Global Expertise
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U.S. Pharmacopeial Convention

DNA Methods for Identification of Botanicals

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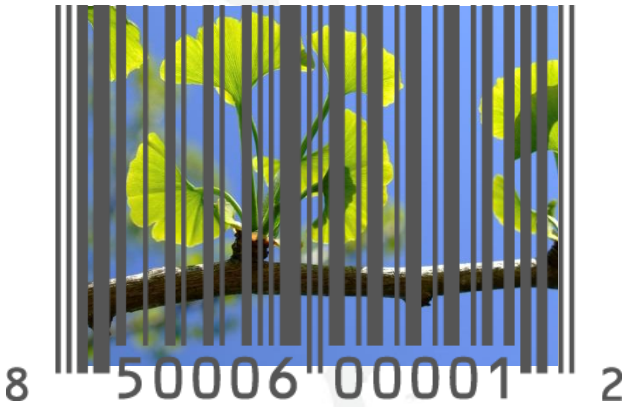


Stakeholder Forum,
June 7, 2017

What did we do so far?

- USP and USDA Workshop on DNA Methods for Quality Control of Botanical Products. October 23 – 24, 2014
- Stimuli article: DNA-Based Methods for Authentication of Articles of Botanical Origin—Compendial Applications. PF 39(5), 2013.
- USP General Chapter <563> Identification of Articles of Botanical Origin

USP Roundtable on DNA Methods for the Identification of Botanicals, May 26, 2016

- Guidelines to appropriately utilize DNA methods
- Library of vouchers of plants (“safe sets”) commonly used in dietary supplements.
- Pilot studies for a set of standard methods for identifying specific botanicals, such as ginseng and others, to include in the compendium.
- Guidance for DNA methods to complement chemical methods.
- Consolidate DNA libraries into a single repository targeted for dietary supplements

http://www.usp.org/sites/default/files/events/stakeholder_forums/2017/face-face-meeting/briefing-material-2017-06-07_0.pdf

USP Stakeholder Forum 2016: What We Heard? DNA-based Methods for Botanical Identification

- DNA testing is an emerging tool of indisputable value. At the current stage of development, nucleic acids techniques are not suitable for regular quality control to determine parts of the plants.
- No single DNA method can fully define a pharmacopeial article; identity must be determined on a case-by-case basis involving orthogonal tests including physical and chemical methods.

USP Stakeholder Forum 2016: What We Heard? DNA-based Methods for Botanical Identification

- USP could take the lead in consolidating information from the various DNA libraries into a single repository targeted for dietary supplements as a reliable resource for researchers and ingredient purchasers. USP could leveraging its experience in other similar library resources supplemental to public standards and explore how to apply that experience to DNA libraries.
- Industry is looking to USP to take a lead role in exploring development of a repository of authenticated plant material that could serve validation purposes in DNA procedures.
- Plants within a single species can be highly variable. Industry is looking for partnerships to ensure that if a DNA method is developed as a standard, it identifies material representative of articles in commerce.

http://www.usp.org/sites/default/files/events/stakeholder_forums/2017/face-face-meeting/briefing-material-2017-06-07_0.pdf

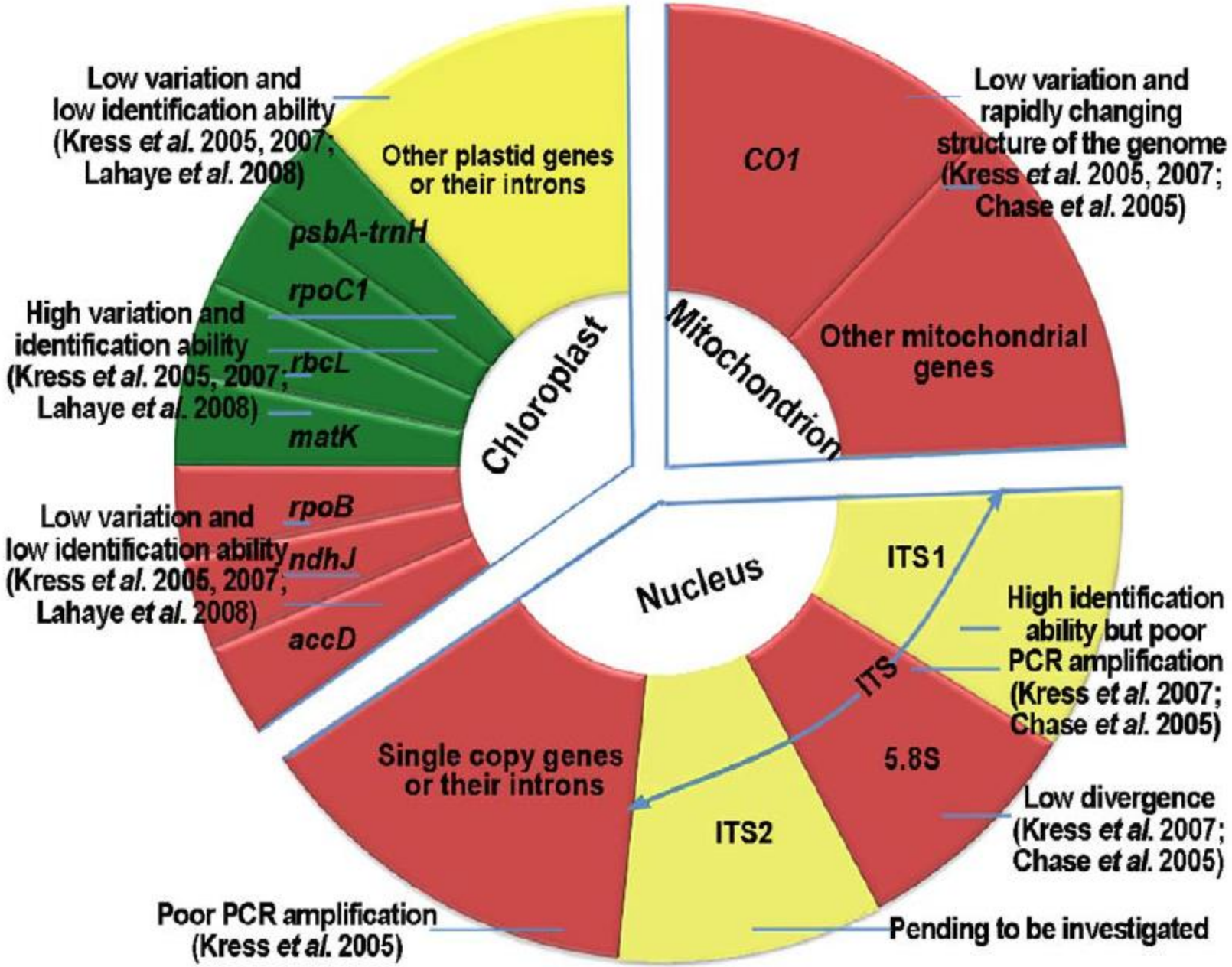


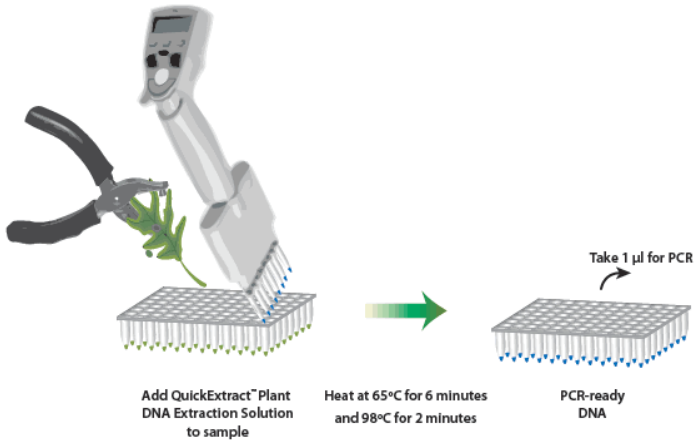
Figure 1. Genes from three genomes in plants that are candidate barcodes. Green markers are potential barcod candidates and yellow markers are pending to be investigated.
doi:10.1371/journal.pone.0008613.g001

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



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CTTGGATTGGATCATTTTATGCATTTCACTATGCATTTCACTATTAGTAAGGTAAGATGCTTGGGTCAATCCCAAGTTGAAGGAATTATTTTAC
ATTAAGTAATCCAATTCTGAACTACCCTAAAGAGGGAGTCGGATGAAGTTTGGGAAGAAATGATCGGACGAGGATAAAGATATAGTCCAATT
CTACACGTCAATGCCAACAACAATGCGAATTGCAGTAAGAGGAAAATCCGTCGGCTTTATAGACCGTGAGG

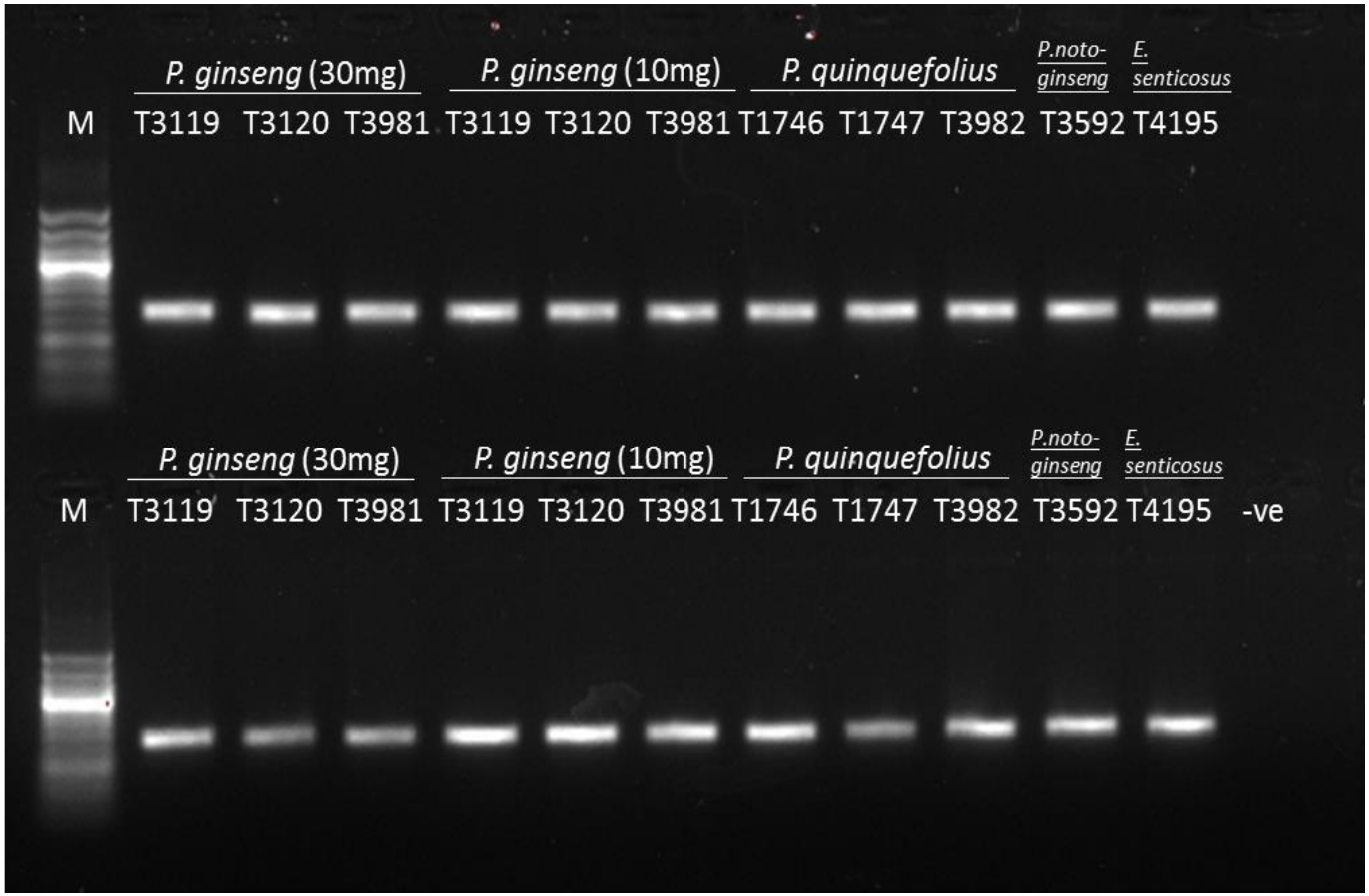
DNA barcoding

- **DNA Extraction**
  - CTAB method or DNeasy Plant Mini Kit.
  - DNA quality check by NanoDrop or by capillary electrophoresis
- **PCR Amplification**
  - Polymerase buffer (1x), MgCl2 (2.5 mM), thermophilic DNA polymerase (1 Unit), Primers (0.1µM each), dNTPs (0.1µM each), DNA (10 ng may be suitable) and molecular grade water.
- **Primer Sequence** (5′ to 3′)
  - ITSP3 (C/T) GACTCTCGGCAACGGATA
  - ITSE4 (A/G) GTTTCTTTCCTCCGCTTA
- **Cycling programme**
  - Initialization step of 5 minutes at 94°C;
  - 35 cycles consisting of: 30s at 94°C; 30s at 56°C; 45s at 72°C; Final elongation step of 10 minutes at 72° C;
- **PCR product purification**: Qiagen PCR purification kit or Biomed DNA Gel Extraction Kit
- **Sequencing**
  - 3730XL sequencer (Applied Biosystems, USA); sequences assembled using CodonCode Aligner 3.0 (CodonCode Co., USA).
- **Species Identification**
  - http://www.tcmbarcode.cn; MMDBD database; GenBank

PCR products from amplification with ITS2 primers

DNeasy Plant Mini Kit

CTAB method



ITS2 retrieved sequence

Panax ginseng

CGCATCGCGTCGCCCCCCAACCCATCACTCCCTTGCGGGAGTTGAGGCGGAGGGG
CGGATAATGGCCTCCCGTGTCTCACCGCGCGGTTGGCCCAAATGCGAGTCCTTGGC
GATGGACGTCACGACAAGTGGTGGTTGTAAAAAGCCCTCTTCTCATGTCGTGCGGTG
ACCCGTCGCCAGCAAAAGCTCTCATGACCCTGTTGCGCCGTCCTCGACGTGCGCTC
CGACCG

Panax quinquefolius

CGCATCGCGTCGCCCCCCAACCCATCACTCCTTTGCGGGAGTCGAGGCGGAGGGG
CGGATAATGGCCTCCCGTGTCTCACCGCGCGGTTGGCCCAAATGCGAGTCCTTGGC
GATGGACGTCACGACAAGTGGTGGTTGTAAAAAGCCCTCTTCTCATGTCGTGCGGTG
ACCCGTCGCCAGCAAAAGCTCTCATGACCCTGTTGCGCCGTCCTCGACGTGCGCTC
CGACCGCG

Panax notoginseng

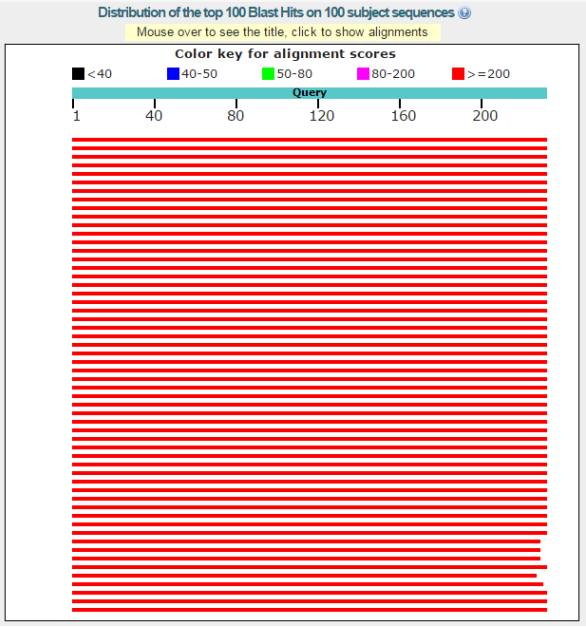
CGCATCGCGTCGCCCCCCAACCCATCATTCCCTCGCGGGAGTCGATGCGGAGGGG
CGGATAATGGCCTCCCGTGTCTCACCGCGCGGTTGGCCCAAATGCGAGTCCTTGGC
GATGGACGTCACGACAAGTGGTGGTTGTTAAAAGCCCTCTTCTCATGTCGTGCGGTG
ACCCGTCGCCAGCAAAAGCTCTCATGACCCTGTTGCGCTGTCCTCGACGCGCGCTC
CGACCGCGACCC

BLAST of the *Panax ginseng*

Sequences producing significant alignments:

Select: All None Selected:0

Alignments Download GenBank Graphics Distance tree of results



Description	Max score	Total score	Query cover	E value	Ident	Accession
Panax ginseng voucher PH05145 internal transcribed spacer 2, partial sequence	425	425	100%	2e-115	100%	KX674877.1
Panax ginseng voucher PH05144 internal transcribed spacer 2, partial sequence	425	425	100%	2e-115	100%	KX674876.1
Panax ginseng voucher PH05137 internal transcribed spacer 2, partial sequence	425	425	100%	2e-115	100%	KX674875.1
Panax ginseng voucher S-RShen-biaozhun internal transcribed spacer 2, partial sequence	425	425	100%	2e-115	100%	KX302822.1
Panax ginseng voucher S-RShen-19 internal transcribed spacer 2, partial sequence	425	425	100%	2e-115	100%	KX302821.1
Panax ginseng voucher S-RShen-18 internal transcribed spacer 2, partial sequence	425	425	100%	2e-115	100%	KX302820.1
Panax ginseng voucher S-RShen-15 internal transcribed spacer 2, partial sequence	425	425	100%	2e-115	100%	KX302819.1
Panax ginseng voucher S-RShen-14 internal transcribed spacer 2, partial sequence	425	425	100%	2e-115	100%	KX302818.1
Panax ginseng voucher S-RShen-13 internal transcribed spacer 2, partial sequence	425	425	100%	2e-115	100%	KX302817.1
Panax ginseng voucher S-RShen-07 internal transcribed spacer 2, partial sequence	425	425	100%	2e-115	100%	KX302815.1
Panax ginseng voucher S-RShen-06 internal transcribed spacer 2, partial sequence	425	425	100%	2e-115	100%	KX302754.1
Panax ginseng voucher S-RShen-01 internal transcribed spacer 2, partial sequence	425	425	100%	2e-115	100%	KX302753.1
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Panax ginseng isolate PB020MT2 internal transcribed spacer 2, partial sequence	425	425	100%	2e-115	100%	KT285156.1
Panax ginseng isolate PB020MT1 internal transcribed spacer 2, partial sequence	425	425	100%	2e-115	100%	KT285155.1
Panax ginseng isolate PB020MT6 internal transcribed spacer 2, partial sequence	425	425	100%	2e-115	100%	KT285106.1
Panax ginseng isolate PB020MT5 internal transcribed spacer 2, partial sequence	425	425	100%	2e-115	100%	KT285105.1
Panax ginseng isolate PB020MT4 internal transcribed spacer 2, partial sequence	425	425	100%	2e-115	100%	KT285104.1
Panax ginseng voucher T3981 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene, complete sequence; and internal transcribed spacer 2, partial sequence	425	425	100%	2e-115	100%	KU886053.1
Panax ginseng cultivar Chunpoong 18S ribosomal RNA gene, internal transcribed spacer 1, 5.8S ribosomal RNA gene, internal transcribed spacer 2, and 26S ribosomal RNA gene, complete sequence	425	425	100%	2e-115	100%	KM036295.1
Panax ginseng cultivar Panax ginseng landrace Jakyung 18S ribosomal RNA gene, internal transcribed spacer 1, 5.8S ribosomal RNA gene, internal transcribed spacer 2, and 28S ribosomal RNA gene, complete sequence	425	425	100%	2e-115	100%	KM207674.1
Panax ginseng cultivar Panax ginseng landrace Hwangsook 18S ribosomal RNA gene, internal transcribed spacer 1, 5.8S ribosomal RNA gene, internal transcribed spacer 2, and 28S ribosomal RNA gene, complete sequence	425	425	100%	2e-115	100%	KM207673.1
Panax ginseng cultivar Sunun 18S ribosomal RNA gene, internal transcribed spacer 1, 5.8S ribosomal RNA gene, internal transcribed spacer 2, and 28S ribosomal RNA gene, complete sequence	425	425	100%	2e-115	100%	KM207672.1
Panax ginseng cultivar Sunpoong 18S ribosomal RNA gene, internal transcribed spacer 1, 5.8S ribosomal RNA gene, internal transcribed spacer 2, and 28S ribosomal RNA gene, complete sequence	425	425	100%	2e-115	100%	KM207671.1
Panax ginseng cultivar Sunone 18S ribosomal RNA gene, internal transcribed spacer 1, 5.8S ribosomal RNA gene, internal transcribed spacer 2, and 28S ribosomal RNA gene, complete sequence	425	425	100%	2e-115	100%	KM207670.1
Panax ginseng cultivar Sunhyang 18S ribosomal RNA gene, internal transcribed spacer 1, 5.8S ribosomal RNA gene, internal transcribed spacer 2, and 28S ribosomal RNA gene, complete sequence	425	425	100%	2e-115	100%	KM207669.1
Panax ginseng cultivar Kopoong 18S ribosomal RNA gene, internal transcribed spacer 1, 5.8S ribosomal RNA gene, internal transcribed spacer 2, and 28S ribosomal RNA gene, complete sequence	425	425	100%	2e-115	100%	KM207668.1
Panax ginseng cultivar Gumpoong 18S ribosomal RNA gene, internal transcribed spacer 1, 5.8S ribosomal RNA gene, internal transcribed spacer 2, and 28S ribosomal RNA gene, complete sequence	425	425	100%	2e-115	100%	KM207667.1
Panax ginseng cultivar Cheongsun 18S ribosomal RNA gene, internal transcribed spacer 1, 5.8S ribosomal RNA gene, internal transcribed spacer 2, and 28S ribosomal RNA gene, complete sequence	425	425	100%	2e-115	100%	KM207666.1
Panax ginseng voucher HI-R3 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene, complete sequence; and internal transcribed spacer 2, partial sequence	425	425	100%	2e-115	100%	KP265318.1

Sequence alignment of *Panax ginseng, P. quinquefolius* and *P. notoginseng*

Sequence ID: Query_67417 Length: 232 Number of Matches: 1

Range 1: 1 to 230 Graphics ▼ Next Match ▲ Previous Match

Score	Expect	Identities	Gaps	Strand
414 bits(224)	7e-121	228/230(99%)	0/230(0%)	Plus/Plus

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Query  1    CGCATCGCGTCGCCCCCCAACCCATCACTCCCTTGCGGGAGTTGAGGCGGAGGGGCGGAT  60
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Sbjct  1    CGCATCGCGTCGCCCCCCAACCCATCACTCCTTTGCGGGAGTCGAGGCGGAGGGGCGGAT  60

Query  61   AATGGCCTCCCGTGTCTCACCGCGCGGTTGGCCCAAATGCGAGTCCTTGGCGATGGACGT  120
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  61   AATGGCCTCCCGTGTCTCACCGCGCGGTTGGCCCAAATGCGAGTCCTTGGCGATGGACGT  120

Query  121  CACGACAAGTGGTGGTTGTAAAAAGCCCTCTTCTCATGTCGTGCGGTGACCCGTCGCCAG  180
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  121  CACGACAAGTGGTGGTTGTAAAAAGCCCTCTTCTCATGTCGTGCGGTGACCCGTCGCCAG  180

Query  181  CAAAAGCTCTCATGACCCTGTTGCGCCGTCCTCGACGTGCGCTCCGACCG  230
            ||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  181  CAAAAGCTCTCATGACCCTGTTGCGCCGTCCTCGACGTGCGCTCCGACCG  230
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Sequence ID: Query_35561 Length: 236 Number of Matches: 1

Range 1: 1 to 230 Graphics ▼ Next Match ▲ Previous Match

Score	Expect	Identities	Gaps	Strand
387 bits(209)	2e-112	223/230(97%)	0/230(0%)	Plus/Plus

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Query  1    CGCATCGCGTCGCCCCCCAACCCATCACTCCCTTGCGGGAGTTGAGGCGGAGGGGCGGAT  60
            ||||||||||||||||||||||||||||| ||||| ||||||||| || ||||||||||||||
Sbjct  1    CGCATCGCGTCGCCCCCCAACCCATCATTCCCTCGCGGGAGTCGATGCGGAGGGGCGGAT  60

Query  61   AATGGCCTCCCGTGTCTCACCGCGCGGTTGGCCCAAATGCGAGTCCTTGGCGATGGACGT  120
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
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Query  121  CACGACAAGTGGTGGTTGTAAAAAGCCCTCTTCTCATGTCGTGCGGTGACCCGTCGCCAG  180
            |||||||||||||||||||||||||||||| |||||||||||||||||||||||||||||||
Sbjct  121  CACGACAAGTGGTGGTTGTTAAAAGCCCTCTTCTCATGTCGTGCGGTGACCCGTCGCCAG  180

Query  181  CAAAAGCTCTCATGACCCTGTTGCGCCGTCCTCGACGTGCGCTCCGACCG  230
            |||||||||||||||||||||||||||||| |||||||||| ||||||||||
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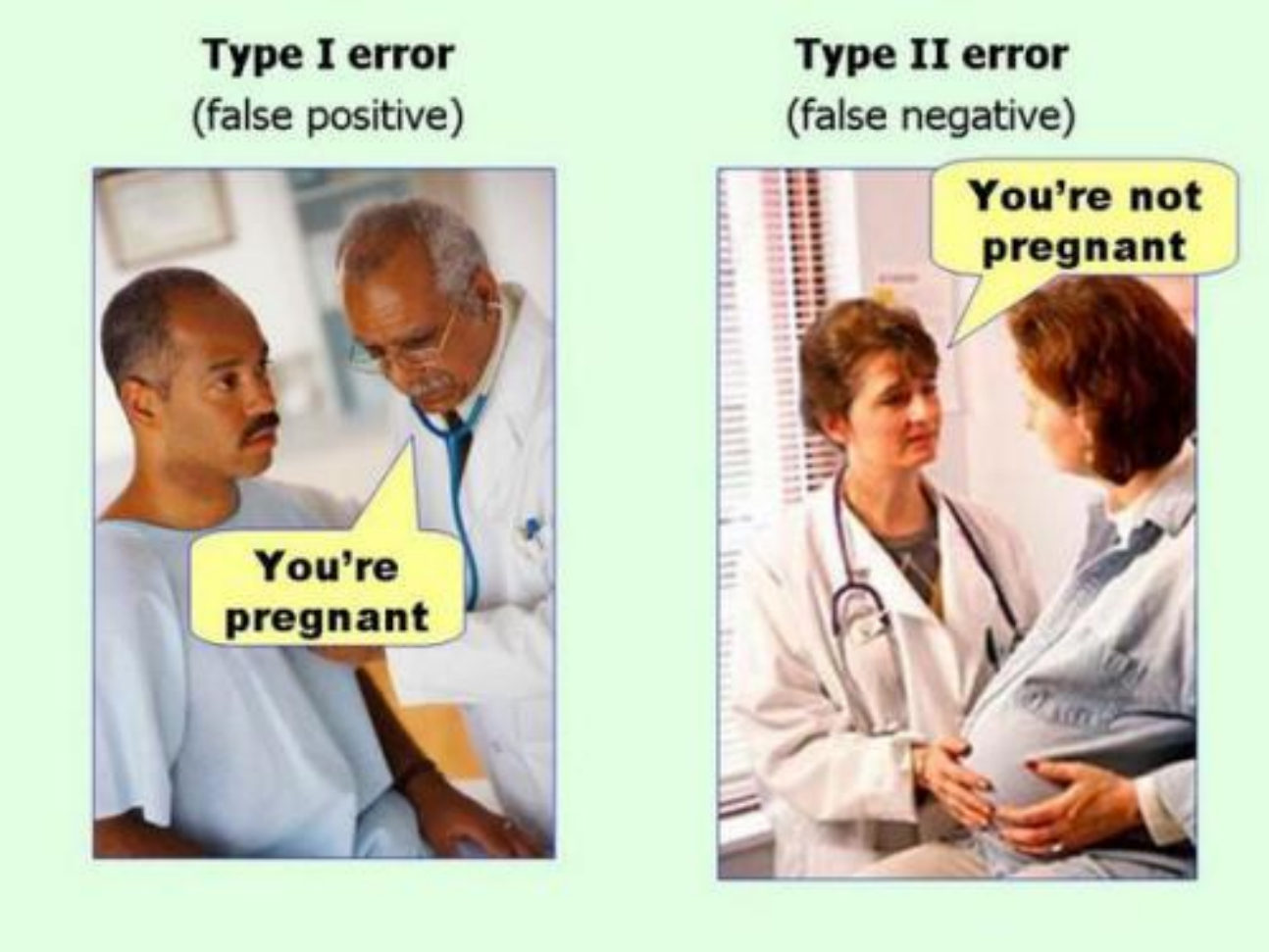
False? Positive?



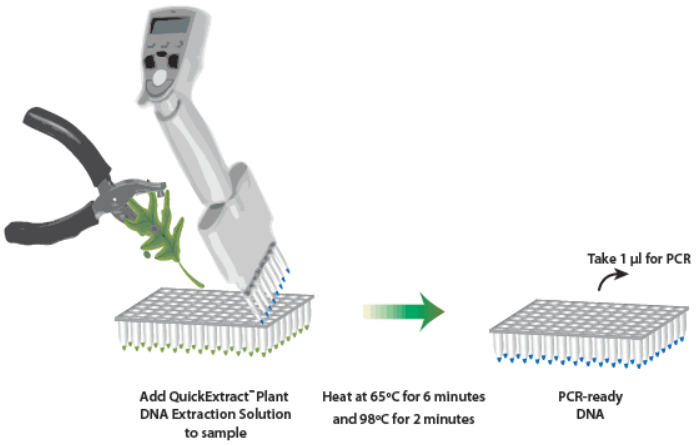
Applied Statistics Lesson and Humour of the Day – Type I Error (False Positive) and Type 2 Error (False Negative)

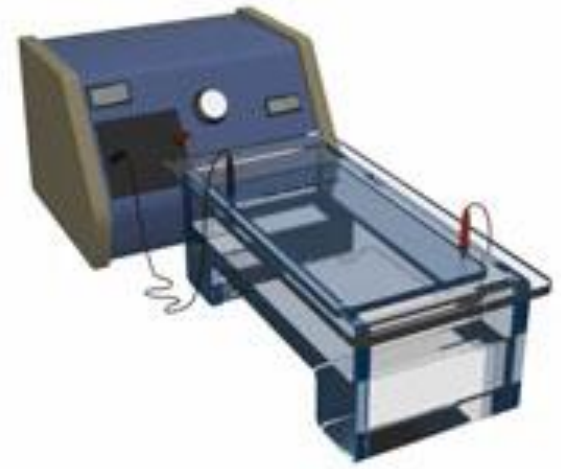
Option 2: PCR-based method

Step 1: Extract DNA

Step 2: PCR amplify with specific validated primers

Step 3: Run Gel electrophoresis and look for specific bands





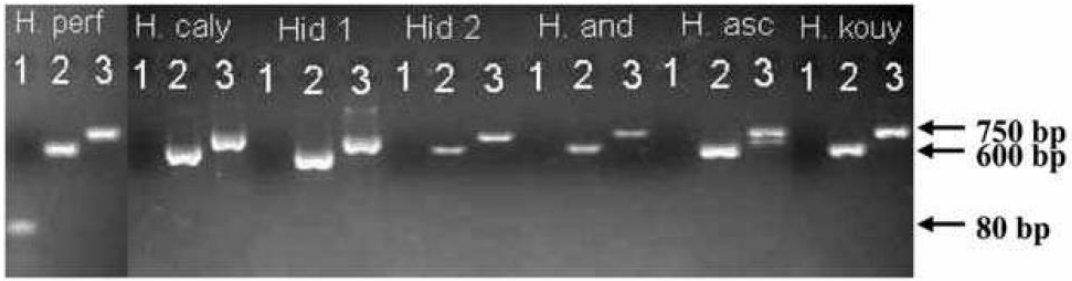
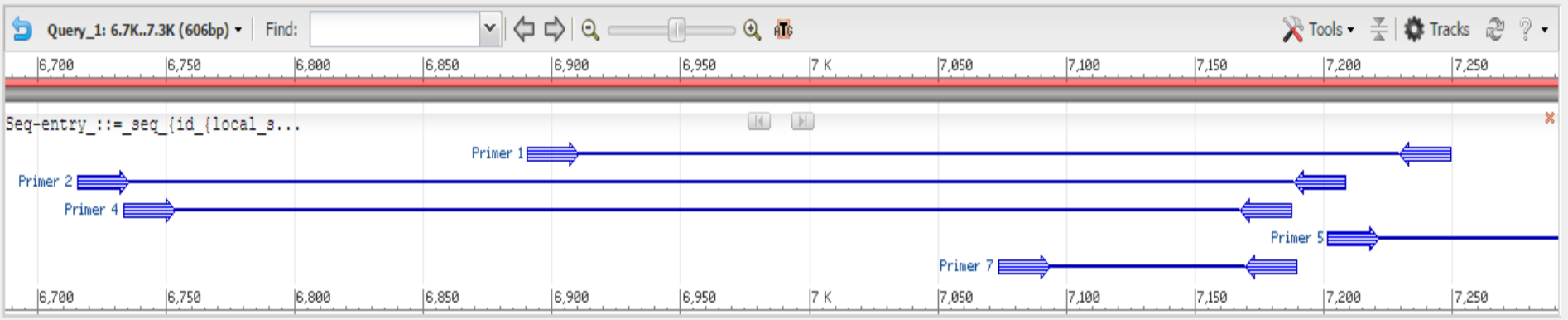


Fig. 3 Fresh leaf DNA samples with primer pairings as indicated by numbered lanes: 1: FO2 and HRI-S; 2: *rpoC* 2 and 4; 3: ITS1 and ITS4. All DNA samples were amplified with both *rpoC* 2 and *rpoC* 4 and ITS1 and ITS4; only the *H. perforatum* sample was amplified with FO2 and HRI-S.

Design specific primers



Primer pair 1

	Sequence (5'->3')	Template strand	Length	Start	Stop	Tm	GC%	Self complementarity	Self 3' complementarity
Forward primer	CATGTGTGAGGCGAAGAGGT	Plus	20	6891	6910	60.04	55.00	4.00	0.00
Reverse primer	GTGTCGAGAAGGGCCATTGA	Minus	20	7249	7230	60.04	55.00	6.00	2.00
Product length	359								

Primer pair 2

	Sequence (5'->3')	Template strand	Length	Start	Stop	Tm	GC%	Self complementarity	Self 3' complementarity
Forward primer	TTAACGAGAGTGTGGGCGTC	Plus	20	6716	6735	60.04	55.00	4.00	2.00
Reverse primer	GTCGACCACAAACGGCTTTC	Minus	20	7208	7189	60.04	55.00	6.00	1.00
Product length	493								

Primer pair 3

	Sequence (5'->3')	Template strand	Length	Start	Stop	Tm	GC%	Self complementarity	Self 3' complementarity
Forward primer	CGCGACGGGGTATTGTAAGA	Plus	20	5735	5754	59.90	55.00	4.00	2.00
Reverse primer	TCAATGGGCAGGTTAGCTGG	Minus	20	6095	6076	60.03	55.00	5.00	3.00
Product length	361								

Next steps

- PHASE I - CREATE LIBRARY
- PHASE II - ESTABLISH PROOF OF CONCEPT; COLLABORATIVE STUDIES
- PHASE III - DETERMINE THE PRODUCT OFFERING

9. Advisory Stakeholder Forums and Project Teams

9.01 Formation

- Formed by CoE Chairperson

9.02 General

- Members serve as representatives of an organization, company, or service provider - **advisory only**

9.03 Stakeholder Forums

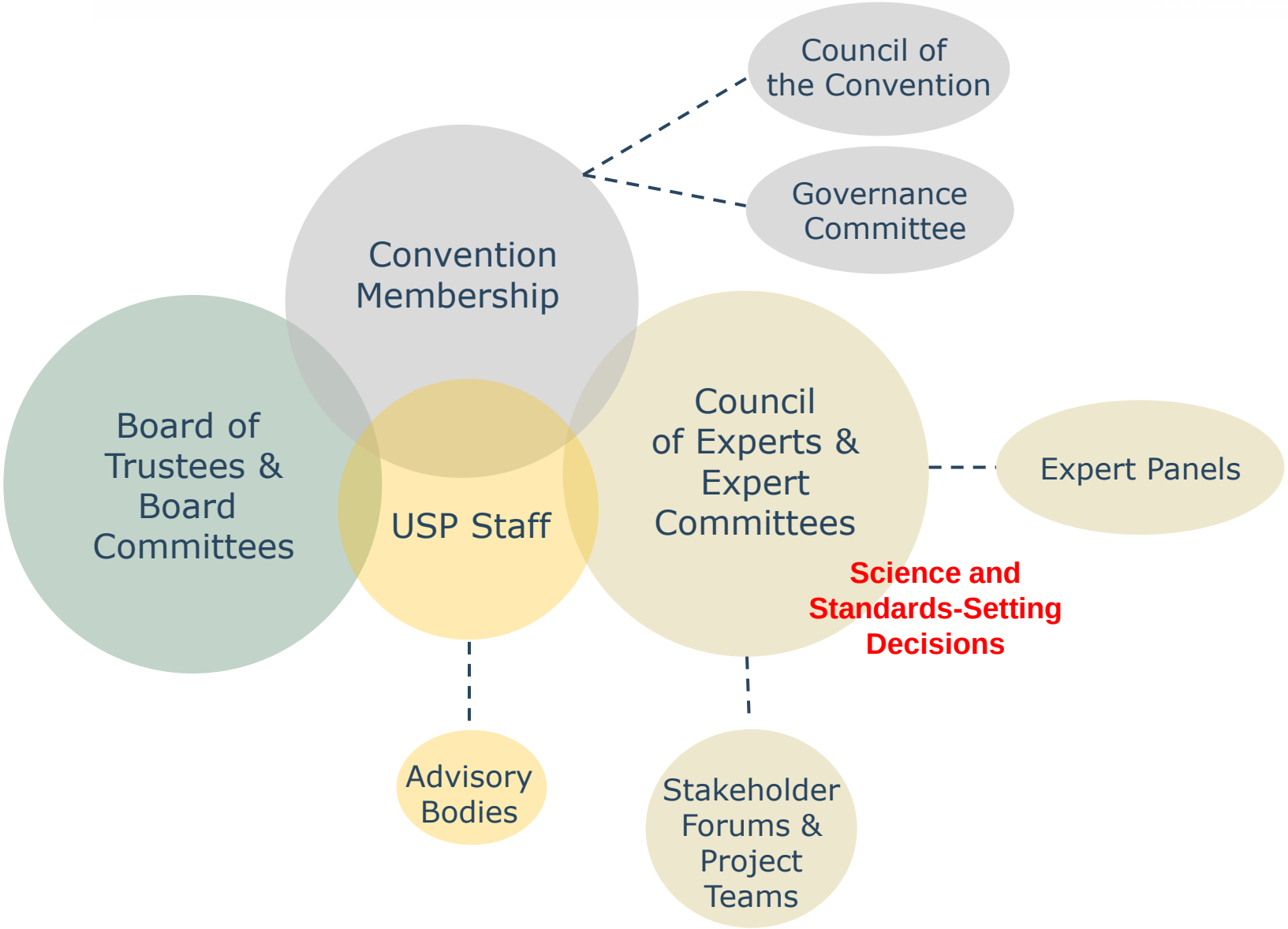
- Formed by CoE Chairperson to enable an exchange of information and perspectives with the ultimate goal of improving USP standards and information

9.04 Project Teams

- Generally formed by CoE Chairperson to address a specific compendial topic (primarily process-oriented) for a particular Stakeholder Forum

https://www.usp.org/sites/default/files/usp_pdf/EN/USPNF/2016-02-01_2015-2020_coe_rules_and_procedures-final.pdf

USP Governing and Advisory Bodies



Project Team

Charge: To provide industry inputs and research collaboration to help USP establish a sample repository (library) for most commonly used botanicals.

These inputs help USP:

- Develop scientifically valid uniform public standards and methodologies (USP monographs or General Chapters) to authenticate botanicals by DNA-based identification methods
- Establish public genetic standards libraries, and
- Establish appropriate Reference Standards based on botanical raw materials or nucleic acid.

Thank You