

Sex, croziers, truffles and variation

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Abstract: We have gone back through the literature on truffles and sought out what is known of their lifecycles. This primarily morphological information was then interpreted in the light of molecular research carried out over the past decade.

Most of the *Ascomycota* are primarily haploid but a dikaryotic state is formed prior to a short sexual phase. This is followed by the formation of the crozier apparatus, nuclear fusion, pairing of the chromosomes and meiosis leading to recombinant variation. The apparent absence of croziers in truffle ascoma and fewer than eight ascospores in asci has led to the belief by some that truffles may have aberrant life cycles. However, the evidence is that *Tuber* ascospores produce a haploid thallus, these form haploid mycorrhizas, the haploid hyphae produce antheridia and ascogonia just prior to ascoma production, and then crozier formation inside the truffles leads to meiosis, recombinant variation and the production of haploid spores.

There is now sufficient information that variation in truffles is substantial. We postulate that this variability may be responsible for poor production in truffières containing plants inoculated with truffles ill adapted to climatic and edaphic conditions at outplant sites.

Zusammenfassung: Wir durchsuchten die Trüffelliteratur, um herauszufinden, was über deren Lebenszyklen bekannt ist. Diese in erster Linie morphologische Information wurde dann unter Berücksichtigung der molekularen Forschung der letzten Dekade interpretiert.

Die meisten *Ascomycota* sind hauptsächlich haploid, aber ein dikaryotisches Stadium wird vor einer kurzen sexuellen Phase gebildet. Diesem folgt Hakenbildung, Kernverschmelzung, Chromosomenpaarung und Meiose mit Rekombination. Das offensichtliche Fehlen von Haken in Trüffelfruchtkörpern und weniger als acht Ascosporen in den Asci führten bisweilen zu dem Glauben, dass Trüffeln aberrante Lebenszyklen haben könnten. Jedoch ist es eine Tatsache, dass aus *Tuber*-Ascosporen haploide Thalli und haploide Mykorrhizen entstehen und sich an den haploiden Hyphen Antheridien und Ascogonien unmittelbar vor der Fruchtkörperbildung entwickeln. Dann führt Hakenbildung innen in den Trüffeln zu Meiose mit Rekombination und zur Entstehung von haploiden Sporen.

Es gibt jetzt genügend Information, dass die Variabilität in Trüffeln beträchtlich ist. Wir postulieren, dass diese Variabilität für mageren Ertrag in jenen Trüffelpflanzen verantwortlich sein könnte, die Pflanzen enthalten, die mit nicht entsprechend an die klimatischen und edaphischen Bedingungen des Kulturstandortes angepassten Trüffelvarianten inokuliert wurden.

The Burgundy or Summer truffle, *Tuber aestivum*, has the widest distribution of any of the edible truffles (Fig. 1). However, it would not be surprising if the prize for the most mobile truffle went to *Tuber maculatum*, which although native to Europe, is also widely distributed in New Zealand from Northland down to Invercargill at the southernmost tip of the South Island (BULMAN & al. 2009). These species also form mycorrhizas with a wider range of host plants than other species of truffle (Appendix 3 in HALL & al. 2007). For *Tuber aestivum* and *Tuber maculatum* to have adapted to so wide a range of biotic, climatic and edaphic environments suggests they should have considerable genetic variability.

A decade ago we were told that molecular techniques had demonstrated there was little variation in *Tuber melanosporum* (Périgord black truffle) (BERTAULT & al. 1998, 2001). However, over the following years FRIZZI & al. (2001) and MELLO & al. (2002, 2005) showed that there is genetic variability in both *Tuber aestivum* and *T. magnatum* and that in *T. melanosporum* there is polymorphism in the ribosomal DNA ITS region (MURAT & al. 2004). Since then BONUSO (2008) has shown there are two cryptic species within *Tuber borchii*, NAPOLI & al. (2010) have found nine new haplotypes in *T. melanosporum*, and recently recombinant variation has been detected in *T. melanosporum* (MURAT & MARTIN 2008, RICCONI & al. 2008).

What we have done here is to step back and take a fresh look at morphological information and other evidence for how variation in truffles is maintained. We also review some of the older research that is becoming available on-line as publishing houses scan older issues of their journals, information that has sometimes been missed by younger members of the scientific community - and some of their supervisors!

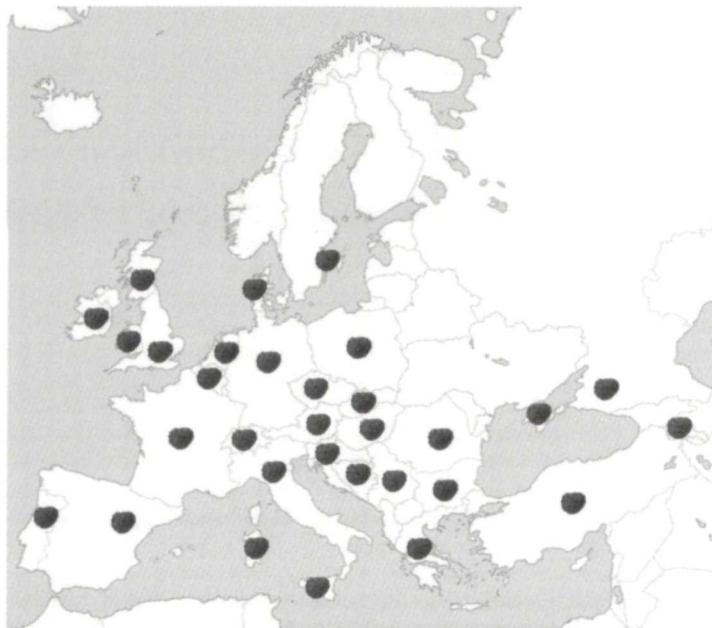


Fig. 1. The distribution of the Burgundy truffle (*Tuber aestivum*) in Europe. From HALL & al. (2007) with the addition of Austria, Greece, Switzerland, Ukraine (Crimea – Nikita Botanic Garden, Yalta, Cherkasy oblast, Zakarpats'ka oblast) and Russia (Adygea, Voronezh oblast, Krasnodarskyi krai) (from D. MINTER, pers. comm., A. URBAN, pers. comm. and www.swissfungi.ch).



Fig. 2. “Revealed! Those secret truffle croziers. Wow!” – the heading of the caption to the above photographs published in the April 1985 British Mycological Society foray programme (ANONYMOUS 1985). The text below the caption was: “Not all of the truffles collected on last year’s truffle hunt went into the pot (burp! Pardon!). A few of each species were put into the herbarium at the CMI, and these excellent photomicrographs were taken of fresh croziers, asci and ascospores of *Tuber aestivum*. They show many of the hymenial features typical of members of the *Tuberaceae* (*Pezizales*): note the prominent croziers (arrows) and the globose asci (typical of fungi with passive ascospore discharge) each bearing a different number of ascospores. Note also that the ascospores are of different sizes, roughly according to how many are in the ascus, and that although they are smooth in their early stages of development, they eventually become beautifully ornamented.”

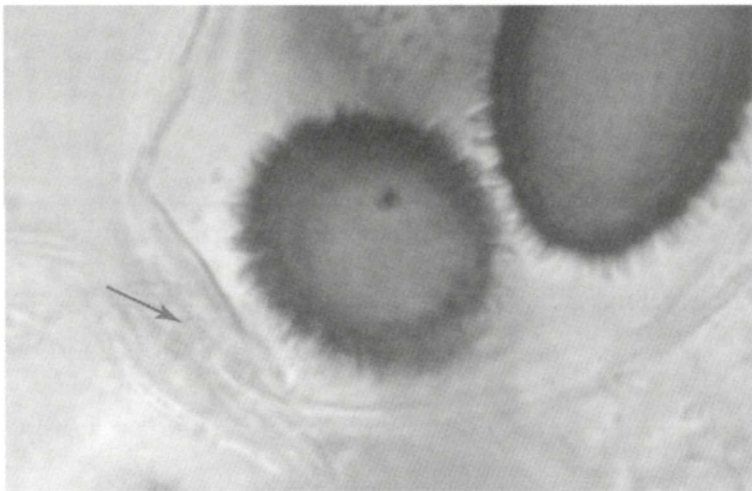


Fig. 3. Two *Tuber melanosporum* asci with the crushed remains of a crozier (arrow) sandwiched between them. – Phot. I. R. HALL.



Fig. 4. A *Tuber aestivum* ascus with the remains of the crozier attached. – Phot. A. ZAMBONELLI.

Croziers and the life cycle of truffles

The truffle genus, *Tuber*, is placed in the *Pezizales*, in the *Ascomycota* where the vast majority of fungi have a haploid thallus. However, prior to fruiting body formation the fusion of an antheridium and an ascogonium forms ascogenous cells, the dikaryotic phase where each cell contains two nuclei derived from each of the partners. In some species ascogenous hyphae form which can branch profusely (LIU & HALL 2004, PÖGGELER & al. 2006). To ensure outcrossing barriers to self fertilization, incompatibility, strains and the like have evolved (KENDRICK 2002).

After the establishment of the dikaryotic phase, croziers (like a shepherd's crook and the hooked staff carried by bishops of Christian churches) form with a terminal cell that contains two haploid nuclei. Eventually the two nuclei merge, the chromosomes pair up and, in heterokaryotic species, there is the opportunity for recombinant variation. Finally, usually eight ascospores are produced, generally each containing a single haploid nucleus with a different combination of genes from those in the antheridium and ascogonium. Illustrations of what happens during crozier formation abound in the literature but a good example can be found on slides 8 and 9 in a downloadable PowerPoint presentation by WONG (2007). So in the classical ascomycete while the ascospores (= meiospores) form from dikaryotic hyphae after karyogamy and meiosis, the tissues of the fruiting body are composed of haploid, non-dikaryotic hyphae (PÖGGELER & al. 2006).

In 1954 Professor LILIAN HAWKER made drawings of croziers of *Tuber aestivum* (Burgundy truffle), *T. brumale* (winter truffle), *T. macrosporum*, *T. rapaeodorum* and *T. rufum* (HAWKER 1954). However, it was not until 1985 that photographs of truffle croziers were published (ANONYMOUS 1985). This was in an ephemeral UK publication not easy to obtain. The illustrations are therefore presented here with the permis-

sion of the British Mycological Society (Fig. 2). Some more photographs of croziers in truffles can be downloaded from Mycotaxon (HANLIN & al. 1989). The remains of croziers can also be seen on photographs of *Tuber brumale*, *T. bellonae*, *T. nitidum*, *T. panniferum*, *T. requienii* and *T. rufum* in the book "Truffes d'Europe et de Chine" (RIOUSSET & al. 2001).

IAN R. HALL first began seeing truffle croziers when he was required to confirm the identity of truffles purported to be *Tuber melanosporum* (Périgord black truffle) that had been imported into New Zealand for the production of truffle infected plants. Many of the truffles were very immature and croziers were not uncommon. However, in less immature truffles the croziers were generally crushed (Fig. 3). Recently ALESSANDRA ZAMBONELLI also went in search of croziers in *Tuber aestivum* truffles and found them to be not uncommon (Fig. 4).

CERUTI & al. (1964) using an electron microscope showed "anteridio" and "oogonio" in *Tuber*. CALLOT (2001) also saw these in the early stages of truffle formation and in chapters 4 and 5 of his book he showed photographs and drawings of ascogone, filaments recouvrants and trichogynes of *Anthracobia nitida* (Pyronemataceae, Pezizales). He contrasted them with similar structures in *T. melanosporum* and hence suggested that the sexual phase in the life cycle occurs at the very start of truffle formation. PAOLOCCI & al. (2006) using polymorphic microsatellites to compare the allelic configurations of asci with hyphae within a *Tuber magnatum* truffle showed that *T. magnatum* outcrosses and that its life cycle is predominantly haploid including the mycorrhiza.

In contrast to the above, ROTH-BEJERANO & al. (2004) found that *Kalaharituber (Terfezia) pfeilii* hyphae growing from sterile glebal hyphae were multinucleate but with the nuclei in pairs. Hyphae originating from two anastomosing hyphae each from a single ascospore produced multinucleate cells which also had paired nuclei. This was discussed by KAGAN-ZUR & ROTH-BEJERANO (2008 a, b) and put forward the evidence for the occurrence of heterokaryons both in the gleba and mantle.

In a review of *Tuber* genetics, POMA & al. (2006), on the basis that there are often fewer than eight spores in some *Tuber* asci, suggested, "*Tuber* reproduction system might be different from that usually accepted for a dikaryotic mycelium produced via heterothallic somatogamy". However, this may not be the case because typically eight ascospores are found in *Tuber malenconii*, *T. panniferum* and *T. regianum* and in other hypogeous *Pezizales* such as *Eremiomyces echinulatus*, *Genabea*, *Genea*, *Geopora*, *Hydnocystis*, *Hydnotrya*, *Kalaharituber pfeilii*, *Mattirolomyces*, *Mycoclelandia*, *Picoa*, *Sepultaria*, *Terfezia* and *Tirmania* (MONTECCHI & LAZZARI 1993, TRAPPE & al. 2009).

From the above information it seems that truffles are fundamentally typical *Ascomycota*: meiosis occurs after the formation of the crozier apparatus, haploid hyphae emerge from ascospores, and then the formation of the dikaryotic state follows the fusion of an antheridium and ascogonium at the time an ascoma is initiated (CALLOT 2001, PAOLOCCI & al. 2006, RUBINI & al. 2007). However, the important point is that the basis for variation, i.e. a heterokaryotic mycelium, is established prior to the most important phase in the life cycle of the truffle-crozier formation, meiosis and recombinant variation.

Fruiting and non-fruited truffières in New Zealand

The first large batch of experimental English oak (*Quercus robur*) and hazelnut (*Corylus avellana*) seedlings, all mycorrhized with Périgord black truffles (*Tuber melanosporum*) from the same source, were produced in New Zealand in 1987 and used the following year to establish eight truffières near to Opotiki (38°S), Gisborne (39°S), Taumarunui (39°S), Paraparaumu (41°S), Havelock (41°S), Amberley (43°S) and Duntroon (45°S) (Fig. 5). A rough climatic comparison of these truffières and climatic conditions in natural truffle producing areas of France, Italy and Spain can be downloaded from HALL & al. (2008).

Despite the wide range of climates in the early New Zealand truffières only two areas did not produce. One of these was the truffière established near Duntroon in North Otago, a dry part of New Zealand, which was not irrigated sufficiently, while the other at Mahau Sound near Havelock was established on a soil where the pH was not properly adjusted. Consequently, both were unlikely candidates for successful Périgord black truffle production. So it would appear that at least some sources of truffles have sufficient genetic plasticity to allow fruiting under quite a wide range of temperatures. However, spectacular yields occurred only in the two truffières in the warmest locations (Opotiki and Gisborne) where summer temperatures most closely matched the source of the truffles. Later batches of trees appear to have been less successful even though the techniques used were essentially the same. The big difference with these later truffières was that the origin of the truffles used to produce the infected plants was unknown and could have been from almost anywhere in France, Italy or Spain.



Fig. 5. Location of the 11 *Tuber melanosporum* truffières established in New Zealand prior to 1990. All except Mahau Sound and North Otago have produced truffles.

Temperature triggers to fruiting in the fungi

Fruiting of some cultivated saprobic mushrooms such as shiitake (*Lentinula edodes*) and oyster mushrooms (*Pleurotus* spp.) is triggered by dropping the temperature of the substrate (e.g., BRUHN & MIHAIL 2009, HALL & al. 2003, OEI 2003, STAMETS 2003). The temperatures at which fruiting occurs in shiitake also varies with strain (CHEN 2001, MATSUMOTO & al. 2003). A temperature trigger was also suggested as a possibility in the fruiting of the mycorrhizal mushroom *Boletus edulis* (HALL & al. 1998) and, of course, many other mushrooms fruit in autumn when temperature falls and the moisture content of the substrate rises. Confirmation that temperatures and autumn rain are important for fruiting comes from the recent work by KAUSERUD & al. (2008) who showed the climatic changes that have occurred in Norway since 1940 has resulted in a delay in fruiting of wild mushrooms by 12.9 days. Other environmental factors that influence sexual development in the *Ascomycota* are pH, carbon dioxide concentration, osmotic pressure, minerals and a variety of nutrients (PÖGGELER & al. 2006).

The natural distribution of the Périgord black truffle in Europe stretches from 39°N to 47°N and from 6°W to 13°E and has retreated south and advanced north with past glaciations (ADAMS 2009, BERTAULT & al. 2001, MURAT & al. 2004). It would, therefore, be very surprising if truffles did not have considerably more genetic variability (e.g., NAPOLI & al. 2010) than has been demonstrated so far using molecular and biochemical techniques (BERTAULT & al. 1998, 2001; FRIZZI & al. 2001; MELLO & al. 2002, 2005). For example, if there were variability in the temperatures required to trigger fruiting, an understandable evolutionary step for a fungus growing in a wide range of climates and almost totally dependent on the production of truffles and animals as a means of medium distance dispersal, then it would have consequences for the truffle grower.

In the late 1800s, and the heyday of truffle cultivation, truffle infected trees were produced using Talon's technique (HALL & al. 2007: 34). Because of transport costs and difficulties in those days, it seems likely that the new seedlings were replanted not so far away from where they were produced. Consequently, these seedlings were also likely to have been infected with strains that suited the biological and edaphic conditions in the new plantation. Nowadays truffles are shipped all around Europe to meet demand with many of the truffles sold in Périgord coming from Spain (HONRUBIA, pers. comm.). So we echo the conclusions of RUBINI & al. (2007) that it would be imprudent for a truffle grower in the coolest parts of the geographic range to grow plants that had been mycorrhized with Périgord black truffles collected from the hottest part of Europe.

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