DNA Barcoding of the Solanaceae Family in Puerto Rico Including Endangered and Endemic Species

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ABSTRACT. The Solanaceae family is one of the largest and well-distributed plant families in the world. It contains species of agricultural and economical importance, such as *Solanum tuberosum*, *Solanum melongena*, *Solanum lycopersicum*, *Nicotiana tabacum*, and *Capsicum annuum*. In Puerto Rico, there are ≈46 species of Solanaceae of which six are endemic: *Brunfelsia densifolia*, *Brunfelsia lactea*, *Brunfelsia portoricensis*, *Goetzea elegans*, *Solanum ensifolium*, and *Solanum woodburyi*. Our objective was to use DNA barcoding to identify the Solanaceae species in Puerto Rico, including the endemics, and to assess the species relationships between them. To accomplish our objective, two chloroplast regions (*psbA-trnH* and *matK*) and a nuclear region [internal transcribed spacer (*ITS*)] were assessed. Pairwise distance and phylogenetic analysis demonstrate that DNA barcoding can be used to discriminate at the species level among these taxa in Puerto Rico. For all three markers, the genus that showed the highest pairwise distance between represented species was *Solanum*, whereas the genus that displayed the least was *Capsicum*. Phylogenetic trees of single and concatenated regions were generated from sequences obtained in this study and from data downloaded from the National Center for Biotechnology Information database. Our results show that this technique can be used to identify species with one, two, or three combinations of DNA barcode markers depending on the taxon. In addition, this is the first study to include the endemic species *S. woodburyi* in a molecular phylogenetic analysis, and it was found to have a close relationship with *S. ensifolium*, also endemic to Puerto Rico, and to *Solanum bahamense* from the Bahamas and Greater Antilles. Therefore, we suggest that *S. woodburyi* might be part of the Bahamense clade.

The Solanaceae family is one of the major groups of angiosperms with ≈2500 species and 100 genera (Filipowicz and Renner, 2012). The family contains species with agricultural and economical importance worldwide such as *Solanum tuberosum*, *Solanum melongena*, *Solanum lycopersicum*, *Nicotiana tabacum*, and *Capsicum annuum*, as well as numerous toxic or poisonous species. Puerto Rico is considered a biodiversity hotspot due to its tropical location and the high concentration of endemic species that have been threatened by habitat loss (Helmer et al., 2002; Myers et al., 2000). According to Axelrod (2011), in Puerto Rico, there are 46 species of Solanaceae from 14 genera. Of these 46 species, *Bunfelsia densifolia*, *Bunfelsia lactea*, *Bunfelsia portoricensis*, *Goetzea elegans*, *Solanum ensifolium*, and *Solanum woodburyi* are endemic. Some of these species are endangered or rare, with only a few studies conducted on them.

Filipowicz and Renner (2012) included the endemic and nonendemic *Brunfelsia* species of Puerto Rico in their systematic study. They hypothesized that these species reached the island 3 to 5 million years ago, and their seeds were dispersed by birds from South America. In research by Muscarella et al. (2014), the Puerto Rican endemic Solanaceae species *G. elegans* and *S. ensifolium* were included as part of the trees of Puerto Rico that were evaluated. They studied 89% of Puerto Rico's trees to analyze the community phylogeny based on regions with different climate areas.

In Puerto Rico, there are two endemic and endangered Solanaceae species: *G. elegans* and *S. ensifolium*. In 1985, *G. elegans* was listed by the U.S. Fish and Wildlife Service under the Endangered Species Act. At that time, there were fewer than 50 individuals in three known sites (U.S. Fish and Wildlife Service, 1985). More recently, this species has increased in number of individuals and has been reported to be present in 10 localities, but it still is considered an endangered species (Vargas, 2013). Comparatively, the species distribution and status of *S. ensifolium* is less known, as it has not been evaluated since 1992. In 1992, 150 individuals were found in the locality of Las Tetras de Cayey (Vargas, 2015). It was hypothesized that the species could be present in other localities, but that has not been confirmed. Unfortunately, during this study, *S. ensifolium* was not found in Las Tetras de Cayey or at other localities where it was thought to be, such as in Florida, Puerto Rico. Its presence at other localities is currently unknown. Due to Hurricane Maria in 2017, there is a high possibility that *S. ensifolium* might be even more critically endangered than in 1992, and in the worst-case scenario, it has gone extinct in the wild. More

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studies and conservation efforts are critically needed to determine the current status of *S. ensifolium*.

In addition, studies of rare species such as the endemic *S. woodburyi* are also needed, especially using molecular tools. This species has not been classified as endangered, but only a few individuals are known to be present in specific localities, such as the Sierra de Luquillo (Axelrod, 2011). Our study is the first to include *S. woodburyi* in a molecular study. Previous research on this species has focused on the description of the species in a synopsis of the endemic species of the West Indies (Howard, 1966; Knapp, 2009). Of the 46 Solanaceae species found in Puerto Rico many, such as *Jaltomata antillana* and *Solanum polygamum*, are not well studied with no molecular data available in public databases such as GeneBank. Therefore, we apply molecular techniques, specifically a DNA barcoding approach, to better understand the species relationships and delimitations for the taxa of Solanaceae in Puerto Rico.

DNA barcoding was proposed as an easy and cost-effective technique for the identification of species. It consists of the amplification of a universal, robust, and standard region. The first region proposed for this analysis was the mitochondrial gene cytochrome c oxidase I (*COI*) for animals (Hebert et al., 2003). It has been successfully used in studies of taxonomy, population genetics, forensics in wildlife crimes, and conservation and in species identification in cases of fish mislabeling, among others (Cywinska et al., 2006; Di Pinto et al., 2015; Hebert et al., 2004; Rolo et al., 2013). In plants, the use of the *COI* region is not recommended for DNA barcoding studies due to its low evolutionary rate (Kress et al., 2005). DNA barcoding for plants has been more challenging than for animals, and more than one barcode region is needed for species identification. Currently, the most accepted and widely used regions in DNA barcoding studies in plants are *rbcL*, *matK*, *psbA-trnH*, and *ITS* (Bolson et al., 2015; CBOL Plant Working Group, 2009; Kress, 2017; Li et al., 2014). Different studies have shown successful identification of species using a combination of these barcode markers, but some studies have also found limited ability to discriminate species within groups consisting of recently diverged species (Collins and Cruickshank, 2013; Kress, 2017; Kress et al., 2009; Muscarella et al., 2014; Spooner, 2009).

In this study, the barcode regions used were two chloroplast regions and a nuclear region, *matK*, *psbA-trnH*, and *ITS*, respectively. The *matK* region is 1500 bp long and encodes for maturase-like polypeptide, which is believed to be involved in Group II intron splicing (Selvaraj et al., 2008). It has an evolutionary rate suitable for distinguishing taxa at high taxonomic levels such as order, family, and, in some cases, at the genus levels (Yu et al., 2011). For a coding region, *matK* has higher variability compared with *rbcL*, but it has not been successfully amplified in all plants (such as gymnosperms). However, it has worked well for angiosperms (CBOL Plant Working Group, 2009; Dunning and Savolainen, 2010; González et al., 2009; Hollingsworth et al., 2011; Kress and Erickson, 2007). Because *matK* cannot distinguish at lower taxonomic levels, it is usually combined with other regions such as *psbA-trnH*, which has a higher substitution rate (Hollingsworth et al., 2011). The *psbA-trnH* region is an intergenic spacer of ≈ 400 to 500 bp in length. Because of its variability, it has been demonstrated to discriminate successfully at the species level when combined with other barcodes (Bolson et al., 2015; Kress and Erickson, 2007; Kress et al., 2009; Štorchová and Olson, 2007).

For discrimination at the species level using DNA barcoding, the nuclear internal transcribed spacer (*ITS*) region been successfully used to complement chloroplast barcodes (Kress et al., 2005; Li et al., 2011). The internal transcribed spacers (*ITS1* and *ITS2*) are between the nuclear ribosomal 18S-5.8S-26S genes, and altogether the marker consists of less than 700 bp (Feliner and Rosselló, 2007). Although *ITS* has helped discriminate at the species level, there are two main concerns when using it in DNA barcoding studies: the possibility of amplifying the *ITS* region from fungal contaminants and the amplification of divergent paralogous copies (Hollingsworth, 2011).

Our overall objectives in this study were 1) to understand the feasibility of using DNA barcoding for identification of Solanaceae species found in Puerto Rico and 2) to study the phylogenetic relationships among the endemic and nonendemic Solanaceae species of Puerto Rico.

Material and Methods

SAMPLE COLLECTION. Permits from the Puerto Rico Department of Natural Resources (2016-EPE-032/O-VS-PVS15-SJ-00854-11072016) and the U.S. Fish and Wildlife Service (41521-2017-08) facilitated the collection of samples from protected areas. Using the location data of previous Solanaceae collections from the University of Puerto Rico Mayaguez's Department of Biology herbarium (MAPR), 53 samples were collected from different field localities in Puerto Rico. Furthermore, 13 samples were collected from the Jardín Botánico of Puerto Rico, Fundación Luis Muñoz Rivera, Guajataca Forest, Rio Abajo Forest, and Maricao Forest. In addition, six samples from the MAPR herbarium were used in this study.

DNA EXTRACTION. The DNA was extracted from fresh samples using the Doyle and Doyle (1990) methodology with a few modifications. Approximately 100 mg of leaf tissue was measured from each sample and pulverized in a mortar chilled with liquid nitrogen. Then, 1.2 mL of 3% CTAB buffer was added and the sample was further crushed. After transferring this solution to a 2 mL microcentrifuge tube, the protocol was followed without further modifications (Doyle and Doyle, 1990). For the DNA extraction from herbarium samples, we used a DNeasy Plant Mini Kit (Qiagen, Germantown, MD) following the manufacturer's recommended procedures.

DNA AMPLIFICATION. The polymerase chain reaction (PCR) amplifications of *matK* and *psbA-trnH* regions were conducted using primers suggested by the Barcode of Life Data System (BOLD). For *matK*: MatK-1RKIM-f and MatK-3FKIM-r (K.J. Kim, unpublished), and for *psbA-trnH*: trnHf_05 (Tate and Simpson, 2003) and *psbA3_f* (Sang et al., 1997) were used (Table 1). For the amplification of the *ITS* region, the primers used were: ITS-u1 and ITS-u2 (Cheng et al., 2016), as well as ITS-Leu1 and ITS4 (Baum et al., 1998; White et al., 1990) (Table 1). The reaction for each sample included 5 μ L of Promega buffer 1X (Promega, Madison, WI), 5 μ L of 5 mM MgCl₂, 1 μ L 0.2 mM dNTPs, 1 μ L of 0.2 μ M forward and reverse primers, 0.2 μ L of Promega GoTaq polymerase (1 unit), and 0.75 μ L of dimethyl sulfoxide (3%) in a final volume of 25 μ L. The thermo-cycling regimens used in the amplification of each region were: 94 °C for 1 min followed by 45 cycles of 94 °C for 30 s, 52 °C for 1 min, 72 °C for 1 min with a final single cycle of 72 °C for 10 min for *matK*; 94 °C for 45 s, followed by 45 cycles of 94 °C for 30 s, 48 °C for 1 min, 72 °C for 40 s with a final

Table 1. Primers used for this study. Primers were selected from the Barcode of Life Data System (BOLD) primer database (Ratnasingham and Hebert, 2007).

Primer	Sequence 5'–3'	Primer length (bp)	Reference
ITS-u1 (f)	GGAAGKARAAGTCGTAACAAGG	22	Cheng et al., 2016
ITS-u4 (r)	RGTTTCTTTTCCTCCGCTTA	20	Cheng et al., 2016
ITS leu	GTCCACTGAACCTTATCATTTAG	23	Urbatsch et al., 2000
ITS 4	TCCTCCGCTTATTGATATGC	20	White et al., 1990
PsbA (f)	GTTATGCATGAACGTAATGCTC	22	Sang et al., 1997
trnH (r)	CGCGCATGGTGGATTACAAATCC	23	Tate and Simpson, 2003
MatK-1RKIM (f)	ACCCAGTCCATCTGGAAATCTTGGTTC	27	K.J. Kim, unpublished data
MatK-3FKIM (r)	CGTACAGTACTTTTGTGTTTACGAG	25	K.J. Kim, unpublished data

single cycle of 72 °C for 10 min for *psbA-trnH*; and 94 °C for 45 s, followed by 45 cycles of 94 °C for 45 s, 48 °C for 40 s, 72 °C for 1 min with a final single cycle of 72 °C for 10 min for *ITS*. Five microliters of each PCR product was visualized in a 1% agarose gel and the rest of the PCR product was then purified using the QIAquick PCR Purification kit (Qiagen) following manufacturer's protocol. The purified samples were then sent to the Genomic Science Laboratory at North Carolina State University (Raleigh) for Sanger sequencing following their protocols.

DATA ANALYSIS. Sequences were aligned and manually edited using BioEdit version 7.1.9 (Hall, 1999). The alignment of each region was performed by ClustalX in BioEdit. A pairwise distance and a neighbor joining (NJ) analysis were then performed for each marker using MEGA version 7.0.21 (Kumar et al., 2016). The pairwise distance data were used to compare relationships between genera and species. Sequence identifier version 1.8 and SequenceMatrix version 1.8 (Vaidya et al., 2011) were used to concatenate the barcode regions, and covert them to Nexus forma. Afterward, the file with the concatenated sequences was exported to PAUP version 4.0 (Swofford, 2002) in which a NJ analysis with 100 bootstraps was performed.

Results

A total of 72 fresh or dried samples were collected (Table 2), of which 66 samples were successfully amplified. PCR amplification success was higher for *matK*, followed by *psbA-trnH* and *ITS* with 91%, 88%, and 87%, respectively. In contrast, sequencing success was higher for *psbA-trnH* followed by *matK* and *ITS* with 89%, 88%, and 85%, respectively. The length of the alignments was 742 bp, 679 bp, and 688 bp for *matK*, *psbA-trnH*, and *ITS*, respectively. Of these regions, *ITS* provided the highest variability, and *matK* was the most conserved region. All sequences from this study have been deposited in GenBank (Table 2).

Pairwise distances

The pairwise distance range was 0.000 to 0.017, 0.000 to 0.054, and 0.007 to 0.088 for *matK*, *psbA-trnH*, and *ITS*, respectively. The outgroup pairwise distance range was 0.166 to 0.296, 0.350 to 0.670, and 0.172–0.467 for *matK*, *psbA-trnH*, and *ITS*, respectively. In all three markers, the genus that showed the highest pairwise distance between its species was *Solanum*, with 0.017, 0.054, and 0.088 for *matK*, *trnH-psbA*, and *ITS*, respectively. The genus that displayed the least pairwise distance between its species was *Capsicum* with 0.000 for the chloroplast barcodes while for *ITS* the genera that exhibited the

least pairwise distance were *Datura* and *Cestrum*, both with 0.007 (Supplemental Tables 1–3).

Neighbor joining analysis

MATK. The *matK* barcode region was able to distinguish 15 specimens at the species level, and in other cases, it was only able to discriminate at genus level (Fig. 1). In the NJ analysis, *matK* was able to distinguish individuals at the species level of *G. elegans* and most *Solanum* species. However, species from *Physalis*, *Capsicum*, *Brugmansia*, *Datura*, *Brunfelsia*, and *Cestrum* could not be differentiated.

PSBA-TRNH. The NJ analysis of *psbA-trnH* showed better discrimination at the species level compared with *matK* (Fig. 2). Most species were separated into their respective clades except for those of the genera *Brugmansia*, *Capsicum*, *Cestrum*, and *Physalis*. For *Physalis* species, this barcode region showed better resolution than *matK* but was still not able to discriminate well at the species level. Other samples that could not be grouped into separate clades were those of the species *S. erianthum*, *S. rugosum*, and *S. polygamum*, which were grouped into a single clade. In addition, an unknown *Physalis* species [Table 2 (no. 27)] was recovered out of the *P. angulata* and *P. pubescens* clades.

ITS. The *ITS* barcode region showed higher variability among individuals in comparison with *matK* and *psbA-trnH* (Fig. 3). It was able to identify, used alone, even more species than *psbA-trnH*. Although more resolution is needed to have a more reliable discrimination between *Cestrum*, *Capsicum*, *Datura*, and *Physalis* species. In contrast with the other barcode regions, the specimens of *S. erianthum*, *S. polygamum*, and *S. rugosum* [Table 2 (no. 39, 46, and 47, respectively)] were able to be separated into clades by species. In contrast, *S. polygamum* and *S. erianthum* grouped into the same clade. Meanwhile, *S. erianthum* grouped in the *Solanum rugosum* clade.

CONCATENATED ANALYSIS. All three barcode regions were concatenated to increase discrimination (Fig. 4). The concatenated sequences had a length range between 1984 to 2109 bp. A total of 43 Solanaceae were included in the concatenated analysis. The genera *Capsicum*, *Solanum*, *Brugmansia*, *Datura*, *Cestrum*, *Brunfelsia*, and *Physalis* were grouped by clades as shown in Fig. 4. *Solanum* was recovered close to *Cestrum*, and these genera were closely related to *Acnistus arborescens* in comparison with the other genera. Similarly, *Brugmansia*, *Datura*, *Solandra*, and *Lycium* were recovered closely related to each other. A notable relationship was *Browallia americana* with *Cestrum*, whereas *Brunfelsia* was more closely related to these two genera in comparison with the rest of the species. Moreover, *Physalis* grouped into a single clade separate from the other clades.

Table 2. GenBank accession numbers of samples used for individual region analysis. Sequences obtained in this study are in bold.

Scientific name		Nonendemic (N) or endemic (E)		matK	psbA-trnH	ITS
1	<i>Acnistus arborescens</i>	N		KU568472.1, MH717175	KJ426590.1, MK381182	DQ314183.1 (1), DQ314181.1 (2), DQ314173.1 (3), MK412103 MK412104
2	<i>Browallia americana</i>	N		EF439050.1, MH717176	MF663708.1, MK381183	JX467600.1 (1), JX467599.1 (2), JX467598.1 (3)
3	<i>Brugmansia x candida</i>	N		KP756823.1 (1), KC146591.1 (2), KC146590.1 (3), MH717177	JX467614.1 (3), KC146626.1 (4), KC146625.1 (5)	
4	<i>Brugmansia suaveolens</i>	N		MF349891.1 (1), KP756824.1 (2), HM851090.1 (3), KC146589.1 (4), MH717178 , MH717179 , MH717180 MH717184	KC146624.1, MK381184 , MK381185 , MK381186 MK381189	JX467597.1 (1), MG693010.1 (2), LC076493.1 (3), HG738853.1 (4), MK412106 , MK412107 , MK412108 MK412105
5	<i>Brunfelsia</i> sp.					
6	<i>Brunfelsia americana</i>	N		KP756837.1, MH717185	KJ426625.1, MK381187 , MK381188	JQ081178.1, MK412109 , MK412110
7	<i>Brunfelsia densifolia</i>	E		MH717181 , MH717182	MK381190 , MK381191	JQ081192.1, MK412111 , MK412112 , MK412113
8	<i>Brunfelsia lactea</i>	E		KJ012480.1, MH717183	KJ426626.1, MK381192	JQ081203.1 (1), JQ081202.1 (2), MK412114
9	<i>Brunfelsia nitida</i>	N		KP756841.1	—	JQ081211.1 (1), JQ081210.1 (2)
10	<i>Brunfelsia portoricensis</i>	E		HM446659.2 (1), KJ012481.1 (2)*, MH717186 MH717187	MF663707.1 (1), KJ426627.1 (2), HM446890.1 (3), MK381193 MK381194	JQ081217.1, MK412115
11	<i>Capsicum chinense</i>	N		EF537310.1 (1), EF537309.1 (2), EF537308.1 (3)	EF537250.1 (1), EF537249.1 (2), EF537248.1 (3)	MK412116 JQ885438.1 (1), JQ885437.1 (2), JQ885436.1 (3), JQ885435.1 (4)
12	<i>Capsicum annuum</i>	N		AB721838.1 (1), AB721834.1 (2), AB721832.1 (3), HQ705990.1 (4), MH717188	EF537187.1 (1), EF537186.1 (2), EF537185.1 (3), JX856331.1 (4), JQ087871.1 (5), MK381195 MK381196	HQ705990.1, MK412117
13	<i>Capsicum frutescens</i>	N				
14	<i>Cestrum</i> sp.			MH717195	MK381196	MK412118
15	<i>Cestrum citrifolium</i>	N		KJ012506.1 (1), KJ012505.1 (2), MH717189 , MH717190	KJ426649.1 (1), KJ426648.1 (2), MK381197 , MK381200	MK412119 , MK412120
16	<i>Cestrum diurnum</i>	N		GU135071.1 (1), KJ652185.1 (2), MH717191 , MH717192	GU135406.2, MK381198 , MK381199	JQ230969.1, MK412121 , MK412122
17	<i>Cestrum macrophyllum</i>	N		HM446667.2, MH717193	HM446901.1, MK381201	MK412123 , MK412124
18	<i>Cestrum nocturnum</i>	N		MF350140.1 (1), KX783646.1 (2), KJ652183.1 (3), DQ508570.1 (4), MH717194	MF348491.1 (1), HG963496.1 (2), JX856333.1 (3), MK381202	DQ508673.1 (1), AY206731.1 (2), JQ230970.1 (3), MK412125
19	<i>Datura innoxia</i>	N		EF438895.1 (1), GQ434218.1 (2), JX996065.1 (3), GQ274313.1 (4), MH717196	JX467623.1 (1), JN244377.1 (2), JN244376.1 (3), JN244375.1 (4), MK381203	HQ658589.1 (1), HQ658588.1 (2), HQ658587.1 (3), AY875750.1 (4), MK412126
20	<i>Datura metel</i>	N		JN244346.1 (1), GQ434221.1 (2), GQ434220.1 (3), JN114747.1 (4)	GQ435287.1 (1), JX467620.1 (2), JN244383.1 (3), JN244382.1 (4)	HQ658594.1 (1), HQ658578.1 (2), LC076492.1 (3), JX467604.1 (4)
21	<i>Datura stramonium</i>	N		KX783664.1 (1), KX258743.1 (2), KU556671.1 (3), KF022401.1 (4)	MF348633.1	DQ006041.1 (1), MG217819.1 (2), MF348633.1 (3), KF454041.1 (4)

Continued next page

Table 2. Continued.

	Scientific name	Nonendemic (N) or endemic (E)	matK	psbA-trnH	ITS
22	<i>Goetzea elegans</i>	E	KJ012611.1 (1), KJ012610.1 (2), HQ384563.1 (3), MH717197 , MH717198 , MH717199	MF663711.1 (1), KJ426751.1 (2), KJ426750.1 (3), MK381204 , MK381205	AY206736.1, MK412127 , MK412128 , MK412129
23	<i>Jaltomata antillana</i>	N	MH717201	MK381206	—
24	<i>Lycianthes virgata</i>	N	AB036627.1	EU742440.1 (1) HQ216167.1 (2), FJ189606.1 (3)	DQ124619.1
25	<i>Lycium americanum</i>	N			
26	<i>Physalis angulata</i>	N	MF159481.1 (1), MF159479.1 (2), EF438950.1 (3), EF438949.1 (4), MH717200	KY263829.1 (1), KY263828.1 (2), MF143763.1 (3), MF143739.1 (4), MK381207	LC076478.1 (1), AY665875.1 (2), MK412130
27	<i>Physalis</i> sp. (1)		MH717202 (1),	MK381208 (1),	MK412131 (1),
28	<i>Physalis</i> sp. (2)		MH717203 (2)	MK381209 (2)	MK412132 (2)
29	<i>Physalis cordata</i>	N		—	AY665886.1
30	<i>Physalis ignota</i>	N	JQ589235.1 (1), JQ589234.1 (2), JQ589233.1 (3)	—	AY665897.1
31	<i>Physalis pubescens</i>	N	EF438977.1 (1), EF438975.1 (2), EF438974.1 (3), EF438944.1 (4)	KY263837.1 (1), KY263836.1 (2), KY263835.1 (3), GQ435283.1 (4)	AY665917.1 (1), AY665916.1 (2)
32	<i>Solandra grandiflora</i>	N	KP756859.1, MH717204 , MH717205 , MH717206	MK381210 , MK381211 MK381212	KT366097.1, MK412133 , MK412134 , MK412135
33	<i>Solanum</i> sp.		MH717220	MK381225	—
34	<i>Solanum americanum</i>	N	HQ235334.1 (1), KJ773158.1 (2), MH717228 , MH717229	MK381213 , MK381214	KC540786.1 (1), KC540784.1 (2), GQ478109.1 (3), GU323361.1 (4), MK412136 , MK412137 , AY996487.1, MK412138 , MK412139 AF244728.1*
35	<i>Solanum bahamense</i>	N	MH717207 , MH717208	MK381215 , MK381216	KU696986.1 (1), JQ638808.1 (2), JQ638806.1 (3), AY561265.1 (4), MK412140 , MK412141
36	<i>Solanum campechiense</i>	N		—	KC539148.1 (1), GQ478110.1 (2), AY996508.1 (3), AF244730.1 (4), MK412142 , MK412143
37	<i>Solanum capsicoides</i>	N	MH717209 , MH717210	MK381217 , MK381218	HG963512.1 (1), KJ426943.1 (2), KP095977.1 (3), KP095978.1 (4)
38	<i>Solanum elaeagnifolium</i>	N	EU983576.1, MH717213 , MH717214	HM016411.1, MK381221 , MK381222	KP093239.1, MK412144 , MK412145
39	<i>Solanum ensifolium</i>	E	KJ012782.1, MH717211 , MH717212	KJ426942.1 (1), KJ426941.1 (2), MK381219 , MK381220	
40	<i>Solanum erianthum</i>	N	KP093367.1 (1), KP093366.1 (2), KJ012783.1 (3)*, MH717215 , MH717216	HG963512.1 (1), KJ426943.1 (2), KP095977.1 (3), KP095978.1 (4), MK381223	
41	<i>Solanum jamaicense</i>	N	GU135067.1 (1), KC312939.1 (2), JQ589259.1 (3), MH717221	GU135400.2, MK381224	GU799064.1 (1), GU799063.1 (2), AF244724.1 (3), MK412146
42	<i>Solanum lycopersicum</i>		JQ412261.1 (1), EF438904.1 (2)	HG963710.1 (1), HQ856112.1 (2)	JN713141.1 (1), DQ314157.1 (2)
43	<i>Solanum mammosum</i>	N	KC312940.1	JX856342.1	AF244721.1 (1), KU696988.1 (2)

Continued next page

Table 2. Continued.

	Scientific name	Nonendemic (N) or endemic (E)	<i>matK</i>	<i>psbA-trnH</i>	<i>ITS</i>
44	<i>Solanum melongena</i>	N	KJ873108.1 (1), KJ652176.1 (2), KC312941.1 (3), KX258752.1 (4), MH717219	HM016410.1 (1), HM016408.1 (2), MF663709.1 (3), HF572794.1 (4), MK381226	KU696991.1 (1), HQ721866.1 (2), JQ638894.1 (3), JQ638897.1 (4), MK412152 KF546021.1 MK412153
45	<i>Solanum nigrescens</i>	N	JQ589579.1, MH717222	—	—
46	<i>Solanum nudum</i>	N	MH717217, MH717218	MK381227	—
47	<i>Solanum polygamum</i>	N	JQ589269.1 (1), JQ589268.1 (2), MH717223	MK381228, MK381229	AY996543.1, MK412147, MK412148
48	<i>Solanum rugosum</i>	N	MH717224	MK381234	JN542598.1, MK412154
49	<i>Solanum seafortianum</i>	N	MF444846.1 (1), GU135088.1 (2), KX258745.1 (3), MH717225, MH717226, MH717227	HG963649.1, MK381233	KT820914.1, MK412156
50	<i>Solanum torvum</i>	N	GU135120.1 (1), GU135073.1 (2), GU135041.1 (3), KC312949.1 (4), MH717230	GU135422.2 (1), JN047230.1 (2), HG963494.1 (3), HG963489.1 (4), MK381230, MK381231, MK381232	KU719933.1 (1), KU696993.1 (2), KU719924.1 (3), MK412149, MK412150, MK412151
51	<i>Solanum viarum</i>	N	—	MF663710.1 (1), GU135408.2 (2), GU135370.2 (3), MK381235	AY561275.1 (1), KU696990.1 (2), KU696989.1 (3), LC076484.1 (4), MK412155 AF244731.1
52	<i>Solanum wendlandii</i>	N	MH717231, MH717232	MK381236, MK381237	—
53	<i>Solanum woodburyi</i>	E	KR024927.1	KR735821.1	GQ478092.1
54	<i>Ipomoea obscura</i>		LN877448.1	AB331298.1	AF245412.1
55	<i>Buxus microphylla</i>				

Discussion

The main purpose of this study was to obtain DNA sequence information from DNA barcoding loci, and to understand the feasibility of using DNA barcoding to identify Solanaceae specimens in Puerto Rico, including endemic and endangered species. An additional objective was to ascertain which DNA barcode marker, individually or in combination, can provide greater discrimination within Solanaceae, which then can help in future studies or conservation efforts.

ENDEMIC SPECIES DISCRIMINATION. Puerto Rico has six endemic Solanaceae species: *B. densifolia*, *B. lactea*, *B. portoricensis*, *G. elegans*, *S. ensifolium*, and *S. woodburyi* [Table 2 (no. 7, 8, 10, 22, 39, and 53)]. DNA barcoding of *Brunfelsia* species has shown discrimination at the species level for both chloroplast and nuclear barcodes, especially when the barcodes are combined (Filipowicz and Renner, 2012). *Brunfelsia* consists of 50 species, high endemism, and a wide distribution in South America and the Antilles. It has been suggested that the adaptation of endemic species of *Brunfelsia* to certain habitats will contribute to the successful species identification within this genus (Filipowicz and Renner, 2012; Filipowicz et al., 2012). Our study also showed good discrimination of the genus *Brunfelsia* using all three barcodes combined (Fig. 4) or using only two barcodes, either *ITS/matK* or *ITS/psbA-trnH* (Supplemental Figs. 1 and 2). In fact, species of *Brunfelsia* could be discriminated only using the ITS barcode (Fig. 3) thus do not require assessment of other DNA barcodes.

Friedrich von Wettstein (1895–1945) proposed that *Goetzea* was part of the Solanaceae. Subsequently, *Goetzea* was classified by different authors in its own family, the Goetzeaceae (Carlquist, 1988; Cronquist, 1981; Dahlgren, 1980). However, morphological and molecular studies have placed *Goetzea* once again as part of the Solanaceae (Fay et al., 1998; Olmstead et al., 1999; Zona, 1989). This demonstrates the important impact molecular studies have had on the taxonomy of this genus. In this study, *G. elegans* [Table 2 (no. 22)] was easily identified using the chloroplast and the nuclear markers (Figs. 1–3). This genus consists of two species, *G. elegans* and *G. ekmanii*, and the latter is endemic to the Dominican Republic (Liogier, 1995), and material was not available to include it in this study. Additional research is needed to determine if DNA barcoding can be successfully used to discriminate between these two species.

The critically endangered Puerto Rican endemic species *S. ensifolium* [Table 2 (no. 39)] was found in neither Cayey nor in Florida (Puerto Rico), thus the status of this plant in the wild is unknown (Vargas, 2015). Further work is needed to propagate the plant and to assess if the species is present in other parts of the island. In the wild, this plant can be confused with *S. bahamense* when there are no flowers or fruit (Strickland-Constable et al., 2010), which makes DNA barcoding important for identifying sterile specimens of this species. In our study, it was demonstrated that *matK* or *psbA-trnH*, individually or in combination, can discriminate between these morphologically similar species (Figs. 1 and 2, Supplemental Fig. 3). Unfortunately, amplification and sequencing of the nuclear region *ITS* did not work for *S. ensifolium*, nor for *Lyciantes virgata*, *S. jamaicensis*, *Solanum* sp. 49, and *S. woodburyi*, even with the use of different primer sets.

NONENDEMIC SPECIES DISCRIMINATION. Although *S. woodburyi* [Table 2 (no. 53)] is not considered an endangered

Fig. 1. Phylogenetic neighbor joining analysis based on the *matK* barcode. Bootstraps scores are shown (100 replicates, $\geq 50\%$) for each branch. Samples with sequences obtained in this study are depicted with an asterisk (*). Discrimination at genus level can be observed using *matK* barcode.

species, it is rare and its known distribution is limited to Sierra de Luquillo (Axelrod, 2011). This is the first time *S. woodburyi* has been sampled in a molecular study. With the successful analysis of the chloroplast barcodes *matK* and *psbA-trnH*, our analysis shows a close phylogenetic relationship between *S.*

woodburyi, *S. ensifolium*, and *S. bahamense* (Figs. 1 and 2). We suggest that *S. woodburyi* might be part of the Bahamense clade. Using either *matK* or *psbA-trnH*, specimens of *S. woodburyi* can be successfully discriminated at the species level. The ITS region of *S. woodburyi* was not able to be amplified.

Fig. 3. Phylogenetic neighbor joining analysis based on the ITS (internal transcribed spacer) barcode. Bootstraps scores are shown (100 replicates, $\geq 50\%$) for each branch. Samples with sequences obtained in this study are depicted with an asterisk (*). Discrimination at species level was observed using ITS barcode.

erianthum for our study. Upon analysis of the ITS region, we are able to identify the sample as *S. rugosum*, which is found in wet or moist areas at high elevations. In contrast, *S. erianthum* is found in dry limestone areas. Because the sample was obtained from Cayey, which is an area of high elevation and

moisture, we were able to reliably identify the sample correctly as *S. rugosum*.

DISCRIMINATION OF AGRICULTURALLY VALUABLE CROPS. This study includes six agriculturally valuable crops, which are *Capsicum annum*, *C. frutescens*, *C. chinense*, *S. lycopersicum*,

Fig. 4. Neighbor joining analysis of the concatenated sequences of *ITS* + *matK* + *psbA-trnH*. Samples with sequences obtained in this study are depicted with an asterisk (*). A consensus of sequences was obtained for each species. Genera grouped by clades were designated with their respectively genus.

S. melongena, and *Physalis pubescens*. The genus that showed least species discrimination in our study was *Capsicum*, which is consistent with previous studies (Olmstead et al., 2008; Walsh and Hoot, 2001). However, the concatenated phylogenetic analysis showed better resolution in species discrimination of the *Capsicum* species compared with the single and double DNA barcode analysis (Fig. 4). *Capsicum* is considered to be native to the new world and domesticated by Native Americans (Moscone et al., 2007). As a result, in comparison with other Solanaceae species, *Capsicum* species have been more recently diverged, thus making species identification with

only chloroplast barcodes not feasible. In Puerto Rico, the most cultivated and consumed *Capsicum* species are *C. chinense*, *C. annuum*, and *C. frutescens* [Table 2 (no. 11, 12, and 13)]. These three species are some of the most economically important crops of this genus (Carrizo García et al., 2016). In the present study, *ITS* showed more discrimination between *C. chinense*, *C. annuum*, and *C. frutescens*, with less variability observed between *C. chinense* and *C. frutescens* (Fig. 3). Previously, Walsh and Hoot (2001) studied the species relationships of *Capsicum* using other chloroplasts regions and the nuclear region, and obtained similar results. Although the chloroplast

region was not able to discriminate between species, the nuclear region was able to provide significant variation to discriminate at species level.

S. lycopersicum is easily discriminated with individual and concatenated DNA barcodes in this study. A close relationship between *S. lycopersicum* with *S. seafortianum* and *S. americanum* was observed in individual and concatenated phylogenetic analysis, similar to previous studies (Marshall et al., 2001; Melotto-Passarini et al., 2008; Weese and Bohs, 2007). In all of these studies the closest species to *S. lycopersicum* was *S. tuberosum* (potato), and as a result, *S. lycopersicum* has been considered part of the potato clade (Bohs, 2005). Moreover, *S. melongena* showed a closer relationship with *S. elaeagnifolium* when evaluated with the chloroplast DNA barcodes. This relationship has been observed in previous phylogenetic analyses that included chloroplast and nuclear DNA barcodes (Weese and Bohs, 2007; Zhang et al., 2013). In contrast, we observed a slightly closer relationship with *S. torvum* in the ITS and the concatenated phylogenetic analyses.

Another agriculturally important crop genus included in this study is *Physalis*, represented by *P. pubescens* and *P. angulata*. Specimens of *Physalis* are difficult to identify to species morphologically. A recent study shows that the chloroplast barcode *psbA-trnH* was able to identify species of this genus (Feng et al., 2018). In our study, the DNA barcode that showed higher variability within *Physalis* was *psbA-trnH*, and when concatenated barcodes were used better discrimination was obtained (Fig. 4, Supplemental Fig. 2).

Additionally, specimens of unknown species were added to our study from different resources to test if the neighbor joining analysis of the different barcodes was capable of identifying them to species. These species were *Solanum* sp. 49, *Physalis* sp. 52, *Physalis* sp. 17, and *Brunfelsia* sp. For the sample *Solanum* sp. 49 [Table 2 (no. 33)] *matK* was able to group it with *S. mammosum*. The unknown samples *Physalis* sp. 52 and *Physalis* sp. 17 [Table 2 (no. 27 and 28)] were identified as *Physalis ignota* and *Physalis pubescens*, respectively (Fig. 4, Supplemental Fig. 2). Traditional taxonomic identification also confirmed that *Solanum* sp. 49 was *Solanum mammosum*. Although the *Solanum* and *Physalis* species were identified with DNA barcoding, the unknown *Brunfelsia* species [Table 2 (no. 5)] could not be matched to any of the Puerto Rico Solanaceae, nor ones from GenBank. This plant was collected from a private citizen's garden, who received it as a gift. Our results suggest that this plant does not correspond to any of the known *Brunfelsia* species from Puerto Rico and is not represented in the GenBank database.

Conclusions

Our study suggests that DNA barcoding can be used to identify the *Solanaceae* species of Puerto Rico. This molecular technique can be useful for future identification of the endemic and nonendemic plants of Puerto Rico for future conservation efforts or other studies. The majority of species were successfully identified with the barcodes individually. *MatK* was the most conserved region, and *ITS* the most variable region. As shown in previous studies, the *psbA-trnH* barcode by itself showed good discrimination in species identification. The concatenated analysis of *matK*, *psbA-trnH*, and *ITS* gave more resolution in comparison with the other analyses.

Literature Cited

- Axelrod, F.S. 2011. A systematic vademecum to the vascular plants of Puerto Rico. Bot. Res. Inst. Texas Press, Fort Worth, TX.
- Baum, D.A., R.L. Small, and J.F. Wendel. 1998. Biogeography and floral evolution of baobabs (*Adansonia*, Bombacaceae) as inferred from multiple data sets. Syst. Biol. 47:181–207.
- Bohs, L. 2005. Major clades in *Solanum* based on *ndhF* sequence data, p. 27–49. In: T. Keating, R. Hollowell, and V. Croat (eds.). A festschrift for William G. D'Arcy: The legacy of a taxonomist. Missouri Bot. Garden Press, Chicago, IL.
- Bolson, M., E. de Camargo Smidt, M.L. Brotto, and V. Silva-Pereira. 2015. ITS and *trnH-psbA* as efficient DNA barcodes to identify threatened commercial woody angiosperms from southern Brazilian Atlantic rainforests. PLoS One 10:1–18.
- Carlquist, S. 1988. Wood anatomy and relationships of Duceodendraceae and Goetzeaceae. Intl. Assn. Wood Anatomists (IAWA) J. 9:3–12.
- Carrizo García, C., M. Barfuss, E.M. Schr, G.E. Barboza, R. Samuel, E.A. Moscone, and F. Ehrendorfer. 2016. Phylogenetic relationships, diversification and expansion of chili peppers (*Capsicum*, Solanaceae). Ann. Bot. 118:35–51.
- CBOL Plant Working Group. 2009. A DNA barcode for land plants. Proc. Natl. Acad. Sci. USA 106:12794–12797.
- Cheng, T., C. Xu, L. Lei, C. Li, Y. Zhang, and S. Zhou. 2016. Barcoding the kingdom Plantae: New PCR primers for ITS regions of plants with improved universality and specificity. Mol. Ecol. Resour. 16:138–149.
- Collins, R.A. and R.H. Cruickshank. 2013. The seven deadly sins of DNA barcoding. Mol. Ecol. Resour. 13:969–975.
- Cronquist, A. 1981. An integrated system of classification of flowering plants. Columbia Univ. Press, New York, NY.
- Cywinska, A., F.F. Hunter, and P.D. Hebert. 2006. Identifying Canadian mosquito species through DNA barcodes. Med. Vet. Entomol. 20:413–424.
- Dahlgren, R.M.T. 1980. A revised system of classification of the angiosperms. Bot. J. Linn. Soc. 80:91–124.
- Di Pinto, A., P. Marchetti, A. Mottola, G. Bozzo, E. Bonerba, E. Ceci, M. Botarro, and G. Tantillo. 2015. Species identification in fish fillet products using DNA barcoding. Fish. Res. 170:9–13.
- Doyle, J.J. and J.L. Doyle. 1990. Isolation of plant DNA from fresh tissue. Focus 12:13–15.
- Dunning, L.T. and V. Savolainen. 2010. Broad-scale amplification of *matK* for DNA barcoding plants, a technical note. Bot. J. Linn. Soc. 164:1–9.
- Fay, M.F., R.G. Olmstead, J.E. Richardson, E. Santiago, G.T. Prance, and M.W. Chase. 1998. Molecular data support the inclusion of *Duceodendron cestroides* in Solanaceae. Kew Bull. 53:203–212.
- Feliner, G.N. and J.A. Rosselló. 2007. Better the devil you know? Guidelines for insightful utilization of nrDNA ITS in species-level evolutionary studies in plants. Mol. Phylogenet. Evol. 44:911–919.
- Feng, S., K. Jiao, Y. Zhu, H. Wang, M. Jiang, and H. Wang. 2018. Molecular identification of species of *Physalis* (Solanaceae) using a candidate DNA barcode: The chloroplast *psbA-trnH* intergenic region. Genome 61:15–20.
- Filipowicz, N., M. Nee, and S. Renner. 2012. Description and molecular diagnosis of a new species of *Brunfelsia* (Solanaceae) from the Bolivian and Argentinean Andes. PhytoKeys 10:83.
- Filipowicz, N. and S.S. Renner. 2012. *Brunfelsia* (Solanaceae): A genus evenly divided between South America and radiations on Cuba and other Antillean islands. Mol. Phylogenet. Evol. 64:1–11.
- González, M.A., C. Baraloto, J. Engel, S.A. Mori, P. Pétronelli, B. Riéra, A. Roger, C. Thebaud, and J. Chavez. 2009. Identification of amazonian trees with DNA barcodes. PLoS One 4:e7483.
- Hall, T.T. 1999. BioEdit: A user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucl. Acids Symp. Ser 41:95–98.

- Hebert, P.D.N., A. Cywinska, S.L. Ball, and J.R. deWaard. 2003. Biological identifications through DNA barcodes. *Proc. Biol. Sci.* 270:313–321.
- Hebert, P.D.N., E.H. Penton, J.M. Burns, D.H. Janzen, and W. Hallwachs. 2004. Ten species in one: DNA barcoding reveals cryptic species in the neotropical skipper butterfly *Astraptes fulgerator*. *Proc. Natl. Acad. Sci. USA* 101:14812–14817.
- Helmer, E.H., O. Ramos, T. López, M. Quinones, and W. Diaz. 2002. Mapping the forest type and land cover of Puerto Rico, A component of the Caribbean biodiversity hotspot. *Caribb. J. Sci.* 38:165–183.
- Hollingsworth, P.M. 2011. Refining the DNA barcode for land plants. *Proc. Natl. Acad. Sci. USA* 108:19451–19452.
- Hollingsworth, P.M., S.W. Graham, and D.P. Little. 2011. Choosing and using a plant DNA barcode. *PLoS One* 6:e19254.
- Howard, R.A. 1966. Notes on some plants of Puerto Rico. *J. Arnold Arbor.* 47:138–139.
- Knapp, S. 2009. Synopsis and lectotypification of *Solanum* (Solanaceae) species endemic in the West Indies. *An. Jardin Botánico Madr.* 1979 66:65–84.
- Kress, W.J. 2017. Plant DNA barcodes: Applications today and in the future. *J. Syst. Evol.* 55:291–307.
- Kress, W.J. and D.L. Erickson. 2007. A two-locus global DNA barcode for land plants: The coding *rbcl* gene complements the non-coding *trnH-psbA* spacer region. *PLoS One* 2:e508.
- Kress, W.J., D.L. Erickson, F. Jones, N.G. Swenson, R. Perez, E. Bermingham, and O. Sanjur. 2009. Plant DNA barcodes and a community phylogeny of a tropical forest dynamics plot in Panama. *Proc. Natl. Acad. Sci. USA* 106:18621–18626.
- Kress, W.J., K.J. Wurdack, E.A. Zimmer, L.A. Weigt, and D.H. Janzen. 2005. Use of DNA barcodes to identify flowering plants. *Proc. Natl. Acad. Sci. USA* 102:8369–8374.
- Kumar, S., G. Stecher, and K. Tamura. 2016. MEGA7: Molecular evolutionary genetics analysis Version 7.0 for bigger datasets. *Mol. Biol. Evol.* 33:1870–1874.
- Li, D.Z., L. Gao, H. Li, H. Wang, X. Ge, J. Liu, and G. Duan. 2011. Comparative analysis of a large dataset indicates that internal transcribed spacer (ITS) should be incorporated into the core barcode for seed plants. *Proc. Natl. Acad. Sci. USA* 108:19641–19646.
- Li, X., Y. Yang, R. Henry, M. Rossetto, Y. Wang, and S. Chen. 2014. Plant DNA barcoding: From gene to genome. *Biol. Rev. Camb. Philos. Soc.* 90:157–166.
- Lioy, H.A. 1995. Descriptive flora of Puerto Rico and adjacent islands. Univ. Puerto Rico Press, Rio Piedras, PR.
- Marshall, J.A., S. Knapp, M.R. Davey, J.B. Power, E.C. Cocking, M.D. Bennett, and A. Cox. 2001. Molecular systematics of *Solanum* section *Lycopersicum* (*Lycopersicon*) using the nuclear ITS rDNA region. *Theor. Appl. Genet.* 103:1216–1222.
- Melotto-Passarin, D.M., I.J. Berger, K. Dressano, V.F. De Martin, G. Conde, G.C. Oliveira, and H. Carrer. 2008. Phylogenetic relationships in Solanaceae and related species based on cpDNA sequence from plastid *trnE-trnT* region. *Crop Breed. Appl. Biotechnol.* 8:85–95.
- Moscone, E.A., M. Scaldaferrro, M. Grabiele, N.M. Cecchini, Y.S. García, R. Jarret, and F. Ehrendorfer. 2007. The evolution of chili peppers (*Capsicum*-Solanaceae): A cytogenetic perspective. *Acta Hort.* 745:137–170.
- Muscarella, R., M. Uriarte, D. Erickson, N.G. Swenson, J.K. Zimmerman, and W.J. Kress. 2014. A well-resolved phylogeny of the trees of Puerto Rico based on DNA barcode sequence data. *PLoS One* 9:e112843.
- Myers, N., R.A. Mittermeier, C.G. Mittermeier, G.A.B. da Fonseca, and J. Kent. 2000. Biodiversity hotspots for conservation priorities. *Nature* 403:853–858.
- Olmstead, R.G., V.F. Garcia, H.A. Migid, S.M. Collier, L. Bohs, and E. Santiago-Valentin. 2008. A molecular phylogeny of the Solanaceae. *Taxon* 57:1159–1181.
- Olmstead, R.G., J.A. Sweere, R.E. Spangler, L. Bohs, and J.D. Palmer. 1999. Phylogeny and provisional classification of the Solanaceae based on chloroplast DNA, p. 111–137. In: J. Nee, M. Symon, D. Lester, and R. Jessop (eds.). *Solanaceae IV: Advances in biology and utilization*. Royal Botanic Gardens, London, UK.
- Ratnasingham, S. and P.D. Herbert. 2007. BOLD: The barcode of life data system. *Mol. Ecol. Notes* 7:355–364.
- Rolo, E.A., A.R. Oliveira, C.G. Dourado, A. Farinha, M.T. Rebelo, and D. Dias. 2013. Identification of sarcosaprophagous diptera species through DNA barcoding in wildlife forensics. *Forensic Sci. Intl.* 228:160–164.
- Sang, T., D.J. Crawford, and T.F. Stuessy. 1997. Chloroplast DNA phylogeny, reticulate evolution, and biogeography of *Paonia* (Paeoniaceae). *Amer. J. Bot.* 84:1120–1136.
- Selvaraj, D., R.K. Sarma, and R. Sathishkumar. 2008. Phylogenetic analysis of chloroplast *matK* gene from Zingiberaceae for plant DNA barcoding. *Bioinformation* 3:24–27.
- Spooner, D.M. 2009. DNA barcoding will frequently fail in complicated groups: An example in wild potatoes. *Amer. J. Bot.* 96:1177–1189.
- Štorchová, H. and M.S. Olson. 2007. The architecture of the chloroplast *psbA-trnH* non-coding region in angiosperms. *Plant Syst. Evol.* 268:235–256.
- Strickland-Constable, R., H. Schneider, S.W. Ansell, S.J. Russell, and S. Knapp. 2010. Species identity in the *Solanum bahamense* species group (Solanaceae, *Solanum* subgenus *Leptoslemonum*). *Taxon* 59:209–226.
- Swofford, D.L. 2002. PAUP*: Phylogenetic analysis using parsimony (*and other methods), version 4.0b3a. Sinauer Associates, Sunderland, MA.
- Tate, J.A. and B.B. Simpson. 2003. Paraphyly of *Tarasa* (Malvaceae) and diverse origins of the polyploid species. *Syst. Bot.* 28:723–737.
- Urbatsch, L.E., B.G. Baldwin, and M.J. Donoghue. 2000. Phylogeny of the coneflowers and relatives (Heliantheae: Asteraceae) based on nuclear rDNA internal transcribed spacer (ITS) sequences and chloroplast DNA restriction site data. *Syst. Bot.* 25:539–565.
- U.S. Fish and Wildlife Service. 1985. Four plants given endangered species act protection. *Endangered Species Tech. Bul.* MB-815.
- Vaidya, G., D.J. Lohman, and R. Meier. 2011. SequenceMatrix: Cladistics multi-gene datasets with character set and codon information. *Cladistics* 27:171–180.
- Vargas, M. 2013. *Matabuey (Goetzea elegans) 5-year review: Summary and evaluation*. 19 Jan. 2019. <https://ecos.fws.gov/docs/five_year_review/doc4257.pdf>.
- Vargas, M. 2015. *Erubia (Solanum drymophilum) 5-year review: Summary and evaluation*. 19 Jan. 2019. <https://ecos.fws.gov/docs/five_year_review/doc4652.pdf>.
- Walsh, B.M. and S.B. Hoot. 2001. Phylogenetic relationships of *Capsicum* (Solanaceae) using DNA sequences from two noncoding regions: The chloroplast *atpB-rbcL* spacer region and nuclear waxy introns. *Intl. J. Plant Sci.* 162:1409–1418.
- Weese, T.L. and L. Bohs. 2007. A three-gene phylogeny of the genus *Solanum* (Solanaceae). *Syst. Bot.* 32:445–463.
- White, T.J., T. Bruns, S. Lee, and J. Taylor. 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In *A guide to methods and applications*. Academic Press, New York, NY.
- Yu, J., J.H. Xue, and S.L. Zhou. 2011. New universal *matK* primers for DNA barcoding angiosperms. *J. Syst. Evol.* 49:176–181.
- Zhang, W., X. Fan, S. Zhu, H. Zhao, and L. Fu. 2013. Species-specific identification from incomplete sampling: Applying DNA barcodes to monitor invasive solanum plants. *PLoS One* 8:e55927.
- Zona, S. 1989. Leaf anatomy of the Goetzeaceae. *Aliso J. Systematic Evol. Bot.* 12:303–312.

Supplemental Fig. 1. Neighbor joining analysis of the concatenated sequences of *ITS* + *matK*. Bootstraps scores are shown (100 replicates, $\geq 50\%$) for each branch.

Supplemental Fig. 2. Neighbor joining analysis of the concatenated sequences of *ITS + psbA-trnH*. Bootstraps scores are shown (100 replicates, $\geq 50\%$) for each branch.

Supplemental Fig. 3. Neighbor joining analysis of the concatenated sequences of *matK*+*psbA-trnH*. Bootstraps scores are shown (100 replicates, $\geq 50\%$) for each branch.

Supplemental Table 1. Interspecific and intraspecific pairwise distance for the *MatK* DNA barcode.

No.	Species	Intraspecific difference	Interspecific difference
1	<i>Acnistus arborescens</i>	0.004	—
2	<i>Browallia americana</i>	0.004	—
3	<i>Brugmansia ×candida</i>	0.000	0.004
4	<i>Brugmansia suaveolens</i>	0.001	0.004
5	<i>Brunfelsia americana</i>	0.004	0.001
6	<i>Brunfelsia densifolia</i>	0.000	0.001
7	<i>Brunfelsia lactea</i>	0.004	0.001
8	<i>Brunfelsia nitida</i>	—	0.001
9	<i>Brunfelsia portoricensis</i>	0.000	0.001
10	<i>Capsicum chinense</i>	—	0.000
11	<i>Capsicum frutescens</i>	0.000	0.000
12	<i>Capsicum annuum</i>	0.000	0.000
13	<i>Cestrum citrifolium</i>	0.000	0.002
14	<i>Cestrum diurnum</i>	0.000	0.002
15	<i>Cestrum macrophyllum</i>	0.002	0.002
16	<i>Cestrum nocturnum</i>	0.001	0.002
17	<i>Datura inoxia</i>	0.002	0.005
18	<i>Datura metel</i>	0.000	0.005
19	<i>Datura stramonium</i>	0.002	0.005
20	<i>Goetzea elegans</i>	0.000	—
21	<i>Lycium americanum</i>	—	—
22	<i>Lycianthes virgata</i>	—	—
23	<i>Physalis angulata</i>	0.000	0.000
24	<i>Physalis pubescens</i>	0.000	0.000
25	<i>Physalis ignota</i>	0.000	0.000
26	<i>Solandra grandiflora</i>	0.017	
27	<i>Solanum americanum</i>	0.000	0.017
28	<i>Solanum bahamense</i>	0.000	0.017
29	<i>Solanum capsicoides</i>	0.001	0.017
30	<i>Solanum elaeagnifolium</i>	0.003	0.017
31	<i>Solanum ensifolium</i>	0.000	0.017
32	<i>Solanum erianthum</i>	0.000	0.017
33	<i>Solanum jamaicense</i>	0.001	0.017
34	<i>Solanum mammosum</i>	—	0.017
35	<i>Solanum melongena</i>	0.000	0.017
36	<i>Solanum nudum</i>	0.009	0.017
37	<i>Solanum polygamum</i>	0.018	0.017
38	<i>Solanum rugosum</i>	0.002	0.017
39	<i>Solanum seafortianum</i>	—	0.017
40	<i>Solanum torvum</i>	0.000	0.017
41	<i>Solanum viarum</i>	0.001	0.017
42	<i>Solanum woodburyi</i>	0.000	0.017
43	<i>Solanum lycopersicum</i>	0.011	0.017
		Outgroups	
		Minimum difference	Maximum difference
<i>Ipomoea obscura</i>		0.200	0.237
<i>Buxus microphylla</i>		0.166	0.296

Supplemental Table 2. Interspecific and intraspecific pairwise distance for the *ITS* DNA barcode.

No.	Species	Intraspecific difference	Interspecific difference
1	<i>Acnistus arborescens</i>	0.008	—
2	<i>Browallia americana</i>	—	—
3	<i>Brugmansia ×candida</i>	0.000	0.015
4	<i>Brugmansia suaveolens</i>	0.015	0.015
5	<i>Brunfelsia americana</i>	0.001	0.010
6	<i>Brunfelsia densifolia</i>	0.000	0.010
7	<i>Brunfelsia lactea</i>	0.000	0.010
8	<i>Brunfelsia nitida</i>	0.000	0.010
9	<i>Brunfelsia portoricensis</i>	0.000	0.010
10	<i>Capsicum chinense</i>	—	0.050
11	<i>Capsicum frutescens</i>	0.008	0.050
12	<i>Capsicum annuum</i>	0.006	0.050
13	<i>Cestrum citrifolium</i>	0.000	0.007
14	<i>Cestrum diurnum</i>	0.005	0.007
15	<i>Cestrum macrophyllum</i>	0.000	0.007
16	<i>Cestrum nocturnum</i>	0.008	0.007
17	<i>Datura inoxia</i>	0.014	0.007
18	<i>Datura metel</i>	0.000	0.007
19	<i>Datura stramonium</i>	0.000	0.007
20	<i>Goetzea elegans</i>	0.000	—
21	<i>Lycium americanum</i>	—	—
22	<i>Lycianthes virgata</i>	—	—
23	<i>Physalis angulata</i>	0.016	0.011
24	<i>Physalis pubescens</i>	0.000	0.011
25	<i>Physalis cordata</i>	—	0.011
26	<i>Physalis ignota</i>	0.000	0.011
27	<i>Solandra grandiflora</i>	0.001	—
28	<i>Solanum americanum</i>	0.005	0.088
29	<i>Solanum bahamense</i>	0.011	0.088
30	<i>Solanum capsicoides</i>	0.000	0.088
31	<i>Solanum elaeagnifolium</i>	0.033	0.088
32	<i>Solanum ensifolium</i>	—	0.088
33	<i>Solanum erianthum</i>	0.013	0.088
34	<i>Solanum jamaicense</i>	0.007	0.088
35	<i>Solanum mammosum</i>	0.000	0.088
36	<i>Solanum melongena</i>	0.002	0.088
37	<i>Solanum nudum</i>	—	0.088
38	<i>Solanum polygamum</i>	0.162	0.088
39	<i>Solanum rugosum</i>	0.012	0.088
40	<i>Solanum seaforthianum</i>	0.284	0.088
41	<i>Solanum nigrescens</i>	—	0.088
42	<i>Solanum torvum</i>	0.045	0.088
43	<i>Solanum viarum</i>	0.011	0.088
44	<i>Solanum wendlandii</i>	—	0.088
45	<i>Solanum lycopersicum</i>	0.000	0.088
		Outgroups	
		Minimum difference	Maximum difference
<i>Ipomoea obscura</i>		0.172	0.392
<i>Buxus microphylla</i>		0.307	0.467

Supplemental Table 3. Interspecific and intraspecific pairwise distance for the *psbA-trnH* DNA barcode.

No.	Species	Intraspecific difference	Interspecific difference
1	<i>Acnistus arborescens</i>	0.000	—
2	<i>Browallia americana</i>	0.000	—
3	<i>Brugmansia ×candida</i>	0.001	0.011
4	<i>Brugmansia suaveolens</i>	0.016	0.011
5	<i>Brunfelsia americana</i>	0.000	0.010
6	<i>Brunfelsia densifolia</i>	0.000	0.010
7	<i>Brunfelsia lactea</i>	0.000	0.010
8	<i>Brunfelsia portoricensis</i>	0.000	0.010
9	<i>Capsicum chinense</i>	—	0.000
10	<i>Capsicum frutescens</i>	0.000	0.000
11	<i>Capsicum annuum</i>	0.000	0.000
12	<i>Cestrum citrifolium</i>	0.002	0.006
13	<i>Cestrum diurnum</i>	0.000	0.006
14	<i>Cestrum macrophyllum</i>	0.005	0.006
15	<i>Cestrum nocturnum</i>	0.004	0.006
16	<i>Datura inoxia</i>	0.000	0.008
17	<i>Datura metel</i>	0.000	0.008
18	<i>Datura stramonium</i>	—	0.008
19	<i>Goetzea elegans</i>	0.000	—
20	<i>Lycium americanum</i>	0.000	—
21	<i>Lycianthes virgata</i>	—	—
22	<i>Physalis angulata</i>	0.013	0.017
23	<i>Physalis pubescens</i>	0.002	0.007
24	<i>Solanandra grandiflora</i>	0.003	—
25	<i>Solanum americanum</i>	0.024	0.054
26	<i>Solanum bahamense</i>	0.000	0.054
27	<i>Solanum capsicoides</i>	0.000	0.054
28	<i>Solanum elaeagnifolium</i>	0.000	0.054
29	<i>Solanum ensifolium</i>	0.000	0.054
30	<i>Solanum erianthum</i>	0.008	0.054
31	<i>Solanum jamaicense</i>	—	0.054
32	<i>Solanum mammosum</i>	—	0.054
33	<i>Solanum melongena</i>	0.000	0.054
34	<i>Solanum nudum</i>	—	0.054
35	<i>Solanum polygamum</i>	0.000	0.054
36	<i>Solanum rugosum</i>	—	0.054
37	<i>Solanum seaforthianum</i>	0.000	0.054
38	<i>Solanum torvum</i>	0.000	0.054
39	<i>Solanum viarum</i>	0.000	0.054
40	<i>Solanum woodburyi</i>	0.000	0.054
41	<i>Solanum lycopersicum</i>	0.000	0.054
		Outgroups	
		Minimum difference	Maximum difference
<i>Ipomoea obscura</i>		0.350	0.580
<i>Buxus microphylla</i>		0.608	0.670