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Aureoboletus projectellus – a fungus once thought to belong to the genus *Boletus*, now reclassified as *Aureoboletus* based on molecular distinctions

Fungi Tell Their Secret Tales

The kingdom of fungi comprises some of the most mysterious, poorly studied, and diverse organisms on our planet. The pioneering DNA-based technology known as the polymerase chain reaction (PCR) is now revolutionizing our understanding of fungal taxonomy, systematics, and ecology.

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Breakthrough discoveries have brought rapid changes to various sciences, in particular biology. Perhaps the first major breakthrough in biology was the introduction of a system of classification of organisms based on their similarities, by Carl Linnaeus (1707–1778). This system, as well as his binomial nomenclature, largely remains in place today, and it has served as a foundation for all biological sciences for many years. It has been the perfect answer to the constant human need to rank and order everything we see around us. The significance of the Linnaeus system is revealed by the vast number of organisms he described and classified. Today, over 12,000 species of plants and animals are annotated with "L", denoting that they were first described by Linnaeus. Furthermore, no one saw a need to any make changes to these descriptions for over 250 years.

Linnaeus's classification system was clear and simple, but there was a single problem: it was entirely artificial. It was wholly based on superficial similarities between organisms and paid no heed to their origins and relationships. This is not strictly a criticism, since the foundations of Darwin's theory of evolution were published almost a century after Linnaeus's death, but even his contemporaries saw the imperfections and the need to modify his system. They needed a tool which would allow them to see more deeply than the superficial external features or even samples viewed through a microscope. In fact, they needed to look at DNA!

DNA chain

The elucidation of the structure of deoxyribonucleic acid (better known as DNA) earned Francis Crick, James Watson, and Maurice Wilkinson the 1962 Nobel Prize in Physiology or Medicine. We came to realize that the life of all organisms – the entire living world – is encoded in a microscopic molecule comprising two polynucleotide chains coiled around each other to form a double helix. From the moment of the discovery, scientists knew that being able to interpret the structure and sequence of nucleotides comprising DNA holds the key to truly understanding the living world. It is fair to say that their discovery marked the birth of molecular biology, and further discoveries followed rapidly. Some of the most important achievements of early molecular biology include describing the mechanism of DNA replication and the discovery of polymerase (the enzyme responsible for the process), DNA sequencing (the order of individual nucleotides in the double helix), and the discovery of the *Thermus aquaticus* bacterium used to create thermostable polymerase known as Taq. We had to wait for the next major breakthrough until 1993 – when the American biochemist Kary B. Mullis described the polymerase chain reaction (PCR), which remains a fundamental technique in molecular biology. Mullis was also awarded the Nobel Prize, this time in Chemistry.

PCR is used to make vast numbers of copies of a specific DNA sample. The genius of the reaction lies its simplicity. The process involves interlinked

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Mycorrhiza of *Cenococcum geophilum* – one of the most common fungi symbiotic with tree roots

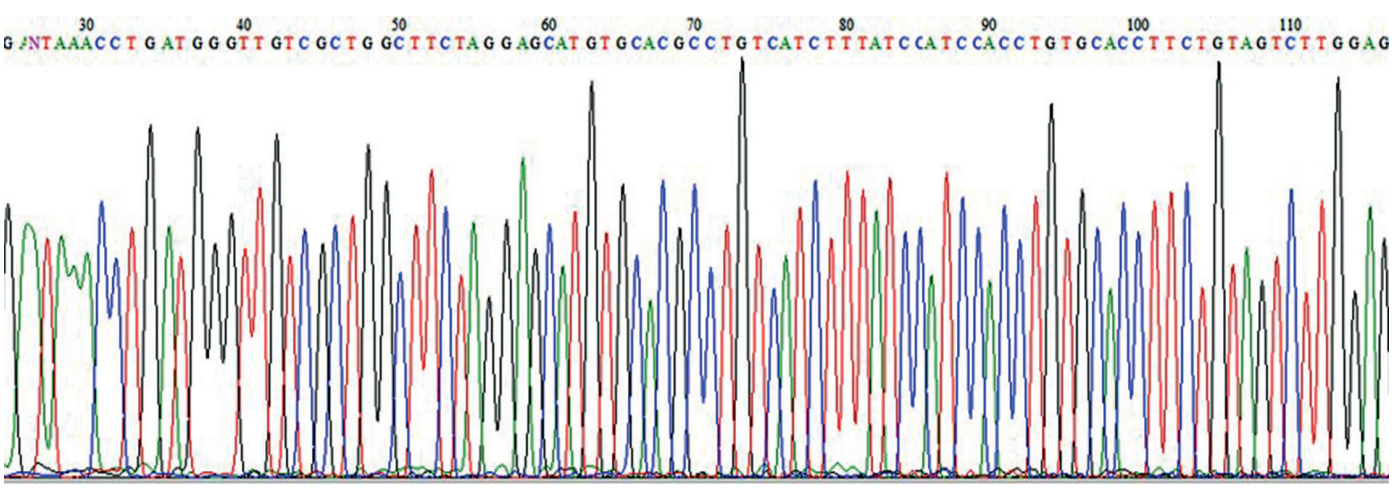
cycles of temperature changes, under certain conditions and in the presence of a few "ingredients." Each cycle starts with denaturation – the separation of the two DNA strands – at a high temperature, typically 90–95°C. In the second step, the strands are bound by primers (short synthetic fragments known as oligonucleotides with a complementary sequence to the target DNA region). This step takes place at a temperature between 50 and 60°C, depending on the primer – each primer requires a specific temperature for maximum efficacy. Next, the complementary strand is elongated in the presence of the Taq polymerase. This involves four different nucleotides added to the reaction mixture which bind in a specific way – adenine with thymine, and cytosine with guanine. As a result of elongation, a second, synthetic nucleic acid strand is formed. There are usually between 35 and 40 repeated cycles, which means each cycle doubles the size of the original DNA fragment. The technique means that a single double DNA strand can generate millions of identical copies, which opens up myriad possibilities for research and analysis. PCR-based molecular biology techniques are currently used in many scientific fields, such as taxonomy, systematics, ecology, forensics, medicine and clinical diagnostics. One of the best recent examples is the use of the technology during the COVID-19 pandemic to test for the presence of the virus.

Molecular mycology

The rapid development of molecular biology over the last thirty or so years has made a major contribution to the development of biological sciences. In this article, I will use the kingdom of fungi – some of the most mysterious, poorly studied, and diverse organisms on our planet – to illustrate how the discovery of the structure of DNA and the development of PCR has pushed science forward. The rapid development of methods improving molecular tools in mycology (the study of fungi) has been used in taxonomical, ecological, and population studies. The development is mainly the result of the use of PCR in mycological research and increased availability of tools for analyzing nucleotide sequences.

A significant development came with the recommendation of the internal transcribed spacer (ITS) as the universal fungal barcode sequence. DNA barcoding is a method of species identification using a short DNA marker. This unique code can be read from various parts of a fungus – including the fruit bodies, mycorrhiza, and mycelium. We need a suitable number of copies of the ITS fragment created using PCR; they are then sequenced and presented as a string of letters corresponding to each nucleotide. The integrated method using PCR and sequencing of the products has revolutionized classical mycology, turned the systematics of the fungi kingdom upside-down and allowed researchers to identify thousands of new species.

Perhaps the best example of this is *Aphyllophorales*, a now-obsolete order of fungi originally classed together as those not having gills. Following numerous revisions based on molecular biology methods, the group has been reclassified into 14 different orders! Currently, the ecological group includes over 500 taxa found all over the globe, representing all functional groups of fungi. Another example is the division of the more familiar genus of *Boletus* fungi. Research done in recent years has shown the genus to have been highly polyphyletic (actually of mixed evolutionary origin) – only a fraction of the species formerly assigned there actually belong to the genus *Boletus*, and so numerous other genera have had to be posited. A similar fate befell the bay bolete, the most widely picked mushroom in Polish forests – formerly a member of the *Xerocomus* genus, it is now classified as *Imleria*.



Chromatogram of a fragment of an ITS rDNA sequence

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Tomentella punicea – an aphyllophoroid fungus currently classified in the *Thelephoraceae* family

Hidden richness

As well as being used in classical taxonomy, PCR has also found a wide range of other applications in mycology. Prior to the era of molecular research, mycological observations were mainly based on classical methods, such as observations of the fruit bodies, which appear irregularly. The method required many years of painstaking field studies, largely dependent on weather. The introduction of molecular research based on ITS analysis has revealed a hidden abundance of fungi in tree roots as mycorrhiza or mycelium in the soil. Research in Estonia found 122 symbiotic fungi species identified as mycorrhiza in the roots of a single aspen tree. One of these species, *Cenococcum geophilum*, was identified in over 20 different genotypes. Molecular research has become essential in fungal ecology, revealing the great diversity of mycobiota, in particular species whose entire life cycle occurs in the soil without producing fruit bodies. Research conducted over the last two decades at the PAS Institute of Dendrology has helped us identify dozens of new fungi species in Poland, even though none of them have formed fruit bodies. The existence of these rare organisms was confirmed by the presence of their mycorrhiza.

The next stage in mycological research has involved the introduction of massive parallel sequencing. In contrast to the method described above, where a single PCR cycle allows us to identify a single fungus species, massive parallel sequencing is a high-throughput method for sequencing millions of short fragments from thousands of species. Studies of 365 soil samples (each weighing 2 grams) collected all over the globe have allowed us to identify 100,000 fungal taxa! The majority had no reference sequences in existing databases of known species. We also discovered many new phylogenetic lineages.

State-of-the-art methods used in contemporary mycology allow us to see even deeper. Genomics studies make it possible to describe entire fungal genomes – to present an entire sequence of genetic information unique for a given species. Whole gene sequences have been published for a few species of fungi, including the *Laccaria bicolor* and the commercial truffle. The genomic approach makes it possible to study the functional ecology of fungi, aiming to better understand what role is played by individual species in ecosystems. This makes use of other state-of-the-art "-omics" methods, such as metabolomics (metabolites), transcriptomics (gene expression), and proteomics (proteins), which dive even more deeply into individual hyphae to try to answer questions about what's happening on the physiological, molecular, and biochemical levels.

Overall, molecular methods have revolutionized mycology and opened up myriad ways of discovering the hidden stories fungi have to tell. However, we should also remember several early breakthroughs in general biology which have been adapted for mycology and which have changed and continue to change how we see fungi. ■

Further reading:

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