffes, nous n'avons encore jamais mis en évidence de polymorphisme de l'ITS au sein d'un même individu. D'autre part, l'absence d'hétérozygotes lors de l'analyse de divers loci (Frizzi et al., 2001 ; Bertault et al., 2001 ; Paolocci et al., 2004 ; Rubini et al., 2004) suggère une reproduction homothallique pour les truffes. Cela indiquerait qu'il n'existe pas de croisements entre individus différents de la même espèce et donc que les processus d'évolution concertée au sein d'une espèce n'auraient pas un rôle important. En d'autres termes, l'apparition d'une mutation chez un individu ne serait pas éliminée par évolution concertée car il n'y a pas de croisement entre cet individu et un autre (Figure 24). Cela pourrait expliquer la présence de plusieurs haplotypes d'ITS chez les truffes.

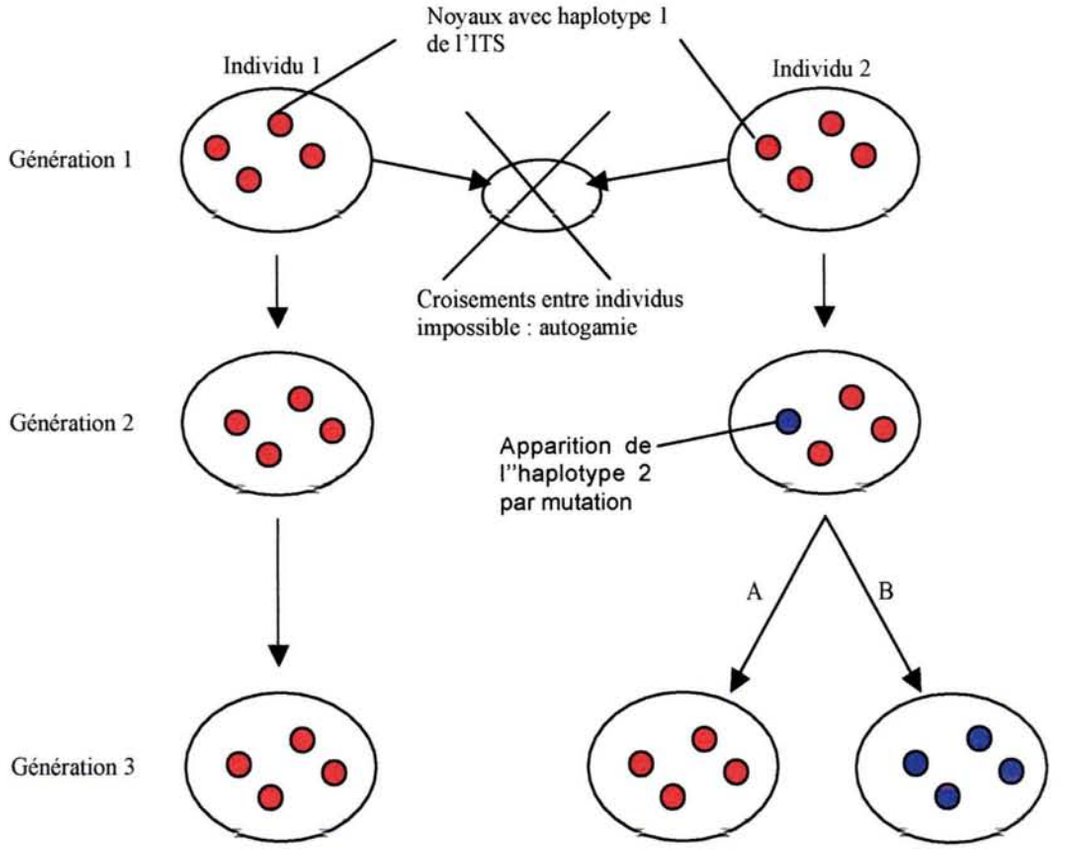

Figure 24. Mécanisme pouvant expliquer l'existence de plusieurs haplotypes de l'ITS chez les truffes. A la génération 1 tous les individus ont le même haplotype ITS dans leurs noyaux (1 en rouge). A la génération 2 apparaît par mutation un autre haplotype (2 en bleu) dans un noyau d'un individu de la lignée 2. Pour cet individu, il existe une homogénéisation des répétitions ITS par évolution concertée, deux voies sont alors possibles: (A) l'haplotype 2 est éliminé ou (B) l'haplotype 2 est conservé. Par contre, étant donné qu'il n'existe pas de croisements entre individus (reproduction homothallique ou autogamie) il n'y a pas d'homogénéisation des haplotypes ITS entre eux, ce qui expliquerait la présence de plusieurs haplotypes ITS au sein des espèces truffes.

3.1.2 Mise en évidence d'une structure génétique chez *T. melanosporum* et *T. magnatum*

L'analyse de la distribution géographique des haplotypes de l'ITS de *T. melanosporum* a mis en évidence une importante différenciation génétique entre populations de l'Est et de l'Ouest de la France (§2.1). En ceci nos résultats divergent des données jusque-là disponibles. En effet, l'analyse RAPD et microsatellite (RAMS et SSR) n'a pas permis d'identifier de structuration génétique pour *T. melanosporum* (Bertault et al., 1998, 2001). D'autre part, contrairement aux études antérieures nous avons étendu notre échantillonnage aux populations septentrionales (Bourgogne, Lorraine). L'existence d'une telle différence entre les populations de l'Est et de l'Ouest de la France indique qu'il existe peu d'échanges génétiques (*Gene flow*) entre ces populations. Tout échange génétique serait empêché par le Massif Central qui jouerait le rôle de barrière géographique, mais aussi écologique car les sols de ces montagnes ne sont pas calcaires.

Étant donné que ces résultats ont été obtenus seulement par l'étude d'un seul locus, sont-ils fiables ?
En effet, chaque partie du génome évolue de façon différente et il est difficile de généraliser les résultats obtenus par l'analyse d'un seul locus. Par exemple, James et al. (2001) montrent que les valeurs de *Fst* (Indice de différenciation génétique) pour *Schizophyllum commune* sont différentes d'un locus à l'autre : de 0,487 pour l'IGS1 à 0,008 pour le locus d'incompatibilité sexuelle A (*mating-type A*). De même, Le Quéré (2004) montre que plusieurs gènes codant des protéines de *Paxillus involutus* soumis à une pression de sélection ont une évolution différente. En effet, le taux de mutation diffère en fonction de la capacité mycorhizogène des différentes souches. En d'autres termes, le taux de mutations de certains gènes régulés lors de la symbiose est supérieur pour une souche ne formant pas de mycorhizes. Ceci s'expliquerait par une baisse de la pression de sélection pour ce locus dans le cas de cette souche fongique. Il serait donc indispensable d'étudier d'autres régions génomiques de *T. melanosporum*, et entre autres des gènes fonctionnels, afin de voir si la structure génétique trouvée avec l'ITS est confirmée.

Cependant, le séquençage de plus de 50 Kpb (SCAR) (Murat, 2001) et de la famille multigénique codant un probable facteur d'incompatibilité (données obtenues en collaboration avec Mirco Iotti et Alessandra Zambonelli) ne présente pas de polymorphisme. D'autre part, j'ai aussi analysé un locus microsatellite sur l'ensemble des 188 ascocarpes (F12I ; Bertault et al., 2001) pour lequel trois haplotypes ont été identifiés. Toutefois, un seul de ces haplotypes a une fréquence supérieure à 5%, ce locus ne peut donc pas être considéré comme polymorphe.

Ces résultats confirment le faible niveau de polymorphisme de T. melanosporum déjà mis en évidence par RAPD, RAMS et les Isoenzymes (cf. § 1.4.3).

Dans le cas de *T. magnatum*, l'analyse de la distribution géographique des haplotypes de l'ITS et de la région SCAR-A21inf suggère aussi une structure génétique (cf. § 2.2). Toutefois, nous ne disposions pas d'un échantillonnage permettant d'analyser la structure macro-géographique de cette espèce.

3.1.3 Phylogéographie des truffes

À partir du Tertiaire (65 millions d'années), la Terre est devenue plus froide, avec des oscillations qui ont augmenté en amplitude jusqu'à une série de glaciations au Quaternaire (2,4 millions d'années) (Hewitt, 2000). Avec l'augmentation des données provenant de carottes de glaces, de sédiments et de pollens fossiles, il est maintenant possible d'avoir une idée sur les effets de ces changements climatiques rapides sur les plantes et les animaux. Des espèces disparaissent, d'autres colonisent de nouveaux habitats et certaines survivent dans des refuges pour ensuite recoloniser le milieu, ceci se produisant à multiples reprises (Hewitt, 2000). Ces expansions ont probablement été produites par des événements de dispersion à longue distance permettant de coloniser les habitats avant d'autres espèces ; cet effet fondateur conduisant à une perte d'allèle et une accumulation d'homozygotes (Hewitt, 1993). En théorie, il existe donc une plus grande richesse en haplotypes au niveau des refuges, alors qu'en s'éloignant de ces régions, le niveau de diversité génétique diminue (Hewitt, 2000 ; Lowe et al., 2000 ; Petit et al., 2003). Toutefois, les caractéristiques propres aux espèces, comme leur mode de migration, et l'existence de refuges plus au nord peuvent altérer ce profil (Petit et al., 2003).

Les événements climatiques ont donc un effet sur la structure des génomes via les phénomènes de recolonisation géographique. C'est pourquoi, l'étude de la structure génétique actuelle d'une espèce peut nous informer sur son histoire et sur ses routes de recolonisation post-glaciaire ; il s'agit de l'analyse phylogéographique.

Quels sont les principaux refuges et les principales voies de recolonisation post-glaciaire en Europe?

Les forêts tempérées étaient limitées aux régions méditerranéennes comme la péninsule Ibérique, l'Italie et les Balkans (Petit et al., 2003). Toutefois, certains arbres auraient trouvé refuge plus au Nord. Par exemple, *Pinus sylvestris* aurait survécu au Sud de l'Irlande (Sinclair et al., 1999). En fait, le Sud de l'Europe a aussi été le refuge de plusieurs animaux comme les hérissons (Santucci et al., 1998 ; Seddon et al., 2001) et les ours (Hewitt, 2000). Hewitt (2000) identifie trois types de comportements différents lors de la glaciation (Figure 25) :

1- Les espèces ayant comme principal refuge les Balkans, (plus rarement l'Italie et l'Espagne). Les migrations à partir de ces trois refuges sont restreintes à cause des barrières géographiques que sont les Alpes et les Pyrénées. L'Aulne, le Hêtre, la Sauterelle et le Triton ont suivi ces voies de recolonisation (Figure 25-1)

2- La colonisation du Nord de l'Europe a eu lieu à partir de trois refuges : l'Espagne, l'Italie et les Balkans ; c'est le cas du Hérisson, du Chêne et du Sapin (Figure 25-2)

3- L'ours a recolonisé l'Europe à partir de l'Espagne et des Balkans ou du Caucase (Figure 25-3).

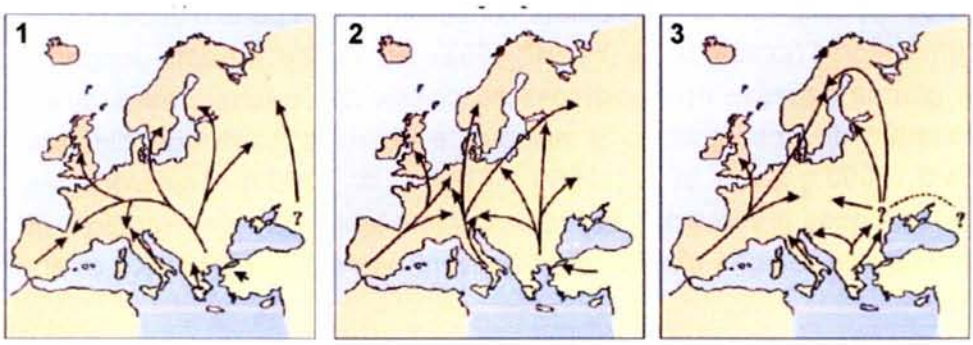

Figure 25. Les trois principaux types de recolonisation post-glaciaire de l'Europe (d'après Hewitt, 2000).

Quand est-il pour <u>T. melanosporum</u> ?

Lors de cette thèse, nous avons identifié une structure génétique pour *T. melanosporum* avec des différences entre les populations de l'Est et de l'Ouest de la France (cf. § 2.1 ; Figure 26).

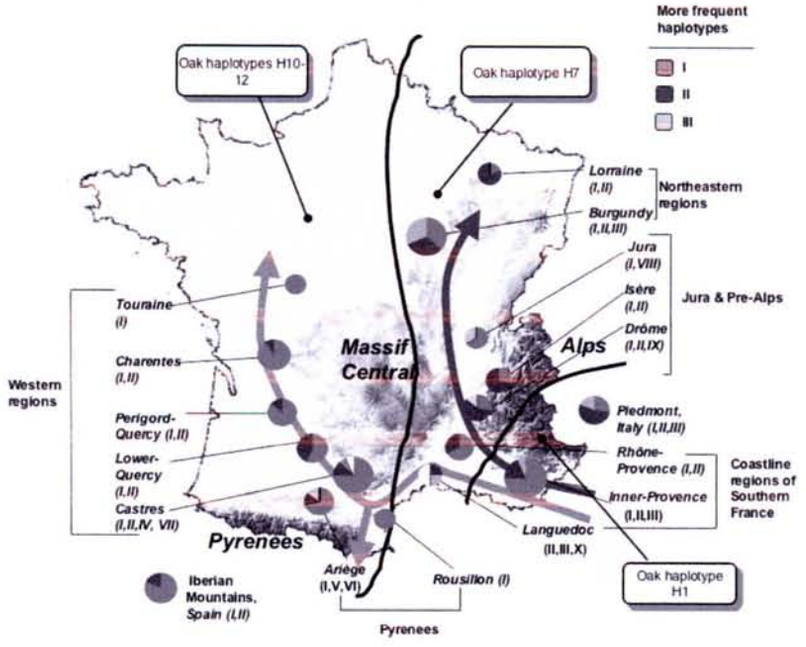

Figure 26. Distribution des 10 haplotypes ITS de T. melanosporum dans les 17 populations analysées (Fig. 2 du paragraphe 2.1).

À partir de ces données, nous avons émis l'hypothèse que les populations de *T. melanosporum* auraient trouvé refuge dans les forêts de l'Espagne ou de l'Italie. Malheureusement, nous ne disposions pas d'un échantillonnage couvrant le sud de l'Italie et de l'Espagne pour savoir exactement quels ont été les refuges de cette truffe durant les glaciations. Alors, pourquoi avons-nous plutôt pensé à l'Italie? Et bien, dans la population piémontaise (Italie) analysée, nous avons retrouvé les trois haplotypes les plus fréquents ; cette observation nous a conduit à considérer l'Italie comme possible refuge. Toutefois, nous n'avons pas de preuves expérimentales pour étayer cette hypothèse. Nous avons aussi suggéré que la truffe aurait recolonisé la France, en suivant les chênes, à partir de cette population italienne et ceci par deux voies principales : la voie de la vallée du Rhône et la voie de l'Atlantique. Mais existe-t-il d'autres scénarii possibles ? Effectivement, nous pouvons imaginer que la Truffe aurait trouvé refuge en Italie mais aussi en Espagne et que les deux lignées françaises résultent de la recolonisation à partir de ces deux refuges. Dans ce cas, il existe plusieurs possibilités :

1- Les populations espagnoles et italiennes sont composées des mêmes haplotypes (I et II). La voie de l'Atlantique (Ouest de la France) correspondrait à la recolonisation par la population espagnole et la voie de la vallée du Rhône à la recolonisation par la population italienne. L'haplotype III proviendrait de la population italienne.

2- L'Haplotype I proviendrait des populations espagnoles et les haplotypes II et III des populations italiennes. Dans ce cas, il faudrait que la population espagnole recolonise l'Italie, or, à ma connaissance, il n'existe pas d'exemple d'une telle voie. Par contre, la colonisation de l'Espagne par l'Italie a été suggérée pour les Chênes (Petit et al. 2000a ; Petit et al. 2000b ; Petit et al., 2003).

Seule une analyse d'un grand nombre d'échantillons de ces zones géographiques permettrait de vérifier notre hypothèse.

Qu'en est-il pour les autres espèces de Tuber ?

Peut-être plus que *T. melanosporum*, *T. magnatum* est une espèce qui a de fortes exigences environnementales (cf § 1.2.2) et son niveau de diversité génétique est aussi très faible (cf. §1.4.3). Il est donc probable que cette truffe ait aussi subi une forte diminution démographique lors d'une récente glaciation suivie par une recolonisation lors du réchauffement climatique. Mais, à la différence de *T. melanosporum*, elle n'aurait pas franchi les Alpes. Sa présence dans les pays de l'Europe de l'Est (Slovénie, Croatie, Hongrie...) peut être expliquée par une recolonisation à partir de l'Italie, ou bien par la persistance d'une population dans les Balkans. Les échantillons Croates étudiés présentent deux haplotypes pour le locus A21inf aussi trouvés en Italie (cf. § 2.2). En revanche, il existe un haplotype 3 de l'ITS leur étant spécifique (cf. § 2.2). Toutefois, ces résultats ne permettent pas de déterminer laquelle des deux hypothèses énoncées précédemment est la bonne. Il faudrait analyser un grand nombre d'échantillons, provenant de tous les pays producteurs, pour savoir si *T. magnatum* a pris refuge en Italie ou bien en Italie et dans les Balkans.

Comme je l'ai dit au paragraphe 1.4.3, il existe des espèces de truffes ayant une plus forte variabilité génétique, c'est le cas de *T. borchii*, *T. uncinatum*, *T. maculatum.* Le fait que ces espèces soient trouvées dans le Nord de l'Europe, montre qu'elles résistent mieux au froid et au gel hivernaux. Il est donc probable que les modifications de température du Quaternaire ont eu moins d'effet sur elles. Nous pouvons aussi imaginer que l'effet « *bottleneck* » aurait été moins important et qu'elles auraient pris refuge non seulement dans le Sud de l'Europe, mais aussi dans plusieurs régions du Nord de l'Europe. Ceci expliquerait leur plus grand niveau de diversité génétique. Toutefois, il n'existe encore aucune étude de la structure génétique de ces espèces sur l'ensemble de leur aire de répartition et donc nous ne pouvons que spéculer sur leur histoire.

La mise en évidence d'un niveau de structuration génétique significatif pour *T. melanosporum* et *T. magnatum* remet en cause la théorie expliquant les différences phénotypiques (qualités organoleptiques) par les conditions pédoclimatiques (effet « terroir ») en excluant d'éventuelles variations génétiques (« inné ») (Bertault et al., 1998). L'existence d'un polymorphisme de l'ITS permettra le développement d'outils de diagnostics moléculaires aptes à identifier l'origine géographique des truffes et, ainsi le développement de normes, telles que l'Appellation d'Origine Géographique Contrôlée (AOC).

3.2 Analyse d'une truffière de *T. magnatum*

T. magnatum est la truffe la plus chère au monde, mais contrairement à *T. melanosporum* sa culture n'est pas encore maîtrisée (cf. § 1.2.3.2). Il est donc fondamental de mieux connaître l'écologie des truffières afin d'en améliorer la gestion et éventuellement espérer maîtriser sa culture. C'est pourquoi, nous avons décidé d'étudier la communauté ectomycorhizienne d'une truffière de *T. magnatum*. En effet, la compétition entre espèces mycorhiziennes est un facteur majeur des processus de mycorhization contrôlée. Par exemple, de nombreuses truffières artificielles de *T. melanosporum* sont contaminées par d'autres espèces comme *T. brumale* (cf. §1.2.3.1). De même, *T. borchii* et *T. maculatum* sont souvent récoltés dans les expérimentations de plants mycorhizés par *T. magnatum* (cf. §1.2.3.2). Pour cela, des ascocarpes de *T. magnatum* ont été récoltés durant cinq saisons dans une truffière naturelle (Montemagno, Asti). Nous avons aussi échantillonné des apex racinaires en Novembre 2001 et Mai 2002 dans des zones productives et non productives de la truffière.

3.2.1 La diversité génétique au sein de la truffière

Au début de cette étude nous ignorions la diversité génétique au sein de la population de *T. magnatum* occupant cette truffière: (i) Existe-t-il un ou plusieurs génotypes et quelle est leur distribution dans la truffière ?; (ii) Le mycélium de ces génotypes se déplace-t-il dans la truffière ?

Lors de l'analyse d'une truffière artificielle de *T. melanosporum*, Bertault et al. (2001) ont identifié jusqu'à six génets autour d'un arbre. Même si la truffière a été étudiée durant deux années successives, aucune information sur l'évolution des génets lors de ces deux saisons n'a été fournie. Ces auteurs ont utilisé une approche multi locus (RAPD), mais aussi simple locus avec l'analyse de loci microsatellites. Ces deux types de marqueurs

se sont montrés polymorphes au sein de la population de la truffière. Le séquençage direct d'une région génomique de *T. magnatum* (SCR-A21inf ; cf. §2.2) a permis d'identifier deux génotypes. Ceux-ci sont toujours trouvés dans la même zone de la truffière, ce qui indique une certaine stabilité. Toutefois, nous ne savons pas combien d' « individus » de *T. magnatum* il existe, puisque nous n'avons qu'un seul locus polymorphe. Afin de répondre à cette question, il aurait fallu utiliser une technique multilocus comme la RAPD ou l'AFLP. Nous avons décidé de ne pas mener ce type d'analyse pour deux raisons :

1- Nous ne disposions que d'ADN stocké depuis plusieurs années, or il semble que ce type d'ADN ne soit pas utilisable pour ces techniques car il est dégradé et produit des bandes polymorphes artéfactuelles.
2- Nous voulions mettre au point des marqueurs co-dominants et spécifiques pouvant être utilisés sur l'ADN extrait de mycorhizes et de l'ADN du sol.

Nous avons donc mis au point une analyse permettant de suivre l'évolution d'une population à l'échelle micro-géographique. Dans le futur, nous souhaiterions utiliser ce marqueur polymorphe (SCAR-A21inf) sur l'ADN extrait de mycorhizes, mais aussi et surtout sur des extraits d'ADN de sol.

3.2.2 Que se passe-t-il dans le sous-sol de la truffière ?

Identification de mycorhizes de T. magnatum

Les deux seules publications traitant de l'identification moléculaire de mycorhizes de *T. magnatum* sont celles de Mello et al. (2001) et Rubini et al. (2001). Toutefois, ces mycorhizes sont retrouvées uniquement sur les plants élevés dans des conditions contrôlées (pépinière). Lorsque les plants sont transférés dans la nature, les mycorhizes de cette truffe ne sont plus détectées. Ces auteurs expliquent cette absence par une faible compétitivité de *T. magnatum* par rapport aux autres espèces mycorhiziennes. En fait, notre connaissance sur la période pendant laquelle *T. magnatum* produit des mycorhizes, sur leur répartition, sur leur fréquence et sur leur dynamique temporelle est très limitée.

Nous avons identifié deux morphotypes correspondant à *T. magnatum* lors de l'échantillonnage de Mai 2002 une période pendant laquelle le mycélium ne produit pas de fructifications (période non productive). Par contre, nous n'en avons pas détecté pendant une période productive (Novembre 2001). En fait, l'autonomie nutritionnelle de l'ascocarpe a déjà été proposée pour *T. melanosporum* (cf. §1.3.3.2 ; Callot, 1999). Toutefois, la présence de

mycorhizes en Mai est intéressante puisqu'il s'agit de la période pendant laquelle se forment les fructifications. Elles pourraient être fondamentales pour la formation du corps fructifère, en leur donnant par exemple l'énergie nécessaire. Malheureusement, nous ne disposons pas de systèmes expérimentaux permettant d'étudier la formation des ascocarpes et de voir quels sont les facteurs conduisant à leur apparition.

Biodiversité ectomycorhizienne au sein de l'écosystème truffier

Les espèces ectomycorhiziennes majoritaires dans le sous-sol de la truffière de Montemagno appartiennent aux *Telephoraceae*, *Pezizales* et *Sebacina*. À ma connaissance, il n'existe pas dans la littérature d'études de la biodiversité ectomycorhizienne d'un écosystème truffier similaire. Si Rossi et al. (2002) identifient des morphotypes *Tuber-like* dans une truffière de *T. magnatum* du centre Italie, ils n'ont pas confirmé l'appartenance de ceux-ci par séquençage de l'ITS. Dans une analyse morphologique d'apex mycorhiziens prélevés dans plusieurs truffières de *T. melanosporum* espagnoles, De Miguel et al. (2002) identifient plusieurs espèces de *Tuber* et de *Tomentella*.

Il semble donc que les Telephoraceae et les Pezizales soient parmi les taxa dominants de l'écosystème truffier.

En fait, plusieurs études montrent que les *Russulaceae* et les *Telephoraceae* sont parmi les taxa ectomycorhiziens les plus répandus dans les communautés de conifères européens et Nord américains (Taylor et Bruns, 1998 ; Jonsson et al., 1999 ; Horton et Bruns, 2001). Les résultats obtenus lors de ce travail de thèse indiquent que les *Telephoraceae* sont aussi abondants dans les communautés du sud de l'Europe composées de peupliers et de chênes. Parmi ceux-ci, les *Tomentella* sont les espèces les plus abondamment identifiées. Elles forment des fructifications souvent trouvées au niveau de débris végétaux et de la litière, ceci avait fait penser que ces champignons étaient saprotrophes (Larsen, 1971). Leurs fructifications sont cachées et tendent donc à être ignorées lors d'études basées sur la récolte des carpophores, ceci contribue à augmenter les discordances entre le prélèvement des fructifications et des mycorhizes. Pour le moment, nous ne savons pas pourquoi ces champignons sont aussi abondants dans les communautés ectomycorhiziennes, mais il est possible qu'ils soient de bons compétiteurs et qu'ils jouent un rôle clef dans le fonctionnement des écosystèmes (Horton et Bruns, 2001).

En ce qui concerne les *Sebacina*, cette famille était encore négligée comme espèce ectomycorhizienne jusqu'à récemment (Agerer 1987-1998 ; Smith et Read, 1997). Actuellement, les *Sebacina* sont reconnues comme formant des symbioses ectomycorhiziennes avec les arbres et les

orchidées (Urban et al., 2003 ; Selosse et al., 2002). Lors de l'étude de la communauté ectomycorhizienne associé à *Eucalyptus marginata* en Australie, Glen et al. (2002) montrent que les *Sebacina* sont abondants dans ce type d'écosystème.

Ces résultats indiquent qu'il existe des similitudes entre les communautés ectomycorhiziennes d'écosystèmes différents (Europe, Amérique et Australie) et que les espèces dominantes appartiennent souvent aux mêmes taxa (par exemple Telephoraceae et Sebacina).

Comme je l'ai déjà évoqué dans le paragraphe 1.3.1, nous ne connaissons pas la distribution du mycélium de truffes dans le sous-sol des truffières. Étant donné que les truffes ne forment pas de rhizomorphes et que leur culture *in vitro* n'est pas aisée, la seule technique pouvant être utilisée pour connaître la distribution du mycélium truffier est l'analyse d'extrait d'ADN provenant de sols. Cette technique a été utilisée avec succès pour étudier la biodiversité ectomycorhizienne dans des extraits de sols (Landeweert et al., 2003), mais aussi la répartition d'un champignon ectomycorhizien, *Hebeloma cylindrosporum* (Guidot et al., 2001, 2002, 2004 ; cf. § 1.4.1.2). C'est pourquoi, lors de ce travail de thèse, j'ai commencé l'analyse du sol de la truffière de Montemagno afin d'essayer d'avoir une image de la répartition de *T. magnatum* dans le sous-sol. Pour le moment, je suis parvenu à obtenir des extraits d'ADN amplifiable avec les amorces universelles ITS1f-ITS4. En revanche, il reste à améliorer les conditions d'amplification de ces extraits d'ADN avec des amorces spécifiques de *T. magnatum.*

En conclusion, les truffières sont des écosystèmes complexes pour lesquels nous avons encore peu de données. Il apparaît fondamental de mieux les connaître afin d'améliorer la culture de ces produits naturels dont la production a fortement décliné.

3.3 Limites rencontrées par l'analyse de *T. melanosporum* et *T. magnatum* et conséquence pour la filière truffe

Comme je l'ai évoqué tout au long de ce manuscrit, *T. melanosporum* et *T. magnatum* sont des espèces économiquement importantes. Ceci rend leur analyse intéressante, mais est aussi à l'origine de nombreux problèmes :

1- Leur coût entraîne pour l'analyse d'un ample échantillonnage un investissement financier important.

2- D'autre part, il est souvent difficile lors d'une analyse de diversité génétique macro-géographique d'avoir des informations précises sur le lieu de récolte, pourtant ces données sont nécessaires pour ce type d'étude.

3- Lors des prélèvements d'apex racinaires et de sol dans les truffières, il est impossible de réaliser un ample échantillonnage et les prélèvements en période productive sont souvent difficiles à obtenir.

Ces problèmes conduisent à penser que même si ces deux espèces sont les plus intéressantes d'un point de vue économique et social (cf. §1.2.1), ce ne sont sûrement pas les meilleurs candidats pour les études de diversité génétique et de génétique des populations. En effet, lors de telles analyses macro-géographiques nous avons besoin d'un grand nombre d'échantillons pour lesquels nous devons connaître avec précision les sites de récolte ainsi que les caractéristiques écologiques dans lesquelles elles sont récoltées (hôte, altitude...etc.). Dans le cas de *T. melanosporum*, 80 % de la production actuelle française provient de truffières artificielles (cf. §1.2.3.1). Il faut donc faire attention et ne pas se limiter qu'aux échantillons dont l'origine est connue. Il en est de même pour l'analyse des truffières. Comme nous l'avons constaté, notre travail est limité par le nombre d'échantillons. Il serait donc plus simple d'échantillonner une truffière naturelle d'une espèce moins prestigieuse mais tout aussi intéressante du point de vue biologique

Malgré ces limites, nous sommes parvenus à obtenir des résultats intéressants pour la filière truffe. Tout d'abord, la mise en évidence d'une structure génétique non négligeable pour *T. melanosporum* et *T. magnatum* est un premier pas vers la réalisation d'Appellations d'Origines Contrôlées (AOC). D'autre part, nous montrons que le choix des inoculums pour la réalisation de plants truffiers ne peut être fait au hasard. En d'autres termes, il est souhaitable de prendre comme inoculum des souches provenant de la zone dans laquelle la plantation sera réalisée. Ceci pour deux principales raisons : (1) ne pas provoquer de flux géniques entre populations, ce qui conduirait à une homogénéisation de celles-ci et rendrait impossible la réalisation de marqueurs régionaux spécifiques et (2) ces souches étant indigènes, elles sont probablement mieux adaptées au microclimat existant.

Références de la discussion générale

Agerer R (ed.) (1987-98) Colour Atlas of Ectomycorrhiza. Einhorn Verlag, Scwäbissch Gmünd.

Bertault G., Raymond M., Berthomieu A., Callot G., Fernandez D., 1998. Trifling variation in truffles. Nature 394, 734.

Bertault G., Rousset F., Fernandez D., Berthomieu A. Hochberg, M. E., Callot G., Raymond M., 2001. Population genetics and dynamics of the black truffle in a man-made truffle field. Heredity 86, 451-458.

Brown D.D., Wensink P.C., Jordan E., 1972. A comparison of the ribosomal DNA's of *Xenopus laevis* and *Xenopus mulleri*: the evolution of tandem genes. Journal Molecular Biology, 63(1), 57-73

Callot G. 1999. La Truffe, la Terre, la Vie. . INRA, Paris, 210 p.

De Miguel A., De Roman M., Etayo M.L., 2002. Mycorrhizal fungi competing with *Tuber melanosporum* Vitt. In cultivated truffle beds in north-eastern Spain. Edible mycorrhizal mushrooms and their cultivation. Proceedings of the Second International Conference on Edible Mycorrhizal Mushrooms, Ed. Hall, I.; Yun Wang; Danell, E.; Zambonelli, A., Christchurch, New Zealand, 3-6 July, 2001

Douet J. P., Castroviejo M., Mabru D., Chevalier G., Dupre C., Bergougnoux F., Ricard J. M., Medina B., 2004. Rapid molecular typing of *Tuber melanosporum*, *T. brumale* and *T. indicum* from tree seedlings and canned truffles. Analytical and Bioanalytical Chemistry 379 (4), 668-673

Dover G., 1982. Molecular drive: a cohesive mode of species evolution. Nature, 299, 111-117

Elder J.F., Turner B.J., 1995. Concerted evolution of repetitive DNA sequences in eukaryotes. Quart. Rev. Biol., 70, 297-320

Frizzi G., Lalli G., Miranda M., Pacioni G., 2001. Intraspecific isoenzyme variability in Italian population of the white truffle *Tuber magnatum*. *Mycol Res* 105 (3), 365-369.

Gandeboeuf D., Dupré C., Roeckel-Drevet P., Nicolas P., Chevalier G., 1997. Typing *Tuber* ectomycorrhizae by polymerase chain amplification of the internal transcribed spacer of rDNA and the sequence characterized amplified region markers. Canadian Journal of Microbiology 43, 723-728

Glen M, Tommerup IC, Bougher NL, O'Brien PA 2002. Are Sebacinaceae common and widespread ectomycorrhizal associates of Eucalyptus species in Australian forests? Mycorrhiza 12 (5): 243-247.

Glen M., Tommerup I.C., Bougher N.L., O'Brien P.A., 2001. Interspecific and intraspecific variation of ectomycorrhizal fungi associated with Eucalyptus ecosystems as revealed by ribosomal DNA PCR-RFLP. Mycological research, 105, 843-858

Guidot A., Debaud J.C., Effosse A., Marmeisse R., 2004. Below-ground distribution and persistence of an ectomycorrhizal fungus. New Phytologist, 161, 539-547

Guidot A., Debaud J.C., Marmeisse R., 2001. Correspondence between genet diversity and spatial distribution of above- and below-ground populations of the ectomycorrhizal fungus *Hebeloma cylindrosporum*. Molecular Ecology, 10, 1121-1131

Guidot A., Debaud J.C., Marmeisse R., 2002. Spatial distribution of the below-ground mycelia of an ectomycorrhizal fungus inferred from specific quantification of its DNA in soil samples. FEMS Microbiology Ecology, 42, 477-486

Hewitt, G. M. 1993. Hybrid zones and Evolutionary Process (ed. Harrison, R. G.) 140–164 (Oxford Univ. Press, Oxford).Hewitt G. 2000. The genetic legacy of the Quaternary ice ages. Nature 405, 907-913.

Horton T.R., Bruns T.D., 2001. The molecular revolution in ectomycorrhizal ecology: peeking into the black-box. Molecular Ecology, 10, 1855-1871

James T.Y., Moncalvo J.M., Li S., Vilgalys R., 2001. Polymorphism at the DNA spacers and its relation to breeding structure of the widespread mushroom *Schizophyllum commune*. Genetics 157: 149-161

Jonsson T, Kokalj S, Finley R, Erland S 1999. Ectomycorrhizal community structure in a limed spruce forest. Mycol. Res. 103 (4): 501-508.

Landeweert R., Leeflang P., Kuyper T.W., Hoffland E., Rosling A., Wernars K., Smit E., 2003. Molecular identification of ectomycorrhizal mycelium in soil horizons. Appl. Environ. Microbiol. 69, 327-333

Lanfranco L., Delpero M., Bonfante P., 1999. Intrasporal variability of ribosomal sequences in the endomycorrhizal fungus Gigaspora margarita. Molecular Ecology, 8 (1), 37-45

Lanfranco L., Wyss P., Marzachi C., Bonfante P. 1993. DNA probes for identification of the ectomycorrhizal fungus *Tuber magnatum* Pico. FEMS Microbiol. Lett., 114, 245-25

Larsen MJ (1971) The genus Pseudotomentella (Basidiomycetes, Thelephoraceae s.str.) Nova Hedwigia, 22: 599-619.

Le Quéré A., 2004. Genome and Transcriptome Analyzes in the Ectomycorrhizal Fungus Paxillus involutus. PhD thesis, University of Lund, Sweden.

Lowe A.J., Gillies A.C.M., Wilson J., Dawson I.K., 2000. Conservation genetics of bush mango from central/west Africa: implications from random amplified polymorphic DNA analysis. Molecular Ecology 9 (7), 831-841

Mabru D., Dupré C., Douet J. P., Leroy P., Ravel C., Ricard J. M., Medina B., Castroviejo M., Chevalier G., 2001. Rapid molecular typing method for the reliable detection of Asiatic black truffle (*Tuber indicum*) in commercialized products : fruiting bodies and mycorrhizal seedlings. *Mycorrhiza* 11 : 89-94.

Mello, A., Fontana, A., Meotto, F., Comandini, O., Bonfante P., 2001. Molecular and morphological characterization of *T. magnatum* mycorrhizas in a long-term survey. Microbiol Res 155: 279-284.

Murat C., 2001. Etude de la diversité génétique de la Truffe noire du Périgord (*Tuber melanosporum* Vittad.). Diplôme d'Etude Approfondie, Université de Nancy.

Paolocci F., Rubini A., Riccioni C., Topini F., Arcioni S., 2004. *Tuber aestivum* and *Tuber uncinatum*: two morphotypes or two species? FEMS Microbiol. Lett. 235 (1), 109-115

Parkin et Butlin, 2004. Whithin- and between-individual sequence variation among ITS1 copies in the meadow grasshopper Chorthippus parallelus indicates frequent intrachromosomal gene conversion. Molecular biology and evolution, 21 (8), 1595-1601

Pawlowska T.E, Taylor J.W, 2004. Organization of genetic variaton in individuals of arbuscular mycorrhizal fungi. Nature, 427, 733-737

Petit R.J., Latouche-Hallé C., Pemonge M.H., Kremer A., 2002a. Chloroplast DNA variation of oaks in France and the influence of forest fragmentation on genetic diversity. Forest Ecology and Management 156: 115-129.

Petit RJ, Brewer S Bordacs *et al.*, 2002b. Identification of refugia and post-glacial colonisation routes of European white oaks based on chloroplast DNA and fossil pollen evidence. Forest Ecology and Management 156: 49-74.Mabru D., Douet J.P., Mouton A., Dupre C., Ricard J.M., Medina B., Castroviejo M., Chevalier G., 2004. PCR-RFLP using a SNP on the mitochondrial Lsu-rDNA as an easy method to differentiate *Tuber melanosporum* (Perigord truffle) and other truffle species in cans.International Journal of Food Microbiology, 94 (1), 33-42

Petit RJ, Aguinagalde I, De Beaulieu JL, et al., 2003. Glacial refugia: hotspots but not melting pots of genetic diversity. *Science* 300: 1563-1565.

Rossi I., Bertini L ;, Amicucci A., Cecchini M., Gregori L., Sacchi A., Zambonelli A., Stocchi V., 2002. Study of a natural *Tuber magnatum* Pico truffière in central Italy. Edible mycorrhizal mushrooms and their cultivation. Proceedings of the Second International Conference on Edible Mycorrhizal Mushrooms, Ed. Hall, I.; Yun Wang; Danell, E.; Zambonelli, A., Christchurch, New Zealand, 3-6 July, 2001

Roux, C., Séjalon-Delmas N., Martins M., Parguey-Leduc A., Dargent R., Bécard G., 1999. Phylogenetic relationships between european and chinese truffles based on parsimony and distance analysis of ITS sequences. FEMS Microbiology Letters 180 : 147-155.

Rubini A., Topini F., Riccioni C., PaolocciF., Arcioni, S., 2004. Isolation and characterization of polymorphic microsatellite loci in white truffle (*Tuber magnatum*). Mol Ecol Notes 4, 116-118.

Santucci F., Emerson B.C., Hewitt G.M., 1998. Mitochondrial DNA phylogeography of European Hedgehogs. Molecular Ecology, 7, 1163-1172.

Seddon J. M., Santucci F., Reeve N. J., Hewitt G. M., 2001. DNA footprints of European hedgehogs, *Erinaceus europaeus* and *E. concolor*: Pleistocene refugia, postglacial expansion and colonization routes. Molecular Ecology 10 (9), 2187-2198

Selosse M-A., Bauer R., Moyersoen B. (2002) Basal hymenomycetes belonging to the Sebacinaceae are ectomycorrhizal on temperate deciduous trees. New Phytologist 155: 183-195.

Sinclair W. T., Morman J. D., Ennos., R. A., 1999. The postglacial history of Scots pine (*Pinus sylvestris* L.) in western Europe: evidence from mitochondrial DNA variation. Molecular Ecology, 8 (1), 83-88

Smith SE, Read DJ (1997) Mycorrhizal Symbiosis. Academic Press, New York.

Taylor DL and Bruns TD 1999. Community structure of ectomycorrhizal fungi in a *Pinus muricata* forest: minimal overlap between the mature forest and resistant propagule communities. Mol. Ecol. 8: 1837-1850.

Urban A., Weiß M., Bauer R. (2003) Ectomycorrhizas involving sebacinoid mycobionts. Mycol Res 107 (1): 3-14.

Vollmer S., Palumbi S., 2004. Testing the utility of internally transcribed spacer sequences in coral phylogenetics. Molecular Ecology, 13, 2763-27

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Résumé

Les truffes sont des champignons ectomycorhiziens du genre *Tuber*. À cause de leurs qualités organoleptiques, certaines espèces de truffes ont une forte valeur économique, c'est le cas de *Tuber melanosporum* et *T. magnatum*. Malgré la multiplication des études, il persiste de nombreuses inconnues sur la biologie (par exemple: phylogéographie et l'écologie) de ces espèces.

L'analyse de la diversité génétique de *T. melanosporum* sur l'ensemble de son aire de répartition en France a mis en évidence un niveau de différenciation génétique non négligeable entre les populations de l'Est et de l'Ouest de la France. Une analyse phylogéographique a permis de suggérer deux voies de recolonisation post-glaciaire : la voie de la vallée du Rhône et la voie de l'Atlantique.

L'analyse des ascocarpes de *T. magnatum* récoltés dans une truffière naturelle a montré l'existence d'au moins deux génotypes dans cette population. D'autre part, la récolte d'apex mycorhiziens indique que les ectomycorhizes de *T. magnatum* sont très rares (deux morphotypes sur 39). Les espèces ectomycorhiziennes majoritaires appartiennent aux *Telephoraceae*, *Pezizales* et *Sebacina*.

Mots clefs : *T. melanosporum*, *T. magnatum*, populations, phylogéographie, diversité

Abstract

Truffles are ectomycorrhizal fungi belonging to *Tuber*. Fructifications of some species, such as *T. melanosporum* and *T. magnatum,* are appreciated by consumers and have important economic value. Despite of numerous studies, some aspects of truffle biology (i.e. phylogeography and ecology) are unknown.

Wide range analysis of genetic diversity in *T. melanosporum* showed important genetic differentiation between East and West French populations. Phylogeographic analysis allowed us to hypothesis that *T. melanosporum* recolonized France by two main routes : Rhône Valley route and Atlantic route.

T. magnatum ascocarps analysis in a natural truffle ground identified at least two genotypes in this population. In order to characterize *T. magnatum* mycorrhizosphere, root tips have been harvested. We found that *T. magnatum* ectomycorrhizas are very rare (two out of 39 morphotypes). Moreover, more abundant species belong to *Telephoraceae*, *Pezizales* and *Sebacina*.

Keywords: *T. melanosporum*, *T. magnatum*, populations, phylogeography, diversity