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AFLP data support the recognition of a new tuber-bearing *Solanum* species but are uninformative about its taxonomic relationships

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Abstract. *Solanum* section *Petota*, containing the cultivated potato and its wild relatives, is a group of around 200 species. Many of these species are morphologically very variable with unclear boundaries, and the group as a whole appears to be somewhat over-classified. Describing a new species in this group should only be undertaken with caution, and molecular data can be used to test the distinctness of any putative new taxon. AFLP markers have shown the ability to reliably distinguish species in several groups within the genus *Solanum*. We tested the distinctness of a new tuber-bearing *Solanum* species using morphological and AFLP data, and tried to establish its affiliation to the series within the section. There was clear support for the species status of the material known as *Solanum hannemanii* in genebank collections, but the AFLP data were inconclusive about its relationships to the other investigated species. Also, the distinction of the series *Tuberosa* and *Megistacroloba*, to which these species belong, was not supported.

Keywords: AFLP; *Solanum* section *Petota*; New species; *Solanum hannemanii*

The secondary genepool of the cultivated potato, *Solanum tuberosum* L., consists of a large number of tuber-bearing species, classified as *Solanum* sect. *Petota* Dumort. The number of species was estimated at 232 by Hawkes (1990), and later reduced to 206 by Spooner and Heijmans (2001), and to 188 by Spooner and Salas (2006). Many of these species are morphologically very similar to each other. Correll (1962) cautioned against describing new species in this group before the nature of already-recognized taxa was better understood. Still, new species are discovered now and again, and morphological studies can be supplemented with molecular analysis to assess the distinctness of any putative new taxon. Repeatedly, AFLP data have been shown to reveal the proper amount of heritable variation to enable delimitations of taxa at the species level (Kardolus 1998; Mace et al. 1999a, b; Koopman et al. 2001; Van den Berg et al. 2002; Wong et al. 2002; Lara-Cabrera and Spooner 2004; Dehmer and Hammer 2004).

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In 1972 and 1973, Andrea Clausen and Armando Okada collected material of tuber-bearing *Solanum* species in Argentina. Some of this material was identified as a possible hybrid between *S. venturii* Hawkes & Hjerting and *S. megistacrolobum* Bitter; other collections were designated as *S. spegazinii* Bitter. Later, the collectors recognised that this material might belong to two unknown taxa. The material was entered into genebank collections under the provisional names *S. hannemanii* and *S. hawkesianum*, as suggested by these collectors. The latter can on morphological grounds be assigned to the *brevicaule/leptophyes/gourlayi* alliance, with its narrow leaflets closely resembling accessions of *S. gourlayi* Hawkes subsp. v*idaurrei* (Card.) Hawkes & Hjerting. The former putative new taxon is distinctive in its morphology, and the present study was set up to assess molecular support for this taxon and compare it with putative closely related species, based mainly on a characteristic of their leaf dissection (i.e. the presence of a large terminal leaflet and only few, small lateral leaflets). To investigate the possibility of a hybrid origin for this putative taxon involving species from series *Megistacroloba* Card. & Hawkes and *Tuberosa* (Rydb.) Hawkes, representatives of those series were included. The narrow-leafed species, *S. infundibuliforme* Phil., was chosen as an outgroup. This species was assigned by Hawkes (1990) to the separate series *Cuneoalata* Hawkes, and is more closely related to members of the *brevicaule* complex than to the species considered here (Kardolus et al. 1998).

Materials and methods

Plant material and measurements. Table 1 lists the 37 accessions used in this study, all but two from the CGN (Centre for Genetic Resources, the Netherlands). The species identification of these accessions was checked by us using the descriptions and keys in Hawkes (1990), but we treat *Solanum toralapanum* Card. & Hawkes as a subspecies of *S. megistacrolobum*, following Giannattasio and Spooner (1994). We remained unsure about the identity of accessions assigned to the very similar species *S. okadae* Hawkes & Hjerting and *S. venturii*. From each accession, three plants were grown in the greenhouse, and 40 morphological characters (Table 2) were measured from each plant. Thirty of these characters are quantitative and ten are qualitative, describing 21 vegetative and 19 generative features. There are missing data for three individuals (*med* 16-2, *mga* 17-2 and *rap* 31-3), because these plants did not flower during the observation period. All data were analysed separately, with the OTU (Operational Taxonomic Unit) representing individual plants.

The AFLP analysis used the same material as the morphological analysis. Three genotypes per accession were used except in five cases where only two genotypes per accession were available (Table 1).

AFLP protocol. Nuclear DNA was extracted from nitrogen-frozen young leaves and sprouts with the CTAB-method (Bernatzky and Tanksley 1986). The AFLP protocol follows the one described in Kardolus et al. (1998) with some small modifications: the dilution of the ligation mixture was 10-fold; the dilution of the first PCR product was 50-fold; and a 0.35 mm sequence gel system (GIBCO BRL, Invitrogen Corporation, Carlsbad, CA) was used. Two primer combinations were used: E + AAC/M + CAC (E32/M48) and E + ACA/M + CAC (E35/M48).

Data analysis

Morphology. Each morphological character was analysed for its mean, standard deviation, minimum, maximum and significance by one-way analysis of variance in JMP (SAS Institute Inc. 1995). Cluster Analysis and Principal Components Analysis were performed with NTSYS-PC, version 2.11T (Rohlf 2000). The procedures SIMINT and SAHN within NTSYS were used. With SIMINT, similarity matrices were generated by using Euclidian Distance (EUCLID), Squared Euclidian Distance (EUCLIDSQ), Manhattan Distance (MANHAT), Average Taxonomic Distance (DIST) and Product-Moment Correlation (CORR). Clustering was performed by using the Unweighted Pair-Group Method (UPGMA), Weighted Pair-Group Method (WPGMA), Single Linkage (SINGLE) and Complete Linkage (COMPLETE) in SAHN.

Table 1. *Solanum* material examined in this study

Code	Species	Series[1]	Source[2]	Collector	Origin	Latitude	Longitude	[3] # GT AFLP
han6	*S. hannemanii*	?	C17854	OKA 4374	Arg-Jujuy	23°36′ S	65°08′ W	3
han7	*S. hannemanii*	?	C17855	OKA 4407 × 4372	Arg-Jujuy	-	-	3
han8	*S. hannemanii*	?	C17856	OKA 4407 × 4481	Argentina	-	-	3
han9	*S. hannemanii*	?	C17858	OKA 4481 × 4374	Argentina	-	-	3
han10	*S. hannemanii*	?	C17996	OKA 4372	Arg-Jujuy	23°36′ S	65°08′ W	3
han11	*S. hannemanii*	?	C17997	OKA 4383A	Arg-Jujuy	23°38′ S	65°06′ W	3
han12	*S. hannemanii*	?	C18001	OKA 4407	Arg-Jujuy	23°38′ S	65°09′ W	3
ifd13	*S. infundibuliforme* Phil.	CUN	C17857	OKA 4457B	Arg-Jujuy	23°08′ S	65°06′ W	3
ifd14	*S. infundibuliforme*	CUN	C17940	OKA 4451	Arg-Jujuy	23°12′ S	65°16′ W	3
ast1	*S. astleyi* Hawkes & Hjerting	MEG	C18207	HAM 203	Bol-Potosi	19°38′ S	65°17′ W	3
ast2	*S. astleyi*	MEG	C18211	HAM 208	Bol-Potosi	19°38′ S	65°17′ W	2
ast3	*S. astleyi*	MEG	C18212	HAM 209	Bol-Potosi	19°38′ S	65°17′ W	3
blv4	*S. boliviense Dunal*	MEG	C17680	ALN 006	-	-	-	3
blv5	*S. boliviense*	MEG	C18208	HAM 204	Bol-Potosi	19°38′ S	65°17′ W	2
mga17	*S. megistacrolobum Bitter* subsp. *megistacrolobum*	MEG	C17726	PEH 0366	Arg-Jujuy	-	-	3 3
mga19	subsp. *megistacrolobum*	MEG	C18184	HAM 072	Bolivia	19°31′ S	65°41′ W	3
mga20	subsp. *megistacrolobum*	MEG	C17727	ROR 0150	Bol-Chuq	-	-	3
mga21	subsp. *megistacrolobum*	MEG	C17985	OKA 3989	Arg-Jujuy	22°23′ S	66°05′ W	3
tor37	*S. megistacrolobum* subsp. *toralapanum* (Card. & Hawkes) R.B. Giannattasio & D.M. Spooner	MEG	C18141	VSAL 118	Bolivia	16°34′ S	67°59′ W	3
tor38	*S. megistacrolobum* subsp. *toralapanum*	MEG	C18147	VSLC 138	Bol-Coch	17°40′ S	66°32′ W	2
tor39	*S. megistacrolobum* subsp. *toralapanum*	MEG	C18145	VSAL 134	Bolivia	17°26′ S	65°32′ W	2
rap29	*S. raphanifolium* Cárdenas & Hawkes	MEG	C17834	ROR 0776B	Peru	-	-	3
rap30	*S. raphanifolium*	MEG	C18089	HVHL 5421	Peru	13°30′ S	72°00′ W	3
rap31	*S. raphanifolium*	MEG	C18320	OCH S-58	Peru	-	-	3
sct32	*S. sanctae-rosae* Hawkes	MEG	B18315	EBS 2691[4]	-	-	-	3
sct33	*S. sanctae-rosae*	MEG	C18090	HOHH 6084	Arg-Tuc	26°46′ S	65°45′ W	3
sct34	*S. sanctae-rosae*	MEG	C18092	HOHH 6092	Arg-Tuc	26°46′ S	65°46′ W	3
sct35	*S. sanctae-rosae*	MEG	C18091	HOHH 6087	Argentina	26°46′ S	65°45′ W	2
sgr36	*S. sogarandinum* Ochoa	MEG	C17601	OCH 1440	Peru	8°09′ S	78°11′ W	3
med15	*S. medians* Bitter	TUBw	C18043	HAM 2489	Peru	-	-	3
med16	*S. medians*	TUBw	C18307	VIL 211	Peru	11°22′ S	77°01′ W	3
mcd22	*S. microdontum* Bitter	TUBw	C17596	OKA 4478	Arg-Salta	23°13′ S	64°55′ W	3
mcd23	*S. microdontum*	TUBw	C17597	OKA 4820	Arg-Salta	25°09′ S	65°45′ W	3
mcd24	*S. microdontum*	TUBw	C18200	HAM177	Bolivia	21°25′ S	64°22′ W	3
oka25	*S. okadae* Hawkes & Hjerting	TUBw	C18109	HOHH 6033	Arg-Salta	25°10′ S	65°50′ W	3
oka26	*S. okadae*	TUBw	B27158	VSA 176	Bolivia	17°01′ S	67°15′ W	3
vnt40	*S. venturii* Hawkes & Hjerting	TUBw	C17755	WAC 3257[4]	Arg-Tuc (Tafi)	-	-	3

[1] Series abbreviations according to Hawkes (1990)
[2] B Braunschweig Genetic Resources Collection; C Center for Genetic Resources, the Netherlands
[3] Number of genoypes studied with AFLP
[4] Donor number, no collector number available

AFLP. The AFLP fragments were scored as present (1) or absent (0); band-intensity differences were not scored. Lane matching and fragment scoring were performed automatically on digital images of the autoradiograms. For scoring the E35/M48 primer combination, the program Phoretix 1D advanced Version 4.00 (Phoretix International, Newcastle upon Tyne, UK) was used, producing 146 bands. The program AFLP Quantar™ Pro 1.0 (Key Gene

Table 2. Morphological characters and states used in the measurements and data analysis

Vegetative characters:
1. width stem (mm)
2. wings on stem: absent (0), present (1), broad (2)
3. pubescence density on stem: absent or few (0), present (1)
4. pubescence length on stem: short (0), long (1)
5. length leaf (mm)
6. width leaf (mm)
7. length petiole leaf (mm)
8. length terminal leaflet (mm)
9. width terminal leaflet (mm)
10. place maximum width terminal leaflet (mm from apex)
11. length petiolule terminal leaflet (mm)
12. apex terminal leaflet: obtuse (1), acute (2), acuminate (3)
13. base terminal leaflet: cuneate (1), truncate (2), cordate (3)
14. pairs lateral leaflets
15. length 1st lateral leaflet (mm)
16. length petiolule 1st lateral leaflet (mm)
17. degree decurrency 1st lateral leaflet (mm from midvein downwards)
18. length 2nd lateral leaflet (mm)
19. length petiolule 2nd lateral leaflet (mm)
20. number interjected leaflets
21. pubescence density upperside leaf: 0 (1), 10 (2), 30 (3), 50 (4), 70 (5) hairs per cm^2

Generative characters:
22. number of flowers per inflorescence
23. length peduncle from stem till leaf (mm)
24. length peduncle from leaf onwards (mm)
25. length pedicel (mm)
26. distance articulation - calyx (mm)
27. length calyx (mm)
28. length calyx acumen (mm)
29. colour corolla outside: white (1), very pale purple (2), purple (3), dark purple (4), very dark purple (5)
30. colour corolla inside: white (1), very pale purple (2), purple (3), dark purple (4), very dark purple (5)
31. corolla pattern outside: absent (0), present (1)
32. corolla pattern inside: absent (0), present (1)
33. radius to apex corolla lobe (mm)
34. radius to base corolla lobe (mm)
35. width corolla lobe at base (mm)
36. length widest point corolla lobe (mm)
37. length corolla acumen (mm)
38. length anther (mm)
39. length style (mm)
40. style: straight (0), curved (1)

Products, Wageningen, The Netherlands) was used to analyze the primer combination E32/M48, producing 66 bands. The latter primer combination produced no bands for one accession of *S. boliviense* Dunal (C18208) and one of *S. hannemanii* (C17858). All but the faintest bands were scored; where necessary, scores were corrected manually.

Phenetic analyses were performed by using the program NTSYS-PC, version 2.11T (Rohlf 2000). By using the procedure SIMQUAL, similarities between OTUs were calculated with

Jaccard's similarity coefficient. The UPGMA clustering algorithm was used and the cophenetic correlation coefficient was calculated by using the procedures COPH and MXCOMP. Neighbour-Joining trees were generated with PAUP 4.0 (Swofford 2002). Trees were calculated for each primer combination separately as well as for the combined dataset.

Cladistic analyses were performed using PAUP* 4.0b10 (Swofford 2002). A Jackknife analysis (10,000 reps) of the dataset resulting from the combined primer combinations was performed, and heuristical searches were run with 1000 random sequence taxon additions with TBR swapping.

Finally, Bayesian analyses were performed with MrBayes 3.1 (Ronquist and Huelsenbeck 2003); the resulting trees were visualized with TreeView 1.6.6 (Page 1996).

Results

Morphology. All the morphological characters showed significant differences between at least some pairs of OTUs in the one-way analysis of variance.

Phenograms were constructed by using different similarity measures and various clustering methods. Values for the cophenetic correlation coefficients of the phenograms resulting from the various combinations of similarity measures and clustering methods were compared. These values ranged between 0.72178 (CORR / UPGMA) and 0.85467 (DIST / UPGMA). In the phenogram with the highest cophenetic correlation coefficient (Fig. 1) all or most of the individuals of the following species grouped together: *S. astleyi* Hawkes & Hjerting, *S. boliviense*, *S. infundibuliforme*, *S. sanctae-rosae* Hawkes, *S. medians* Bitter, *S. raphanifolium* Cardenas & Hawkes, *S. microdontum* Bitter and *S. venturii*. Also, representatives of the putative species *S. hannemanii* cluster together, with one exception. Accessions or individuals of *S. megistacrolobum* subsp. *megistacrolobum*, *S. megistacrolobum* subsp. *toralapanum* (Card. & Hawkes) R.B. Giannattasio & D.M. Spooner, *S. sogarandinum* Ochoa and *S. okadae* show less than perfect clustering. Branch lengths in the phenogram (Fig. 1) show that there is a substantial amount of variation within all species.

In the Principal Components Analysis (not shown), the first three principal components accounted for 29.8, 12.6 and 9.7% of the variation, respectively, for a total of 52%. The characters with the highest loadings on these first three components are all dimensions of the leaf and its leaflets. The scatterplots of the first three principal components do not show much differentiation, except for all of the representatives of *S. microdontum*, which are separated from a large cloud of points containing the other species. Within that cloud, the representatives of each species are not intermixed but do form subgroups.

AFLP

Phenetic analysis. The UPGMA tree (not shown) of the combined data set of the two primer combinations (cophenetic correlation coefficient [UPGMA, J] = 0.96222) shows perfect clustering of the accessions in their species, with the exception of the two accessions of *S. okadae*, which are separated by *S. venturii*, and the accessions of *S. astleyi* and *S. boliviense* which are intermixed. A number of subgroups can be distinguished. The putative new species *S. hannemanii* is most similar to *S. microdontum*; *S. raphanifolium* clusters with *S. medians*; and *S. infundibuliforme* is linked to an *astleyi/boliviense* group. Finally, *S. megistacrolobum* subsp. *megistacrolobum*, *S. megistacrolobum* subsp. *toralapanum* and *S. sanctae-rosae* Hawkes form a group, with *S. megistacrolobum* subsp. *megistacrolobum* closer to *S. sanctae-rosae* than to *S. megistacrolobum* subsp. *toralapanum*.

The Neighbour-Joining tree from PAUP (not shown) with *S. infundibuliforme* as outgroup shows the same groups but places *S. sogarandinum* close to the *megistacrolobum /toralapanum/sanctae-rosae* cluster.

Cladistic analysis. The Jackknife analysis (not shown) supports many but not all of the 13 taxa included in this study. The grouping of all

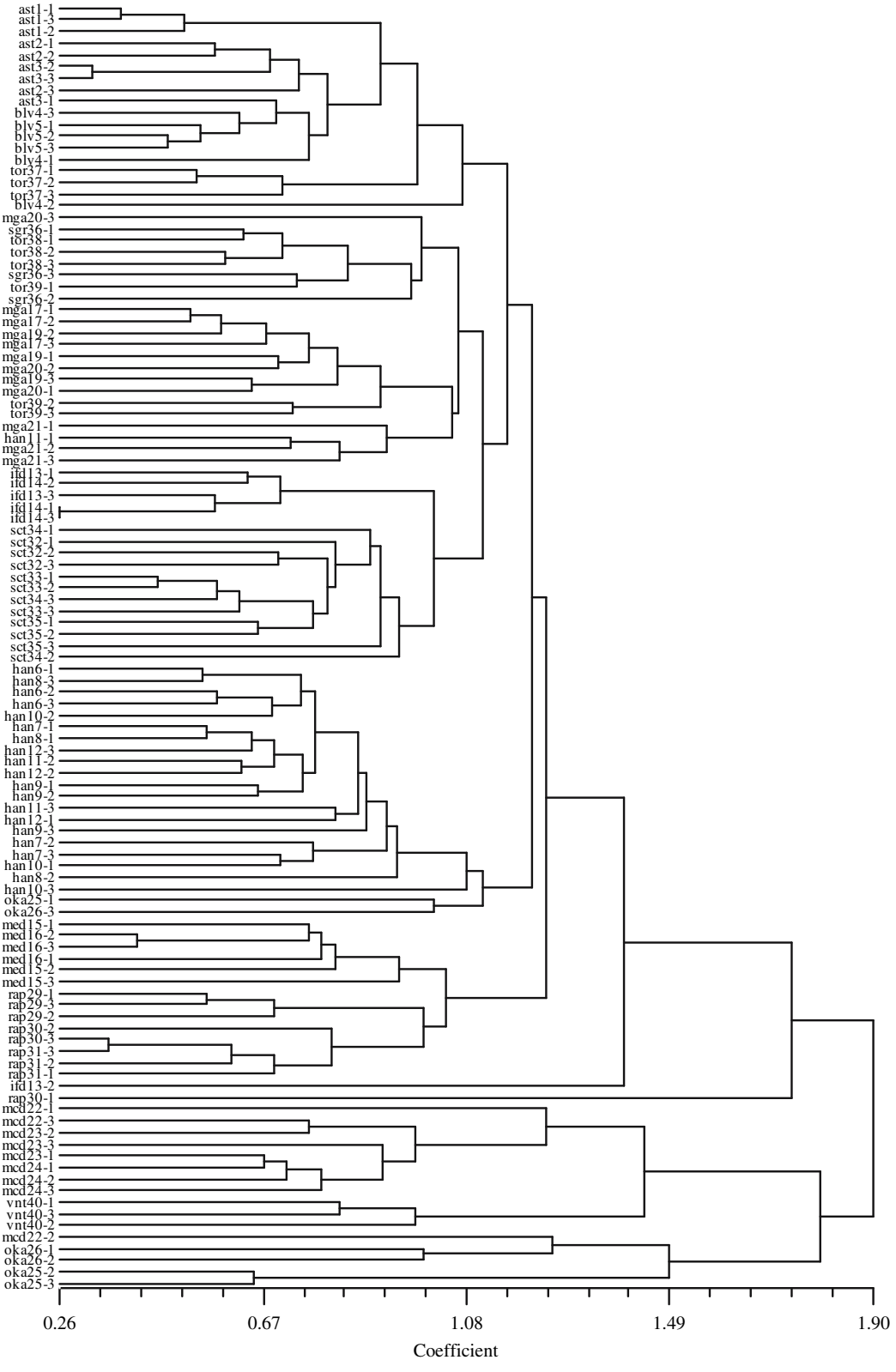

Fig. 1. UPGMA tree based on morphological characters

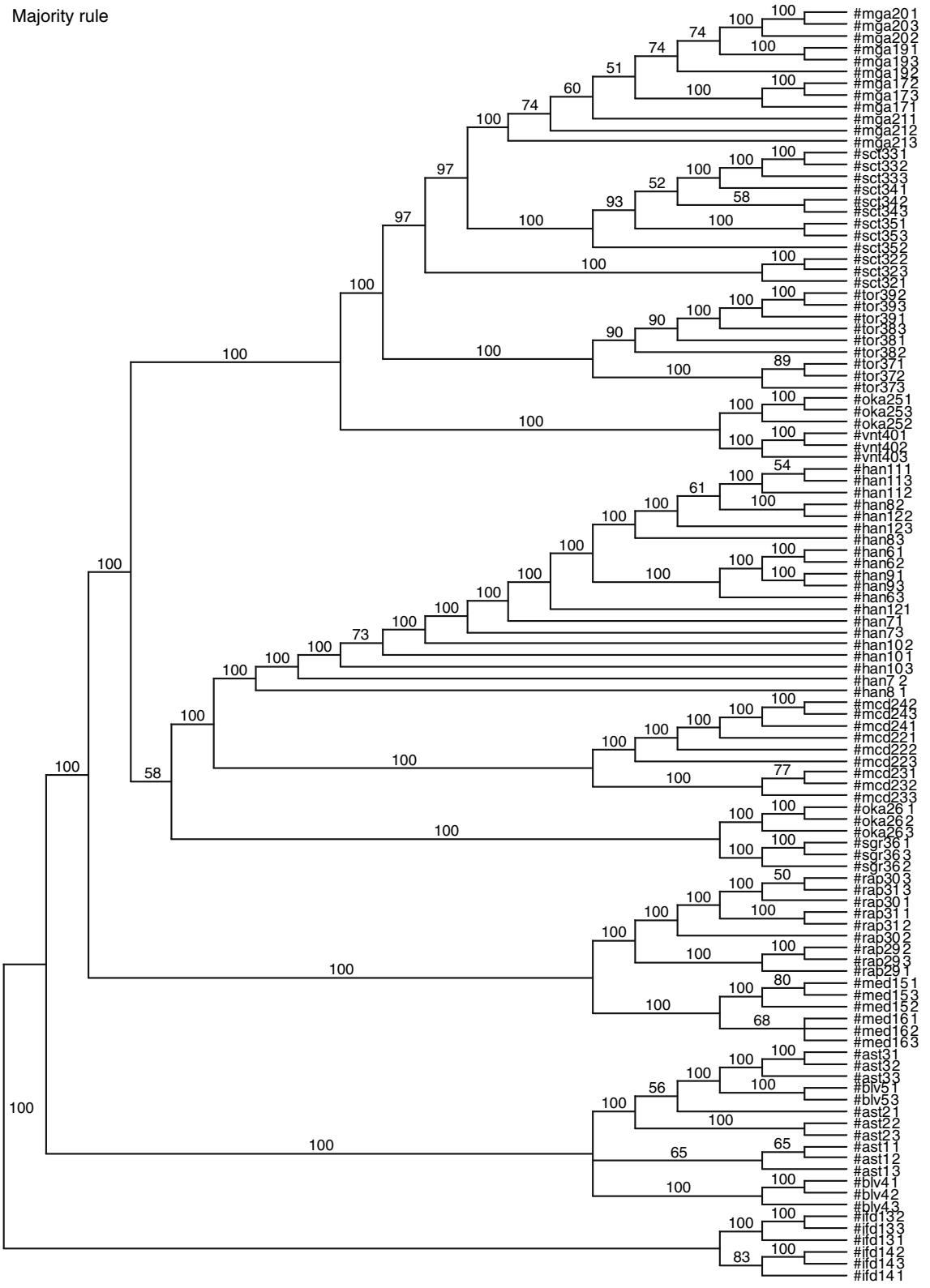

Fig. 2. Maximum Parsimony 50% majority rule consensus tree based on AFLPs

individuals of the species(names) *S. hannemanii*, *S. medians*, *S. raphanifolium*, *S. venturii*, and *S. sogarandinum* receive 100% support, that of *S. microdontum* slightly less (95%), but *S. megistacrolobum* subsp. *megistacrolobum*, *S. megistacrolobum* subsp. *toralapanum*, and *S. sanctae-rosae* are less well supported (69–72% for the individual taxa, 65% for this group), while accessions of *S. astleyi* and *S. boliviense* are intermixed, and the two accessions of *S. okadae* are separated from each other. Furthermore, the Jackknife tree is inconclusive with respect to the interrelationships of the species: in the ingroup, all species except *S. astleyi* / *S. boliviense* are in one group with 94% support, but their relationships to each other are completely unresolved.

The topology of the cladograms resulting from the heuristic searches largely conform with each other. Figure 2 gives a Maximum Parsimony majority-rule consensus tree. After the outgroup, *S. infundibuliforme*, the first clade to branch off consists of *S. astleyi* and *S. boliviense* (not separated from each other but mixed), then a group consisting of *S. medians* and *S. raphanifolium*, a group consisting of *S. hannemanii* and *S. microdontum* with one of the *S. okadae* accessions and *S. sogarandinum*, and, finally a clade consisting of 2 subgroups: 1) the remaining *S. okadae* accession and *S. venturii* and 2) the *megistacrolobum/toralapanum/sanctae-rosae* cluster, with *S. megistacrolobum* subsp. *megistacrolobum* closer to *S. sanctae-rosae* than to *S.megistacrolobum* subsp. *toralapanum*, identical to the results of the phenetic analysis.

Bayesian analysis. In the results of the Bayesian analyses (Fig. 3), the same clusters of species representatives are retrieved with posterior probability scores between 81 and 100%, grouping *S. infundibuliforme* with *S. astleyi* and *S. boliviense* (88%), and including a *megistacrolobum/toralapanum/sanctae-rosae* cluster (93%), but now with *S. megistacrolobum* subsp. *megistacrolobum* closest to *S. megistacrolobum* subsp. *toralapanum*. However, the results of these analyses are also inconclusive about most of the interrelationships among the various groups, as is especially evident in the unrooted tree shown (Fig. 3).

Discussion

The group of tuber-bearing *Solanum* species is probably somewhat over-classified (Spooner and Van den Berg 1992), even with the 188 species currently recognized by Spooner and Salas (2006). Many species are morphologically extremely similar and difficult to distinguish, even by experts (Spooner et al. 2003). Under these circumstances, the description of a new species can only be undertaken exercising sufficient caution. However, one can test the status of taxa based on levels of support from molecular data. The AFLP marker system has been shown to provide resolution at the species level in studies of *Solanum* sect. *Petota*, usually displaying clustering of individual plants in their genebank accessions and of accessions in species (Kardolus 1998; Kardolus et al. 1998; Van den Berg et al. 2001, 2002; Lara-Cabrera and Spooner 2004).

There is clear support in the AFLP data for the recognition of *S. hannemanii* at the species level, since a cluster of all representatives of this putative species was retrieved in all analyses. Consistently, this cluster is placed closest to the cluster of *S. microdontum* accessions. This close relationship between *S. hannemanii* and *S. microdontum* is not apparent from the morphological data, where *S. microdontum* is relatively more distant and *S. hannemanii* more closely resembles the *S. megistacrolobum*-like species.

In the present study, both morphological data and the AFLP results indicate the presence of a clear hierarchical structure, with representatives of most species clustering coherently. Exceptions are the intermixing of *S. astleyi* and *S. boliviense*, the uncertainty of the placement of various accessions of *S. okadae* and *S. venturii*, and the unexpectedly close relationship between *S. megistacrolobum* subsp. *megistacrolobum* and *S. sanctae-rosae*, with *S. megistacrolobum* subsp. *toralapanum* sister to both (but this is contradicted by the Bayesian results).

Interestingly, earlier studies using RAPD data to evaluate relationships between *S. astleyi* and *S. boliviense* (Spooner et al. 1997) and between *S. megistacrolobum* subsp. *megistacrolobum* and *S. megistacrolobum* subsp. *toralapanum* (Giann-

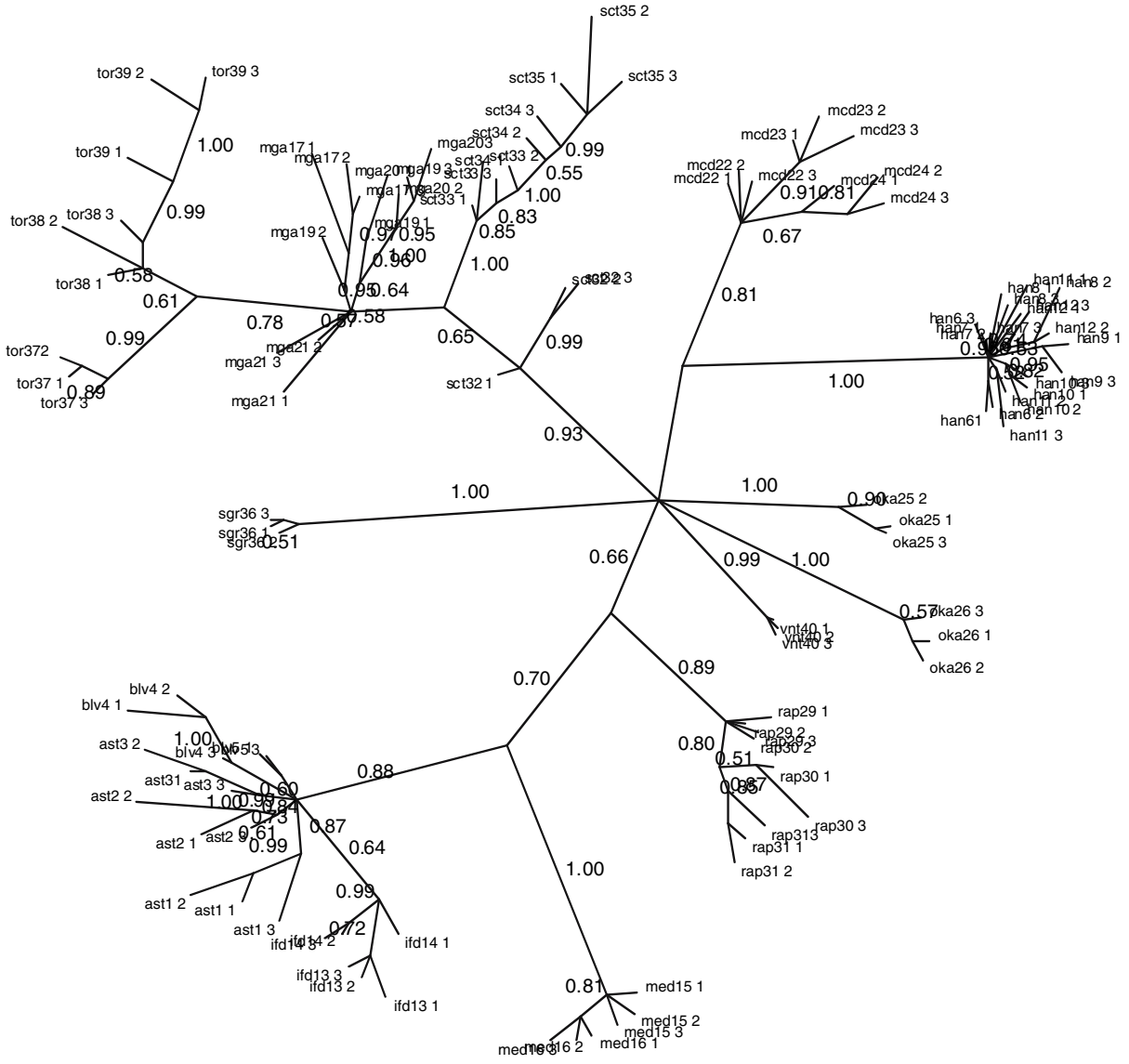

Fig. 3. Bayesian unrooted tree based on AFLPs

attasio and Spooner 1994) highlighted the uncertainties about the species status of these taxa, concluding that they might be best recognized as subspecies or even varieties. The present AFLP results confirm the close affinity or conspecificity of *S. astleyi* and *S. boliviense* and indicate the need to re-investigate the status of *S. sanctaerosae*, and the identity and boundaries of *S. okadae* and *S. venturii*. The uncertain placement of the latter species could also be due to problems with the identity of the genebank material, since we experienced difficulties in distinguishing these species by using the keys in Hawkes (1990). Although the status of the other investigated species seems clear, the AFLP data do not provide enough information on interrelationships among them, irrespective of the method of analysis (phenetic, cladistic, or Bayesian). Neither is there any convincing evidence in support of the hypothesis that *S. hannemanii* was the result of hybridisation between species from series *Tuberosa* and *Megistacroloba*, as was

suggested from its morphology by the original collectors.

All of the investigated species (except the outgroup *S. infundibuliforme*) have been classified into two series: *S. medians*, *S. microdontum*, *S. okadae* and *S. venturii* in series *Tuberosa* and *S. megistacrolobum*, *S. sanctae-rosae*, *S. raphanifolium*, *S. sogarandinum*, *S. astleyi* and *S. boliviense* in series *Megistacroloba* (Hawkes 1990). In contrast to this, Correll (1962) considered *S. boliviense* to belong to series *Tuberosa*. The present results do not support the distinction of these two series, as illustrated by the close relationship between *S. medians* and *S. raphanifolium*, and it might be better to recognize informal species groups instead of formal series, following the practice of Whalen (1984), Knapp (2000) and Spooner et al. (2004) in *Solanum* taxonomy.

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