INNER WORKINGS

The mysterious parentage of the coveted black truffle

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In the winter of 2016, ecologist Laure Schneider-Maunoury went truffle hunting in France. But she wasn't looking to add the fungus to a culinary delicacy. Schneider-Maunoury, a graduate student at the National Museum of Natural History in Paris, was on the hunt for the truffle's missing father, the form that contributes genes to generate the aromatic, edible fruiting body. Researchers still don't know where these paternal truffles live or how the maternal and paternal partners find each other.

It's a mystery with major implications for farmers, chefs, and foodies enamored with the pungent, expensive black truffle (*Tuber melanosporum*). Slivers of the fungus, which can run hundreds of dollars per pound, grace dishes from risottos to pizzas. Although farmers can raise truffles in orchards of oak or hazelnut, where the fungus joins with the tree roots, the crop remains what some call "protodomesticated" because growers can't control its reproduction. Complicating matters, yields from truffle grounds have plummeted in the past century, likely a result of dwindling habitat.

"For most truffle growers, it's a game—you never know if you're going to get something out," says Aurélie Deveau, a researcher in the field of ecogenomics who studies truffles at the French National Institute for Agricultural Research (INRA) in Champenoux, France. If researchers could work out the reproductive cycle, it might suggest more effective farming practices.

Scientists are on the hunt for the paternal partner that sires the spores within the edible fruiting body of the black truffle. Image courtesy of Shutterstock/Vitalina Rybakova.

On the Lookout

The mystery and the potential ramifications have led researchers such as Schneider-Maunoury and her advisor, mycological ecologist Marc-André Selosse of the Paris museum, to go looking for the truffle father. Their recent work follows a decade of investigations into *T. melanosporum* reproduction.

The part of the truffle people eat is the reproductive organ, or fruiting body. But it's a small part of the much larger organism, which forms a symbiotic association with tree roots. The fungus grows as filaments that wind their way throughout the surrounding soil, forming a network called a mycelium. These filaments collect water and nutrients to share with the tree. In return, the tree provides sugar, made via photosynthesis, to the fungus (see www.pnas.org/content/114/46/12089).

Within the aromatic fruiting body, white flesh surrounds dark spores. If animals eat the truffles, they usually deposit the spores elsewhere through feces. A spore can then germinate to make a new fungus, under a new tree.

What researchers still don't understand is exactly how the truffles mate to produce a new fruiting body. Scientists used to think truffles could self-fertilize—largely because, when they analyzed the DNA of a fruiting body, they only ever found one genome (1).

Francesco Paolocci, a plant molecular biologist in Italy, turned that idea on its head in 2008. With colleagues at Italy's National Research Council in Perugia, Paolocci carefully isolated DNA from the white flesh and black spores, separately. It was easy to extract the genetic material from the pale flesh, as others had done before. But the spores were tougher; they're meant to survive an animal's digestive tract, after all. But by breaking them open with tiny ceramic beads, the team was able to isolate *T. melanosporum* spore DNA for the first time.

They found that the DNA of the spores contained genetic sequences not present in the flesh (2). These sequences must have come from another parent. Because the main fruiting body was formed and nourished

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by the surrounding mycelium, that mycelium came to be considered the mother. The mystery father seemed to provide DNA, but nothing more, to the spores in a fruiting body.

Another major clue came in 2010, with the sequencing of the black truffle genome (3). Researchers discovered that, like some other fungi, a single *T. melanosporum* will be one of two possible mating types, MAT1-1 or MAT1-2 (4). In such fungi, both mating types and both sexes are required to reproduce. A germinating *T. melanosporum* of either mating type can act as mother or father, but hermaphrodites that do both are rare. That means that for, say, a paternal, MAT1-1 truffle to sire a future generation, it would have to find its way to a maternal, MAT1-2 truffle.

And that's not as easy as it sounds, because of the way *T. melanosporum* arranges itself around a tree. Paolocci found that, in a wild truffle ground in central Italy, a tree or group of trees close together, tended to host one mating type or the other, not both. In young plants inoculated with *T. melanosporum* in nurseries, both mating types were present at first. But over time, one strain with one mating type gained precedence, outcompeting the other (5).

Paolocci confirmed this distribution pattern in truffle orchards in collaboration with Claude Murat, a microbiologist at INRA. They visited orchards in Italy and France and mapped the locations of each mating type. The researchers found patches of one type and patches of the other but little overlap (6). "It was a sort of mating type-dependent war between strains," says Paolocci.

Why? The leading hypothesis is that truffles practice vegetative incompatibility. That's when a fungus tends to associate with closely related kin, sharing resources and even fusing together, to the exclusion of more distantly related fungi. Mating type could be one of the signals *T. melanosporum* uses to identify kin. This system would be good for mycelia growing on one tree, but it does seem like it might put a damper on sexual reproduction.

It's possible that the father produces a sort of fertilization structure that could reach the mother through the air or other means. Other fungi do this, but it's never been observed for *T. melanosporum*.

Mystery Parent

Recent studies further complicated the mystery of truffle mating and the missing father. Elisa Taschen, as a PhD student with Selosse, collaborated with truffle growers to collect several years' worth of samples from both orchards and wild truffle grounds. The earthy, garlicky aroma of truffles permeated the lab and aroused complaints as she processed more than 1,000 samples for DNA. Even Taschen, who loves truffles, grew weary of the smell. What she discovered is that the mother and father of a truffle fruiting body tended to be closely related (7). That argues against paternal structures carried a long distance on the breeze. Yet how could the mother and father be near relatives if two parents of opposite mating types would be separated in space by vegetative incompatibility?

Researchers conducted population genetic analyses of truffles' male and female genetic structure at this experimental site in the Grand Est region of France. An electric fence surrounds the plot to fend off wild boars in the area. Image courtesy of Claude Murat (French National Institute for Agricultural Research, Nancy, France).

Moreover, says Taschen, mothers stuck around year after year, unlike in many other fungi. In contrast, the fathers usually contributed to only one or a few fruiting bodies in one year; then they disappeared. Murat's group, meanwhile, is midway through a 10-year investigation of truffle genetics in an orchard in northeastern France; they also find that fathers are less likely to stick around for several seasons (8).

These studies suggest the absentee father is both small and short-lived, making him hard for researchers to spot. Perhaps, Selosse speculates, when a spore lands near a mycelium of the opposite mating type, it can't set up its own mycelium—it will be outcompeted by the resident mating type. In order to pass on its genes, its best bet is to act as a paternal partner. Then it can mate with the local mycelium. It would be a short, though eventful, life cycle, says Selosse: "Birth, then a drop of sex, then nothing."

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> **—Richard Splivallo**

The soil may also contain a sort of "bank" of ungerminated spores that could serve as fathers as needed. But Selosse and Schneider-Maunoury have another theory. Perhaps, they muse, the father does not interact with the roots of towering oaks or hazelnuts. Perhaps, instead, he hitches himself to the roots of herbaceous, nonwoody plants nearby. He wouldn't have to compete with the local, maternal mycelium, but he'd be close at hand when it comes time to mate.

Hidden Gems

That's the hypothesis that Schneider-Maunoury has been investigating. For a study the team recently published, Taschen had collected truffle fruiting bodies from three orchards in the winter of 2015. In June of that year, Schneider-Maunoury went to the same orchards to gather the roots of herbaceous plants surrounding the truffle trees. Using the genetic data from the fruiting bodies, she could figure out which genetic sequences were associated with the maternal and paternal parents in that area and look for those sequences with the herbaceous plant samples.

She was pleased to find some confirmation of the hypotheses: *T. melanosporum* genetic material occurred within nearly 80% of the herbaceous plants, suggesting those plants may be a previously undiscovered niche for the fungus (9). It's not clear what the fungus is doing there, whether it's helping or harming the plant. But there was no paternal genetic material to be found. "We find, again, the mothers," laments Selosse.

Although Schneider-Maunoury was disappointed to find no paternal partners, she and Selosse aren't giving up yet. If the father is transient, perhaps Schneider-Maunoury collected her roots at the wrong time, and she missed him. He may have died after fathering the truffles Taschen gathered earlier that year. In Schneider-Maunoury's current study, she's collecting the roots with potential fathers first in the spring and then the truffle fruiting bodies the following winter.

Taschen, now a soil ecologist at INRA in Montpelier, France, is skeptical that the fathers live on roots for more than a very short period of time—based both on the inbreeding observed and on the lack (thus far) of any paternal truffles on nearby herbaceous plants. "I am pretty sure about this now, they are present in the soil as spores," she says. To test this idea, Selosse and collaborators are seeding truffle grounds with spores of known genetic provenance to see if they will father the next generation.

"Understanding where the males come from, or where they hide, will surely help to increase the yield of harvested truffles," says Richard Splivallo, a chemical ecologist who studies truffles at Goethe University Frankfurt in Germany but hasn't been involved in the *T. melanosporum* sex studies. "Eventually it will be possible to find him, as molecular techniques to detect tiny bits of truffle filaments in the soil are getting cheaper and more sensitive. It's just a matter of time."

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