National Institute of Standards & Technology

Report of Investigation

Reference Material 8650

Ground Kudzu (*Pueraria montana* var. *lobata*) Rhizome

This Reference Material (RM) is intended primarily for use in evaluating analytical methods for the determination of isoflavones and elements in kudzu rhizomes and similar matrices. RM 8650 provides a common matrix to those in the botanical supplements community who may wish to conduct research studies. This RM has also been characterized for its DNA sequence. A unit of RM 8650 consists of five packets, each containing approximately 3 g of kudzu rhizome powder.

The development of RM 8650 was a collaboration among the National Institute of Standards and Technology (NIST), the National Institutes of Health Office of Dietary Supplements (NIH ODS), and the Food and Drug Administration Center for Drug Evaluation and Research (FDA CDER). The addition of genetic information was accomplished through collaboration among NIST, NIH ODS, the U.S. Department of Agriculture Agricultural Research Service (USDA ARS), NSF International (formerly AuthenTechnologies®, Richmond, CA), and American Herbal Pharmacopoeia (Scotts Valley, CA).

Taxonomic Identification: The taxonomic identity is *Pueraria montana* var. *lobata* established through identification by a trained botanist using an herbarium specimen from the original material and from associated DNA sequence analysis from botanically authenticated *Pueraria montana* var. *lobata*. The associated DNA sequences are available in companion FASTA-formatted files [1]. The uncertainty associated with each nucleotide in the sequence, and in turn the uncertainty associated with the DNA sequence as an identifier of species, is expressed in an ordinal scale that represents the confidence estimates of the assigned value (Tables 1 and 2) [2]. These DNA sequences are used as a source of identity data for *Pueraria montana* var. *lobata*.

Reference Mass Fraction Values: Reference mass fraction values of isoflavones and elements in RM 8650, reported on a dry-mass basis, are provided in Tables 3 and 4. A NIST reference value is a noncertified value that is the best estimate of the true value based on available data; however, the value does not meet the NIST criteria for certification [3] and is provided with an associated uncertainty that may not include all sources of uncertainty. The reference values in this material are the means of measurements provided by NIST using one technique, and the associated uncertainty values represent a 95 % level of confidence [4–6].

Expiration of Value Assignment: RM 8650 is valid, within the measurement uncertainty specified, until **30 June 2029**, provided the RM is handled and stored in accordance with the instructions given in this Report of Investigation (see "Instructions for Storage and Use"). The report is nullified if the RM is damaged, contaminated, or otherwise modified.

Maintenance of RM: NIST will monitor this RM over the period of its validity. If substantive technical changes occur that affect the value assignments before the expiration of this report, NIST will notify the purchaser. Registration (see attached sheet or register online) will facilitate notification.

Coordination of the technical measurements leading to the issuance of this RM was performed by C.A. Rimmer and L.J. Wood of the NIST Chemical Sciences Division.

Support for the development of RM 8650 was provided in part by NIH ODS and FDA CDER.

Carlos A. Gonzalez, Chief
Chemical Sciences Division

Gaithersburg, MD 20899
Report Issue Date: 18 May 2021

Steven J. Choquette, Director
Office of Reference Materials

Analytical measurements at NIST were performed by C.A. Barber and L.J. Wood of the NIST Chemical Sciences Division and M.R. Ale, K.D. Chieh, and J.A. Lippert, formerly of NIST.

Statistical analysis was provided by J.H. Yen of the NIST Statistical Engineering Division.

Support aspects involved in the issuance of this RM were coordinated through the NIST Office of Reference Materials.

NOTICE AND WARNING TO USERS: RM 8650 IS INTENDED FOR RESEARCH USE; NOT FOR HUMAN CONSUMPTION.

INSTRUCTIONS FOR STORAGE AND USE

Storage: The RM should be stored at controlled room temperature (20 °C to 25 °C) in the original unopened packet until required for use. For elemental analyses, the packet can be opened and resealed; test portions can be removed and analyzed until the material reaches its expiration date. For isoflavone analyses, the packet can be resealed, stored under refrigeration, and test portions removed and analyzed for one week after the packet was initially opened.

Use: Before use, the contents of a packet of material should be mixed thoroughly. To relate analytical determinations to the values in this Report of Investigation, the following masses used for NIST analyses should be used as the minimum sample size to ensure valid results: 0.4 g for elements and 10 mg for isoflavones (see "Source and Preparation" below). Test portions should be analyzed as received and results converted to a dry-mass basis. The moisture conversion factor given below (see "Determination of Moisture") can be used for the sample(s) when using an unopened packet for the first time. If using a previously opened and resealed packet, moisture must be determined using one of the recommended techniques described below. Analytical results should include their own estimates of uncertainty and can be compared to the reference values using procedures described in reference 7.

Determination of Moisture: Moisture content of RM 8650 was determined at NIST by (1) drying over magnesium perchlorate in a desiccator at room temperature for 21 d and (2) drying for 3 h in a forced-air oven at 80 °C. The means from both techniques were averaged to determine a dry-mass proportion of (0.9421 ± 0.0033) gram dry-mass per gram as-received mass; the uncertainty shown on this value is an expanded uncertainty to represent a 95 % level of confidence. The conversion factor used to convert data from an as-received to a dry-mass basis is the inverse of the dry-mass proportion. A relative uncertainty component of 0.2 % for the conversion factor obtained from the moisture measurements is incorporated in the uncertainties of the assigned values, reported on a dry-mass basis, that are provided in this report.

SOURCE, PREPARATION AND ANALYSIS[(1)]

Source and Preparation: The material for production of RM 8650 is a kudzu rhizome powder. The original plant material was harvested from Boone County, WV by a trained botanist. The powdered material was transferred to High-Purity Standards (Charleston, SC) where it was blended, aliquoted, and heat-sealed inside nitrogen-flushed 4 mil polyethylene bags, which were then sealed inside nitrogen-flushed aluminized plastic bags along with two packets of silica gel each. Following packaging, RM 8650 was irradiated (Neutron Products, Inc., Dickerson, MD) to an absorbed dose of 6.8 kGy to 8.5 kGy.

Analytical Approach for Determination of Isoflavones*:* Value assignment of the mass fraction of puerarin in RM 8650 was based on measurements provided by NIST using liquid chromatography with ultraviolet absorbance detection (LC/UV-absorbance). Value assignment of the mass fractions of daidzin, and daidzein in RM 8650 was based on measurements provided by NIST using liquid chromatography with mass spectrometry detection (LC-MS).

NIST Analyses for Isoflavones using LC/UV-Absorbance and LC-MS: The mass fractions of puerarin was measured by LC/UV-absorbance, and daidzin and daidzein were measured by LC-MS in duplicate 10 mg test portions taken from each of ten packets of RM 8650. Methanol/water (80/20 (v/v)) and an internal standard solution of 0.60 mL caffeine, 0.50 mL $^{13}C_6$-daidzin, and 0.60 mL $^{13}C_6$-daidzein was added to each test portion as an internal standard, mixed well and extracted using ultrasonication and centrifugation. The supernatant was saved and the extraction process using only methanol/water (80/20 (v/v)) was repeated with the supernatant added to the previous portion. Sodium hydroxide was added to the supernatant to convert acetyl- and malonyl-glycosides to free glycosides. A gradient mobile phase was used to separate the isoflavones; caffeine was monitored at 274 nm and the puerarin

[(1)]Certain commercial instruments, materials, or processes are identified in this report to adequately specify the experimental procedure. Such identification does not imply recommendation or endorsement by the National Institute of Standards and Technology, nor does it imply that the instruments, materials, or processes identified are necessarily the best available for the purpose.

at 251 nm. Daidzin and $^{13}C_6$-daidzin were monitored at *m/z* 417 and *m/z* 423 respectively. Daidzein and $^{13}C_6$-daidzein were monitored at *m/z* 255 and *m/z* 261, respectively. Four stock calibration solutions were prepared gravimetrically at levels intended to approximate the levels of the isoflavones in the RM following extraction. The purity of the isoflavone calibrant materials was determined at NIST using quantitative proton nuclear magnetic resonance spectroscopy (qNMR).

Analytical Approach for Determination of Elements: Value assignment of the mass fractions of elements in RM 8650 was based on NIST results using inductively coupled plasma mass spectrometry (ICP-MS).

NIST Analysis for As, Cd, and Pb Using ICP-MS: Mass fractions of arsenic, cadmium, and lead were determined by ICP-MS from duplicate, nominal 0.4 g test portions taken from each of eight packets of the RM. Test portions were digested in sealed vessels with a HNO_3/HF mixture using a microwave digestion system. Quantitation was based on the method of standard additions using calibration solutions prepared from the SRM 3100 series of single-element standard solutions.

ASSIGNMENT OF IDENTITY

Molecular Approach for Species Identity: Sanger sequencing was used on two independent chloroplast gene regions, *trnL-trnF* intron [8−10], and the nuclear ribosomal gene internal transcribed spacer (ITS2) sequence for authentication of RM 8650. Complete sequencing of the strands of DNA for the two gene regions was performed on triplicate samples, the sequence reads were independently hand-curated, and the confidence values were estimated as described below. DNA sequences are available in companion FASTA-formatted files [1]. To certify the species identity, validation studies were performed based on the "Probability of Identification (POI)" model using an Inclusivity and Exclusivity Panel [11]. Multiple samples of authenticated herbarium vouchers, botanical identity reference materials, and published literature were used to comprise the panels from the target and most closely related species (see Appendix A for more information on the reference samples). These samples were analyzed a minimum of ten times to ensure consistency in methods from replicate to replicate. Maximum Likelihood (ML) phylogenetic analyses were performed using the phylogenetic estimation using ML (PhyML) algorithm, with a General Time Reversible (GTR) substitution model, a fixed transition-transversion ratio, and 1,000 bootstrap replicates. ML phylogenies, and the DNA aligned matrices, which can be found in Appendices B and C, were used to determine the species identity of RM 8650 and to estimate the confidence (as described below). The confidence levels for both the *trnL-trnF* intron and the ITS2 sequence were "Confident (2)."

The taxonomic identification is *Pueraria montana* var. *lobata* and the associated chloroplast DNA sequences from the *trnL-trnF* intron regions and from the nuclear ribosomal gene internal transcribed spacer (ITS2) sequence are available in companion FASTA-formatted files [1]. The confidence associated with each nucleotide in the sequence, and in turn the confidence associated with the DNA sequence as an identifier of species, is expressed in an ordinal scale that represents the strength of the belief in the assigned value (Table 1) [2]. In the absence of a fully developed metrology for identity, these DNA sequences are used as a source of identity data for *Pueraria montana* var. *lobata.* Chloroplast and nuclear ribosomal DNA sequences from botanically authenticated *Pueraria montana* var. *lobata* samples are used to establish inclusivity; chloroplast and nuclear ribosomal DNA sequences from close relatives are used to establish exclusivity.

Nucleotide Identity: A set of heuristic, experience-based, rules (see Table 2) were used to establish confidence estimates for the nucleotides comprising the DNA sequences obtained from RM 8650, and their use in identifying the species (see Table 1). The DNA sequences with curated confidence estimates are available in companion FASTA-formatted files [1]. The DNA aligned matrices used to determine the species identity and their confidence estimates are provided in Appendices B and C. The confidence associated with each nucleotide in the sequence in the FASTA files provided [1], and in turn the confidence associated with the DNA sequence as an identifier of species, is expressed in an ordinal scale that represents the strength of the belief in the assigned value (Table 2) [2]. Characteristics of sequence data and phylogenetic data used for species identity associated with the levels of the ordinal scale are described in Tables 1 and 2. The confidence estimates for the *trnL-trnF* intron region and ITS2 sequences are available in companion FASTA-formatted files [1].

Table 1. Definitions of Heuristic Rules for Confidence Estimates of Species Identity

Confidence Level	Species Identity
Most Confident (0)	Have very well-supported and well-resolved phylogeny and/or multiple diagnostic nucleotides differentiating species from closest relatives; have data from multiple samples of both an inclusivity and exclusivity panel; data from multiple independent gene regions agree.
Very Confident (1)	Have reasonably well-supported and well-resolved phylogeny and/or a few diagnostic nucleotides differentiating species from close relatives; have data from multiple samples of both an inclusivity and exclusivity panel; data from one gene, or data from multiple independent gene regions agree.
Confident (2)	Have reasonably well-supported and well-resolved phylogeny and/or one or a few diagnostic nucleotides differentiating species from close relatives; have data from a few samples of both an inclusivity and exclusivity panel; data from one gene, or data from multiple independent gene regions generally agree.
Ambiguous (3)	Have a poorly supported and poorly resolved phylogeny and/or no diagnostic nucleotides differentiating species from close relatives; have data from a few or multiple samples of both an inclusivity and exclusivity panel; data from one gene, or data from multiple independent gene regions generally disagree.

Table 2. Definitions of Heuristic Rules for Confidence Estimates of DNA Nucleotide Identity

Confidence Level	Nucleotide Identity
Most Confident (0)	Have good answers (fully reliable, unambiguous base calls) on both strands; all data from both strands agree.
Very Confident (1)	Have good answer on one strand; poor answer (less than fully reliable, potentially ambiguous base call) on the second/alternate strand; base calls from both strands typically agree, and there is biochemical context that explains the anomalous sequence data.
Confident (2)	Have good answer on one strand; anomalous sequence data that may give rise to a conflicting base call on the alternate strand; judgment required to resolve anomaly.
Ambiguous (3)	No clear mutually supporting results; unambiguous base calls disagree; or no unambiguous base calls on either strand; data from the two opposing strands could not be authoritatively reconciled.

Homogeneity Assessment: The homogeneity of isoflavones and elements was assessed at NIST using the methods and test portion sizes described above; analysis of variance with 5 % significance showed possible inhomogeneity for daidzin. The uncertainty for this analyte incorporates a component for possible inhomogeneity based on the standard deviation. The homogeneity of the DNA sequences was evaluated by sequencing the DNA from multiple samples from three randomly-selected packets, revealing the degree of homogeneity in DNA sequence or species identity. The DNA data were homogeneous.

Reference Mass Fraction Values for Isoflavones: Each reference mass fraction value is the mean result from a single NIST analysis using LC/UV-absorbance or LC-MS. Values are expressed as $x \pm U_{95\%}(x)$, where x is the estimated value and $U_{95\%}(x)$ is the expanded uncertainty of the value. The uncertainty of the estimate incorporates a component for moisture correction and a Type B relative component of 5 %. The method-specific value of the analyte lies within the interval $x \pm U_{95\%}(x)$ with about a 95 % confidence [4,5]. The measurands are the total mass fraction of each isoflavone listed in Table 3, on a dry-mass basis, as determined by the method indicated. Metrological traceability is to the measurement processes and standards used by NIST.

Table 3. Reference Mass Fraction Values for Isoflavones in RM 8650

	Mass Fraction (mg/g)		
Puerarin	32.2	±	3.2
Daidzin	4.21	±	0.43
Daidzein	4.17	±	0.43

Reference Mass Fraction Values for Elements: Each reference mass fraction value is the mean result of NIST analyses using ICP-MS. Values are expressed as $x \pm U_{95\%}(x)$, where x is the estimated value and $U_{95\%}(x)$ is the expanded uncertainty of the value. The method-specific value of the analyte lies within the interval $x \pm U_{95\%}(x)$ with about a 95 % confidence [4,5]. The measurands are the total mass fraction of each element listed in Table 4 on a dry-mass basis as determined by the method indicated. Metrological traceability is to the SI measurement unit for chemical mass fraction expressed as milligrams per kilogram, as realized by the method used.

Table 4. Reference Mass Fraction Values for Elements in RM 8650

	Mass Fraction (mg/kg)		
Arsenic (As)	0.156	±	0.014
Cadmium (Cd)	0.348	±	0.014
Lead (Pb)	1.159	±	0.046

REFERENCES

[1] Reference data can be downloaded from the "Data and Information Files" link for the RM available at https://www-s.nist.gov/srmors/view_detail.cfm?srm=8650.
— The reference sequence data file for *trnL-trnF* intron is:
RM8650 *trnL-trnF* Sequence_v1 FASTA.docx
— The reference confidence estimates for every base of *trnL-trnF* intron region sequence data file is:
RM8650 *trnL-trnF* Nucleotide Confidence_v1.rtf
— The reference sequence data file for ITS sequence is:
RM8650 ITS2 Sequence_v1 FASTA.docx
— The reference confidence estimates for every base of ITS sequence data file is:
RM8650 ITS2 Nucleotide Confidence_v1.rtf

[2] SRM 2374; *DNA Sequence Library for External RNA Controls*; National Institute of Standards and Technology; U.S. Department of Commerce: Gaithersburg, MD (20 March 2013); available at https://www-s.nist.gov/srmors/view_detail.cfm?srm=2374 (accessed May 2021).

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[4] JCGM 100:2008; *Evaluation of Measurement Data — Guide to the Expression of Uncertainty in Measurement* (GUM 1995 with Minor Corrections); Joint Committee for Guides in Metrology (2008); available at https://www.bipm.org/en/publications/guides (accessed May 2021); see also Taylor, B.N.; Kuyatt, C.E.; *Guidelines for Evaluating and Expressing the Uncertainty of NIST Measurement Results*; NIST Technical Note 1297; U.S. Government Printing Office: Washington, DC (1994); available at https://www.nist.gov/pml/nist-technical-note-1297 (accessed May 2021).

[5] JCGM 101:2008; *Evaluation of Measurement Data — Supplement 1 to the "Guide to Expression of Uncertainty in Measurement" — Propagation of Distributions Using a Monte Carlo Method*; JCGM (2008); available at https://www.bipm.org/en/publications/guides (accessed May 2021).

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[12] Nicolalde-Morejon, F.; Vergara-Silva, F.; Gonzalez-Astorga, J.; Stevenson, D.W.; Vovides, A.P.; Sosa, V.; *A Character-based Approach in the Mexican Cycads Supports Diverse Multigene Combinations for DNA Barcoding*; Cladistics, Vol. 27, pp. 150–164 (2011).

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Users of this RM should ensure that the Report of Investigation in their possession is current. This can be accomplished by contacting the SRM Program: telephone (301) 975-2200; e-mail srminfo@nist.gov; or via the Internet at https://www.nist.gov/srm.

Appendix A

Reference Samples and Sequences Used in the Specificity Validation Study for RM 8650

Table A1. Inclusivity Panel for *Pueraria montana* var. *lobata*

Pueraria montana var. *lobata*	Sample Source[a]	Voucher Information or GenBank Accession #	ITS2[b], *trnL-trnF*[c]
1	UC	1475595	+, +
2	UC	922190	+, +
3	UC	M310360	+, +
4	UC	1740708	+, +
5	UC	M288626	+, +
6	UC	M237220	+, +
7	UC	1335813	+, +
8	UC	1410692	+, –
9	UC	1400152	+, –
10	UC	M163653	+, –
11	UC	612430	+, –
12	Genbank	AB685325.1	–, +
13	See reference 1	EU717326.1	–, +
14	See reference 2	EF543431.1	–, +

[a] UC = University Herbarium, University of California, Berkeley; Genbank = unpublished on Genbank; Reference 1 = Stefanovic, S.; Pfeil, B.E.; Palmer, J.D.; Doyle, J.J.; *Relationships Among Phaseoloid Legumes Based on Sequences From Eight Chloroplast Regions*; Syst. Bot., Vol. 34(1), pp. 115–128 (2009); Reference 2 = Egan, A.N. and Crandall, K.A.; *Incorporating Gaps as Phylogenetic Characters Across Eight DNA Regions: Ramifications for North American Psoraleeae (Leguminosae)*; Mol. Phylogenet. Evol., Vol. 46(2), pp. 532–546 (2008).
[b] Internal Transcribed Spacer Sequence is included when a plus sign (+) is present, not included when a minus (–) is present.
[c] Intron sequence is included when a plus sign (+) is present, not included when a minus (–) is present.

Table A2. Exclusivity Panel for *Pueraria montana* var. *lobata*

Species	Sample Source[a] or Reference	Voucher Information or GenBank Accession #	ITS2[b], *trnL-trnF*[c]
Pueraria phaseoloides	UC	M082749	+, +
Pueraria javanica	UC	1361309	+, +
Pueraria caerulea	UC	M082310	+, –

[a] UC = University Herbarium, University of California, Berkeley.
[b] Internal Transcribed Spacer Sequence is included when a plus sign (+) is present, not included when a minus (–) is present.
[c] Intron sequence is included when a plus sign (+) is present, not included when a minus (–) is present.

trnL-trnF DNA Aligned Matrix for *Pueraria montana* var. *lobata* and Relatives

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RM 8650          TGGGCAATCCTGAGCCAAATCCCGTTTTCCGAAAACAAAGAAAAGTTCGGAAAGTGATAA
Pueraria lobata  TGGGCAATCCTGAGCCAAATCCCGTTTTCCGAAAACAAAGAAAAGTTCGGAAAGTGATAA
Pueraria hirsuta ------------------------------------------------------------
Pueraria javanica TGGGCAATCCTGAGCCAAATCCCGTTTTCCGAAAACAAAGAAAAGTTCAGAAAGTGATAA
Pueraria phaseoloides TGGGCAATCCTGAGCCAAATCCCGTTTTCCGAAAACAAAGAAAAGTTCAGAAAGTGATAA

RM 8650          TAAAAAAGGGATAGGTGCAGAGACTCAATGGAAGCTGTTCTAACAAACGGAGTTGACGAT
Pueraria lobata  TAAAAAAGGGATAGGTGCAGAGACTCAATGGAAGCTGTTCTAACAAACGGAGTTGACGAT
Pueraria hirsuta ---------------------GAGACTCAATGGAAGCTGTTCTAACAAACGGAGTTGACGAT
Pueraria javanica TAAAAAAGGGATAGGTGCAGAGACTCAATGGAAGCTGTTCTAACAAACGGAGTTGACGAT
Pueraria phaseoloides TAAAAAAGGGATAGGTGCAGAGACTCAATGGAAGCTGTTCTAACAAACGGAGTTGACGAT

RM 8650          TTTTCCTTTTTGCATTAGGAAAAGAATCCGTCCATCAAAATTCCAGGAATGGATCAAAGA
Pueraria lobata  TTTTCCTTTTTGCATTAGGAAAAGAATCCGTCCAYCAAAATTCCAGGAATGGATCAAAGA
Pueraria hirsuta TTTTCCTTTTTGCATTAGGAAAAGAATCCGTCCATCAAAATTCCAGGAATGGATCAAAGA
Pueraria javanica TTTTCCTTTTTGTATTAGGAAAAGAATCCTTCCATCAAAATTCCAGGAATGGATCAAAGA
Pueraria phaseoloides TTTTCCTTTTTGTATTAGGAAAAGAATCCTTCCATCAAAATTCCAGGAATGGATCAAAGA

RM 8650          TAAACATATATATACTGAAATACTATTTCAATTGATTAATGAAGATCCATTTGTGATAAA
Pueraria lobata  TAAACATATATATACTGAAATACTATTTCAATTGATTAATGAAGATCCATTTGTGATAAA
Pueraria hirsuta TAAACATATATATACTGAAATACTATTTCAATTGATTAATGAAGATCCATTTGTGATAAA
Pueraria javanica TAAACATATATATACTGAAATACTATTTCAGTTGATTAATGAAGATCCATTTGTGATAAA
Pueraria phaseoloides TAAACATATATATACTGAAATACTATTTCAGTTGATTAATGAAGATCCATTTGTGATAAA

RM 8650          AATATTCACAAATGAAAGATGTGAA-----TCAATTCCAAGTTGAAGAAAAGATGGAATA
Pueraria lobata  AATATTCACAAATGAAAGATGTGAA-----TCAATTCCAAGTTGAAGAAAAGATGGAATA
Pueraria hirsuta AATATTCACAAATGAAAGATGTGAA-----TCAATTCCAAGTTGAAGAAAAGATGGAATA
Pueraria javanica AATATTCACAAATGAAAGATGTGAATCAAATCAATTCCAAGTTGAAGAAAAGATGGAATG
Pueraria phaseoloides AATATTCACAAATGAAAGATGTGAATCAAATCAATTCCAAGTTGAAGAAAAGATGGAATG

RM 8650          TTCATTGATCAAATTATTCACTCCATCATAATCTGATAGATCCCTTGAAGAACTGATTAA
Pueraria lobata  TTCATTGATCAAATTATTCACTCCATCATAATCTGATAGATCCCTTGAAGAACTGATTAA
Pueraria hirsuta TTCATTGATCAAATTATTCACTCCATCATAATCTGATAGATCCCTTGAAGAACTGATTAA
Pueraria javanica TTCATTGATCAAATTATTCACTCCATCATAATCTGATAGATCCCTTGAAGAACTGATCAA
Pueraria phaseoloides TTCATTGATCAAATTATTCACTCCATCATAATCTGATAGATCCCTTGAAGAACTGATCAA

RM 8650          TCAGACGAGAATAAAGATAGAGTCCTATTCTACATGTCAATACCGACAACAATGAAATTT
Pueraria lobata  TCAGACGAGAATAAAGATAGAGTCCTATTYTACATGTCAATACCGACAACAATGAAATTT
Pueraria hirsuta TCAGACGAGAATAAAGATAGAGTCCTATTCTACATGTCAATACCGACAACAATGAAATTT
Pueraria javanica TCAGACGAGAATAAAGATAGAGTCCTATTCTACATGTCAATACCGACAACAATGAAATTT
Pueraria phaseoloides TCAGACGAGAATAAAGATAGAGTCCTATTCTACATGTCAATACCGACAACAATGAAATTT

RM 8650          ATAGTAAGAGGAAAATCCGTCGACTTAAGAAATCGTGAGGGTTCAAGTCCCTCTATCCCC
Pueraria lobata  ATAGTAAGAGGAAAATCCGTCGACTTAAGAAATCGTGAGGGTTCAAGTCCCTCTATCCCC
Pueraria hirsuta ATAGTAAGAGGAAAATCCGTCGACTTAAGAAATCGTGAGGGTTCAAGTCCCTCTATCCCC
Pueraria javanica ATAGTAAGAGGAAAATCCGTCGACTTAAGAAATCGTGAGGGTTCAAGTCCCTCTATCCCC
Pueraria phaseoloides ATAGTAAGAGGAAAATCCGTCGACTTAAGAAATCGTGAGGGTTCAAGTCCCTCTATCCCC

RM 8650          AAAAGACCTGGTTAACTTTCTAATT-TTTTCCCATATCCTCTCTATCTTTAAGTCGTTAT
Pueraria lobata  AAAAGACCTGKTTAACTTTCTAATT-TTTTCCCATATCCTCTCTATCTTTAAGTCGTTAT
Pueraria hirsuta AAAAGACCTGTTTAACTTTCTAATT-TTTTCCCATATCCTCTCTATCTTTAAGTCGTTAT
Pueraria javanica AAAAGGCCTGTTTGAGGTTCTAATTTTTTTCCTATATCCTCTCTATCTTTAAGTCGTTAT
Pueraria phaseoloides AAAAGGCCTGTTTGAGGTTCTAATTTTTTTCCTATATCCTCTCTATCTTTAAGTCGTTAT

RM 8650          TTATGTGTTTTATTCAGTTTATATTCAGTTTATTCTTTCACAACTAAATTGGAATTTGTC
Pueraria lobata  TTATGTGTTTTATTCAGTTTATATTCAGTTTATTCTTTCACAACTAAATTGGAATTTGTC
Pueraria hirsuta TTATGTGTTTTATTCAGTTTATATTCAGTTTATTCTTTCACAACTAAATTGGAATTTGTC
Pueraria javanica TTATGTATTCTATTCAGTTTATATTCAGTTTATTCTTTGACAACTAAATTGGAATTTTTC
Pueraria phaseoloides TTATGTATTCTATTCAGTTTATATTCAGTTTATTCTTTGACAACTAAATTGGAATTTTTC

RM 8650          TTTTTATTTTCAAAAATTTCTTATCATAATTACAAGTCACAAGTTTTGAAATATATATGT
Pueraria lobata  TTTTTATTTTCAAAAATTTCTTATCATAATTACAAGTCACAAGTTTTGAAATATATATGT
Pueraria hirsuta TTTTTATTTTCAAAAATTTCTTATCATAATTACAAGTCACAAGTTTTGAAATATATATGT
Pueraria javanica TTTTTATTTTCAAAATTTTCTTATCATAATTACAAGTCACAAGTTTTGAAATATATATGT
Pueraria phaseoloides TTTTTATTTTCAAAATTTTCTTATCATAATTACAAGTCACAAGTTTTGAAATATATATGT

RM 8650          GAAAGACAGAGAATTTTTTTTAATGATAAACGTACAAATGAATATCTTATTTTTGAGCAA
Pueraria montana GAAAGACAGAGAATTTTTTTTAATGATAAACGTACAAATGAATATCTTATTTTTGAGCAA
Pueraria lobata  GAAAGACAGAGAATTTTTTTTAATGATAAACGTACAAATGAATATCTTATTTTTGAGCAA
Pueraria hirsuta GAAAGACAGAGAATTTTTTTTAATGATAAACGTACAAATGAATATCTTATTTTTGAGCAA
Pueraria javanica GAAACACATATCATTTTTTTTAATGATAAACGTAGAAATTAATATCTTATTTTTGAGCAA
Pueraria phaseoloides GAAACACATATCATTTTTTTTAATGATAAACGTAGAAATTAATATCTTATTTTTGAGCAA
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RM 8650          GGAATTCTCATATGCGTGATTAACAAACAATACAAAATAATTACTACTACTGAAACTAA
Pueraria lobata  GGAATTCTCATATGCGTGATTAACAAAACAATACAAAATAATTACTACTACTGAAACTAA
Pueraria hirsuta GGAATTCTCATATGCGTGATTAACAAAACAATACAAAATAATTACTACTACTGAAACTAA
Pueraria javanica GGAATTCCCATATGTGTGATT-----AACAATACAAAATAATTACTACTACTGAAACTAA
Pueraria phaseoloides GGAATTCCCATATGTGTGATT-----AACAATACAAAATAATTACTACTACTGAAACTAA

RM 8650          CTTACAAACTTTTATTTTTCGTC-TTTTTTTTTTTACTTAGTTGATATAGATTCATTGACA
Pueraria lobata  CTTACAAACTTTTATTTTTCGTC-TTTTTTTTTTTACTTAGTTGATATAGATTCATTGACA
Pueraria hirsuta CTTACAAACTTTTATTTTTCGTCTTTTTTTTTTTACTTAGTTGATATAGATTCATTGACA
Pueraria javanica CTTACAAATTTTTATTTTTCGTC---TTTTTTTTAGTTAGTTGATAGAGATTCATTGACA
Pueraria phaseoloides CTTACAAATTTTTATTTTTCGTC---TTTTTTTTAGTTAGTTGATAGAGATTCATTGACA

RM 8650          TAGACTCCAGTAATCTTTTAAAA
Pueraria lobata  TAGACTCCAGTAATCTTTTAAAA
Pueraria hirsuta TAGACTCCAGT------------
Pueraria javanica TATACTCCAGTAATCTTTTAAAA
Pueraria phaseoloides TATACTCCAGTAATCTTTTAAAA
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Figure B1. *trnL-trnF* DNA Aligned Matrix for *Pueraria montana* var. *lobata* and Relatives. The results from the chloroplast region demonstrates that this region does distinguish this species from its relatives and does authenticate *Pueraria montana* var. *lobata*. The four bases of DNA are represented as A = Adenine, T = Thymine, G = Guanine, and C = Cytosine. Polymorphic bases are represented by standard IUPAC codes with Y = C/T, K = G/T, and "-" = missing data. The confidence estimate for the species identification of RM 8650 as *Pueraria montana* var. *lobata* is Confident (2).

ITS2 DNA Aligned Matrix for *Pueraria montana* var. *lobata* and Relatives

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RM 8650          CCCGTGAACCATCGAGTCTTTGAACGCAAGTTGCGCCCGAAGCCATTTAGGCAGAGGGCA
Pueraria lobata  CCCGTGAACCATCGAGTCTTTGAACGCAAGTTGCGCCCGAAGCCATTTAGGCAGAGGGCA
Pueraria hirsuta CCCGTGAACCATCGAGTCTTTGAACGCAAGTTGCGCCCGAAGCCATTTAGGCAGAGGGCA
Pueraria caerulea CCCGTGAACCATCGAGTCTTTGAACGCAAGTTGCGCCCGAAGCCA-TTAGGTGGAGGGCA
Pueraria javanica ---------------------------------TGCGCCCGAAGCCA-TTAGGTTGAGGGCA
Pueraria phaseoloides CCCGTGAACCATCGAGTCTTTGAACGCAAGTTGCGCCCGAAGCCA-TTAGGCCGAGGGCA

RM 8650          CGCCTGCCTGGGTGTCACACATCGTTACC--CC-AACGCAAACAGACGTCCCACACGACG
Pueraria lobata  CGCCTGCCTGGGTGTCACACATCGTTACC--CC-AACGCAAACAGACGTCCCACACGACG
Pueraria hirsuta CGCCTGCCTGGGTGTCACACATCGTTACC--CC-AACGCAAACAGACGTCCCACACGACG
Pueraria caerulea CGCCTGCCTGGGTGTCACACATCGTTACC--CCAAACGCAAACAAATGTCTCACCGGACG
Pueraria javanica CGCCTGCCTGGGTGTCACACATCGTTACCCTCCTCACACATGCATGCGT-------GCAT
Pueraria phaseoloides CGCCTGCCTGGGTGTCACACATCGTTACC--CC-CACGCAAA----------------

RM 8650          GCCGTTGCGTGGTAGGGTGCACGCTGACCTCCCGCGAGCGGCGTCTCGCGGTTGGTTGAA
Pueraria lobata  GCCGTTGCGTGGTAGGGTGCACGCTGACCTCCCGCGAGCGGCGTCTCGCGGTTGGTTGAA
Pueraria hirsuta GCCGTTGCGTGGTAGGGTGCACGCTGACCTCCCGCGAGCGGCGTCTCGCGGTTGGTTGAA
Pueraria caerulea ACCGTTGCGCAGTGGGGTGCACGCTGACCTCCCGCGAGCACCGTGTCGTGGTTGGTTGAA
Pueraria javanica GCAGT----AAGCAGGGTGTATGCTGGCCTCCCACGAGCGACCTCTCGCGGTTGGTCGAA
Pueraria phaseoloides ----ATGCCTGG--GGGCGCATGCTGACCTCCCGCGAGCACCGTCTCGTGGCTGGTTGAA

RM 8650          AATCGAGTTCGCGGCCGAGCACGCCGTGATAAAATGGTGGATGAG---CAACGCTCGAGA
Pueraria lobata  AATCGAGTTCGCGGCCGAGSACGCCGTGATAAAATGGTGGATGAG---CAACGCTCGAGA
Pueraria hirsuta AATCGAGTTCGCGGCCGAGSACGCCGTGATAAAATGGTGGATGAG---CAACGCTCGAGA
Pueraria caerulea AATCGAGTTCGTGGCCGAGTACGCCGTGATAGAATGGTGGATGAGTAACAACGCTCGAGA
Pueraria javanica AATCGAGTTCGTGGCCGAGTGTGCCGTGATACAATGGTGGATGAGTAATTACGCTCGAGA
Pueraria phaseoloides AACCGGGTTCATGGTCGGGCGCGCCGTGATAAAGTGGTGGATGAG---TCATGCTCGAGA

RM 8650          CCAATCACG---CGCTGCGACTCGGTCCGCGAAGGACTCCCTGATTGATGACGACCCTAC
Pueraria lobata  CCAATCACG---CGCTGCGACTCGGTCCGCGAAGGACTCCCTGATTGATGACGACCCTAC
Pueraria hirsuta CCAATCACG---CGCTGCGACTCGGTCCGCGAAGGACTCCCTGATTGATGACGACCCTAC
Pueraria caerulea CCGATCACG---CGCAGCGACTCGGTCGGCTACGGACTCCTTC---G----CGACCCTAC
Pueraria javanica CCGATCAC-----GC-GCGTCTCGTCCAGCTCCGGACTCC----------ACGACCCCTC
Pueraria phaseoloides CCAATCGCGTCACGC-GCGACCCTGCCGGTTGTGGACTCCC----------CGACCC-AC

RM 8650          AGTGCGCCTC--CTCTCC-----GGAGACGCTCTCKACGAGACCTCAGGTCAGGCGGGGC
Pueraria lobata  AGTGCGCCTC--CTCTCC-----GGAGACGCTCTCKACGAGACCTCAGGTCAGGCGGGGC
Pueraria hirsuta AGTGCGCCTC--CTCTCC-----GGAGACGCTCTCKACGAGACCTCAGGTCAGGCGGGGC
Pueraria caerulea GGTGC--CTC--CTCTTC-----GGAGACGCCCTCAACGAGACCTCAGGTCAGGCGGGGC
Pueraria javanica GGTG--TCTCTGCACAGC-----AAAGACGCTCTCAACGAGACCTCAGGTCAGGCGGGGC
Pueraria phaseoloides -GCGCGTCTCTGCATTCCGTGCGGGAGACGCTCTCGACGAGACCTCAGGTCAGGCGGGGC

RM 8650          TACCCGCTGAGTTTAAGCATATCAATAAGCGGAGGAAAAGAAACTAACAAGGATTCCCCT
Pueraria lobata  TACCCGCTGAGTTTAAGCATATCAATAAGCGGAGGAAAAGAAACTAACAAGGATTCCCCT
Pueraria hirsuta TACCCGCTGAGTTTAAGCATATCAATAAGCGGAGGAAAAGAAACTAACAAGGATTCCCCT
Pueraria caerulea TACCCGCTGAGTTTAAGCATATCAATAAGCGGAGGAAAAGAAACTAACAAGGATTCCCCT
Pueraria javanica TACCCGCTGAGTTTAAGCATATCAATAAGCGGAGGAAAAGAAACTAACAAGGATTCCCTT
Pueraria phaseoloides TACCCGCTGAATTTAAGCATATCAATAAGCGGAGGAAAAGAAACTAACAAGGATTCCCCT

RM 8650          AGTAACGGCGAGCGAACCGGGAAGAGCCCACCATGAGAATCGGTCGCCCCGGCGTCCG
Pueraria lobata  AGTAACGGCGAGCGAACCGGGAAGAGCCCACCATGAGAATCGGTCGCCCCCGGCGTCCG
Pueraria hirsuta AGTAACGGCGAGCGAACCGGGAAGAGCCCACCATGAGAATCGGTCGCCCCCGGCGTC--
Pueraria caerulea AGTAACGGCGAGCGAACCGGGAAGAGCCCACCATGAGAATCGGTCGCCCTCGGCGTCCG
Pueraria javanica AGTAACGGCGAGCGAACCGGGAATAGCCCACCAT-----------------------
Pueraria phaseoloides AGTAACGGCGAGCGAACCGGGAAAAGCCCACCATGAAAATCGGTCGTCTCCGGCGTCCG
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Figure C1. ITS2 DNA Aligned Matrix for *Pueraria montana* var. *lobata* and Relatives. The results from the nuclear ribosomal gene region demonstrates that this region does distinguish this species from its relatives and is sufficient for identification of *Pueraria montana* var. *lobata*. The four bases of DNA are represented as A = Adenine, T = Thymine, G = Guanine, and C = Cytosine. Polymorphic bases are represented by standard IUPAC codes with S = G/C, K = G/T, and "-" = missing data. The confidence estimate for the species identification of RM 8650 as *Pueraria montana* var. *lobata* is Confident (2).