Conserv Genet (2008) 9:381–387
DOI 10.1007/s10592-007-9350-y

ORIGINAL ARTICLE

The use and limits of AFLP data in the taxonomy of polyploid wild potato species in *Solanum* series *Conicibaccata*

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Received: 26 October 2006 / Accepted: 21 May 2007 / Published online: 12 July 2007

Abstract *Solanum* sect. *Petota* (tuber-bearing wild and cultivated potatoes) are a group of approximately 190 wild species distributed throughout the Americas from the southwestern United States south to Argentina, Chile, and Uruguay. *Solanum* series *Conicibaccata* are a group of approximately 40 species within sect. *Petota*, distributed from central Mexico to central Bolivia, composed of diploids ($2n = 2x = 24$), tetraploids ($2n = 4x = 48$) and hexaploids ($2n = 6x = 64$); the polyploids are thought to be polysomic polyploids. This study initially was designed to address species boundaries of the four Mexican and Central American species of series *Conicibaccata* with AFLP data with the addition of first germplasm collections of one of these four species, *Solanum woodsonii*, as a follow-up to prior morphological, chloroplast DNA, and RAPD studies; and additional species of series *Conicibaccata* from South America. AFLP data from 12 primer combinations (1722 polymorphic bands) are unable to distinguish polyploid species long thought to be distinct. The data suggest a complex reticulate history of the tetraploids or the need for a broad downward reevaluation of the number of species in series *Conicibaccata*, a trend seen in other series of sect. *Petota*. Separately, through flow cytometry, we report the first ploidy level of *S. woodsonii*, as tetraploid ($2n = 48$).

Keywords AFLP · *Solanum* section *Petota* · *Solanum* series *Conicibaccata* · Taxonomy

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Introduction

Solanum sect. *Petota* (tuber-bearing wild and cultivated potatoes) are a group of approximately 190 wild species distributed throughout the Americas from the southwestern United States, south to Argentina, Chile, and Uruguay (Spooner and Salas 2006). *Solanum* series *Conicibaccata* Bitter are a group of approximately 40 of these species, distributed from central Mexico to central Bolivia. They are generally distinguished by conical fruits and pinnately dissected leaves with a somewhat parallel-sided morphology and narrowly ovate to elliptical leaflets, but these characters vary, and the limits of the series are not always clear (Castillo and Spooner 1997; Spooner and Salas 2006). The series contains diploids ($2n = 2x = 24$), tetraploids ($2n = 4x = 48$) and hexaploids ($2n = 6x = 64$); all four species from Mexico and Central America are tetraploids. The polyploids are believed to be polysomic polyploids, but their genomic contributors (if extant) are controversial (Matsubayashi 1991). Based on chloroplast DNA restriction site data the diploids and polyploids are members of different clades (Castillo and Spooner 1997).

The initial purpose of our study was to explore the utility of AFLPs to elucidate phylogenetic relationships of the four wild species of ser. *Conicibaccata* in Mexico and Central America (*Solanum agrimonifolium* Rydb., *S. longiconicum* Bitter, *S. oxycarpum* Scheide, and *S. woodsonii* Correll). The distribution and morphological

differences of these species are outlined in Spooner et al. (2004). Populations of all of these species in nature are highly variable, and they are distinguished, sometimes with difficulty, only with character states that overlap in extent. RAPD data from the first three of these species (Spooner et al. 2001; *S. woodsonii* was not then available as germplasm) distinguished them as species-specific groups, and distinguished them from South American diploids of the series, and these from the outgroups *S. piurae* Bitter and *S. bulbocastanum* Dunal (Fig. 1). The primary goal of our study was to determine the phylogenetic relationships of newly available *S. woodsonii* (tetraploid, collected in Panama), and two unidentified tetraploid members of ser. *Conicibaccata* that we suspected were the first collections of *S. woodsonii* from Costa Rica. We used nearly the same species and accessions as those from Spooner et al. (2001) but additionally added seven accessions of four tetraploid South American species of ser. *Conicibaccata* (*S. colombianum* Bitter, *S. garcia-barrigae* Ochoa, *S. otites* Dunal, and *S. lobbianum* Bitter). Our expectation, based on the long-held acceptance of these South American species, and the results of Spooner et al. (2001), was that all of these tetraploid members of ser. *Conicibaccata* would be separated into clades (cladistic analyses) or groups (phenetic analyses).

The Amplified Fragment Length Polymorphism (AFLP) technique combines restriction enzyme reactions with the Polymerase Chain Reaction (PCR), revealing high levels of polymorphism (Vos et al. 1995). AFLP data have been shown to typically have high congruence with many other marker types (Powell et al. 1996; Milbourne et al. 1997; Russell et al. 1997), and generally show results concordant to existing taxonomic hypotheses. They are useful to study relationships within species and of closely related species within genera (Spooner et al. 2005c). Within section *Petota*, AFLPs have been used to study the phylogeny of different sections (Kardolus et al. 1998), species within *Solanum* series *Longipedicellata* Buk. (van den Berg et al. 2002), species within Mexican diploid wild potatoes (Lara-Cabrera and Spooner 2004), accessions of the *Solanum brevicaule* complex and related cultivars (Spooner et al. 2005a), and diversity within *Solanum tuberosum* (Kim et al. 1998). Outside of sect. *Petota*, Mace et al. (1999b) and Furini and Wunder (2004) used AFLPs to examine relationships of *Solanum melongena* L. (eggplant) and related species; Spooner et al. (2005b) to study all wild tomato (*Solanum* sect. *Lycopersicon*) species; and Prohens et al. (2006) to examine species of *Solanum* sect. *Basarthrum* (Bitter) Bitter. Elsewhere in Solanaceae, Mace et al. (1999a) studied members of the solanaceous tribe *Datureae* (*Datura* L. and *Brugmansia* Pers.).

Materials and methods

Plants

Similar to the RAPD and morphological study of series *Conicibaccata* from Mexico and Central America, we analyzed seven accessions *S. agrimonifolium*, ten *S. longiconicum*, seven *S. oxycarpum*, one *S. woodsonii*, and two newly-collected unidentified species from Costa Rica that we suspected to be *S. woodsonii*; all the above are from Mexico and Central America. We additionally studied four tetraploid species of the series from South America (four accessions of *S. colombianum*, one *S. garcia-barrigae*, one *S. lobbianum*, one *S. otites*). Like Spooner et al. (2001) we also studied one accession each of four diploid species of the series from South America (*S. buesii* Vargas, *S. laxissimum* Bitter, *S. santolallae* Vargas, and *S. violaceimarmoratum* Bitter). To root the tree for cladistic analyses we used the same species in Spooner et al. (2001) of one species from series *Bulbocastana* (Rydb.) Hawkes (*S. bulbocastanum*, 2*x*; designated as outgroup), and additionally used one species from series *Piurana* Hawkes (*S. piurae*, 2*x*). The *S. woodsonii* accessions came from CIP, or from recent collections from author Brenes in Costa Rica, and all other accessions came from the US Potato Genebank in Sturgeon Bay Wisconsin (the six-digit accession numbers on Figs. 2, 3). The locality data of the US Potato Genebank can be found on-line (http://www.ars-grin.gov/nr6/) and in Spooner et al. (2004), and herbarium vouchers are deposited at the US Potato Genebank herbarium in Sturgeon Bay Wisconsin (herbarium code PTIS; http://sciweb.nybg.org/science2/IndexHerbariorum.asp). The new collections from Costa Rica are maintained as herbarium samples and in-vitro plants at the Universidad de Costa Rica, and their locality data are reported in Table 1.

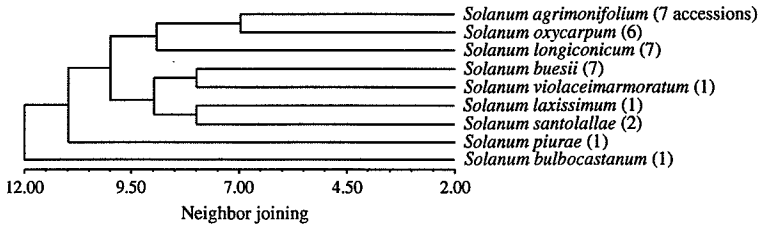

Fig. 1 Abstracted phenetic results (Neighbor-joining) of RAPD analysis of the North and Central American members of *Solanum* series *Conicibaccata* from Spooner et al. (2001)

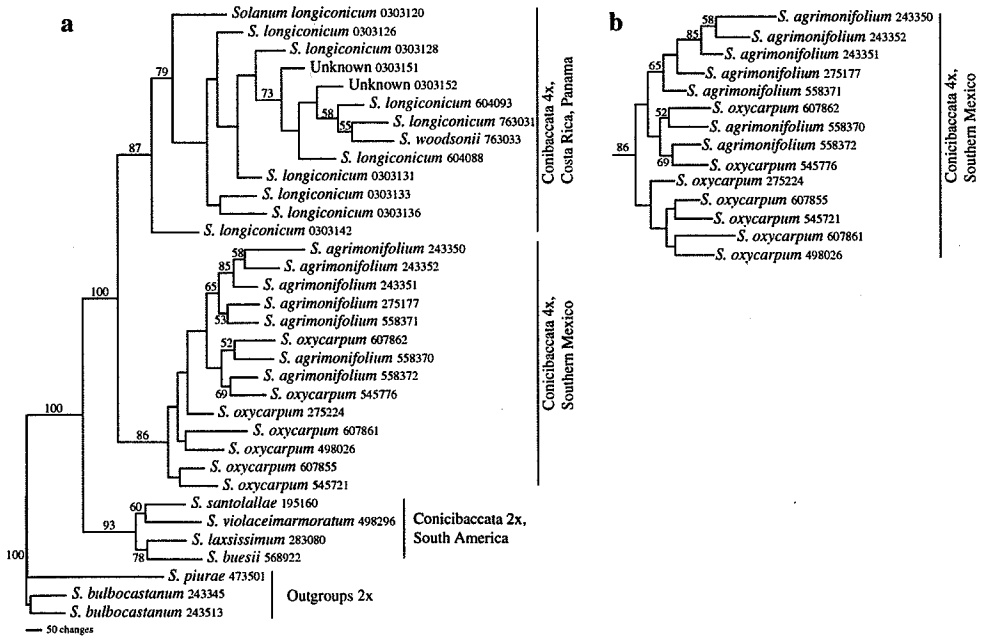

Fig. 2 (**a**) One of two equally-parsimonious Fitch trees of AFLP data of only the Mexican and Central American accessions and diploid outgroups (as in Spooner et al. 2001). (**b**) The only portion of the second equally-parsimonious Fitch tree from Fig. 2a that differs from this tree

Plant growth conditions, DNA extraction, AFLP generation, ploidy determination

Plants were grown in the greenhouse and DNA was extracted from lyophilized leaf tissue from single individuals of 2-month-old plants, using the medium-scale DNA extraction procedure from the International Potato Center, CIP (1999). The restriction and ligation reaction of 1 × T4 Ligase buffer (with ATP), 0.05 M NaCl, 0.045 mg/ml BSA, 1 μM *Eco*RI or *Pst*I adapter, 5 μM *Mse*I adapter, 5 U *Eco*RI or *Pst*I and *Mse*I, 1 U T4 DNA Ligase, and 500 ng of DNA in a total of 11 μl was incubated overnight at room-temperature. The preselective amplification (PSA) in a total of 13 μl containing 1 × PCR Buffer with 20 mM $MgCl_2$, 0.2 mM dNTPs, 0.3 μM each *Eco*RI + 1 or *Pst*I + 1 and *Mse*I + 1, 0.5 U *Taq* DNA polymerase (Takara *Ex Taq*, Madison, WI, USA), and 3 μl of a 1:10 diluted reaction amplified at 2 min at 72°C, 20 cycles of 94°C for 20 s, 56°C for 30 s, and 72°C for 2 min, followed by 72°C for 2 min, and a final extension of 60°C for 30 min. Next followed the selective amplification (SA) in 8 μl with 2 μl of a 1:20 diluted PSA in 1 × PCR buffer, 2 mM $MgCl_2$, 0.2 mM dNTPs, 0.625 μM each D4-PA labeled *Eco*RI + 3 or *Pst*I + 3 and unlabeled *Mse*I + 3, and 0.2 U *Taq* DNA polymerase (SIGMA® JumpStart *Taq* polymerase, Saint Louis, MO, USA); amplified at 94°C for 2 min, 10 cycles of 94°C for 20 s, 66°C for 30 s, decreasing 1°C per cycle, and 72°C for 2 min, 25 cycles 94°C for 30 s, 56°C for 30 s, and 72°C for 3 min, and a final extension of 60°C for 30 min. AFLP assays were performed using the following 12 primer combinations, following advice of collaborators from the Scottish Crop research Institute: (EAAC + MCCA, EACA + MCAC, EAAC + MCAC, EACA + MCCA, PAC + MACT, PAC + MAAC, PAG + MACC, PAG + MACT, PAT + MAAC, PAT + -MAGG, PCA + MAGG, PCA + MAAC).

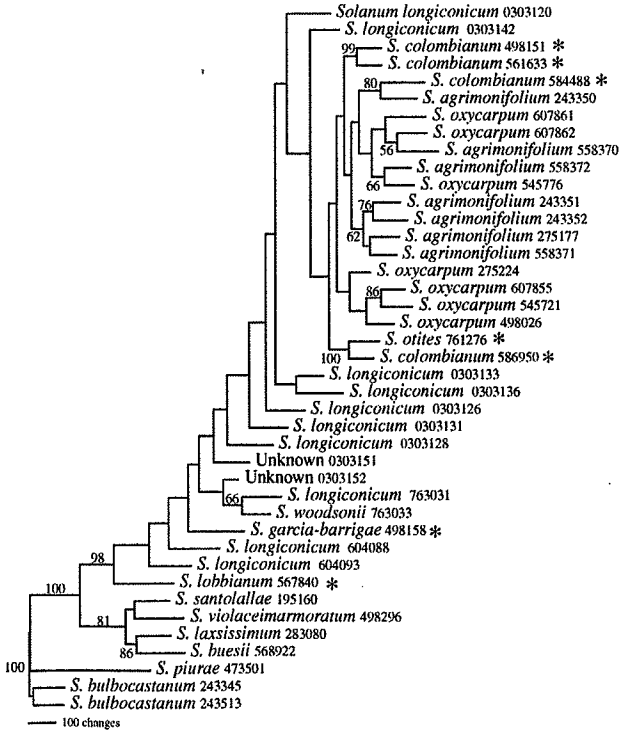

Fig. 3 The single most-parsimonious Fitch tree of AFLP data from all examined accessions. Asterisks denote the tetraploid members of series *Conicibaccata* from South America, interdigitated with the tetraploid accessions from Mexico and Central America

Table 1 New collections of the wild potato species *S. longiconicum* and accessions likely to be *S. woodsonii*

S. longiconicum. Costa Rica. Cartago: on slope of Irazú, on road to San Gerardo, on side of road, 9°59′10.1″N, 83°50′21.1″W, 3177 m, *A. Brenes 0303120*

S. longiconicum. Costa Rica. Cartago: on slope of Irazú, on road to San Gerardo, on side of road, 9°59′10.1″N, 83°50′21.1″W, 3177 m, *A. Brenes 0303120*

S. longiconicum. Costa Rica. Alajuela: Volcán Poás, 100 m from public cafeteria, 10°11′2.6″N, 84°14′10.9″W, 2573 m, *A. Brenes 0303126*

S. longiconicum. Costa Rica. Cartago: Tapantí Reserve, near town of Orosi, 15 km after entrance, 9°41′18″N, 83°45′31.2″W, 1695 m, *A. Brenes 0303131*

S. longiconicum. Costa Rica. Heredia: Volcán Barva, on the Varo Trail about 600 m from its beginning at the ranger station, 10°7′54.8″N, 84°7′21.8″W, 2675 m, *A. Brenes 0303133*

S. longiconicum. Costa Rica. San José: on Cerro Chompipe, 4 ½ km from entrance to Monte de la Cruz Recreation Center, 10°5′12.6″N, 84°4′15.7″W, 2117 m, *A. Brenes 0303136*

S. longiconicum. Costa Rica. San José: near town of Altamira, at 56 km marker of Pan American Highway., 9°41′43.4″N, 83°55′26.5″W, 2555 m, *A. Brenes 3030142*

S. woodsonii? Costa Rica. San José: on trail to Cerro de Chirripo, near Monte Sin Fe, between 9 km and 10 km mark, 9°26′38.1″N, 83°31′58.2″W, 3157 m, *A. Brenes 0303151*

S. woodsonii? Costa Rica. San José: On trail to Cerro de Chirripo, near Llano Bonito, between 6 km and 7 km mark, 5 m before wooden bridge over small swamp, 9°27′27.3″N, 83°33′8.9″W, 2511 m, *A. Brenes* 0303152

Samples were prepared as follows for the CEQ™ 8000; 1 μl of SA, 35 μl SLS (Beckman Coulter, Inc., Fullerton, California, USA), and 0.66 of DNA size standard 400 (Beckman Coulter, Inc.). On the CEQ™ 8000, samples denatured at 90°C for 2 min, injected at 1 kV for 30 s, and separated at 5 kV for 55 min. To analyze the results, the Analysis Parameter Set was modified as cubic for the model and PA ver.1 for the Dye Mobility Calibration. The fragment list was then filtered to exclude all dyes but D4 and rfu values less than 3600. Data were converted to 0 (absent) and 1 (present).

Flow cytometry determines nuclear DNA content by measuring the fluorescence emission of isolated nuclei, then relating this observed fluorescence to DNA amount by comparison to a known standard. Similar to the analysis of the North and Central American species of ser. *Conicibaccata* by Spooner et al. (2001) we used flow cytometry to assess ploidy levels of six accessions of *S. longiconicum* (*A. Brenes 0303120, 0303126, 0303131, 0303133, 0303136, 0303142*), and two of *S. woodsonii* (*A. Brenes 0303151, 0303152*). Leaf samples were prepared for flow cytometric analysis of DNA content according to the method of Dickson et al. (1992). Fifty milligrams of plant leaves were placed in a 55 mm plastic Petri dish. Then, we added 400 μl extraction buffer and chopped for 30–60 s with a single edged razor blade, and incubated these for 30 s. The solution was filtered through a Partec 50 μm CellTricks disposable filter, later added 1.6 ml Staining Solution to the test tube and incubated for 30 to 60 s. Finally, analyzed in the flow cytometer in the blue fluorescence channel. The internal standard was a diploid sample of *S. commersonii* $2n = 2x = 24$ (CIP 761086).

Data analyses

Data were analyzed cladistically and phenetically. Cladistic analyses were performed using PAUP version 4.0b8 (Swofford 2001), using Fitch parsimony (Farris 1970). *Solanum bulbocastanum* was designated as outgroup, following results of Spooner et al. (1993). To find multiple tree islands, we used a four-step search strategy (modified from Olmstead and Palmer 1994): 1) one hundred thousand replicates initially were run using random order entry starting trees with nearest-neighbor interchange (NNI), 2) the shortest trees from this analysis were used individually as starting trees with the tree-bisection reconnection (TBR) method, 3) the resulting trees were searched with NNI, retaining all most parsimonious trees (MULPARS), 4) The resulting trees were searched with TBR and MULPARS. A bootstrap analysis was conducted on 1,000 replicates with TBR and MULPARS. Phenetic analyses used NTSYS-pc® version 2.02k (Rohlf 1997). Distance matrices (in SIMQUAL) were generated using Jaccard's matrix, which places no weight on shared absent bands, as an appropriate algorithm for AFLPs that are predominately dominant markers. Clustering was performed using the unweighted pair group method (UPGMA) and the Neighbor-Joining method in SAHN.

Results

The 12 AFLP primer combinations produced 1722 polymorphic bands and there were no missing data; raw data are available from authors (DMS). We examined three subsets of species: 1) only the Mexican and Central

American accessions and diploid outgroups (as in Spooner et al. 2001), 2) all species, 3) only the polyploids (all ser. *Conicibaccata*, no outgroups). Cladistic and phenetic analyses defined the same main clades or groups in all three analyses and only the cladistic analyses are presented here.

Parsimony analysis of subset 1 produced two equally parsimonious trees (Fig. 2) with a tree length of 5,921, consistency index of 0.2775, rescaled consistency index of 0.1144, and retention index of 0.4123. Both trees produced two well defined (>85% bootstrap support) clades of the polyploid members of ser. *Conicibaccata* from Mexico and Central America: 1) the accessions from Costa Rica and Panama (*S. longiconicum*, *S. woodsonii*, and the newly collected accessions suspected to be *S. woodsonii*), and 2) the species from southern Mexico south to Honduras (*S. agrimonifolium*, *S. oxycarpum*). However, there were no species-specific clades within these two clades, and AFLP data support only two species, not the four species that are currently accepted. Sister to these two clades is a clade of the four diploid South American members of ser. *Conicibaccata*, followed by outgroups. This result is extremely similar to the RAPD results of these accessions (Spooner et al. 2001) in almost every respect (Fig. 1), except *S. agrimonifolium* and *S. oxycarpum* are not separated into species-specific clades and the diploid species of ser. *Conicibaccata* form different species pairs clades (compare Figs. 1, 2). *S. woodsonii* is a new addition relative to this prior study, but neither the Panamanian accession of this species, nor the two accessions from Costa Rica we suspected to be this species are distinguished from *S. longiconicum*.

Parsimony analysis of subset 2 (all accessions, with the addition of seven accessions of four tetraploid South American species of ser. *Conicibaccata* relative to Fig. 2) produced a single most parsimonious tree (Fig. 3) with a tree length of 7,032, consistency index of 0.2442, rescaled consistency index of 0.0968, and retention index of 0.3964. The addition of these four tetraploid species drastically altered the topology of Fig. 2. The only remaining concordance of these two trees is the topology of the outgroups, but good bootstrap support for the outer nodes of the polyploid members of ser. *Conicibaccata* disappears relative to Fig. 2, the two clades (*S. agrimonifolium* + *S. oxycarpum*, *S. longiconicum* + *S. woodsonii*) disappear, and there is extensive intermixture of all polyploid species on the cladogram.

Parsimony analysis of subset 3 (only the polyploids containing all ser. *Conicibaccata*), retained the topological structure of intermixed *Conicibaccata* species.

All nine accessions *S. longiconicum* or *S. woodsonii* analyzed by flow cytometry proved to have a DNA content within a very narrow range, and each averaged 2.99 pg/2C nucleus. This included a determination for Spooner et al. 7413, collected in Panama in 2000 at the type locality of this species (Spooner et al. 2004) and maintained as germplasm at CIP. The very narrow range of DNA contents in all 10 wild potato accessions, and relationship of these to prior counts of $2n = 4x = 48$ in *S. longiconicum* make our data easy to interpret as all species to be $2n = 4x = 48$. This is the first reported ploidy determination of *S. woodsonii*, and the only remaining species of sect. *Petota* remaining undetermined for ploidy is *S. donachui* (Ochoa) Ochoa (Hijmans et al., in press).

Discussion

The taxonomy of *Solanum* section *Petota* has always been notoriously difficult, and differing taxonomic interpretations of the number of species and their interrelationships are common. For example, Hawkes (1990) recognized 232 species of sect. *Petota*, divided into 21 taxonomic series. Despite the description of 10 new species since Hawkes (1990; raising the total to 242 species), Spooner and Salas (2006) summarize the reduction of species of sect. *Petota* to 189, and predict yet further reductions in species with continuing research.

We used AFLPs rather than RAPDS in our study because of problems of RAPDs with low reproducibility (Schierwater and Ender 1993) and non-homology across unrelated germplasm. For example, a study of genetic diversity in two phylogenetically diverse species of potato (*Solanum fendleri* A. Gray = *S. stoloniferum* Schltdl. and *S. jamesii* Torr.) by Del Rio et al (1997) required different sets of RAPD primers for each species. We also chose AFLPs because of their superior marker index (the product of heterozygosity and individual markers produced per marker type and a measure to evaluate the overall information content of a marker system) in potato.

Our study was initiated to use AFLPs to discover taxonomic affinities of newly collected accessions from Costa Rica and Panama that were the first putative germplasm collections of *S. woodsonii*, and to test the concordance of AFLP data to prior RAPD data (Spooner et al. 2001) for the Mexican and Central American accessions of ser. *Conicibaccata*. South American polyploid accessions were added for comparative purposes, since based on RAPD results (Spooner et al. 2001) we suspected AFLPs would define species-specific clades. The results of the North and Central American polyploids were largely concordant with these prior results, except AFLP data were unable to distinguish *S. agrimonifolium* and *S. oxycarpum*, unlike RAPD results. However, most other topological details of the trees were concordant. Based on the RAPD data, Spooner et al. (2001, 2004) recognized *S. agrimonifolium* and *S. oxycarpum* as distinct, but the present AFLP data

suggest a need to reevaluate this decision, and to question *S. longiconicum* and *S. woodsonii* as distinct. The failure to distinguish these species is unlikely to be a result of insufficient AFLP data. Our 12 primer combinations producing 1722 polymorphic bands is by far much larger than any prior AFLP study in the Solanaceae, that used a range of 2–8 primer combinations (Introduction) to produce 232 to 512 bands.

The failure to distinguish these species, as well as the drastic loss of species-specific clades with the addition of the four South American species of ser. *Conicibaccata* has various possible explanations: 1) improper species circumscriptions, 2) local gene flow, 3) complex reticulate histories. 1) The taxonomy of sect. *Petota* is in great need of a comprehensive taxonomic treatment throughout its entire range to force a more rigorous comparison of species boundaries from all geographical areas. Many species in the series are variable and overlap in ranges of character states with other currently recognized species, and species circumscriptions may be too narrow. 2) Gene flow may be combining some of the AFLP markers among nearby species that are morphologically defined. For example, Schmidt-Lebuhn et al. (2006) discovered that samples from different species in the genus *Polylepis* (Rosaceae) sometimes clustered according to geographic proximity rather than systematic affiliation. They interpreted these results as indicative of frequent hybridization and introgression, as may be the case in nearby species pairs *S. agrimonifolium* and *S. oxycarpum*; and fully sympatric species pairs *S. longiconicum* and *S. woodsonii*. 3) Polyploid members of ser. *Conicibaccata* are putative polysomic polyploids, and anonymous AFLP data could provide false phylogenetic signal of accessions with complex histories of multiple origins from different progenitor species. Despite this fact, AFLPs have been used to show concordant results in other polyploid groups (Van den Berg et al. 2002; Spooner et al. 2005a; Schmidt-Lebuhn et al. 2006).

AFLPs highlight potential taxonomic problems of species in ser. *Conicibaccata*, and the resolution of their species status and relationships require: 1) numerical morphological studies of the readily available germplasm accessions of these species, 2) herbarium studies from specimens from throughout the range of the group, and 3) the use of cloned DNA sequences of the single-copy nuclear genes from polyploids compared to diploids to tease apart possible reticulate origins as has been done in other groups (Ge et al. 1999; Cronn et al. 2002; Ford and Gottlieb 2002; Senchina et al. 2003; Doyle et al. 2004; Mason-Gamer 2004). We are currently conducting such morphological and molecular studies in the context of an NSF-funded worldwide taxonomic treatment of *Solanum* http://www.nhm.ac.uk/research-curation/projects/solanaceaesource//.

Acknowledgments We thank CONICIT, MICIT (Costa Rica) and the University of Costa Rica for funding J.P. Jiménez's AFLP studies in D.M. Spooner's laboratory, and NSF DEB 0316614 (Spooner), "A world-wide treatment of *Solanum*," for funding laboratory work.

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