The Capabilities and Limitations of DNA Barcoding of Botanical Dietary Supplements

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Summary

DNA testing has been regarded as incontrovertible evidence for forensic investigations, medical diagnostics, and paternity testing in humans for more than two decades. However, its use as a routine tool for authentication of botanical (herbal) dietary supplements is relatively recent and far less established. As a result, a general lack of understanding exists—even by those who claim to perform DNA testing—of the complexities of the methods, especially as they relate to finished dietary supplement products containing botanical extracts. This lack of awareness has resulted in both the misuse of the technologies and misinterpretation of test results. "DNA barcoding" is a commonly used term that in the public eye has become synonymous with "DNA testing" or "DNA authentication." However, DNA barcoding is one of many DNA testing methods and is not the most appropriate one for authenticating finished products, especially those containing botanical extracts [1].

The objective of this paper is to provide an expert assessment of the capabilities and limitations of DNA barcoding for botanical dietary supplement authentication to inform assessments of the applicability and accuracy of DNA test results. We explain what DNA barcoding is and how it is performed, discuss its capabilities and limitations especially in regard to finished dietary supplements and botanical extracts, provide guidance on how to perform DNA barcoding properly, and finally, offer a critique of the DNA testing methods used in a recent investigation of botanical dietary supplements by the New York Attorney General's Office (NY AG) [2].

What is DNA Barcoding?

All organisms, including plants, animals, fungi, and bacteria, contain DNA within their cells. DNA comprises nucleotide "bases" adenine, cytosine, guanine, and thymine (A, C, G, T), which are arranged in patterns, like words in a book. DNA barcoding refers to examining the sequences from a standard DNA region ("gene") for species identification [3-5]. The idea is similar to the concept of using a supermarket barcode scanner to identify groceries, where each item on the shelf can be identified by its unique barcode on the label. By examining the specific arrangement of these nucleotide bases, scientists are able to identify patterns that correspond to species, populations, or even individuals depending on the region of the genome (the complete set of all genes in an organism) analyzed. While closely related organisms share many of the same genes, for example, the genomes of humans and chimpanzees are nearly 99% identical [6], certain genes tend to vary greatly between closely related species and generally can serve as genetic markers for identification.

The term "DNA barcoding" was first introduced as a means to identify animal species [3]. The application of DNA barcoding is particularly appropriate for distinguishing fresh or living tissue obtained from distinct species such as cows and pigs, or for fish such as tuna or snapper and has been validated by the FDA for this purpose [7]. DNA barcoding of plant materials has also been used to identify major plant groups such as grasses and pine trees, where identifying the exact species is not necessary [5, 8].

However, over the past 10 years, the use of DNA barcoding for the specific identification of plants has sparked considerable debate in the academic community, because plants are extremely complex and cannot be successfully identified to the species level with one or a few standard regions, as is possible with animals [9-12].

Very little research has been conducted on the use of DNA barcoding for materials other than those that are fresh or living, especially on botanical extracts in dietary supplements where the DNA can be removed or degraded (see further discussion below). Therefore the use of DNA barcoding for finished dietary supplements is largely misunderstood and misapplied—even by those who claim to perform it. Despite several reports indicating the successful use of DNA barcode methods on botanical extracts [13-14], the results are erroneous and likely due to cross-contamination by raw or fresh materials either in manufacturing or in the laboratory conducting testing [15].

How Does DNA Barcoding Work?

The process of DNA barcoding typically follows the following process: (1) sample materials are homogenized or ground to a fine powder; (2) the genomic DNA is extracted; (3) specific gene regions are amplified using PCR (Polymerase Chain Reaction); and (4) the genes are sequenced and the electronic DNA sequence data are further analyzed against a known reference material to identify the sample [1]. (Figure 1) DNA sequences can be identified in a number of ways by comparing them to known reference DNA sequences using a variety of computer algorithms; the specific reference sequences and algorithms used are critical for accurate identification.

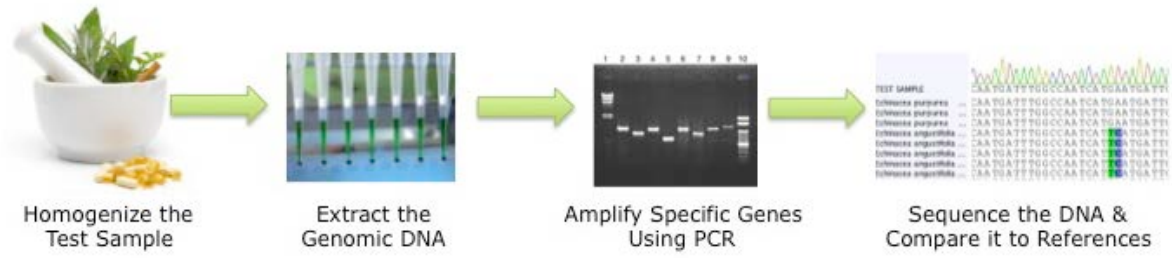

Figure 1. A general overview of the DNA barcoding process. *First, test samples are homogenized or ground using mechanical methods. Then, the genomic DNA is extracted and purified. Next, one or a few specific gene regions are amplified using PCR and visualized on an agarose gel to confirm amplification. Finally the DNA from the PCR amplifications are sequenced and compared to known reference DNA sequences to identify the test sample.*

Within each step of this process, opportunities arise for error or misinterpretation. Typical DNA barcoding analyzes gene regions that are approximately ~500-1000 bases in length, which can be detected using primers (probes that select for the specific genes) designed to work across all plant species. Primers can be designed to any desired level of specificity:

on one end of the spectrum, primers can amplify any plant, animal, fungi and bacteria DNA in a sample; while on the other end, primers can be designed to amplify only one species or even one variety or lineage within it.

While DNA barcoding using a single gene or standard set of genes may be appropriate for animals, it is not so for plants due to their dramatically different life history characteristics, evolutionary histories, and hybridization. For example, "living fossils" like *Ginkgo biloba* are slow growing trees with no close relatives, so their genes are unique and can be easily differentiated from those of other plants. However, dynamic lineages of closely related species, such as *Echinacea* are difficult to distinguish genetically due to widespread hybridization.

Therefore, the most critical step in using DNA for plant species identification is to locate the genes that contain the appropriate level of variation to allow for differentiation of species. This often requires extensive research into the evolutionary history and biology of the plants being tested. Once specific genes are identified that distinguish species, true identification can be achieved. That said, the method underlying DNA barcoding—sequencing of genes for identification—is not debated, but rather the controversy is whether a single or standard set of a few genes is enough to differentiate between species.

What are the Capabilities and Limitations of DNA Barcoding?

Over the past ten years, numerous scientific articles have been published on the use of DNA sequencing for authentication of herbs of commerce, especially in their raw or fresh form [16-21]. While DNA sequencing can be a useful tool for authentication of raw herbs (if the appropriate genes are used), its application for finished herbal/botanical dietary supplements is limited due to the generally low quality of DNA in those products. Few studies have been performed using DNA methods of authentication on finished products, and generally they do not explicitly state whether or not materials being examined contained botanical extracts [13-14, 22-25]; the failure to appreciate the effects of extraction on botanical material indicates the general lack of understanding by scientists performing DNA testing on finished products and the importance of the distinction between raw and processed materials, especially extracts. Because of the relatively new application of DNA barcoding to finished supplements, there is a general lack of understanding of its capabilities and limitations on these materials.

Knowledge of the specific processes and ingredients used in the production of botanical dietary supplements is critical in evaluating whether DNA barcoding is an appropriate method of authentication. Many finished botanical dietary supplements contain botanical extracts, which can be developed using different extraction methods (e.g., solvents, super-critical CO_2, treatment with heat or pressure, etc.). Thus an awareness of the extraction method for a particular botanical allows evaluation of the appropriateness of DNA barcoding for that product. Depending on the specific process used, differing quantity and quality of DNA will pass through to the finished product [17]. Typically most, if not all, of the material containing cells (with the DNA) is removed during extraction, leaving the phytochemicals but not the DNA. Any DNA that does remain in a

botanical extract is generally low in quality and concentration. Moreover, the original DNA strands that once were long will have become fragmented into short pieces. (Figure 2) Therefore, the relatively long gene regions required for DNA barcoding (ranging from ~500-1000 bases in length) are not intact in most botanical extracts. As a result, attempts to sequence and analyze such material typically would lead to false negative results, as suspected in the recent investigation by the NY AG (see below).

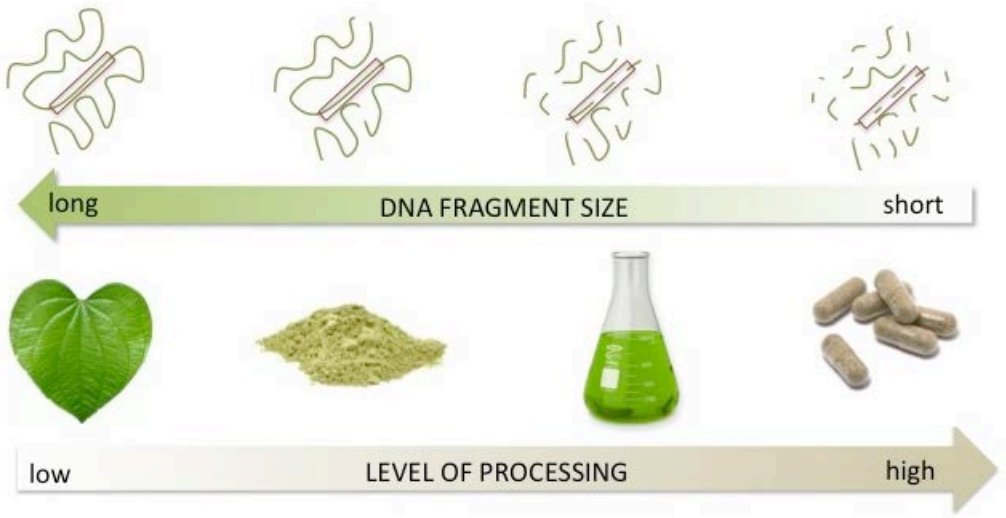

Figure 2. *The relationship between the level of processing of botanical dietary supplements and the DNA fragment size. As materials are more highly processed the DNA becomes more fragmented. The rectangular boxes overlaid on the DNA fragment diagrams represent a DNA barcode region; if the DNA has fragmented within an area targeted by the barcode primers, the DNA will not be detected.*

For finished products containing extracts, instead of typical DNA barcoding methods that require relatively intact long chain DNA, "specific DNA authentication" methods are required that are designed to detect small fragments of DNA (~100-200 bases in length). The method of "specific DNA authentication" has been used successfully in some botanical extracts, oils, and tinctures [26-28]. However, specific DNA authentication methods are not publically available (through peer-reviewed publications) for most commercially used species, including some of those tested in the NY AG study so independently developed and validated methods for these materials would be necessary to assure accurate results.

While specific DNA authentication is useful for some botanical dietary supplements containing extracts, not all botanical extracts contain DNA. Moreover, DNA barcoding—like all DNA methods—is not capable of identifying the chemical constituents or plant parts, nor is it able to quantify the amount of plant material used in the product. Therefore, the use of additional methods (i.e., microscopic and chemical) is necessary to control the overall quality of a botanical dietary supplement and to verify label claims by identifying chemicals of interest, confirming the plant part, and/or ensuring the potency of the product.

All methods, however, have different inherent strengths and weaknesses, especially in more highly processed materials. For most authentication methods, the use of fresh and/or whole plants is preferred to increase the confidence in identification. When this is not practical to obtain, the use of multiple methods on processed materials is necessary to increase the level of confidence in the identity and quality of the material.

How is DNA Testing Performed Correctly?

DNA testing methods, including DNA barcoding, are sensitive to contamination, so it is critical to perform testing in a well-controlled laboratory. Cross-contamination from the laboratory can lead to false positive results, confounding the results and making it impossible to identify the source of the contamination, which can also occur at multiple stages in the harvesting and processing of the botanical material. Moreover, using methods that have not been validated for their intended purpose and on the specific materials tested, can lead to erroneous results. For botanical identification, DNA barcoding is limited to raw materials and requires specific knowledge of botanical taxonomy and relationships of species in order to be performed correctly. It must be executed by experienced plant scientists well versed in the different types of plant materials used by supplement manufacturers (e.g., raw materials vs. extracts vs. finished products in complex matrices).

Below are key areas that must be addressed in order to perform accurate species identification using any DNA method, including DNA barcoding:

1. *Perform Method Validation Specific to the Intended Application:* Before test samples are analyzed using DNA, it is imperative to determine if the species and types of products to be tested (i.e., the botanical extracts or other finished dietary supplements) are fit-for-purpose using DNA for authentication. To accomplish this, multiple representatives of known, authentic materials must be analyzed to determine that the method is capable of providing repeatable, reliable, positive results.

2. *Identify the Most Appropriate Genes*: Because most genes are identical from plant to plant, it is difficult to identify the few regions of the genome that vary enough to identify them. Even if DNA can be detected in a sample, if it is from a gene region that is identical across many species of plants, it is not useful for identification and will provide ambiguous results. Therefore, numerous genes must be tested in order to identify the gene(s) that uniquely occur in one species, and in no other plant species on earth.

3. *Run Positive and Negative Control Samples*: In order to validate results, it is imperative to run sufficient control samples throughout the testing process to rule out false positive and negative results. All steps performed throughout the testing process, from DNA extraction, to PCR, to sequencing, must contain appropriate positive and negative controls.

4. *Follow Strict Quality Control Procedures*: DNA can be found in many materials and on surfaces in the laboratory, including the test samples, the microscopic organisms living in water and reagents, the fungal spores in dust, and even pens, desks, and notebooks. Strict quality control and contamination elimination procedures must be followed in order to avoid erroneous results. Most botanical dietary supplement ingredients are in powder form, which increases the risk of contamination with airborne particles. When contamination has been detected, specific criteria for re-testing must be followed to avoid false positive results.

5. *Use Authentic Reference Sequences*: Once DNA sequences are obtained from test materials, they must be compared to known reference sequences for identification. The best source for DNA sequences is from fresh or dried whole plants, such as those in botanical gardens or plant museums, known as herbaria; whole plants contain the characteristics that botanists need to identify the species with certainty. Without use of reliable reference sequences, identification of unknown test materials may not be accurate. DNA barcoding methods often use a publically available web-based database called GenBank to obtain reference sequences. However, this often leads to inaccurate identification due to the presence of incorrect and missing DNA sequence data in GenBank [29-32].

6. *Understand Acceptable Variation:* Plant species are highly variable, especially in species that have a broad geographic distribution. As a result, the genes within a particular species may not be consistent, even if they are considered the same thing taxonomically and chemically. Therefore, it is important to understand the acceptable variation that exists within the species, so that test samples are not falsely rejected. To do this, multiple reference sequences should be obtained from across the morphological, chemical, and geographical diversity within each species.

7. *Identification Algorithms:* Using an appropriate algorithm for analysis of DNA sequences is extremely important for accurate identification. Specific algorithms should be used that are designed to take into account the specific diagnostic DNA characters for a given species [33-36], rather than on an overall percent similarity as generally used to examine sequences on GenBank, which can be highly misleading.

What Conclusions Can Be Drawn From the NY AG's Investigation?

In February 2015, the New York Attorney General ordered four major dietary supplement retailers to remove products from store shelves based on results of DNA barcoding testing apparently performed in an academic laboratory [2]. The investigation by the NY AG's office concluded that a majority of the products tested lacked the target botanical and/or contained unlabeled species. To date, the NY AG has declined to release for evaluation and peer review the actual test results, the specific methods used, or other details about the test. Although very limited information is available, a review of information contained in the NY AG's press release and the letters to the four retailers provides some important insights into the appropriateness of the testing. Below is a

scientific critique of the investigation, focused on the questions that should be answered in order to make an accurate assessment of the NY AG's study and conclusions:

1. *Were the DNA barcoding methods inappropriately used on extracts?* According to the investigation, which reportedly used DNA barcoding methods, "a large number of the tests did not reveal any DNA from a botanical substance of any kind" [2]. However, as discussed previously, DNA in botanical dietary supplements containing extracts is of relatively poor quality—or absent altogether. If specific DNA authentication methods were not used targeting short DNA fragments, a high rate of false negative results would have been highly probable. The NY AG has not released for public examination its testing methods; however, because the NY AG reported testing six gene regions across all of the products tested, it is not likely that the investigator used specific methods for each species as would have been appropriate.

2. *Did the laboratory performing the test have sufficient knowledge of the complexities of testing botanical dietary supplements?* As stated earlier in this paper, identifying species, especially in botanical extracts where the DNA has been removed or is highly degraded, is extremely complex and challenging. Specialized training and extensive experience in the field of plant species identification on top of a solid understanding of the various processing and extraction techniques used by each manufacturer are necessary to obtain reliable results from testing botanical dietary supplements. While the scientist hired to conduct the investigation appears to be experienced with DNA analysis of reptiles [37], there is no indication that he has experience with identification of botanicals, especially finished products, nor is there any indication that the laboratory is ISO accredited or certified to perform testing in compliance with federal dietary supplement current good manufacturing practices (cGMPs) or other internationally recognized standards for quality. DNA barcoding cannot be adapted from reptilian biology to authentication of complex botanical products.

3. Were adequate procedures used to avoid contamination of samples in the testing facility? The NY AG study reported that, "contaminants identified include rice, beans, pine, citrus, asparagus, primrose, wheat, houseplant, wild carrot, and others" [4]. Additionally, garlic was identified in a number of samples not listed to contain that species. While rice and wheat are not wholly unexpected fillers in botanical dietary supplements, the widespread discovery of plants not typically known as adulterants such as asparagus, houseplants, and garlic across independent samples from different suppliers suggests the possibility of environmental and/or cross contamination between samples in the laboratory. As discussed previously, it is imperative to have procedures to avoid and eliminate contamination and to retest samples with unexpected results so that results do not represent false positives.

4. Were validated reference sequences and appropriate analytical tools used to identify species? Most of the species under investigation by the NY AG cannot be accurately identified without a proprietary database of reference sequences, as several of the species under investigation—including St. John's wort and *Echinacea*—do not have publically available reference sequences from the genes necessary to identify them. Therefore, using

GenBank to identify *Echinacea* sequences from most commonly used DNA barcoding regions will provide highly ambiguous results, with high percent similarity with plants such as feverfew and sunflower. This is clearly an issue with the NY AG's study, which provided highly ambiguous results including reports of identifying *Allium* (which includes both onion and garlic), as well as legumes (which constitute a wide range of species including soy and pinto beans), as well as houseplant (which could be any number of things).

5. *Were valid conclusions made from the results?* The NY AG made public pronouncements with potentially far-reaching legal and market implications that a majority of the dietary supplement products were devoid of the labeled ingredients and contained a myriad of unlabeled ones. However, these conclusions are not valid for a number of reasons. First, the absence of DNA cannot by itself indicate whether a botanical product originated from a plant, because the DNA can be removed while retaining the phytochemicals from the plant source; additional testing must be employed when no DNA is identified to provide conclusive results. Second, because some DNA testing methods are capable of detecting trace levels of DNA, the amount of plant material that is present cannot be extrapolated (only that it is present in the sample), and the significance of the species found cannot be determined. Moreover, the *source* of the contaminants cannot be concluded—they could have arisen from the lab itself, or from any one of a number of steps in the process from harvest to milling and packaging.

Conclusions

Popular media—through television, radio, and newspapers—have created the widespread perception that DNA testing is the pinnacle of quality and reliability, especially when it comes to identification of humans (e.g., forensic investigations). As a result, society has developed an uncritical—and sometimes, misplaced—acceptance of the ability of DNA testing to be 100% reliable, no matter how it is performed and who performs it. However, the fact is that we would not expect DNA testing of humans to be performed by a botanist. The same holds true in DNA testing of botanical dietary supplements; the specific laboratory and equipment, the particular methods used, and the individual scientists all play an important role in the accuracy and validity of DNA test results.

Numerous questions and concerns have been raised regarding the NY AG's investigation, including the specific methods used and the experience of the scientists, and ultimately, the NY AG's conclusions and actions based on the results. Without access to the complete methodology utilized, testing procedures and analytics employed, and the full test results, it is impossible to place any confidence in the announced outcomes of those tests. However, we can assert confidently that the conclusions—that a majority of supplements lacked any botanical at all—based only on the results of DNA barcoding tests were unjustified.

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This scientific white paper was commissioned by four trade associations—the American Herbal Products Association (AHPA); the Consumer Healthcare Products Association (CHPA); the Council for Responsible Nutrition (CRN); and the United Natural Products Alliance (UNPA)—representing the dietary supplement industry.