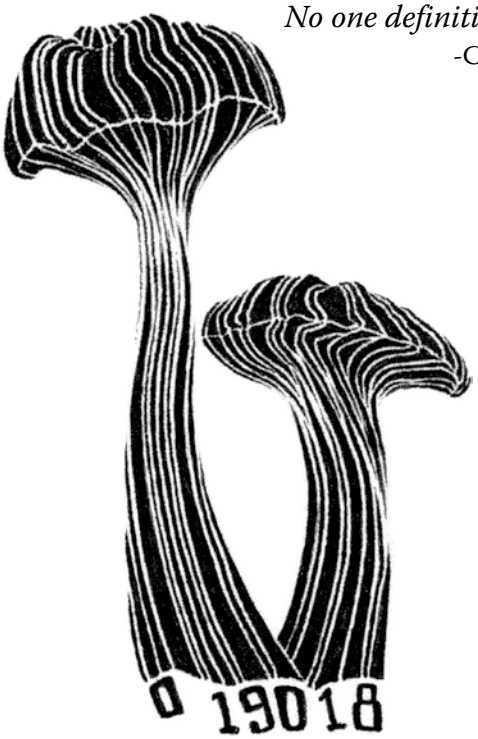
BARCODING FUNGI:

HOW TO TELL A KLOTZ FROM A GLOTZ

by Britt A. Bunyard

There are no such things as sharply defined families or genera or species in nature; such exist only in books.
-George Massee, *British Fungi and Lichens* (1911)

No one definition [of what a species is] has satisfied all naturalists.
-Charles Darwin, *On the Origin of Species* (1859)



One day, during last summer's 2010 North American Mycological Association (NAMA) Annual Foray in the beautiful mountains of Colorado, Gary Lincoff and I split off from the main group of hikers and happened upon a particularly memorable patch of huge boletes. "Porcini!" I exclaimed, and began filling my basket. Gary was quick to point out that although they looked every bit like *Boletus edulis*, the "true" king bolete, these were subtly different and probably *B. pinicola.* The markings and color seemed quite a bit different as I analyzed a second mushroom; a third seemed just as variable as the previous two. "How can you be certain? Is there a difference in smell…or taste?" I asked. "Well, it's tough to be certain. Do you have any chemicals to do a test—potassium hydroxide, iron sulfate?" "All I got's my knife and a hand lens, Gary." "Well, if we could look at the spores under the scope…," his voice trailed off.

About that time, Erin and Noah bounded down the path. I asked them if they had any chemicals to perform some tests for ID. Erin quipped, "You two are so '90s!" "Right," Noah agreed, "I have an app for that!" And with that, took out a cell phone and attached something to a side port. The plug-in device was about the size of a USB flashdrive. Erin knew the routine and held out a pea-sized slice from a freshly plucked bolete. Noah smeared it on the top of the tiny plug-in device. "This will take about fifteen seconds." Gary and I watched, dumbstruck. Noah shot a knowing grin at Erin, "Ha! They're both wrong. It's *Boletus huronensis.* No one would have suspected that to be fruiting out here! Britt, I guess you won't be eating those, afterall."

Wouldn't such a technology be wonderful? (It may be closer than you think, but more on that later…) Welcome to the Age of Biotechnology! Many consumers simply have to own the latest gizmos and would run right out and purchase such a device. But to many more of us, new technology is a constant bugaboo. (Make no mistake about it: science has conjured up a few demons and unleashed them upon the planet… though, overall, I think the record's pretty good.) Our fears of science and technology running out of control—founded or not—have been preyed upon in numerous books and movies. Michael

How to tell a Klotz from a Glotz

Well, the Glotz, you will notice,
has lots of black spots.

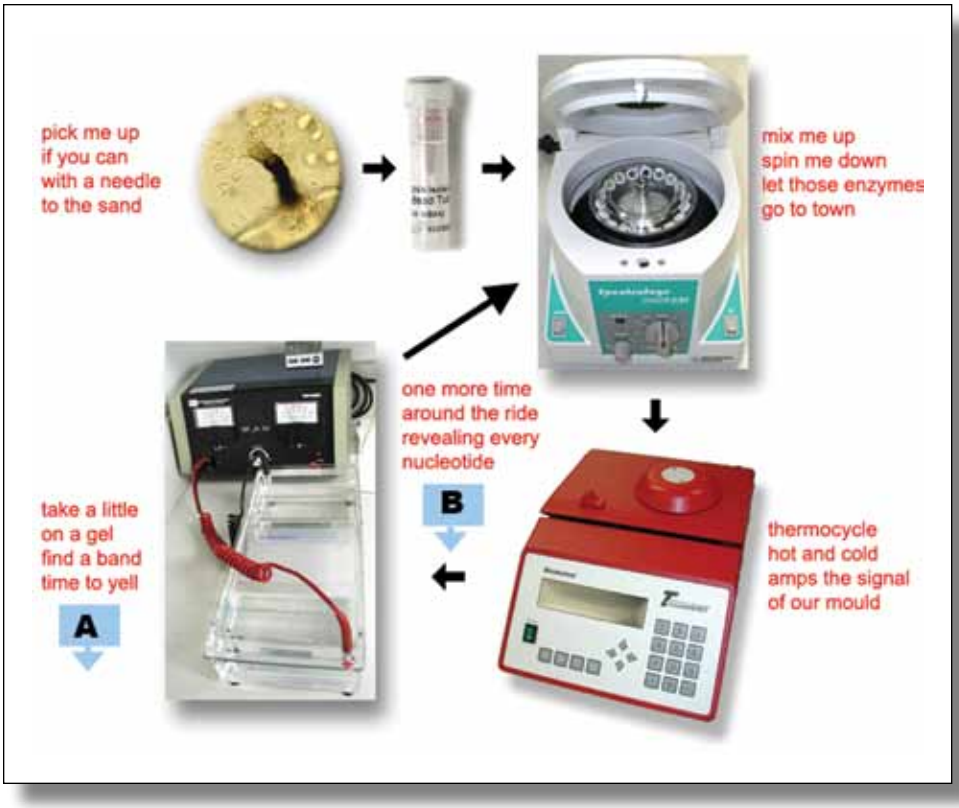
The Klotz is quite different
with lots of black dots.

But the big problem is
that the spots on a Glotz
are about the same size
as the dots on a Klotz.

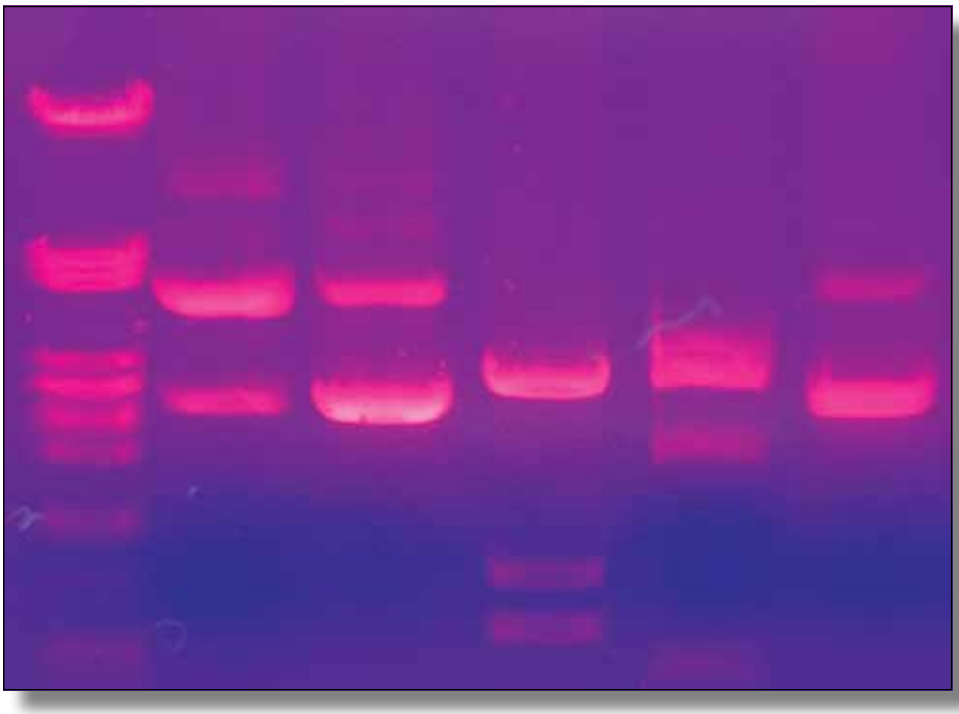
So you first have to spot
who the one with the dots is.

Then it's easy to tell
who the Klotz or the Glotz is.

-Dr. Seuss,
Oh Say Can You Say (1979)



Bar coding in the lab. Mycelium of fungus is grown and harvested. Mycelial cells are digested with the help of enzymes and DNA is extracted. A tiny amount of DNA is then precipitated in centrifuge tubes. The minute amount of fungal DNA is tremendously increased, or amplified, in a thermocycler. Different regions of the genome are separated and made "visible" (the glowing pink bands) via gel electrophoresis. "Reading" the DNA sequence from the electrophoresis gel banding patterns is then carried out with the assistance of a computer.



Creighton's gotten wealthy writing in this genre. And some of the greatest stories of all time come to mind. Mary Shelley's *Frankenstein* played up fears of science during the Industrial Revolution. Even before this, Goethe wrote *The Sorcerer's Apprentice* (which later became the basis for the beautifully animated Disney movie, *Fantasia*; who can forget the out-of-control mops getting chopped into even smaller, faster, mops!). Goethe's famous poem was likely based on a much earlier tale (*Philopseudes*, written around AD 150) by the Assyrian scribe Lucian, regarded as possibly the first to write science fiction. And there have been many others since. The wonders of man's scientific creations always seem to turn into golems that wreak havoc and destruction.

And so it goes with taxonomy. The field of taxonomy has entered the DNA era as well. Much to the consternation of many amateur (and more than a few professional) mycologists. Molecular phylogenetics (as opposed to the earlier kind, based on morphology, or characteristics you could see) uses the results of DNA analysis (in conjunction with traditional taxonomic methods) to put things in order; who is related to whom and to answer questions about evolution. The results of this new technology mirror those of the earlier pioneers of mycology who used little more than hand lenses or microscopes (plus collections of thousands of specimens) in about 90% of the cases. Still, harrumphing continues from the masses.

Now comes something called "DNA barcoding." Most people don't know what it is…but know they're definitely not going to like it.

What is DNA barcoding?

Briefly, DNA barcoding utilizes a single, short section of DNA sequence to identify species. That is, any and all species—plant, animal, fungal, bacterial, and everything in between. At least that's the goal. Neither the idea of, nor the technology behind, DNA barcoding is novel. The underlying concept can be traced to Carl Woese, of the University of Illinois, who first showed some 30 years ago that DNA sequences could be used to reconstruct the evolutionary Tree of Life. What is new (and controversial) is the idea (first proposed in 2003 by Hebert et al.) of using short DNA sequences from a single, standardized region of the genome to identify species from a wide taxonomic range across kingdoms. Advances in sequencing technology allow DNA sequences nowadays to be obtained rapidly and cheaply (Hebert's

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From this single short sequence of the CO1 gene, individuals have been identified down to species level with a success rate ranging from 98 to 100% in birds, fish, and insects. Though, not so well for plants. Or fungi.

lab sequences 1,000 specimens per day!) so that a system of barcoding life across all kingdoms (and for all species) appears both plausible and worthwhile. Indeed, when Paul Hebert first came up with the idea and coined the term "barcoding" he envisioned that the target stretch of DNA sequence, the As, Ts, Cs, and Gs, could be written as an actual barcode strip and read by devices like those used to keep track of every item stocked in a grocery store or to track every cargo shipping container in an expansive sea port.

Not everyone is on board with the idea just yet. Barcoding has created more than a little controversy in the taxonomic community (Ebach and Holdrege, 2005; Petersen and Hughes, 2009). Traditional taxonomists use multiple morphological traits (macro- and microscopic) as well as mating studies to delineate species. Today, such traits are increasingly being supplemented with DNA-based information taken from several regions of the genome; more and more routinely, the entire genome is analyzed. In contrast, the DNA barcoding identification system is based on a portion of one gene, the mitochondrial cytochrome c oxidase subunit I gene (sometimes called COXI or CO1) for most groups of organisms. Mostly because the system relies on such a small portion of a given organism's entire genome, some critics have quickly dismissed barcoding results as unreliable. (This gets at the age-old debate over **what is a species?** Barcoding isn't likely to resolve this...but then again, the debate's been going on for a long time now—since before Darwin—which is why we're in a constant state of flux over fungal species names.)

Not so fast say proponents. Research papers demonstrating the accuracy of the technique have been piling up for nearly a decade. From this single short sequence of the CO1 gene, individuals have been identified down to species level with a success rate ranging from 98 to 100% in birds, fish, and insects. Though, not so well for plants. Or fungi. Problems with CO1 in plants seem to have been circumvented by using a different region of the genome. (For plants, a short stretch of DNA from the chloroplast genome has been used with good success.) With fungi, there have been some problems finding a region stable enough, but not too stable, evolutionarily speaking, to provide delineation among all species. (This is mostly due to the tremendous diversity among fungi; only the insects match fungi in diversity of life on the planet.) Progress is being made; the familiar nuclear ITS region seems promising (Seifert, 2009). But besides fungi, overall barcoding seems to be working well and giving reliable results. As well as correctly identifying known species, a number of cryptic species have been discovered within what had previously been thought to be single morphologically based species. This is turning up more and more commonly among all the groups of organisms analyzed. And here is an area where barcoding can really be a boon to mycology.

Barcoding's apparent success has fuelled speculation that accurate species identification is now possible by anyone with access to DNA sequencing even if they lack taxonomic expertise. An inexpensive handheld device in the future would further simplify this. The goal of the international Consortium for the Barcode of Life is to catalogue and barcode 500,000 of the known species on the planet (now at 1.7 million; 80,000 of them fungal if you include Myxomycetes and Oomycetes), by 2015. Hebert has stated publicly, "Any species humans encounter frequently will be barcoded by 2025."

How does it work?

The methodology used in DNA barcoding is straightforward. DNA is extracted from organisms, alive or long dead, pathogens living in host tissue or even extracted from soil samples. (This demonstrates one of the real exciting uses of DNA technology: we can study and even identify microbes in the environment that we've never been able to culture, and thus have never actually seen.) The DNA is analyzed and sequences of the barcoding target region are obtained from the specimens. For most organisms, the DNA barcoding region is a portion of one gene, comprising around 650 base pairs (the As, Cs, Gs, and Ts of the stringy DNA molecule) from the first half of the mitochondrial CO1 gene.

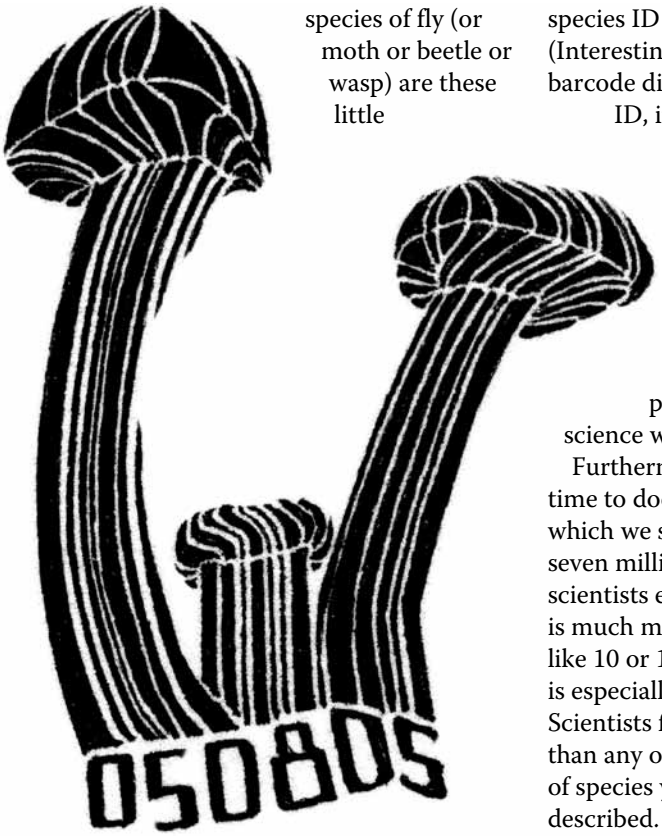
The resulting DNA sequence data are then used to construct a phylogenetic tree with related individuals clustered closely together. DNA barcode sequences themselves vary extensively between species but hardly at all within them, thus can be used to distinguish one species from another. As a result, when we look at the resulting phylogenetic tree, each cluster of closely related individuals that we see, are assumed to represent a separate species.

Do we need barcoding?

Many seem to think so and I'll steal a line from Thomas Hardy's book *Jude*

the Obscure to sum up why: "Because we are too many." We, the living creatures—animals, plants, fungi—are too vast in diversity for scientists to describe and catalogue. Traditional morphology works great (to wit we now have 1.7 million named species on the planet) but morphology has limits. Based on appearances obvious to our eye (aided with a microscope or not), we cannot "see" all the species around us. A tremendous number of species remain cryptic, that is indistinguishable from close relatives. Which is, of course, exactly what you'd expect if evolution really works.

Just being able to know and discuss members of a closely related group of organisms requires a tremendous amount of scientific specialization. Look around you at the next bioblitz you may attend. You'll see a number of scientists or other experts from many different fields. A survey of insects or fungi will require many scientists with expertise across many orders of species just to make a decent account of what the surveyors collect and bring in to document. And imagine doing an ecology study across orders, classes, or kingdoms of life. My own research interests have long been with the mycophagous true flies that live, as maggots, within mushrooms. What species of fly (or moth or beetle or wasp) are these little despoilers of that perfect, prized basidiocarp you hold in your hands? You won't know unless you can get the mushroom home intact and allow the insect to complete its life cycle to adulthood. And much of the time, despite your best efforts, this won't happen. Identification to species of immature insects from most groups is all but impossible. Now to be able to ID the insect (dipteran, hymenopteran, coleopteran, lepidopteran) from that mushroom (as well as to ID the species of mushroom) is an even tougher task. A tool that could ID the mushroom and all the organisms inside would be a godsend. One could then really get down to the business of studying the interactions between all the organisms involved. Currently, we know very little about the association between mycophagous insects and their mushroom hosts. A similar study (of tropical moth species, the host, and their tachinid fly parasites) turned up amazing results (Smith et al., 2008; 2007). Although they had no handheld devices at their disposal (which would have been a tremendous benefit in the steamy mountains of Costa Rica), they collected samples of DNA from host and parasite, did the barcode analysis and found that the DNA barcode analysis quickly determined all the species of moth involved—agreeing with morphological species ID in nearly all cases. (Interestingly, in the few cases where the barcode disagreed with morphological ID, it was later shown that each one of those moth specimens was previously misidentified in the field.) The DNA barcoding really proved its mettle in identifying the parasitic flies; identification was many times faster using barcoding plus several species unknown to science were revealed.



Furthermore, we are running out of time to document what organisms with which we share our planet. One point seven million species seems like a lot; scientists estimate that the actual number is much much higher…probably more like 10 or 100 times that number. This is especially true with insects and fungi. Scientists feel these two groups, more than any others, feature vast majorities of species yet to be discovered and described. There simply aren't enough taxonomists to do the work of discovery and identification and won't likely ever be enough. And every day we lose some species forever, mostly to loss of habitat.

> Why use a gene sequence from mitochondria rather than from the nucleus of the cell? Mitochondria are organelles within living cells and function to produce and distribute the energy molecules of the cell. Likely as a result of their evolutionary origins, mitochondria (and chloroplasts in plants) have their own tiny chromosomes. Mitochondrial genomes in general evolve at a much faster rate than much of the nuclear genome of the cell...seemingly at a rate making them ideal for use for some taxonomic purposes. Like distinguishing among just about every species on the planet.

The problem of cataloguing all the fungi is more similar to the challenge of enumerating bacteria than it is to collecting insects. Compiling this catalogue will provide the major challenge to and application for fungal DNA barcoding. As hinted at above, studies of environmental DNA (yes, the DNA left behind in the soil after organisms die and fall apart) reveal the existence of many microorganisms, including fungi, that have not yet been successfully cultured in the lab. Thus we have yet to observe, describe, and name them. A visit to the GenBank "Top Organisms" page lists "uncultured fungus," "uncultured soil fungus" and "uncultured endophytic fungus" among the top five most frequently reported "species," with nearly 8,000 records in total, compared to about 5,000 for *Homo sapiens* and nearly 3,000 for "uncultured bacterium." So, what are these fungi? Obviously they're of importance to the environment. It's likely some of these groups of fungi are the dominant organisms in nature—maybe even the most numerous. And we have almost zero knowledge of them. In the future, fungi very well may be most effectively detected and identified by studies of their DNA alone.

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WILL DNA BARCODING BE THE NEXT FRANKENFOOD?

Scientists nowadays routinely genetically "engineer" novel varieties of organisms for food and fiber and medicines. Opponents have used the term "frankenfoods" to described many such genetically altered organisms. The list of species whose complete genomes have been deciphered continues to grow, almost monthly. These are exciting times to be a biologist. But this rapid upsurge in technology leaves many in society puzzled and at times fearful. Is this justifiable?

To be clear, human civilization has always practiced genetic modification of organisms. Civilization began, some ten thousand years ago, when humans decided to switch from that of a hunter-gatherer to one concerned with practicing agriculture. People started to select which plants they liked to eat, removed competitors, saved some seed for the next year, and domestication was underway. Over time, land races (varieties) of given species were selected on the basis of which performed better in a given region. About the same time, domestication of animals began around the world. Early on, these plant and animal crops were "improved" to suit human desires for yield, taste, or other traits. These improvements were a result of intentional mixing of varieties or even species of plants or animals—selective breeding. Genetic manipulation. Selective breeding by the old fashioned way or modern genetic modification results in the same thing, qualitatively, in that the genotype and—more importantly to the grower—phenotype (larger peaches, disease resistant roses, whiter button mushrooms) of the crop is altered to bring about a desired trait.

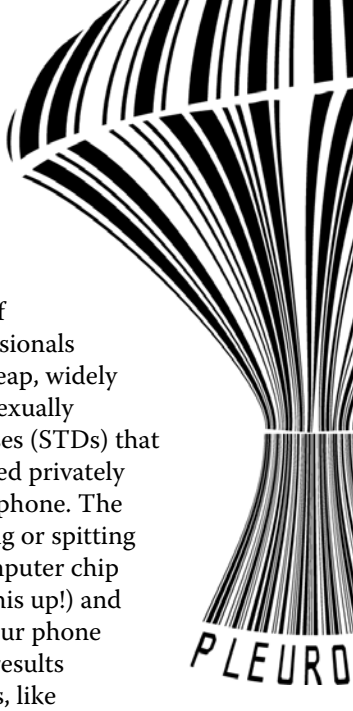
Will barcoding put classically trained taxonomists out of a job?

I don't think so. And neither do a lot of taxonomists. The great ecologist Daniel Janzen has stated that if we are to catalogue all the planet's living organisms it will require the combined efforts of the entire "taxasphere"—the collective intellectual might of taxonomists, museums, collections, and their centuries of literature (Janzen, 2004).

Many feel that with the influx of unknown and cryptic species that will inevitably turn up as a result of widespread use of DNA barcoding, the need for trained taxonomists to make formal description of new species will actually increase manyfold.

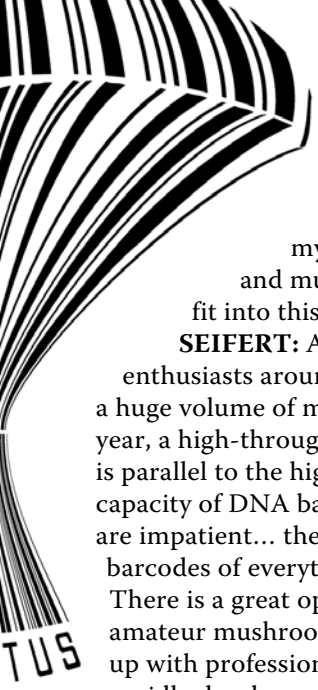
DNA barcoding has come along at a critical time in biology. For a number of reasons, species extinction rates are ever increasing and this makes cataloguing the organisms of the planet ever more important. Likewise there is an ever increasing need to be able to identify organisms rapidly from situations as diverse as economically important invasive species showing up at our borders, to emerging infectious disease outbreaks, to verifying the safety and authenticity of the food we eat (you would be surprised to find out how often the fish you think you're eating turns out to be something else!). And with an already over burdened taxonomic community, DNA barcoding could be a boon to museum curators swamped under backlogs of specimens awaiting analysis.

And what about that handheld DNA barcode scanner at the beginning of our story? Just fantasy? Think again. It's today…well, almost. A group of U.K. health professionals is developing a cheap, widely available test for sexually transmitted diseases (STDs) that can be administered privately through a mobile phone. The test involves peeing or spitting onto a special computer chip (I'm not making this up!) and plugging it into your phone for analysis. Test results for common STDs, like gonorrhea, chlamydia and herpes, will appear in minutes. The software also has the potential to include treatment recommendations, links to additional informational websites, and directions to your nearest doctor. Previous tests were performed in a clinic, in person, and can be embarrassing or are simply inconvenient. Results often take days to receive. This new test offers many obvious benefits and results are known in minutes. The test is also geared towards tech-savvy youths who may be too shy or lazy to get tested. Researchers affiliated with the new program plan to make the tests widely accessible and cheap, hoping to distribute the tests in nightclub vending machines, pharmacies, and supermarkets for as little as about $0.80 each. In a recent essay (King, 2011) marveling at the latest breakthroughs of living in the Genomics Age, University of Washington professor Mary-Claire King noted that, "Genetics is a way of thinking, genomics is a set of tools, and if we think rigorously about genetics and use these tools well, [our discoveries] will be bounded only by our imaginations."



Interview: Dr. Keith A. Seifert

Dr. Keith A. Seifert, a Research Scientist with Agriculture and Agri-Food Canada, and a well-known molecular mycologist was instrumental in putting together this paper. He agreed to share some additional commentary from his own personal experiences.



FUNGI: Barcoding and mushrooms… where do mycological societies and mushroom enthusiasts fit into this?

SEIFERT: Amateur mushroom enthusiasts around the world collect a huge volume of mushrooms every year, a high-throughput practice that is parallel to the high-throughput capacity of DNA barcoding. Barcoders are impatient… they want DNA barcodes of everything immediately. There is a great opportunity for amateur mushroomers to partner up with professional mycologists to rapidly develop a comprehensive DNA barcode database for mushrooms. With the appropriate lobbying, the barcoding infrastructure should be able to accommodate 10,000 mushroom barcode sequences per year… that's a million sequences in ten years. Then anyone with access to DNA sequencing can identify mushrooms reliably, as long as the barcodes themselves are reliably identified. Ecologists will be able to detect, identify and study mushrooms directly in the soil or in wood without waiting for the mushroom themselves to appear. And amateur mushroomers will be able to be proud that they have made a significant contribution to an international, big science initiative.

FUNGI: What would you tell skeptics of the increased use of DNA sequence analysis, in general, and specifically DNA barcoding?

SEIFERT: I was a skeptic about DNA sequencing for a long time, was probably considered a heretic (or Luddite) by some gene jockeys for awhile, but gradually I came around. I now think that most of the anti-barcoding sentiment is a knee jerk reaction derived from anti-reductionist tendencies. I understand this myself. Many naturalists have an inbuilt mystical streak, and taxonomists (amateur or professional) often operate in a kind of impressionistic manner. We like the fuzziness and subjectivity of it, and resent the thought that a machine or a bunch of chemical reactions might deliver more accurate answers than we can despite all our experience and knowledge. Well, we can still go about our identifications and our nature loving this way, and each of us will have our own inherent error rate. I can pretty well guarantee that the error rate for barcoding will be lower than it will be for most taxonomists, amateur or professional. It seems to me that if anyone can carry a small cell phone sized device into the field and reduce their error rate in identifying mushrooms, and that if beginners who lack confidence can get more confidence, that more people will be enabled, more will be interested, and this will mostly be a good thing. Like any tool, barcoding needs to be used with intelligence and common sense… if the mushroom identified has never been found on your continent, the tool should tell you that, and you should realize there is a problem.

FUNGI: The world does seem to be gravitating toward handheld devices—GPS, smart phones and the like. I see there are now apps for iPhones for identifying mushrooms.

SEIFERT: Actually, this relationship with cell phones is interesting. I saw a month or so ago an article about someone who has invented a lensless microscope that works in a cell phone body, and sends images of blood parasites from field workers to far away doctors. There is an iPhone app that sends GPS coordinates and photographs from the field directly into specimen databases. And the anthrax attacks in your country led to the development of portable laboratories and fully automated systems that look for anthrax DNA puffing out from letters in your post offices. I have seen the future and it was already here, yesterday, almost.

Acknowledgement

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