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DNA Barcode Authentication of Saw Palmetto Herbal Dietary Supplements

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Herbal dietary supplements made from saw palmetto (*Serenoa repens*; Arecaceae) fruit are commonly consumed to ameliorate benign prostate hyperplasia. A novel DNA mini–barcode assay to accurately identify [specificity = 1.00 (95% confidence interval = 0.74–1.00); sensitivity = 1.00 (95% confidence interval = 0.66–1.00); *n* = 31] saw palmetto dietary supplements was designed from a DNA barcode reference library created for this purpose. The mini–barcodes were used to estimate the frequency of mislabeled saw palmetto herbal dietary supplements on the market in the United States of America. Of the 37 supplements examined, amplifiable DNA could be extracted from 34 (92%). Mini–barcode analysis of these supplements demonstrated that 29 (85%) contain saw palmetto and that 2 (6%) supplements contain related species that cannot be legally sold as herbal dietary supplements in the United States of America. The identity of 3 (9%) supplements could not be conclusively determined.

Serenoa repens (W.Bartram) Small–commonly known as saw palmetto–is a palm (Arecaceae) indigenous to the southeastern United States of America (Alabama, Florida, Georgia, Louisiana, Mississippi, and South Carolina)[1]. The closest living relative of *S. repens*[2–4], *Acoelorrhaphe wrightii* (Grisebach & H.Wendland) H.Wendland ex Beccari, occurs in United States of America (southern Florida), Bahamas, Cuba, southeastern México (Campeche, Chiapas, Tabasco, Veracruz, Yucatán, and Quintana Roo), Belize, Guatemala, Honduras, Nicaragua, Colombia (Isla de Providencia), and Costa Rica[1,5,6]. Although morphological and molecular data strongly support the close relationship between *S. repens* and *A. wrightii*, until recently their relationship to the other species of tribe Livistoneae could not be resolved[2–4]. New data suggest that *S. repens* and *A. wrightii* are sister to subtribe Livistoninae (*Johannesteijsmannia, Lanonia, Licuala, Livistona, Pholidocarpus, Pritchardiopsis*, and *Saribus*) and that the *Acoelorrhaphe*/*Serenoa*/Livistoninae clade is in turn sister to *Brahea* and subtribe Rhapidinae (*Chamaerops, Guihaia, Maxburretia, Rhapidophyllum, Rhapis*, and *Trachycarpus*)[4].

The fruit (drupe) of *S. repens* are ellipsoid, about 2 cm in length, 1 cm wide, smooth, blue–black when mature (green to yellow–orange when immature)[1,7,8]. The fruits are eaten by an assortment of wild animals, livestock, and people[7–9]. When labeled as saw palmetto, *S. repens* can be legally sold in the United States of America as an herbal dietary supplement[10]. In 2011, it was the third most popular supplement with sales totaling more than US$ 18 million[11]. Although the fruits of *S. repens* are reported to be useful in the treatment of 51 different medical ailments[7], the fruits are most frequently taken to ameliorate benign prostate hyperplasia[7–9,12]. Extracts of *S. repens* fruit inhibit the conversion of testosterone to dihydrotestosterone by 5α-reductases[13–16]. Benign prostate hyperplasia is associated with elevated concentrations of dihydrotestosterone[17]. Clinical studies report few adverse events from *S. repens* consumption (mostly mild)[18,19], but treatment outcomes vary greatly–on average little success has been reported[19].

Wild *S. repens* grows abundantly on as many as 450,000 hectares[7] of costal sand dunes, mesic hammocks, pine flatwoods, and sand–pine scrub[1,7–9]. Each hectare annually produces an average of 200 kg of fruit (range = 100–1,500 kg/hectare)[9]. The magnitude of annual fruit harvest is unknown, but estimates are as high as 6,800,000 kg[7]. Almost all of the fruit is harvested from wild plants[7,8] and approximately half is picked by independent wild-crafters[8]. Fruit is often harvested when immature: a final product with a minimum of 10% mature (blue–black) and 60% partially ripened (yellow–orange) fruit is commercially acceptable[8].

DNA barcode researchers collectively aim to produce a global public reference library of standardized, high–quality, vouchered DNA sequences that can be used to identify specimens. The protein coding plastid genes *matK* and *rbcL* have been sanctioned by the Consortium for the Barcode of Life for use in plant DNA barcoding[20,21]. By using standard genomic regions, data and protocols can be shared thus maximizing scarce research funds.

We aim to (*i*) generate and test a DNA barcode reference library for *S. repens*, (*ii*) devise a barcode assay capable of unambiguously identifying *S. repens*, and (*iii*) estimate the frequency of mislabeled saw palmetto herbal dietary supplements on the market in the United States of America.

Results

For this study, 27 *matK* and 37 *rbcL* barcode sequences where generated from 37 morphologically identifiable specimens (Table 1; GenBank KF746442–KF746505). Median sequence quality (B_{30})[22] of the newly generated sequences was 0.891 (IQR = 0.829–0.928) for *matK* and 0.909 (IQR = 0.756–0.939) for *rbcL*. Trimmed and edited *matK* sequences were 840 bp in length for *A. wrightii* and 837 bp in length for all other species examined (*A. wrightii* has a lysine$^{(AAG)}$ inserted at nucleotide position 306). All Trimmed and edited *rbcL* sequences are 607 bp in length.

When the newly generated sequences were analyzed in concert with publicly available sequences (Table 2)[3,4,23–31], no unambiguous *matK* sequence variation was detected within *S. repens* ($n = 12$) or *A. wrightii* ($n = 15$). Variation was detected at two *rbcL* nucleotide positions in *S. repens* ($n = 18$): GenBank sequence AJ621936[25] had a 'C' at nucleotide position 60 whereas all other sequences examined had an 'A' at that nucleotide position and GenBank sequence M81815[23] had a 'C' at nucleotide position 234 whereas all other sequences examined had a 'T'. Both nucleotide substitutions are predicted to result in amino acid substitutions. Neither nucleotide substitution has been detected in more than one individual. No *rbcL* sequence variation was detected in *A. wrightii* ($n = 17$).

Serenoa repens and *A. wrightii* can be consistently distinguished from *Brahea*, Livistoninae, and Rhapidinae by a combination of *matK* nucleotide positions 802 and 818 (Fig. 1). *Serenoa repens*, *A. wrightii,* and *Pholidocarpus majadum* Becc. (tribe Livistoneae) have a 'G' at nucleotide position 818 whereas all other examined species have an 'A'. *Pholidocarpus majadum* has an 'A' at nucleotide position 802 and thus can be differentiated from *S. repens* and *A. wrightii* which have a 'T' at that nucleotide position. *Serenoa repens* and *A. wrightii* can be differentiated from one another by a three–base insertion in *A. wrightii* at *matK* nucleotide position 306. *Serenoa repens* and *A. wrightii* can also be differentiated from one another by *rbcL* nucleotide positions 292 (*S. repens* has a 'C', *A. wrightii* has a 'T') and 398 (*S. repens* has an 'A', *A. wrightii* has a 'C'; Fig. 1).

Preliminary attempts to PCR amplify full–length barcode markers from saw palmetto herbal supplements were unsuccessful. Fragmented DNA was determined to be the primary cause of PCR failure–the barcode regions are larger than the average fragment size in DNA extracts of saw palmetto herbal supplements. To overcome DNA fragmentation, novel mini–barcode PCR primers were designed to amplify positions diagnostic of *S. repens* while limiting the amplicon size to 200 bp or less (Fig. 1). Unfortunately, there are no regions less than 200 bp within *matK* or *rbcL* that can consistently distinguish *S. repens* from the other species examined. A novel *matK* mini–barcode was designed to span nucleotide positions 802 and 818 and can thus distinguish *S. repens* and *A. wrightii* from all of the other species examined. A novel *rbcL* mini–barcode was designed to span nucleotide positions 292 and 398 and can thus distinguish *S. repens* from *A. wrightii* (Fig. 1). In combination these novel mini–barcodes can distinguish *S. repens* from all of the other species examined.

PCR amplification with the novel mini–barcode primer sets worked well on the 31 morphologically identifiable validation samples as well as saw palmetto herbal supplements. Median sequence quality (B_{30}) of validation mini–barcode sequences was 0.633 (IQR = 0.455–0.732) for *matK* and 0.530 (IQR = 0.386–0.689) for *rbcL*. All validation samples were correctly identified using the combination of *matK* and *rbcL* mini–barcodes [$n = 13$ *S. repens*; $n = 18$ *A. wrightii*; specificity = 1.00 (95% confidence interval = 0.74–1.00); sensitivity = 1.00 (95% confidence interval = 0.66–1.00)].

Of the 37 saw palmetto herbal supplements examined, amplifiable DNA could be extracted from 34 (92%). At least one mini–barcode could be PCR amplified and sequenced from all 34 samples. Both *matK* and *rbcL* mini–barcodes could be PCR amplified and sequenced from 30 of the samples (81%). Mini–barcode analysis conclusively demonstrates that 29 (85%) saw palmetto herbal supplements contain *S. repens* (Fig. 1, supplement type A). The identity of 3 (9%) supplements could not be definitively determined due to failure of the *rbcL* mini–barcode to amplify and sequence (Fig. 1, supplement type B). These supplements could be composed of *S. repens*, they could contain *A. wrightii*, or a mixture of *S. repens* and *A. wrightii*. Two (6%) supplements contain related species that cannot be legally sold as herbal dietary supplements in the United States of America[10]–one supplement (Fig. 1, supplement type C) is definitively *A. wrightii*; the other cannot be conclusively identified to species (Fig. 1, supplement type D; it is a species of *Brahea, Chamaerops, Guihaia, Johannesteijsmannia, Lanonia, Licuala, Livistona, Maxburretia, Rhapidophyllum, Rhapis, Saribus*, or *Trachycarpus*).

Discussion

All newly generated *matK* and *rbcL* reference sequences exceed the quality requirements of the BARCODE data standard (version 2.3)[32].

Intraspecific sequence variation was detected at two *rbcL* nucleotide positions in previously published[23,25] *S. repens* sequences. Such barcode variation is uncommon in plants–particularly in *rbcL*[20,33–41]. From the available data, we cannot determine if the variation is real or the result of sequencing error. If genuine, both of these nucleotide substitutions would result in amino acid substitutions. The fact that these variable sites have not been found in more than one individual each strongly suggests that the variation is artifactual. The *rbcL* mini–barcode does not include these, possibly variable, nucleotide positions and thus these nucleotide positions had no influence on the resulting species identifications (Fig. 1).

Our inability to PCR amplify full–length barcodes from saw palmetto herbal supplements was not unexpected: the processing of plant materials frequently results in highly fragmented DNA, particularly if the samples are heated[42–51]. Failure of PCR amplification from degraded DNA samples is frequently reported when amplicons are greater than 200 bp[42–51], thus one cannot expect full–length barcodes to reliably amplify from processed materials given that the median full–length *matK* barcode is 889 bp (IQR = 880–889)[21] and *rbcL* is uniformly 654 bp[21]. Mini–barcodes were thus designed to ensure PCR amplification from degraded samples.

Amplifiable DNA could not be extracted from three saw palmetto herbal supplements. It is possible that amplifiable DNA belonging to *S. repens* (or closely related species) was absent from the herbal supplements because (*i*) the supplements did not contain any *S. repens* (or closely related species); (*ii*) alternatively the herbal supplements contained *S. repens* (or closely related species), but the material was processed in such a way that all amplifiable *S. repens* DNA was destroyed; or (*iii*) amplifiable DNA was present, but PCR inhibitory compounds were co–purified with the DNA. The successful PCR amplification and sequencing of only the *matK* mini–barcode from four saw palmetto herbal supplements cannot be conclusively explained without assuming that region containing the *rbcL* mini–barcode is more sensitive to DNA degradation than the region containing the *matK* mini–barcode.

The validation experiment conclusively demonstrates that it is possible to distinguish between *S. repens* and closely related species using a combination of *matK* and *rbcL* mini–barcodes (specificity = 1.00; sensitivity = 1.00). Samples can be unambiguously identified provided that both mini–barcodes can be PCR amplified and sequenced. Without the *matK* mini–barcode, it is not possible to distinguish among *S. repens*, *Brahea* and most Rhapidinae (Fig. 1). Without the *rbcL* mini–barcode, it is not possible to distinguish between *S. repens* and *A. wrightii* (Fig. 1).

Table 1 | Voucher information

Species	Provenance	Voucher specimen	Sample type
Acoelorrhaphe wrightii (Grisebach & H.Wendland) H.Wendland ex Beccari	Belize (Cayo)	Atha et al. 957 (NY)	*matK* and *rbcL* reference
	Belize (Cayo)	Ratter 5191 (NY)	*rbcL* reference
	Cuba (Isla de la Juventud)	Curtiss 449 (NY)	validation
	Cuba (La Habana)	Britton et al. 13344 (NY)	validation
	Cuba (La Habana)	Shafer 223 (NY)	*matK* and *rbcL* reference; validation
	Cuba (Mayabeque)	Ekman 908 (NY)	*matK* and *rbcL* reference; validation
	Cuba (Mayabeque)	Leon 14264 (NY)	validation
	Cuba (Pinar del Río)	Britton et al. 9614 (NY)	*matK* and *rbcL* reference; validation
	Cuba (Pinar del Río)	Shafer 10620 (NY)	*matK* and *rbcL* reference; validation
	Cuba (Pinar del Río)	Shafer 426 (NY)	validation
	Cuba (Pinar del Río)	Van Hermann 594 (NY)	validation
	Cuba (unknown)	Avarca 4208 (NY)	validation
	Cuba (Villa Clara)	Britton et al. 10269 (NY)	validation
	Cuba (Villa Clara)	Combs 465 (NY)	*rbcL* reference; validation
	Guatemala (Petén)	Contreras 4012 (NY)	validation
	Guatemala (Petén)	Contreras 5362 (NY)	*rbcL* reference; validation
	Guatemala (Petén)	Lundell 17741 (NY)	*matK* and *rbcL* reference; validation
	Guatemala (Sololá)	Lentz 2010 (NY)	*matK* and *rbcL* reference
	Honduras (Colón)	Saunders 413 (NY)	*matK* and *rbcL* reference
	México (Campeche)	Gutierrez 5120 (NY)	validation
	México (Quintana Roo)	Sanders and Frame 1719 (NY)	*matK* and *rbcL* reference; validation
	México (Veracruz)	Nee 32437 (NY)	*matK* and *rbcL* reference
	Nicaragua (Región Autónoma del Atlántico Norte)	Reveal 7365 (NY)	*matK* and *rbcL* reference
	Nicaragua (Región Autónoma del Atlántico Norte)	Stevens 8558 (NY)	validation
	United States of America (Florida)	Cooley 9332 (NY)	*matK* and *rbcL* reference
Brahea aculeata (Brandegee) H.E.Moore	México (Sonora)	Felger 5023 (NY)	*matK* and *rbcL* reference
Brahea dulcis Mart.	Guatemala (Huehuetenango)	Castillo et al. 2699 (NY)	*matK* and *rbcL* reference
Chamaerops humilis L.	Morocco (Tangier–Tetouan)	Lauria 2006-402 (NY)	*matK* and *rbcL* reference
Licuala tansachana Hodel	Thailand (Narathiwat)	Hodel 1601 (NY)	*rbcL* reference
Livistona humilis R.Br.	Australia (Northern Territory)	Mumir 5590 (NY)	*matK* and *rbcL* reference
Rhapidophyllum hystrix (Frazer ex Thouin) H.Wendl. & Drude	United States of America (Mississippi)	Bryson 22098 (NY)	*matK* and *rbcL* reference
Serenoa repens (W.Bartram) Small	United States of America (Florida)	Atha et al. 2530 (NY)	*matK* and *rbcL* reference; validation
	United States of America (Florida)	Coker s.n. 1939 December 26 (NY)	validation
	United States of America (Florida)	Cooley 2540 (NY)	*matK* and *rbcL* reference
	United States of America (Florida)	Cooley 2665 (NY)	*rbcL* reference; validation
	United States of America (Florida)	Curtiss 6195 (NY)	validation
	United States of America (Florida)	Fortsch et al. 17 (NY)	*matK* and *rbcL* reference; validation
	United States of America (Florida)	Hess et al. 8487 (NY)	*matK* and *rbcL* reference
	United States of America (Florida)	Hill 13337 (NY)	*rbcL* reference; validation
	United States of America (Florida)	Hill 1337 (NY)	*matK* and *rbcL* reference
	United States of America (Florida)	Laconte et al. 861 (NY)	*matK* and *rbcL* reference
	United States of America (Florida)	Moldenke 331 (NY)	*rbcL* reference; validation
	United States of America (Florida)	Nash 644 (NY)	*rbcL* reference; validation
	United States of America (Florida)	Nelson 17035 (NY)	*matK* and *rbcL* reference; validation
	United States of America (Florida)	Nolan 31 (NY)	*matK* and *rbcL* reference
	United States of America (Florida)	Small 2111 (NY)	validation
	United States of America (Florida)	Small 2267 (NY)	validation
	United States of America (Florida)	Standley 152 (NY)	*rbcL* reference; validation
	United States of America (Georgia)	Harper 1817 (NY)	validation
	United States of America (South Carolina)	Radford 11512 (NY)	*matK* and *rbcL* reference
	unknown	anonymous [Museum of Wesleyan University, Barratt herbarium] s.n. (NY)	*rbcL* reference
Trachycarpus fortunei (Hook.) H.Wendl.	Cultivated	Hill 22260 (NY)	reference

Table 2 | Previously published reference sequences deposited in GenBank that were analyzed alongside newly generated sequences

Subtribe	Genus	*matK*	*rbcL*
–	*Brahea*	AM114580 HQ720245 HQ720246 HQ720247 HQ720248 HQ720249 HQ720250	AJ829853 AM110198
Rhapidinae	*Chamaerops*	AM114568 HQ720251 HQ720307	AJ404754 AY012456 GQ120444 HM849886
Rhapidinae	*Guihaia*	AM114569 HQ720273 HQ720274 HQ720275	AJ404755
Rhapidinae	*Maxburretia*	AM114572 HQ720297 HQ720311	AJ829884
Rhapidinae	*Rhapidophyllum*	AM114571 HQ720323	AJ404753
Rhapidinae	*Rhapis*	AM114573 HQ720308 HQ720309 HQ720310 HQ720316 HQ720317 HQ720318 HQ720319 HQ720320 HQ720321 HQ720322	AJ404756 AY012458
Rhapidinae	*Trachycarpus*	AM114570 HQ619794 HQ720312 HQ720313 HQ720314 HQ720315	AJ404752 AY012460 GQ436761 HE963709 HQ619730
–	*Acoelorrhaphe*	AM114579 HQ720241 HQ720242	AJ829845 AM110197
–	*Serenoa*	AM114585 HQ720325 HQ720326	AJ404760 AJ621936 M81815
Livistoninae	*Johannesteijsmannia*	AM114576 HQ720276 HQ720277 HQ720278 HQ720279 HQ720281 HQ720282	AJ404758
Livistoninae	*Lanonia*	HQ720173 JF292980 JF292982 JF292983 JF292985 JF292986 JF292987	–
Livistoninae	*Licuala*	AM114575 HQ720156 HQ720158 HQ720161 HQ720167 HQ720168 HQ720171 HQ720172 HQ720176 HQ720179 HQ720180 HQ720182 HQ720183 HQ720185 HQ720187 HQ720285 HQ720286 HQ720287 HQ720288 HQ720290 HQ720291 HQ720292 HQ720293	AJ404759 AY012462 JF738686 JF738690 JF738960
Livistoninae	*Livistona*	AM114574 HQ720190 HQ720191 HQ720306 HQ720330 HQ720331 HQ720332 HQ720333 HQ720334 HQ720335 HQ720336 HQ720337 HQ720338 HQ720339 HQ720340 HQ720341 HQ720342 HQ720343 HQ720344 HQ720345	AJ404757 AM903191 AY012463 GU135214
Livistoninae	*Pholidocarpus*	AM114577 HQ720294	AJ829894
Livistoninae	*Pritchardiopsis*	AM114578	AM110196
Livistoninae	*Saribus*	HQ720192 HQ720193 HQ720195 HQ720348 HQ720349 HQ720350	AY012464

Two saw palmetto herbal supplements (6%), in our sample, were unambiguously mislabeled. One of these supplements contained *A. wrightii* (Fig. 1). Given the relative rarity of *A. wrightii* within the native geographic range of *S. repens*[1,6] and the distinct macro–morphological differences (*S. repens* is an acaulous to short stemmed palm whereas *A. wrightii* grows in clusters of tall slender stems)[1,6] it is difficult to imagine such a misidentification occurring at the point of harvest. It seems most likely that fruits–which appear similar in both species–were misidentified post harvest. We cannot explain the other mislabeled saw palmetto herbal supplement.

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                                        matK                rbcL
                                        66667777777778888    222233
                                        78893455668890111    558909
                                      n 85958437565682678  n 130218
Brahea                                9 YGGCCTCCCAACCTCGA  4 GGTCAA
Rhapidinae Chamaerops                 4 C..Y..YY....Y....  5 ......
Rhapidinae Guihaia                    4 C................  1 ......
Rhapidinae Maxburretia                3 C................  1 ......
Rhapidinae Rhapidophyllum             3 C................  2 ......
Rhapidinae Rhapis                    11 C................  2 ..C...
Rhapidinae Trachycarpus               7 C................  6 ......
Livistoninae Johannesteijsmannia      7 C....W...RR......  1 .....C
Livistoninae Lanonia                  7 CR...............  0 ??????
Livistoninae Licuala                 23 CR..Y...Y.....M..  6 RR...M
Livistoninae Livistona               21 C................  5 .....M
Livistoninae Pholidocarpus            2 C..........Y.A..R  1 ....GC
Livistoninae Pritchardiopsis          1 C..............T.  1 .....C
Livistoninae Saribus                  6 CRK............K.  1 .....C
Acoelorrhaphe wrightii               15 C...............G 17 ...T.C
Serenoa repens                       12 C...............G 18 ......
supplement type A                    29 C...............G 29 ......
supplement type B                     3 C...............G  0 ??????
supplement type C                     1 C...............G  1 ...T.C
supplement type D                     1 C................  0 ??????
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Figure 1 | Variable nucleotide positions for mini–barcode sequences. Diagnostic positions that, in combination, unambiguously differentiate *Serenoa repens* from its close relatives are highlighted. Nucleotide positions are numbered in reference to Britton et al. 9614 (NY). Periods indicate nucleotides identical to *Brahea*. Question marks indicate unsequenced positions. The four sequence types (A, B, C, and D) found in herbal supplements are reported.

Table 3 | Amplification and sequencing primers used

Marker	Forward primer (5′-3′)	Reverse primer (5′-3′)
matK	ACCCAGTCCATCTGGAAATCTTGGTTC	CGTACAGTACTTTTGTGTTTACGAG
rbcL	ATGTCACCACAAACAGAGACTAAAGC	GAAACGGTCTCTCCAACGCAT
matK mini–barcode	TCTAAAGCTAAATTTTGTACCGTATCG	GCCAAAGTTCTAGCACACGAA
rbcL mini–barcode	GCTATCACATCGAAACCGTTG	GCCTTGGAAAGTTTTGGAATAAG

Variation in the chemical composition of *S. repens* fruit and fruit extracts[52,53] is commonly cited to explain the mixed treatment outcomes observed in clinical studies[19]. An alternate explanation is species misidentification. Between 4 and 15% of the samples we examined were misidentified. If our sample is representative, misidentification may account for a substantial portion of the variation observed in clinical studies. To ensure that misidentified materials are not inadvertently used, clinical researchers should authenticate all saw palmetto herbal supplements using the DNA barcode methodology described here.

Methods

Plant material. Reference and validation samples were morphologically identified by the authors (Table 1). Validation samples were arbitrarily selected from the set of morphologically identified samples. Herbal supplements were purchased in retail stores or on–line. The herbal supplements consisted of dry, cut, and sifted plant materials (gelatin capsules or compression tablets).

DNA extraction. Samples (10 mg) of dried leaf tissue or herbal supplements were disrupted in a 1.6 mL tube using two stainless steel ball bearings (3 mm) and a TissueLyser (Qiagen) at 30 Hz (2 × 1.5 min). Samples were incubated for 18 h at 42°C with 40 rpm horizontal shaking in 600 μL extraction buffer (8 mM NaCl, 16 mM sucrose, 5.8 mM EDTA, 0.5% [w/v] sodium dodecyl sulphate, 12.4 mM tris [pH 9.1], and 200 μg/mL proteinase K)[54]. After incubation, 200 μL of 3 M potassium acetate (pH 4.7) was added to each sample. Following 10 min of incubation at 0°C, samples were centrifuged at 14,000 g for 5 min. 600 μL of each sample's aqueous phase was mixed with 900 μL 2 M guanidine hydrochloride in 95% (v/v) ethanol. The mixtures were applied to silica spin columns (Epoch Life Science), 500 μL at a time, by centrifugation at 7,000 g for 1 min. Wash buffer (50% [v/v] ethanol, 10 mM tris [pH 7.4], 0.5 mM EDTA, and 50 mM NaCl)[55] was applied twice as described above. Columns were dried by centrifugation at 7,000 g for 2 min. Total DNA was eluted in 200 μL 10 mM tris (pH 8.0) by centrifugation at 7,000 g for 1 min.

DNA amplification and sequencing. Markers were amplified in 15 μL Polymerase Chain Reactions (PCR). Each reaction contained 1.5 μL PCR buffer (200 mM tris [pH 8.8], 100 mM KCl, 100 mM $(NH_4)_2SO_4$, 20 mM $MgSO_4$, 1% [v/v] Triton X-100, 50% [w/v] sucrose, 0.25% [w/v] cresol red), 0.2 μM dNTPs, 48 mM betaine (*rbcL* mini–barcode only), 0.5 (*rbcL* only) or 1.0 μM/mL of each amplification primer (Table 3), 0.25 units of *Taq* polymerase, 0.025 mg/mL bovine serum albumin, and 0.5 μL purified DNA.

The *matK* reaction mixtures were incubated for 150 sec at 95°C, cycled 10 times (30 sec at 95°C, 30 sec at 56°C, 30 sec at 72°C), cycled 25 times (30 sec at 88°C, 30 sec at 56°C, 30 sec at 72°C), and incubated 10 min at 72°C. The *rbcL* reaction mixtures were incubated for 150 sec at 95°C, cycled 35 times (30 sec at 95°C, 30 sec at 58°C, 30 sec at 72°C), and incubated 10 min at 72°C. The *matK* mini–barcode and *rbcL* mini–barcode reaction mixtures were incubated for 150 sec at 95°C, cycled 35 times (30 sec at 95°C, 30 sec at 60°C), and incubated 10 min at 60°C.

PCR products were treated with ExoSAP-IT (USB) and bidirectionally sequenced on a 3730 automated sequencer (Life Technologies) using the amplification primers and BigDye v3.1 (Life Technologies; High–Throughput Genomics Unit, University of Washington).

Data analysis. Raw chromatograms were processed with KB (version 1.4; Life Technologies) and contigs were created and edited with Sequencher (version 4.10; Gene Codes). Sequence quality was evaluated using B (version 1.2)[22] with the quality threshold (q) set to 30.

Publicly available reference sequences where analyzed along with the sequences generated for this study (Tables 1 and 2)[3,4,23–31]. Diagnostic nucleotide positions were located in multiple sequence alignments constructed with MUSCLE (version 3.8)[56]. Novel mini–barcode primers spanning diagnostic positions were designed with PRIMER3 (version 1.1)[57].

Sequences from validation samples and herbal supplements were taxonomically identified using BRONX (version 2.0)[58].

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Author contributions

D.P.L. and M.L.J. designed the study. M.L.J. identified morphological vouchers. D.P.L. and M.L.J. conducted laboratory experiments. D.P.L. analyzed the data and wrote the manuscript.

Additional information

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