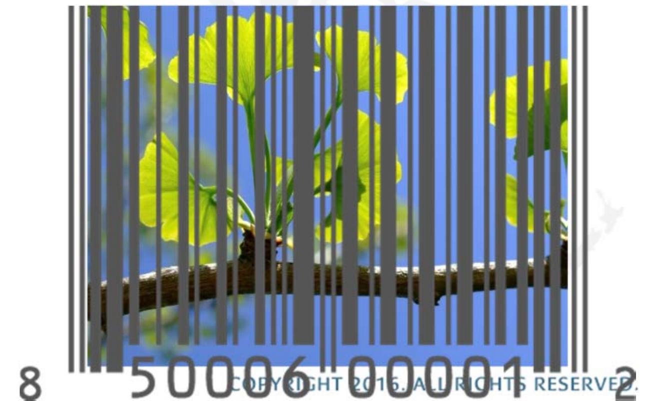
USP Dietary Supplements Stakeholder Forum
Wednesday, June 1, 2016

DNA-Based Methods for Botanical Identification

Nandakumara Sarma, PhD, RPh.



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Workshop on DNA Methods for Quality Control of Botanical Products

Co-sponsored by USP & USDA

October 23-24, 2014
USP Headquarters, Rockville, Maryland

The main messages:

- DNA-based methods could be used to comply with regulatory requirements for identification of botanical articles if “validated”
- Use orthogonal chemical methods to complement the DNA-based methods
- Multiple reference databases, need for communication between the databases.

http://www.usp.org/sites/default/files/usp_pdf/EN/dietarySupp/newsletter/dna-workshop.pdf

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STIMULI TO THE REVISION PROCESS

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DNA-Based Methods for Authentication of Articles of Botanical Origin—Compendial Applications

Christopher Okunji,[a-d] Gabriel I Giancaspro,[a] Nandakumara Sarma,[a] Danica Harbaugh Reynaud,[b] Damon P Little[c]

ABSTRACT The suitability of DNA markers for providing unequivocal identification of botanicals and for detecting adulterants has become a growing area of research with implications for industry and compendial science. Molecular markers have been exploited as diagnostic tools to identify complex ingredients used in foods and dietary supplements, including raw materials, extracts, vegetable oils, and other products. The rising global demand for botanicals and the wide variety of items available to consumers have led to increases in adulteration and substitution. In turn, these practices have been linked to economic losses and diminished consumer confidence, which are serious concerns for both the dietary supplement industry and consumers. A variety of DNA-based methods have potential for authentication of foods and dietary supplements, including both raw materials and finished products. These tools vary in their complexity and cost. Recently, DNA-based methods such as DNA barcoding, which uses short sequences of specific plastid loci, have been developed for identification of plant species and also can be used to identify botanical materials. This *Stimuli* article provides a summary of DNA-based methods for botanical authentication and adulterant detection and discusses the pros and cons of each method for compendial application. These methods may be adopted in *USP* as a general chapter with applications to botanical dietary supplement monographs.

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DNA-BASED METHODS FOR AUTHENTICATION OF ARTICLES OF BOTANICAL ORIGIN

Because morphological identification often is not possible when the original plant material consists of dried, cut and shifted, or processed plant parts or when the material consists only of a whole, single plant part containing no taxonomic characters, additional identification methods, such as DNA-based identification, often are required for these sample types. DNA-based methods have been shown to be efficient in distinguishing genuine plant materials from adulterants in complex botanical matrixes and can complement traditional botanical identification methods that rely on morphological features or chemistry. In addition, DNA-based methods often are more reliable than traditional methods, especially when applied to single-organ specimens that lack diagnostic taxonomic characters, to powdered materials in which the distinguishing characteristics are no longer visible, or when it is difficult to distinguish among closely related or morphologically similar species.

DNA Barcoding

DNA barcoding is a particular type of DNA sequence-based identification method that uses short sequences of specific nuclear or plastid DNA loci for identification of plant species. The assays rely on comparison of nucleotide sequences from a specific stretch of DNA (DNA sequences or DNA barcode) to perform DNA sequence-based identification. Further, DNA-based methods, such as next-generation sequencing (NGS) technologies, are able to identify multiple species in a mixture, including expected and unexpected species.

Botanical Identification Using DNA (Sanger) Sequencing

The process for botanical identification using DNA (Sanger) sequencing includes marker selection, DNA extraction, polymerase chain reaction (PCR) primers and amplification, DNA sequencing, and comparison with reference materials, as described in the following sections. See *Nucleic Acid-Based Techniques—Extraction, Detection, and Sequencing* ⟨1126⟩, *Nucleic Acid-Based Techniques—Amplification* ⟨1127⟩, and *Nucleic Acid-Based Techniques—Genotyping* ⟨1129⟩ for additional information.

MARKER SELECTION

The chosen sequence must be sufficiently specific to capture any potential primary and adulterant species in the sample but also sufficiently universal to avoid false-negative reactions for closely related species. For example, a species-specific primer is not appropriate for most identification procedures because adulterants cannot be detected and amplification failure can result because of either the absence of the species or degraded DNA. In many cases, a single marker may be sufficient for identification, but multiple markers from different parts of the genome (e.g., plastid or nuclear material) ensure that hybrids can be detected. Typically, regions used for DNA sequence identification range from 100–1500 base pairs in length. Smaller DNA fragments may be less susceptible to DNA degradation.

DNA EXTRACTION

Before amplification of the desired marker can be performed, the total genomic DNA must be extracted. The suitability of a procedure for genomic extraction depends on the starting material and the purity of the DNA required for downstream appli-

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STATE OF NEW YORK
OFFICE OF THE ATTORNEY GENERAL

ERIC T. SCHNEIDERMAN
ATTORNEY GENERAL

DIVISION OF REGIONAL AFFAIRS

February 2, 2015

Michael G. Archbold, CEO
GNC Holdings, Inc.
300 Sixth Avenue
Pittsburgh, Pennsylvania 15222

Certified—Return Receipt Requested

Re: ***CEASE & DESIST NOTIFICATION***
Herbal Plus—GNC Distributed Herbal Dietary Supplements

Dear Mr. Archbold:

This letter constitutes a demand to cease and desist engaging in the sale of adulterated and/or mislabeled herbal dietary supplements, and in particular to immediately stop the sale of five "Herbal Plus" dietary supplements as identified by lot number in the exhibit annexed hereto.

Be advised that the Attorney General is authorized by Executive Law § 63(12) to investigate allegations and prosecute businesses which perpetuate fraud upon consumers or engage in illegality in their business practices. General Business Article 22-b further authorizes this office to redress deceptive business acts and practices and false advertising. Of late, the topic of purity (or lack thereof) in popular herbal dietary supplements has raised serious public health and safety concerns,[1] and also caused this office to take steps to independently assess the validity of industry representations and advertising.

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NY AG observations

Gingko Biloba. Negative. No gingko biloba DNA was identified. The only DNA identified was allium (x5), "oryza"(x4)(commonly known as rice), spruce, and asparagaceae. Nine of the tests revealed no plant DNA whatsoever.

St. John's Wort. Negative. No St. John's Wort DNA was identified. Of the 20-tests performed, only three identified any DNA, and it included allium, oryza, and dracaena (tropical houseplant).

Ginseng: Negative. No ginseng DNA was identified. The testing yielded identification of oryza, dracaena, pinus strobus, wheat/grass, and citrus spp., with 15 of the tests identifying no genetic material at all.

Garlic: Positive. All 20 tests yielded DNA from allium.

Echinacea: Negative. Five tests identified oryza DNA, one other yielded the DNA of pinus or ranunculacae. Fourteen tests detected no plant DNA of any sort in the product labeled Echinacea.

Saw Palmetto: Qualified negative. Only 6 of 20 tests did identify the presence of saw palmetto, but the positive results were principally from one sample. The results did not replicate in the three other samples. One sample demonstrated no plant DNA, another revealed the presence of asparagaceae, and oryza, while a fourth was positive for DNA from the primrose family as well as saw palmetto.

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Considerations for DNA-based identification

- DNA-based methods are useful for botanical ingredient ID
  - High degree of specificity and sensitivity
  - Can distinguish closely related or morphologically similar species
- DNA-based methods are not suitable as the sole basis for ID
  - May not be suitable for botanical extracts, unless validated
  - False negative results from processed ingredients or due to interferences from the matrix
  - False positive results from foreign organic matter naturally occurring in the plant material at low but allowable levels (NMT 2%)
  - No guarantee of appropriate Good Agricultural Practices
  - May be fooled by deliberate addition of small amounts of raw material
- **Suitable as an orthogonal test which measures a unique attribute**

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Considerations for DNA-based identification

- Need: Scientific Validation of DNA Test Methods
  - Inclusion / exclusion panels to assure specificity
  - Demonstrate fitness for the purpose for the ID of an analyte
- Need for reference standards
  - system suitability requirements
- Guidance on sampling methods
- Qualitative vs Quantitative methods

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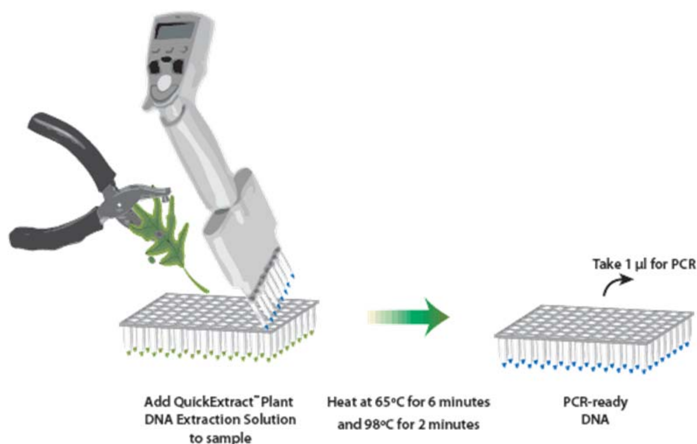
Option 1: Barcode method

Step 1: DNA extraction

Step 2: PCR amplification with universal primers (*psbA-trnH*, *matK*, *rbcL*, *rpoC1*, *ycf5*, ITS2, and ITS)

Step 3: Sequence the PCR product



ACTCGATCGAATTGAGCCTTGGTATGGAAACCTACTAAGTGATAGCTTTCAAATCCAGGGAACCCCGGGATATTTCGAATGGGCAATCCTGA
GCCAAATCCGGTTTACGGAGACATTATTCTCCCAGGAAGAGAAGGGATAGGTGCAGAGACTCGATGGAAGCTATTCTAACGAATAAAGATC
GTTTTACCCAGTACTGTATCTATAGAAAAATCTCTCCATTTACACTTTGGAAGTGGGGTTGGTATATACTACCAAAAAAGATCATGATCAGGA
CTTGGATTGGATCATTTTATGCATTTCACTATGCATTTCACTATTAGTAAGGTAAGATGCTTGGGTCAATCCCAAGTTGAAGGAATTATTTTAC
ATTAAGTAATCCAATTCTGAACTACCCTAAAGAGGGAGTCGGATGAAGTTTGGGAAGAAATGATCGGACGAGGATAAAGATATAGTCCAATT
CTACACGTCAATGCCAACAACAATGCGAATTGCAGTAAGAGGAAAATCCGTCGGCTTTATAGACCGTGAGG

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Option 1: Barcode method

Crude herbal drugs coverage of the DNA barcoding database compared to the coverage of major pharmacopeia worldwide.

Pharmacopeia (references)	No. of species in the DNA barcoding database			No. of crude herbal drugs[a]		
	ITS2	*psbA–trnH*	ITS2 + *psbA–trnH*	In pharmacopeia	In the DNA barcoding database	Percentage (%)[b]
Chinese Pharmacopoeia National Pharmacopoeia Committee (2010)	1037	763	1137	510	505	99.0
Japanese Pharmacopoeia Japanese Pharmacopoeia Editorial Committee (2011)	289	269	362	154	148	96.1
Korean Pharmacopoeia The Korea Food and Drug Administration (2012)	259	182	284	161	159	98.8
Indian Pharmacopoeia Commission IP (2010)	47	36	48	52	50	96.2
U.S. Pharmacopeia U.S. Pharmacopeial Convention (2013)	43	34	45	42	40	95.2
European Pharmacopoeia European Pharmacopoeia Commission (2013)	349	335	441	187	184	98.4

[a] Crude herbal drugs indicate herbal materia medica, which do not include plant extraction, plant preparation, animal and mineral drugs.
[b] Percentage = No. of crude herbal drugs in the DNA barcoding database*100/No. of crude herbal drugs in pharmacopeia.

S. Chen et al. / Biotechnology Advances 32 (2014) 1237–1244

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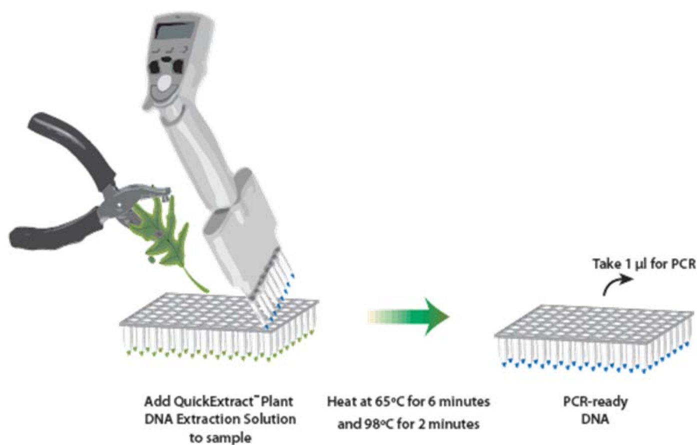
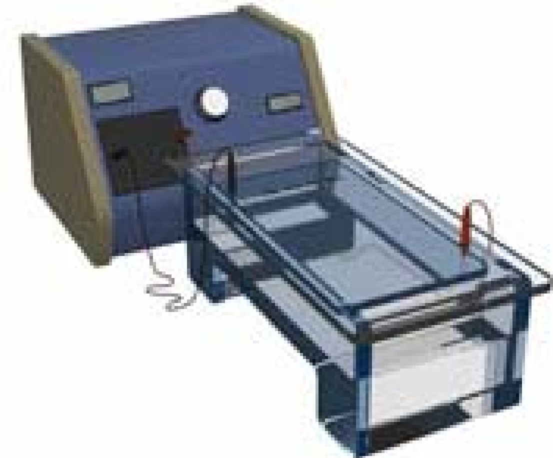
Option 2: PCR-based method

Step 1: Extract DNA

Step 2: PCR amplify with <u>specific</u> validated primers

Step 3: Run Gel electrophoresis and look for specific bands





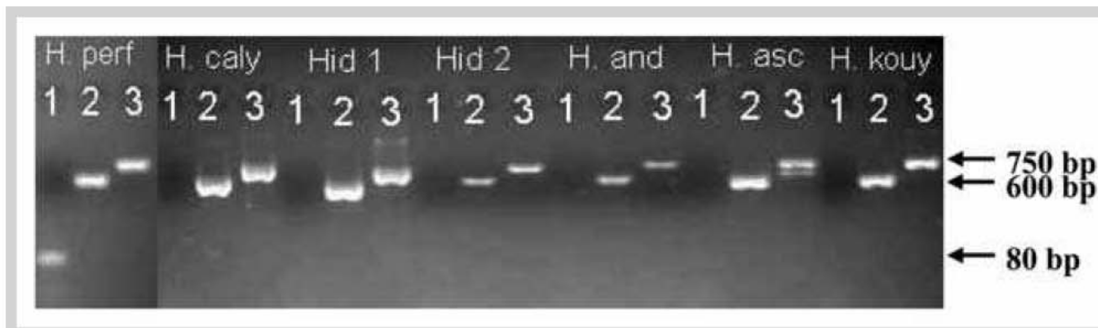


Fig. 3 Fresh leaf DNA samples with primer pairings as indicated by numbered lanes: 1: FO2 and HRI-S; 2: *rpoC* 2 and 4; 3: ITS1 and ITS4. All DNA samples were amplified with both *rpoC* 2 and *rpoC* 4 and ITS1 and ITS4; only the *H. perforatum* sample was amplified with FO2 and HRI-S.

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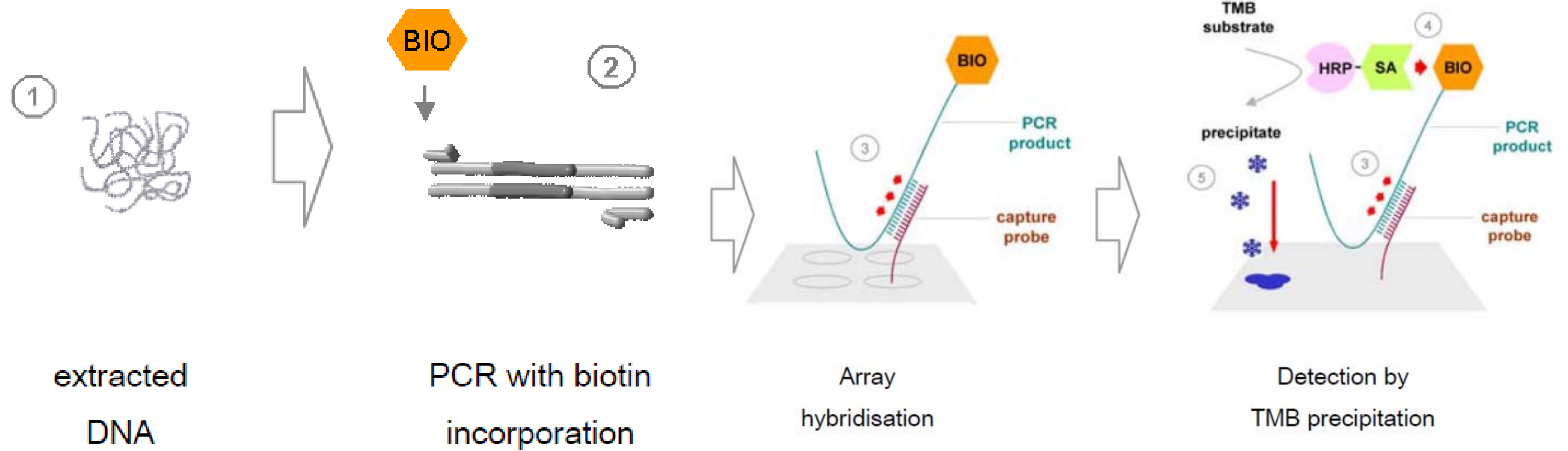
Option 3: Microarray-based method



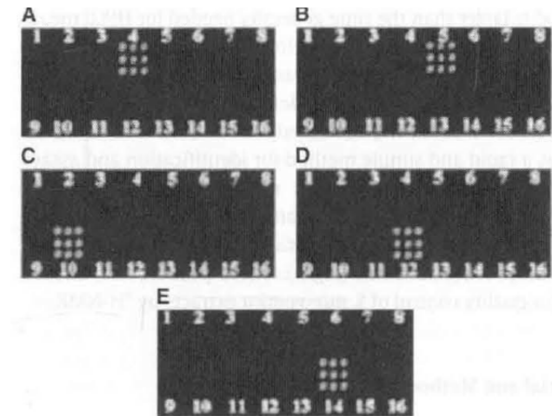


Fig. 1 Identification of Herba Dendrobii by DNA microarray. Panels 1 – 16: spotted ITS1-5.8S-ITS2 sequences from *D. densiflorum*; *D. moniliforme*; *D. chrysotoxum*; *D. loddigesii*; *D. fimbriatum*; *D. jenkinsii*; *D. falconeri*; *D. lindleyi*; *D. primulinum*; *D. nobile*; *D. moschatum*; *D. chrysanthum*; *D. pendulum*; *D. officinale*; *D. lohohense*; *D. crystallinum*. Slides **A – E**: Hybridization of the slides to fluorescent-labeled ITS2 sequences of *D. loddigesii*, *D. fimbriatum*, *D. nobile*, *D. chrysanthum* and *D. officinale*, respectively.

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USP DNA Methods Roundtable, May 26, 2016

Goals and Anticipated Outcomes

- Discuss the issues related to the application of DNA technologies to identification of botanical ingredients
- Discuss criteria for determining the appropriate use of DNA methods for botanical identification purposes.
- Discuss what current DNA technologies are available to address botanical identification and whether or not they meet the criteria determined above.
- Discuss the validation parameters applicable to DNA methods for botanical identification (accuracy, reproducibility and specificity).
- Determine next steps that USP or others should take to incorporate validated DNA methods into USP standards for the identification of botanical articles.
- What other roles can USP play related to DNA methods for botanical identification (database coordination, sequence repository, etc.).

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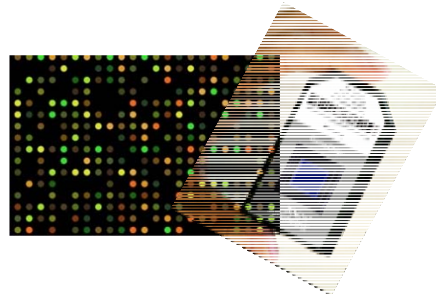
What’s next?

Consideration:

- Introduce DNA-based methods as one of the orthogonal ID tests in select botanical monographs based on science. Method development and validation for ginseng (Asian / American / Tienchi) is underway.

New technologies?



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