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DNA BARCODING WILL FREQUENTLY FAIL IN COMPLICATED GROUPS: AN EXAMPLE IN WILD POTATOES[1]

DAVID M. SPOONER

USDA, Agricultural Research Service, Department of Horticulture, University of Wisconsin, 1575 Linden Drive, Madison, Wisconsin 53706-1590 USA

DNA barcoding ("barcoding") has been proposed as a rapid and practical molecular method to identify species via diagnostic variation in short orthologous DNA sequences from one or a few universal genomic regions. It seeks to address in a rapid and simple way the "taxonomic impediment" of a greater need for taxonomic identifications than can be supplied by taxonomists. Using a complicated plant group, *Solanum* sect. *Petota* (wild potatoes), I tested barcoding with the most variable and frequently suggested plant barcoding regions: the internal nontranscribed spacer of nuclear ribosomal DNA (ITS) and the plastid markers *trnH-psbA* intergenic spacer and *matK*. These DNA regions fail to provide species-specific markers in sect. *Petota* because the ITS has too much intraspecific variation and the plastid markers lack sufficient polymorphism. The complications seen in wild potatoes are common in many plant groups, but they have not been assessed with barcoding. Barcoding is a retroactive procedure that relies on well-defined species to function, is based solely on a limited number of DNA sequences that are often inappropriate at the species level, has been poorly tested with geographically well-dispersed replicate samples from difficult taxonomic groups, and discounts substantial practical and theoretical problems in defining species.

Key words: DNA barcoding; ITS; *matK*; molecular phylogeny; potato; *Solanum* sect. *Petota*; *trnH-psbA* intergenic spacer.

DNA barcoding, here referred to as barcoding, recently has been proposed as a practical method to identify species by their diagnostic variation in short orthologous DNA sequences from one or a small number of universal genomic regions. The use of DNA sequence or DNA marker data as a method to identify species has long been a goal of molecular systematists. However, the proposal to accomplish this goal through universal DNA sequences for all life forms was only recently proposed as a comprehensive research program by Hebert et al. (2003a), who proposed a 645-bp stretch of the mitochondrial cytochrome *c* oxidase I [*cox1* or COI]. This region has been used for barcoding animals because of its generally conserved priming sites and third-position nucleotides with a greater incidence of base substitutions than other mitochondrial genes.

Cox1 was found to be inappropriate for barcoding in plants because of its much slower rate of evolution than in animals (Cho et al., 2004) and its rapidly changing gene content and structure (Adams and Palmer, 2003), making it difficult to design universal primers. Therefore, Kress et al. (2005) proposed two other genomic regions, the internal transcribed spacer region of nuclear ribosomal DNA (ITS), and the chloroplast *trnH-psbA* intergenic spacer. Other genomic regions such as plastid *rpoC1*, *rpoB*, and *matK* are also currently being considered for plants (Chase et al., 2007; Lahaye et al., 2008). The ITS is a multiple-copy region that frequently undergoes concerted evolution that "homogenizes" variant copies arising from hybridization, introgression, and gene duplication (Álvarez and Wendel, 2003). However, concerted evolution in ITS is not complete in all groups, and therefore, recent plant barcoding efforts have focused on plastid regions.

Two consortia arose to coordinate global barcoding efforts: The Barcode of Life (http://www.barcoding.si.edu/) and the Barcode of Life Data Systems (http://www.barcodinglife.org/views/login.php). Proposed activities include a worldwide effort to collect DNA and herbarium and museum vouchers, DNA sequencing, public deposition of these sequences, and development of barcoding analysis software to associate barcodes with species. Barcoding proponents envision the use of hand-held "barcorders," currently under development, for rapid determinations of DNA sequences, with the goal of making field identifications available to everyone (Savolainen et al., 2005; Lahaye et al., 2008; Stoeckle and Hebert, 2008). Practical applications would be enormous, including rapid screening of samples at port quarantine, screening for potential bioterror organisms, for poisonous organisms for medical emergencies, and for biodiversity surveys (Valentini et al., 2009). Essentially, the goal of barcoding is to make taxonomic identifications a readily accessible technical skill open to all user groups, bypassing the need for specialists that have limited time or do not exist for many groups, a problem termed the "taxonomic impediment" (Rodman and Cody, 2003).

Critiques of the rationale, methodology, and interpretation of barcoding results were soon advanced (Moritz and Cicero, 2004; Meyer and Paulay, 2005; Will et al., 2005), ranging from negative empirical tests to theoretical arguments. Despite these critiques, barcoding is rapidly becoming an accepted research program. For example, the Proceedings of the National Academy of Sciences has published three commentaries (Herre, 2006; Miller, 2007; Kress and Erickson, 2008) and 10 empirical tests of barcoding on groups including lepidoptera (Hebert et al., 2004a; Hajibabaei et al., 2006; Burns et al., 2008), diptera (Smith et al., 2006, 2007), hymenoptera (Smith et al., 2008), arthropods (Song et al., 2008), fungi (Seifert et al., 2007), and plants (Kress et al., 2005; Lahaye et al., 2008). All but one of these papers (Song et al., 2008) provide a positive view of barcoding.

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TABLE 1. Species, series affiliations (Hawkes, 1990), ploidy, and USDA Plant Introduction Number (http://www.ars-grin.gov/nr6/) of accessions examined for ITS and plastid *trnH-psbA* intergenic spacer variation in *Solanum* sect. *Petota* and outgroups.

Species	Series[a]	ITS	*trnH-psbA*	Ploidy (2*N* = 24)	Voucher[b] (no. clones sequenced for ITS)
Outgroups					
Capsicum pubescens Ruíz Lopez and Pavón		X	X	24	*G. Anderson 1554*
Datura inoxia Miller		X		24	*D. Spooner 2989*
Solanum diploconos (Mart.) L. Bohs		X		24	*L. Bohs 2335*
S. dulcamara L.		X		24	*D. Spooner 2988*
S. villosum Mill.		X	X	24,48[c]	*L. Bohs 150*
S. lycopersicum L. (cultivated tomato)		X		24	GenBank[d]
S. lycopersicoides Dunal		X		24	LA 1972
S. etuberosum Lindl.		X	X	24	PI 498311
S. palustre Poepp. 1		X	X	24	PI 558233
S. palustre 2		X	X	24	PI 558253
Ingroup (*Solanum* sect. *Petota* Dumort.)					
Solanum acaule Bitter 1	*Acaulia* Buk. and Kameraz	X	X	48	PI 310923
S. acaule 2	*Acaulia*	X	X	48	PI 498277
S. acaule 3	*Acaulia*	X	X	48	PI 500016
S. achacachense Cárdenas	*Tuberosa* (Rydb.) Hawkes 3	X	X	24	PI 558032
S. acroscopicum Ochoa	*Tuberosa* 2	X	X	24	PI 365314
S. agrimonifolium Rydb. 1	*Conicibaccata* Bitter	X	X	48	PI 243349
S. agrimonifolium 2	*Conicibaccata*	X	X	48	PI 243351 (15)
S. alandiae Cárdenas 1	*Tuberosa* 3	X	X	24	PI 498086 (11)
S. alandiae 2	*Tuberosa* 3	X	X	24	PI 498088 (6)
S. albicans (Ochoa) Ochoa	*Acaulia*	X	X	72	PI 266381
S. ambosinum Ochoa	*Tuberosa* 2	X	X	24	PI 498213
S. andreanum Baker	*Tuberosa* 1	X	X	24	PI 320345
S. arnezii Cárdenas	*Tuberosa* 3	X	X	24	PI 545846
S. avilesii Hawkes and Hjert.	*Tuberosa* 3	X	X	24	PI 498091
S. berthaultii Hawkes 1	*Tuberosa* 3	X	X	24	PI 442689
S. berthaultii 2	*Tuberosa* 3	X		24	PI 498075 (5)
S. berthaultii 3	*Tuberosa* 3	X		24	PI 498100 (7)
S. berthaultii 4	*Tuberosa* 3	X		24	PI 545920 (8)
S. brevicaule Bitter group	*Tuberosa* 2	X	X	48	PI 234009
S. bukasovii Juz. 1	*Tuberosa* 2	X	X	24	PI 210042
S. bukasovii 2	*Tuberosa* 2	X	X	24	PI 210055
S. bukasovii 3	*Tuberosa* 2	X	X	24	PI 442700
S. bulbocastanum Dunal	*Bulbocastana* (Rydb.) Hawkes	X	X	24	PI 347757
S. candolleanum P. Berthault	*Tuberosa* 2, 3	X	X	24	PI 545972
S. cardiophyllum Lindl. 1	*Pinnatisecta* (Rydb.) Hawkes	X	X	24	PI 595465
S. cardiophyllum 2	*Pinnatisecta*	X		24	*A. Rodríguez 2534*
S. cardiophyllum 3	*Pinnatisecta*	X	X	24	PI 347759
S. chacoense Bitter 1	*Yungasensa* Correll	X	X	24	PI 472809
S. chacoense 2	*Yungasensa*	X		24	PI 472821 (5)
S. chacoense 3	*Yungasensa*	X		24	PI 472813 (8)
S. chacoense 4	*Yungasensa*	X		24	PI 498321 (5)
S. chomatophilum Bitter	*Conicibaccata*	X	X	24	PI 365339
S. circaeifolium Bitter var. *capsicibaccatum* (Cárdenas) Ochoa 1	*Circaeifolia* Hawkes	X	X	24	PI 498117
S. circaeifolium var. *capsicibaccatum* 2	*Circaeifolia*	X	X	24	PI 545974
S. circaeifolium var. *capsicibaccatum* 3	*Circaeifolia*	X	X	24	PI 498120
S. clarum Correll 1	*Bulbocastana*	X	X	24	PI 275202
S. clarum 2	*Bulbocastana*	X	X	24	PI 283099
S. colombianum Bitter 1	*Conicibaccata*	X	X	48	PI 473462
S. colombianum 2	*Conicibaccata*	X		48	PI 498152 (14)
S. commersonii Dunal	*Commersoniana* Buk. and Kameraz	X	X	24	PI 458317
S. demissum Lindl. 1	*Demissa* Buk. and Kameraz	X	X	72	PI 275211
S. demissum 2	*Demissa*	X	X	72	PI 558482
S. doddsii Correll	*Tuberosa* 3	X		24	*D. Spooner 6651*
S. ehrenbergii (Bitter) Rydb.	*Pinnatisecta*	X	X	24	PI 595480
S. guerreroense Correll	*Demissa*	X	X	72	PI 161730
S. hougasii Correll 1	*Demissa*	X		72	PI 161174
S. hougasii 2	*Demissa*	X	X	72	PI 161727
S. immite Dunal	*Tuberosa* 2	X	X	24	PI 458401
S. incamayoense K. A. Okada and A. M. Clausen	*Tuberosa* 3	X	X	24	PI 473060
S. infundibuliforme Phil.	*Cuneoalata* Hawkes	X	X	24	PI 472857
S. iopetalum (Bitter) Hawkes	*Demissa*	X	X	72	PI 275183
S. jamesii Torr.	*Pinnatisecta*	X	X	24	PI 458424
S. kurtzianum Bitter and Wittm.	*Tuberosa* 3	X	X	24	PI 472923
S. laxissimum Bitter	*Conicibaccata*	X	X	24	PI 283088

TABLE 1. Continued.

Species	Series[a]	ITS	*trnH-psbA*	Ploidy (2*N* = 24)	Voucher[b] (no. clones sequenced for ITS)
S. leptophyes Bitter 1	*Tuberosa* 2, 3[c]	X	X	48	PI 442670 (10)
S. leptophyes 2	*Tuberosa* 2, 3	X	X	24	PI 458378 (6)
S. leptophyes 3	*Tuberosa* 2, 3	X		24	PI 545986 (6)
S. leptophyes 4	*Tuberosa* 2, 3	X		24	PI 545989 (6)
S. leptophyes 5	*Tuberosa* 2, 3	X		24	PI 545993 (7)
S. lignicaule Vargas	*Lignicaulia* Hawkes	X	X	24	PI 473351
S. longiconicum Bitter	*Conicibaccata*	X	X	48	PI 186568
S. marinasense Vargas	*Tuberosa* 2	X	X	24	PI 458380
S. megistacrolobum Bitter	*Megistacroloba* Cárdenas and Hawkes	X		24	PI 545927 (6)
S. microdontum Bitter 1	*Tuberosa* 3	X	X	24	PI 500036 (9)
S. microdontum 2	*Tuberosa* 3	X	X	24	PI 500040 (7)
S. microdontum Bitter 3	*Tuberosa* 3	X		24	PI 473167 (8)
S. microdontum Bitter 4	*Tuberosa* 3	X		24	PI 458355 (6)
S. morelliforme Bitter and G. Muench	*Morelliformia* Hawkes	X	X	24	PI 275218
S. multiinterruptum Bitter	*Tuberosa* 2	X	X	24	PI 365336
S. okadae Hawkes and Hjert.	*Tuberosa* 3	X	X	48	PI 498065
S. oplocense Hawkes 1	*Tuberosa* 3	X	X	72	PI 435079
S. oplocense 2	*Tuberosa* 3	X	X	24	PI 473365
S. oxycarpum Schltdl.	*Conicibaccata*	X	X	48	PI 498026
S. pampasense Hawkes	*Tuberosa* 2	X	X	24	PI 275274
S. piurae Bitter	*Piurana* Hawkes	X	X	24	PI 310997
S. polyadenium Greenm.	*Polyadenia* Correll	X	X	24	PI 161728
S. raphanifolium Cárdenas and Hawkes 1	*Megistacroloba*	X	X	24	PI 265862
S. raphanifolium 2	*Megistacroloba*	X	X	24	PI 473369
S. santolallae Vargas	*Conicibaccata*	X	X	24	PI 195168
S. scabrifolium Ochoa	*Tuberosa* 2	X	X	24	PI 365363
S. schenckii Bitter 1	*Demissa*	X	X	72	PI 275261
S. schenckii 2	*Demissa*	X	X	72	PI 498280
S. schenckii 3	*Demissa*	X		72	PI 558456 (19)
S. schenckii 4	*Demissa*	X	X	72	PI 558457
S. sparsipilum (Bitter) Juz. and Bukasov	*Tuberosa* 2, 3	X	X	24	PI 310957
S. spegazzinii Bitter 1	*Tuberosa* 3	X	X	24	PI 472976
S. spegazzinii 2	*Tuberosa* 3	X		24	PI 473214 (5)
S. stoloniferum Schltdl. 1	*Longipedicellata* Buk. and Kameraz	X	X	48	PI 251740
S. stoloniferum 2	*Longipedicellata*	X	X	48	PI 283108
S. stoloniferum 3	*Longipedicellata*	X	X	48	PI 558453 (11)
S. subpanduratum Ochoa	*Conicibaccata*	X	X	48	PI 498289
S. tuberosum L.	*Tuberosa* cultivated	X	X	24	PI 195188
S. tundalomense Ochoa	*Conicibaccata*	X	X	72	PI 473474
S. ugentii Hawkes and K. A. Okada 1	*Tuberosa* 3	X	X	48	PI 546030 (9)
S. ugentii 2	*Tuberosa* 3	X	X	48	PI 546032 (7)
S. vernei Bitter and Wittm. 1	*Tuberosa* 3	X	X	24	PI 458370 (7)
S. vernei 2	*Tuberosa* 3	X	X	24	PI 458371 (7)
S. vernei 3	*Tuberosa* 3	X	X	24	PI 473309 (6)
S. vernei 4	*Tuberosa* 3	X		24	PI 473310 (7)
S. vernei 5	*Tuberosa* 3	X		24	PI 500062 (5)
S. verrucosum Schltdl.	*Tuberosa* 1	X		24	PI 558488
S. violaceimarmoratum Bitter	*Conicibaccata*	X	X	24	PI 473396
S. yungasense Hawkes 1	*Yungasensa*	X		24	*D. Spooner 6738*
S. yungasense 2	*Yungasensa*	X	X		PI 614703

[a] Series designations from Hawkes (1990). Hawkes designated three informal groups of ser. *Tuberosa* based on distribution: group 1 from Mexico, Venezuela, Colombia, and Ecuador; group 2 from Peru; group 3 from Bolivia, Argentina, and Chile. *Solanum candolleanum*, *S. leptophyes*, and *S. sparsipilum* occur in both Peru and Bolivia (Hawkes, 1990).

[b] Voucher specimens for the Anderson and Bohs specimens are deposited at herbaria at the University of Connecticut (CONN) and University of Utah (UT), respectively; all other vouchers are at the Potato Introduction Station (PTIS).

[c] Counts of both 24 and 48 are known for *S. villosum*; we do not know the ploidy of this collection.

[d] Deposited at the GenBank database (http://www.ncbi.nlm.nih.gov/Genbank/index.html).

The purpose of the current study is to test the utility of barcoding in a well-studied but complicated plant group, *Solanum* sect. *Petota*, wild and cultivated potatoes. The section is widely distributed in the Americas from the southwestern United States to Panama (Spooner et al., 2004) and from Venezuela to the Southern Cone of South America (Hawkes and Hjerting 1969, 1989; Ochoa, 1990, 1999). The latest comprehensive taxonomic treatment of the group by Hawkes (1990) recognized 232 tuber-bearing and nontuber-bearing species divided into 21 taxonomic series. Plastid DNA restriction site studies (Spooner and Sytsma, 1992; Spooner et al., 1993; Castillo and Spooner, 1997; Spooner and Castillo, 1997; Rodríguez and Spooner, 2002), and nuclear DNA sequencing studies (Spooner et al., 2008b; Rodríguez and Spooner, 2009) have greatly

changed our understanding of ingroup relationships in sect. *Petota*. Relative to the last complete taxonomic treatment of the section by Hawkes (1990), the section now excludes the nontuber-bearing species, reclassified as sect. *Etuberosum* (Buk. and Kameraz) A. Child, sect. *Juglandifolia* (Rydberg) A. Child, and sect. *Lycopersicoides* (A Child) Peralta (Contreras and Spooner, 1999; Peralta et al., 2008). Many of Hawkes's (1990) 21 series are shown to be unnatural and the tuber-bearing species are supported to be divided into four clades (1–4) based on plastid restriction site data or three clades based on nuclear DNA sequencing data, with both results similar except that the nuclear DNA sequencing data fail to distinguish clades 1 and 2, and the allopolyploids combine sequences from different clades.

MATERIALS AND METHODS

Plant materials, cloning, and sequence replicates—One hundred-four accessions of 63 ingroup species and 10 accessions of nine outgroup species (in the genera *Solanum*, *Capsicum*, and *Datura*) were examined in this study. These covered 16 of the 19 tuber-bearing series of Hawkes (1990) and all four plastid DNA clades of sect. *Petota* (Spooner and Castillo, 1997). For *trnH-psbA*, all but three of the 63 ingroup species were examined. Seventy-two of these ingroup accessions are diploid, 18 tetraploid, and 12 hexaploid. Twenty-three species were represented by more than one accession for ITS and 17 for *trnH-psbA* (Table 1).

DNA extraction, cloning, sequencing primers and protocols—Total genomic DNA was isolated from young leaves of single plants following Doyle and Doyle (1987). For ITS, 16 of the 63 ingroup species were sequenced after cloning to uncover possible paralogs (4–19 clones per accession). In total, 332 sequences were generated for 114 accessions of 72 species for ITS. The primers "ITS leu1" and "ITS4" (White et al., 1990) were used to amplify the ITS region, and primers "ITS5" and "ITS3B" (Baum et al., 1994) were used for cycle-sequencing. Reaction constituents followed Baum et al. (1994). Thermal cycler conditions followed Baum et al. (1994) but with a higher annealing temperature of 55°C. Reactions were cleaned with a QIAquick spin-columns (QIAgen, Valencia, California, USA), cycle-sequenced with dye-terminator chemistry (Applied Biosystems, Foster City, California), and analyzed on an ABI 377 automated sequencer.

For sequencing *psbA-trnH*, primers psbAF (Sang et al., 1997) and trnH2 (Tate and Simpson, 2003) were used. PCR was performed using GoTaq (Promega, Madison, Wisconsin, USA), following White et al. (1990). Fragments ranging from 560 to 590 bp were gel purified using Zymoclean (Zymo Research, Orange, California), and cloned into the pGEM-T (Promega). Four colonies were sequenced and plasmids were purified with the Wizard Plus SV (Promega). Cycle sequencing used the ABI PRISM BigDye Terminators V 3.1 (Applied Biosystems). Sequences were obtained using a modified M13 reverse primer (5′-GAA ACA GCT ATG ACC ATG-3′). Cycle sequencing was conducted at 96°C for 3 min; followed by 75 cycles of 96°C for 20 s and 60°C for 2 min; then by 72°C for 2 min. Sequencing products were cleaned using Agencourt CleanSEQ (Beverly, Massachusetts, USA) and electrophoresed on a Applied Biosystems 3730*xl* automated DNA sequencer, with 50 cm capillaries containing POP 7 Polymer.

DNA sequences of the *matK* plastid gene for the wild potato species *Solanum bulbocastanum* and *S. tuberosum* determined by Daniell et al. (2006) were downloaded from GenBank and aligned as described later.

Data analyses—Sequences were edited with Staden package version 2003.0 beta (Staden, 1996) and aligned using the program CLUSTAL_X version 1.81 (Thompson et al., 1997) at default parameters, except for the "percentage of delay divergence sequences", which was set to 15% after tests of various parameters. Further manual alignments were done in the program MacClade 4.06 PPC (Maddison and Maddison, 2001) minimizing the number of gaps and preferring transitions instead of transversions. Indels were scored by the simple gap scoring method (Simmons and Ochoterena, 2000) using the program SeqState (available at http://systevol.nees.uni-bonn.de/software/SeqState). The aligned ITS and *trnH-psbA* intergenic spacer files are available as Appendix S1 and Appendix S2 (see Supplemental Data with the online version of this article), respectively, and are deposited in the database TreeBase (http://treebase.org). Phylogenetic analyses based on maximum parsimony (MP) were performed using the program PAUP* version 4.0b10 (Swofford, 2002). Branch support was evaluated with bootstrap analysis in PAUP* using 1000 replicates, tree-bisection-reconnection (TBR), MulTrees, and run separately with and without gaps. Neighbor-joining analysis (Posada and Crandall, 1998) was performed in PAUP*.

RESULTS

ITS tree—The aligned data matrix of ITS 1 and ITS 2 spacers and 5.8S was 724 bp; gaps added 159 characters (883 total); 356 of these (40%) were parsimony informative. Parsimony analysis (114 accessions, 72 species, 332 clones) produced 5000 equally parsimonious trees (the upper limit saved), with tree lengths of 2121 steps, consistency index (CI) of 0.424, consistency index excluding uninformative characters (CE) of 0.325, retention index (RI) of 0.569, and rescaled consistency index (RC) of 0.241. A strict consensus tree with overlaid bootstrap values (Fig. 1) shows 99% bootstrap support for the far outgroups, but all other bootstrap support above 50% is only in the terminal clades, and tomato and its outgroup (sect. *Lycopersicon*, sect. *Lycopersicoides*) are included in sect. *Petota*, within which only clades 1+2 match prior phylogenetic reconstructions (Castillo and Spooner, 1997; Spooner et al., 2008b). Relative to barcoding, replicate samples from direct sequencing or cloning form clades or sister accessions for 10 species: *S. acaule*, *S. agrimonifolium*, *S. circaeifolium*, *S. clarum*, *S. demissum*, *S. megistacrolobum, S. palustre*, *S. raphanifolium*, *S. schenckii*, and *S. stoloniferum*. Fourteen species: *S. alandiae*, *S. berthaultii*, *S. bukasovii*, *S. cardiophyllum*, *S. chacoense*, *S. colombianum*, *S. hougasii*, *S. leptophyes*, *S. microdontum*, *S. oplocense, S. spegazzinii*, *S. ugentii*, *S. vernei*, and *S. yungasense* do not form species-specific clades. A separate analysis excluding the gap codes (not shown) provides even less resolution. A neighbor-joining analysis (Appendix S3, see Supplemental Data with the online version of this article) has minor differences in topology but relative to barcoding also intermixes the same species.

Plastid trnH-psbA tree—The aligned data matrix consisted of 618 bp; gap codes added an additional 13 characters (631 total); 38 of these (6%) were parsimony informative. Most accessions had 15-bp stretches of poly T, sometimes interspersed with a 1-bp insert of another nucleotide, which complicated obtaining good sequence from direct sequencing. A parsimony analysis of the entire data set produced 5000 equally parsimonious trees (the upper limit saved), with tree lengths of 150 steps, CI of 0.827, CE of 0.639, RI of 0.750, and RC of 0.620. A strict consensus tree with overlaid bootstrap values shows an almost complete polytomy in ingroup sect. *Petota* (Fig. 2). Only two of the 17 replicate samples within species (*S. cardiophyllum*, *S. clarum*) form clades, and five clades group different species together rather than grouping replicates within a species (*S. acaule*, *S. agrimonifolium*, *S. bukasovii*, *S. leptophyes*, *S. palustre*).

Plastid matK DNA sequences—Lahaye et al. (2008) recently claimed that a 936-bp region of *matK* served as a universal DNA barcode for plants. Daniell et al. (2006) generated complete plastid genome sequences for *S. bulbocastanum* and *S. tuberosum*, encompassing species from two highly diverse clades in section *Petota* (Spooner and Castillo, 1997). There are

only two polymorphisms in the entire 1530 bp of this gene, showing even less polymorphism and ability to distinguish species than *trnH-psbA* (Fig. 2).

DISCUSSION

The taxonomy of sect. *Petota* is complicated by interspecific hybridization, introgression, allopolyploidy, a mixture of sexual and asexual reproduction, and possible recent species divergence (Spooner and Salas, 2006). The DNA barcoding results highlighted in this study are useless to investigate the many and complicated problems of species boundaries in sect. *Petota* or to serve as markers for the boundaries for the majority of its constituent species. ITS has too much intraspecific variation, and the plastid markers *trnH-psbA* and *matK* lack sufficient polymorphism and fail to cluster some well-supported species. Section *Petota* needs a variety of systematic approaches for an understanding of their species boundaries.

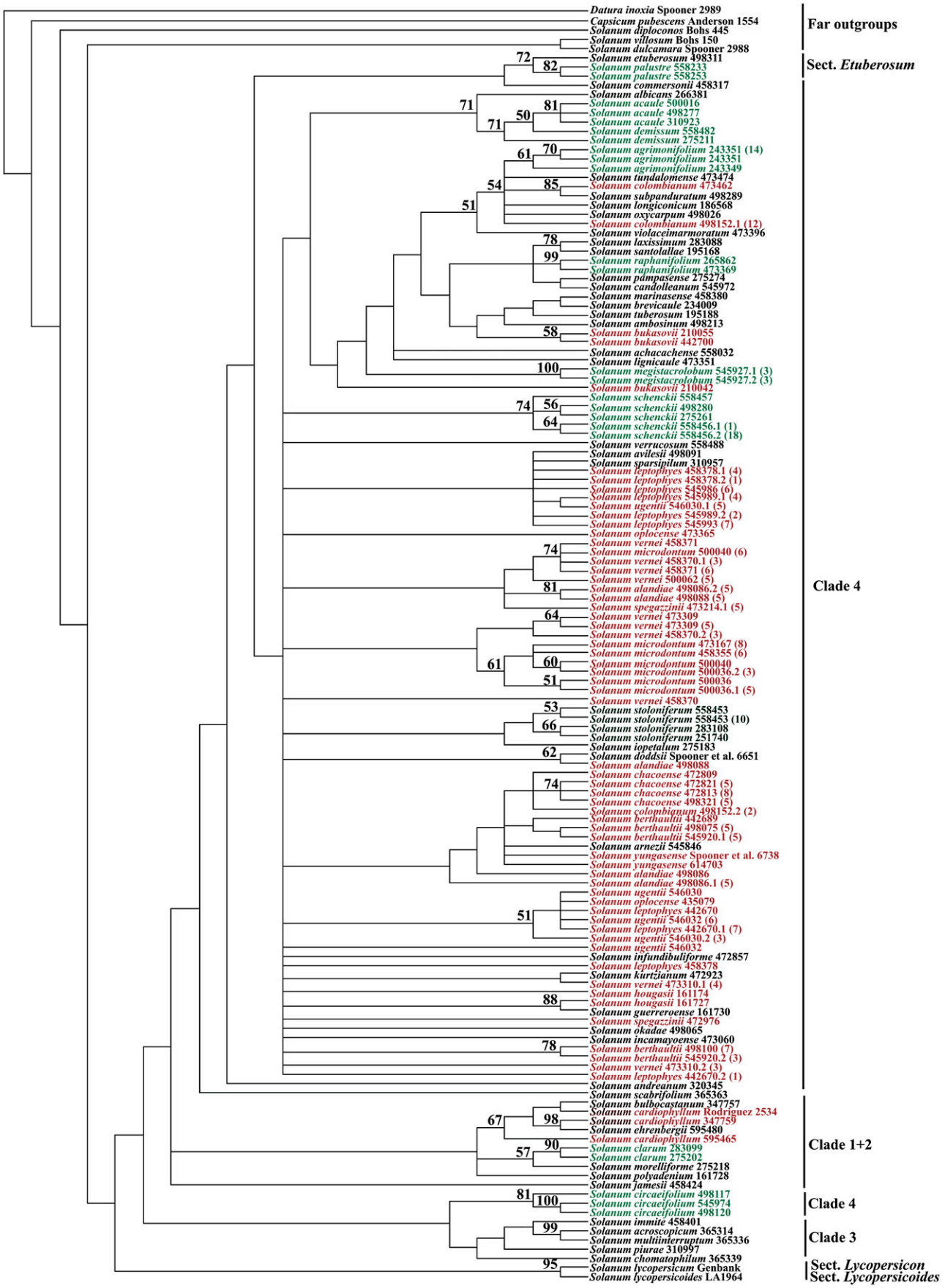
Section *Petota* has been the subject of intensive taxonomic study since the early 1920s (Rydberg, 1924), with research continuing to the present day. Taxonomists of potato traditionally have applied a variety of species concepts, but mainly have used morphological ones. Recent reinvestigations of species boundaries in potato have employed extensive field work throughout the range of the group (Spooner and Salas, 2006), numerical taxonomic investigations of morphological data in common gardens (e.g., van den Berg and Spooner, 1992; Giannattasio and Spooner, 1994a; van den Berg et al., 1998; Spooner et al., 2007a, 2008a; Alvarez et al., 2008; Ames et al., 2008; Fajardo et al., 2008), single- to low-copy nuclear DNA restriction sites (e.g., Hosaka and Spooner, 1992; Giannattasio and Spooner, 1994b; Miller and Spooner, 1999), isozymes (e.g., Spooner et al., 1992), microsatellites (Raker and Spooner, 2002; Spooner et al., 2007b), and AFLPs (Lara-Cabrera and Spooner, 2004; Spooner et al., 2005; Jacobs et al., 2008). An account of post-1990 taxonomic decisions of many workers published in Spooner and Salas (2006) reduced the 232 species of Hawkes (1990) to 190, but a taxonomic decision in my laboratory is converging on about 110 species.

Studies of species boundaries in the wild potato landraces and their progenitors in the *Solanum brevicaule* complex serve to illustrate the need for a variety of morphological and molecular approaches to comprehensively address complex problems of species limits. The *Solanum brevicaule* complex contains about 30 taxa and has long attracted the attention of biologists because of its similarity to cultivated potatoes (Correll, 1962; Grun, 1990; Ugent, 1970). Some members of this complex, endemic to central Peru, Bolivia, and northern Argentina, were considered ancestors of the landraces (Ugent, 1970). The species in the complex share pinnately dissected leaves, round fruits, rotate to rotate-pentagonal corollas, and are largely sexually compatible with each other and with cultivated potato (Hawkes, 1958; Hawkes and Hjerting, 1969, 1989; Ochoa, 1990, 1999). They include diploids, tetraploids, and hexaploids, with some species recognized by Hawkes (1990) possessing multiple levels (*S. gourlayi* with diploids and tetraploids, and *S. oplocense* with diploids, tetraploids, and hexaploids). Members of the complex are so similar that even experienced potato taxonomists Hawkes and Hjerting (1989) and Ochoa (1990) provided different identifications for identical collection numbers of the *Solanum brevicaule* complex in fully 38% of the cases (Spooner et al., 1994). Field studies in Peru (Spooner et al., 1999; Salas et al., 2001), Bolivia (Spooner et al., 1994), and Argentina (Spooner and Clausen, 1993); phenetic analyses of morphological data in the United States (van den Berg et al., 1998) and Peru (Alvarez et al., 2008); single- to low-copy nuclear restriction fragment length polymorphism (nRFLPs) and random amplified fragment length (RAPD) data (Miller and Spooner, 1999); and amplified fragment length polymorphism (AFLP) data, failed to clearly differentiate many wild species in the complex, but defined two geographic subsets: (1) the Peruvian populations, (2) the Bolivian and Argentinean populations. However, even these two groups could only be distinguished by computer-assisted analysis of widely overlapping character states, and not by species-specific characters. A formal taxonomic treatment of the group awaits data from sequencing multiple nuclear genes and from study of herbarium specimens, including types.

Similar taxonomic difficulties have been found in the cultivated potato species that had their progenitors in the *Solanum brevicaule* complex (Spooner et al., 2005). Indigenous primitive cultivated (landrace) potatoes are grown throughout mid to high (about 3000–3500 m a.s.l.) elevations in the Andes from western Venezuela to northern Argentina, and then in lowland south-central Chile, concentrated in the Chonos Archipelago. The widely used recent classification of Hawkes (1990) divided cultivated potato into seven species and seven subspecies, but the Russian potato taxonomists Bukasov (1971) and Lechnovich (1971) recognized 21 species, and Ochoa (1990, 1999) recognized nine species and 141 infraspecific taxa for the Bolivian cultivated species alone. Like the *Solanum brevicaule* complex, the cultivars contained an extensive ploidy series, of diploids, triploids, tetraploids, and pentaploids. Investigating species boundaries of this group used data from morphological phenetics from a field plot in Peru (Huamán and Spooner, 2002), microsatellites (Raker and Spooner, 2002; Ghislain et al., 2006; Spooner et al., 2007b), and plastid DNA deletion data (Hosaka, 2003; Ames and Spooner, 2008). These results supported a reclassification of the cultivated potatoes into four species: (1) *S. tuberosum*, with two cultivar groups (Andigenum group of upland Andean genotypes containing diploids, triploids, and tetraploids and the Chilotanum group of lowland tetraploid Chilean landraces), (2) *S. ajanhuiri* (diploid), (3) *S. juzepczukii* (triploid), and (4) *S. curtilobum* (pentaploid).

Similar studies using a variety of morphological and molecular data have been needed in other species groups in sect. *Petota* (see Spooner et al., 2004 for a summary of how such studies have been applied for species determinations of the species from North and Central America). In summary, a variety of data sets have been necessary to address the considerable and complex taxonomic problems in sect. *Petota*, but DNA sequences from barcoding regions fail completely for these problems.

The taxonomic problems described for sect. *Petota* are not unique in plants and highlight how taxonomists are far from agreement on species boundaries. For example, Harlan and de Wet (1971) showed huge differences in the number of species recognized by different taxonomists in crops, ranging from 100 to 200 in wild relatives of potato, 2 to 24 in wheat, and 1 to 31 in sorghum. Such taxonomic disagreements continue unabated as is evident in contemporary systematic journals. Many taxonomic changes involve long-accepted species circumscriptions from well-studied groups such as the recent recognition of four species within the former wild tomato species *Solanum peruvianum* (Peralta et al., 2005). A primary problem for barcoding results from unresolved definitions of what constitutes a species. A variety of species concepts exist, from the most utilitarian


Datura inoxia Spooner 2989
Capsicum pubescens Anderson 1554
Solanum diploconos Bohs 445
Solanum villosum Bohs 150
Solanum dulcamara Spooner 2988
Solanum etuberosum 498311
Solanum palustre 558233
Solanum palustre 558253
Solanum commersonii 458317
Solanum albicans 266381
Solanum acaule 500016
Solanum acaule 498277
Solanum acaule 310923
Solanum demissum 558482
Solanum demissum 275211
Solanum agrimonifolium 243351 (14)
Solanum agrimonifolium 243351
Solanum agrimonifolium 243349
Solanum tundalomense 473474
Solanum colombianum 473462
Solanum subpanduratum 498289
Solanum longicomicum 186568
Solanum oxycarpum 498026
Solanum colombianum 498152.1 (12)
Solanum violaceimarmoratum 473396
Solanum laxissimum 283088
Solanum santolallae 195168
Solanum raphanifolium 265862
Solanum raphanifolium 473369
Solanum pampasense 275274
Solanum candolleanum 545972
Solanum marinasense 458380
Solanum brevicaule 234009
Solanum tuberosum 195188
Solanum ambosinum 498213
Solanum bukasovii 210055
Solanum bukasovii 442700
Solanum achacachense 558032
Solanum lignicaule 473351
Solanum megistacrolobum 545927.1 (3)
Solanum megistacrolobum 545927.2 (3)
Solanum bukasovii 210042
Solanum schenckii 558457
Solanum schenckii 498280
Solanum schenckii 275261
Solanum schenckii 558456.1 (1)
Solanum schenckii 558456.2 (18)
Solanum verrucosum 558488
Solanum avilesii 498091
Solanum sparsipilum 310957
Solanum leptophyes 458378.1 (4)
Solanum leptophyes 458378.2 (1)
Solanum leptophyes 545986 (6)
Solanum leptophyes 545989.1 (4)
Solanum ugentii 546030.1 (5)
Solanum leptophyes 545989.2 (2)
Solanum leptophyes 545993 (7)
Solanum oplocense 473365
Solanum vernei 458371
Solanum microdontum 500040 (6)
Solanum vernei 458370.1 (3)
Solanum vernei 458371 (6)
Solanum vernei 500062 (5)
Solanum alandiae 498086.2 (5)
Solanum alandiae 498088 (5)
Solanum spegazzinii 473214.1 (5)
Solanum vernei 473309
Solanum vernei 473309 (5)
Solanum vernei 458370.2 (3)
Solanum microdontum 473167 (8)
Solanum microdontum 458355 (6)
Solanum microdontum 500040
Solanum microdontum 500036.2 (3)
Solanum microdontum 500036
Solanum microdontum 500036.1 (5)
Solanum vernei 458370
Solanum stoloniferum 558453
Solanum stoloniferum 558453 (10)
Solanum stoloniferum 283108
Solanum stoloniferum 251740
Solanum iopetalum 275183
Solanum doddsii Spooner et al. 6651
Solanum alandiae 498088
Solanum chacoense 472809
Solanum chacoense 472821 (5)
Solanum chacoense 472813 (8)
Solanum chacoense 498321 (5)
Solanum colombianum 498152.2 (2)
Solanum berthaultii 442689
Solanum berthaultii 498075 (5)
Solanum berthaultii 545920.1 (5)
Solanum arnezii 545846
Solanum yungasense Spooner et al. 6738
Solanum yungasense 614703
Solanum alandiae 498086
Solanum alandiae 498086.1 (5)
Solanum ugentii 546030
Solanum oplocense 435079
Solanum leptophyes 442670
Solanum ugentii 546032 (6)
Solanum leptophyes 442670.1 (7)
Solanum ugentii 546030.2 (3)
Solanum ugentii 546032
Solanum infundibuliforme 472857
Solanum leptophyes 458378
Solanum kurtzianum 472923
Solanum vernei 473310.1 (4)
Solanum hougasii 161174
Solanum hougasii 161727
Solanum guerreroense 161730
Solanum spegazzinii 472976
Solanum okadae 498065
Solanum incamayoense 473060
Solanum berthaultii 498100 (7)
Solanum berthaultii 545920.2 (3)
Solanum vernei 473310.2 (3)
Solanum leptophyes 442670.2 (1)
Solanum andreanum 320345
Solanum scabrifolium 365363
Solanum bulbocastanum 347757
Solanum cardiophyllum Rodriguez 2534
Solanum cardiophyllum 347759
Solanum ehrenbergii 595480
Solanum cardiophyllum 595465
Solanum clarum 283099
Solanum clarum 275202
Solanum morelliforme 275218
Solanum polyadenium 161728
Solanum jamesii 458424
Solanum circaeifolium 498117
Solanum circaeifolium 545974
Solanum circaeifolium 498120
Solanum immite 458401
Solanum acroscopicum 365314
Solanum multiinterruptum 365336
Solanum piurae 310997
Solanum chomatophilum 365339
Solanum lycopersicum Genbank
Solanum lycopersicoides LA1964
Far outgroups
Sect. Etuberosum
Clade 4
Clade 1+2
Clade 4
Clade 3
Sect. Lycopersicon
Sect. Lycopersicoides

ones based on morphology (Cronquist, 1978) to those based on intercrossability (Mayr, 1942), ecology (Van Valen, 1976), phylogeny (Olmstead, 1995), or a combination of these (Templeton, 1989). Some (Ehrlich and Raven, 1969; Levin, 1979) argue that gene flow is too limited among conspecific plant populations to allow them to evolve as evolutionary units, that the population is the only objective reality, and that species are artificial human constructs. Rieseberg et al. (2006) disagree with this view by arguing for much greater gene flow than previously supposed and by summarizing phenetic and crossability studies that support the majority of plant species as morphologically discrete and reproductively independent lineages. Another view of species is that a clear distinction needs to be made between the data used to define species (e.g., morphology, isolating mechanisms, molecular data) and the methods applied to analyze these data (e.g., phenetics, cladistics, population biological methods) (Mallet, 2001). Many taxonomists today equate species with lineages, and De Queiroz (2007) recently proposed a "unified species concept" by distinguishing the conceptualization of species (as separately evolving lineages) from the criteria used to delimit them, such as reproductive isolation, diagnosability, and monophyly, and showing how these various criteria are expected to appear at different times and in different orders in the evolution of different groups of species. New concepts and debates of species continue to arise, such as one arguing that many autopolyploid derivatives of diploid species should be provided separate species status (Soltis et al., 2007).

In addition to the disagreement of how to classify species, many species simply are not yet known in order for their association with barcodes. Current global species estimates range from 3.6 million to 100 million, with a reasonable guess to be 10 million, but science has only identified about 2 million (Wilson, 2003). Our ignorance of species diversity is not confined to difficult to access and understudied "hotspots," but occurs in some of the best-studied areas on the earth. For example, Hartman and Nelson (1998) documented an average of 60 new plant taxa described each year from 1978 to 1998 for North America, one of the best-studied floristic regions on earth, and taxonomic discoveries in this area are continuing at this pace (R. Hartman, University of Wyoming, personal communication).

Some barcoding advocates propose to address this fundamental species problem by using barcodes themselves to flag potential new species through the recognition of "molecular operational taxonomic units" (MOTUs) that they suggest frequently correspond to species and when they do not, have intrinsic value in themselves (Blaxter et al., 2005). Two criteria have been proposed to recognize MOTUs, "reciprocal monophyly" (Wiens and Penkroft, 2002) and the "10 × rule" (Hebert et al., 2004b). Through simulations and empirical data, Hickerson et al. (2006) show that these criteria are subject to high error rates, especially for recently diverged lineages (<4 million years old). There is no objective level of sequence divergence in any gene that is either necessary or sufficient to delimit species. The attempt to search for a universal marker to quantify minimum standard levels of species divergences ignores studies quantifying varying levels of genetic divergence associated with different life forms and reproductive strategies (Hamrick and Godt, 1989). Attempting to use any individual DNA sequence to define species would be a major retrogressive trend in systematics, approaching long-outdated typological species concepts. In character-poor organisms (e.g., asexual stages of fungi), I believe that a reliance on barcoding regions would inevitably lead to taxonomic decisions based on narrow criteria, poorly circumscribed species, and species of reduced predictive value.

Many barcoding studies lack robust assessments of intraspecific polymorphism or assessments of all species within a genus that are needed to assess the species-specific nature of barcodes. Barcoding advocates recommend sampling 10 individuals per species (Stoeckle and Hebert, 2008), but this is rarely done (Prendini, 2005), and the lack of species-specific barcodes was shown here with even fewer replicates. Almost all proposed plant barcoding regions are plastid markers, but intraspecific and intrapopulational plastid DNA polymorphism is widespread (Soltis et al., 1992). Plastid polymorphism generally has been assessed in narrow geographic regions with few replicates (Lahaye et al., 2008). However, intraspecific plastid variation can sometimes be extensive in plants, especially in sympatric zones undergoing hybridization and backcrossing that maintains plastid polymorphism within and between populations (Okuyama et al., 2005). Not only is plastid polymorphism frequent in areas of sympatry (Govindaraju et al., 1989), but it is even more common over broad geographic space (Caron et al., 2000), matching observations of variation with morphological data (Mallet, 2001).

To date, only plastid markers (except for ITS) have been proposed as barcodes, but these often will not be effective to highlight species boundaries. Morjan and Rieseberg (2004) argue that the spread of advantageous alleles among populations of low gene flow is probably the primary mechanism by which species are held together. Consequently, unless plastid DNA is subject to a species-wide selective sweep, it probably will not be very effective as a barcode. Additionally, even if an advantageous mutation arises in the plastid, the speed of the sweep will be quite low relative to nuclear genes because pollen dispersal rates are on average double that of seeds (Morjan and Rieseberg, 2004). Realizing problems in plastid markers, some barcoding advocates (Chase et al., 2005; Kress and Erickson, 2008) propose that nuclear DNA regions be explored for barcodes. Such regions would be ineffective in sect. *Petota*, where allopolyploids retain alleles of both parents, necessitating extensive cloning to reliably recover all alleles that are shared by both parents and the allopolyploid (Spooner et al., 2008b; Rodríguez and Spooner, 2009). Estimates of the incidence of polyploidy in angiosperms vary from 30 to 70% (Levin, 2002). Heterozygosity is not limited to allopolyploids, but is a problem with diploid hybrid species and nonhybrid outcrossers. An additional complication of nuclear loci is often low copy number making amplification difficult, and a variety of primer pairs would be needed to be constructed for different groups.

Another problem is poor conservation of priming sites, such as is documented in some organisms for *Cox1* (Meier et al., 2006), or amplification of pseudogenes, as is documented in

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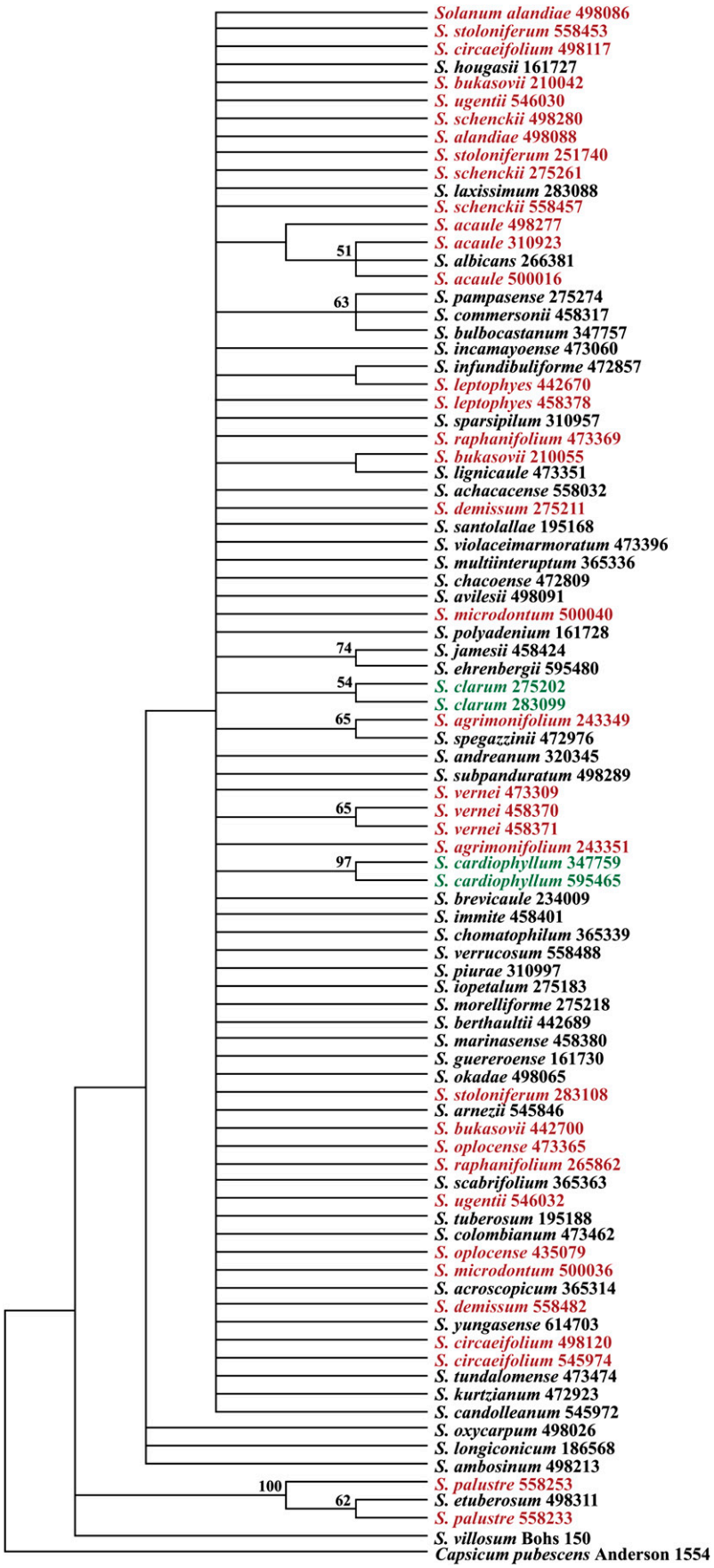
Fig. 1. ITS consensus tree of 5000 equally parsimonious 2251-step Fitch trees of 114 accessions of 72 species of section *Petota* and outgroups. Numbers above the branches are bootstrap values above 50%. Numbers in parentheses after the species refer to numbers of cloned variants falling on that branch. Accessions in red highlight accessions or cloned sequences that do not group species, green that do group species as clades or sister accessions, and black with only one accession examined per species. Clade designations follow Spooner and Castillo (1997).


Solanum alandiae 498086
S. stoloniferum 558453
S. circaeifolium 498117
S. hougasii 161727
S. bukasovii 210042
S. ugentii 546030
S. schenckii 498280
S. alandiae 498088
S. stoloniferum 251740
S. schenckii 275261
S. laxissimum 283088
S. schenckii 558457
S. acaule 498277
S. acaule 310923
51
S. albicans 266381
S. acaule 500016
63
S. pampasense 275274
S. commersonii 458317
S. bulbocastanum 347757
S. incamayoense 473060
S. infundibuliforme 472857
S. leptophyes 442670
S. leptophyes 458378
S. sparsipilum 310957
S. raphanifolium 473369
S. bukasovii 210055
S. lignicaule 473351
S. achacacense 558032
S. demissum 275211
S. santolallae 195168
S. violaceimarmoratum 473396
S. multiinteruptum 365336
S. chacoense 472809
S. avilesii 498091
S. microdontum 500040
S. polyadenium 161728
74
S. jamesii 458424
S. ehrenbergii 595480
54
S. clarum 275202
S. clarum 283099
65
S. agrimonifolium 243349
S. spegazzinii 472976
S. andreanum 320345
S. subpanduratum 498289
S. vernei 473309
65
S. vernei 458370
S. vernei 458371
S. agrimonifolium 243351
97
S. cardiophyllum 347759
S. cardiophyllum 595465
S. brevicaule 234009
S. immite 458401
S. chomatophilum 365339
S. verrucosum 558488
S. piurae 310997
S. iopetalum 275183
S. morelliforme 275218
S. berthaultii 442689
S. marinasense 458380
S. guereroense 161730
S. okadae 498065
S. stoloniferum 283108
S. arnezii 545846
S. bukasovii 442700
S. oplocense 473365
S. raphanifolium 265862
S. scabrifolium 365363
S. ugentii 546032
S. tuberosum 195188
S. colombianum 473462
S. oplocense 435079
S. microdontum 500036
S. acroscopicum 365314
S. demissum 558482
S. yungasense 614703
S. circaeifolium 498120
S. circaeifolium 545974
S. tundalomense 473474
S. kurtzianum 472923
S. candolleanum 545972
S. oxycarpum 498026
S. longiconicum 186568
S. ambosinum 498213
100
S. palustre 558253
62
S. etuberosum 498311
S. palustre 558233
S. villosum Bohs 150
Capsicum pubescens Anderson 1554

some organisms for *Cox1* (Song et al., 2008). This problem is also shared by *matK,* which has recently been proposed as a "universal" barcoding region for plants (Lahaye et al., 2008), but with primer pairs that apparently are not effective outside of the angiosperms and that recover only 80–90% of angiosperm species as monophyletic, depending on the analytical procedures used. Lahaye et al. (2008) argue that not all species need to be distinguished for barcoding to be effective, but the resulting ambiguity of species determinations for these unknown taxa will necessitate checking identifications by specialists, and the taxonomic impediment will not be solved at all.

Many papers that advocate barcoding do not discuss in depth, or sometimes at all, the data showing where it fails. *Cox1* sequences have already been shown to fail in some animals, such as *Cnidaria* (Hebert et al., 2003b), amphibians (Vences et al., 2005), and some diptera (Meier et al., 2006). In plants, *matK* is not effective as a barcoding marker in *Protea* (Chase et al., 2007) and *Solanum* sect. *Petota* and can barely distinguish species from many genera in the Magnoliaceae subfamily Magnolioideae (Shi et al., 2000) and the Lauraceae (Rohwer, 2000). Other well-known taxonomically difficult genera include *Aquilegia*, *Citrus*, *Crepis*, *Diplacus*, *Elymus*, *Geum*, *Gilia*, *Hieracium*, *Iris*, *Oenothera*, *Poa*, *Potentilla*, *Quercus*, *Rubus*, *Senecio*, and *Taraxacum*, to name only a few of scores of well-documented examples. These genera include some of the complicating biological problems mentioned for sect. *Petota*, in addition to aneuploidy, apomixis, and clinal variation (Grant, 1981). Such complicated groups, however, simply have not been subject to in-depth barcoding studies that analyze many accessions within speciose clades with geographically dispersed replicates needed to provide statistically valid tests of barcoding.

Systematic studies typically encounter discordance among diverse data sets that can be attributed to a variety of causes ranging from technical problems (e.g., sequencing error, insufficient taxon sampling, improper gene choice), organism-level processes (e.g., introgression, hybridization, unequal rates of diversification, lineage sorting, convergent or rapid morphological evolution), or genome level processes (e.g., use of unrecognized paralogous loci, concerted evolution) (Wendel and Doyle, 1998). These problems affect the ability of any gene sequence to be properly chosen, aligned (Rokas, 2008), and interpreted, and typically take considerable time to work out for any group. The idea that a limited set of universal barcodes can be selected to address such a fundamental problem as species limits simply ignores these problems that are regularly encountered by those working at the species level.

Proponents of barcoding advocate its use in many fields to include high impact applications such as forensics (Savolainen et al., 2005). But barcoding data entered as legal evidence could be effectively countered simply by challenging existing barcoding data for assessment of species specificity throughout the entire species range, assessment of all possible sister taxa, and all possible hybrids.

Barcoding advocates envision rapid taxonomic surveys via hand-held "barcorders" (discussed earlier). However, rapid assays are frequently not possible for *trnH-psbA* and likely other regions because of long adenosine or thymine strings that require cloning (Winkworth and Donoghue, 2005). I also had to clone this region in my study because most accessions had 15-bp stretches of poly T, or all T with the insert of a single base that precluded getting good sequence from direct sequencing. This cloning adds a minimum of two days and considerable expense to the process, and precludes the potential future use of a barcoder.

A comprehensive international barcoding program is estimated to cost anywhere from $2 billion US (Whitfield, 2003) to $10 billion US (Cameron et al., 2006), and take over 20 years. With an estimated global investment in taxonomic research of $10 million US today (Cameron et al., 2006), a comprehensive barcoding effort would require a 20-year commitment of a 2.5–12.5-fold increase in investment in taxonomic research, devoted to barcoding alone. The taxonomic impediment is a serious problem with no easy solution, even if addressed with such funds to barcoding. Many elements proposed by barcoding, to include a global and coordinated collection of vouchered biological samples, associated DNA collections, cooperation and coordination across political boundaries, and taxonomic study using molecular markers have been proposed before (Rodman and Cody, 2003; Wilson, 2003). Unlike the barcoding proposals, the earlier propositions additionally encompass the integration of a variety of traditional and modern systematic approaches, which are required to truly assess biological diversity effectively and comprehensively.

When used as part of an integrated systematic program, barcoding regions can uncover previously unrecognized species. For example, Hebert et al. (2004a) demonstrated the utility of *cox1* sequences to discover 10 previously unrecognized cryptic species of *Astraptes fulgerator*, a neotropical skipper butterfly (but see Brower, 2006), and Smith et al. (2008) discovered well over 100 new species of tropical parasitoid wasps. This recognition, however, was possible only in the context of coauthor Janzen's 25 years of natural history observations that were used to associate *cox1* variants with a range of cryptic morphological differences and feeding preferences. Barcodes can have other positive uses, for example, in well-studied and well-defined groups of species with sufficient divergence to preclude the many problems highlighted above. In the absence of such divergence at the species level, barcodes may be useful at the genus level, where, in concert with other data, such as digital images of species, or type specimens, species can be more easily identified. Another proposal is to use a "traffic-light" approach to barcoding, that recognizes the inherent problems in obtaining effective barcodes for difficult species and seeks additional data when necessary for such groups (Chase et al., 2005).

The rapid development of massive parallel sequencing technologies has encouraged some to suggest that the problems of DNA barcoding can be overcome by more data (Deppewolf and Cronk, 2008). I predict that many of the problems of barcoding discussed here will frustrate the elusive barcoding goal of simple, cheap, and reliable molecular techniques to identify the large majority of life's organisms.

In summary, DNA barcoding fails in a large group of *Solanum*, section *Petota*, and has problems in many other groups. The use of few universal DNA markers for species determinations is impeded by common complicating biological phenomena,

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Fig. 2. Plastid *trnH-psbA* consensus tree of 5000 equally parsimonious 150-step Fitch trees of 88 accessions of 65 species of section *Petota* and outgroups; bootstrap and color codes as in Fig. 1.

is a retroactive procedure that relies on well-defined species to function, is based solely on DNA sequences that are often ineffective for discovering species, has been inadequately tested with replicate samples from broad geographic regions and from all members of speciose clades of difficult groups, and discounts substantial practical and theoretical problems in defining species. There is no level of sequence divergence in any single or limited set of genes that is either necessary nor sufficient to delimit species, and a sole reliance of barcoding for character poor organisms, while convenient, will likely lead to poorly circumscribed species. Barcode regions are often useful to detect new species, but often not, and different DNA regions will be required to identify species in different groups. The search for a limited suite of universal markers for all species will be an impossible goal, especially for many groups with the biological complications discussed here.

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