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AFLP markers support separation of *Solanum nodiflorum* from *Solanum americanum* sensu stricto (Solanaceae)

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Abstract. This study was aimed at examining the relationships between the African material of *Solanum americanum* (also designated as *S. nodiflorum*), accessions of this taxon from other geographical areas, and American *S. americanum* using AFLP markers. 96 individuals representing 39 accessions of *S. americanum* sensu lato and related diploid species from the widest possible geographical range, and one accession of *S. dulcamara* (as outgroup) were used. The AFLP results suggested that American *S. americanum* differs from *S. nodiflorum* and that the material investigated in this study can be assigned to three different species: *S. americanum* sensu stricto, *S. nodiflorum* and a *Solanum* species from Brazil. These species can be differentiated based on a combination of floral and fruit characteristics.

Key words: Africa, AFLP, nomenclature, *Solanum americanum*, *Solanum nodiflorum*.

The importance of *Solanum* L. section *Solanum* species in Africa cannot be overestimated. Species in this section constitute one of the largest groups of leafy vegetables, and are an important source of income for "Mnafu" (the Swahili name for section *Solanum* species) growers in both rural and urban areas (Edmonds and Chweya 1997, Schippers 2000, Manoko and van der Weerden 2004). Furthermore, in Africa, where about 80% of people still live in rural areas, section *Solanum* species are used in traditional medicine, the sole source of primary health care in these areas. The section is one of the largest and most variable species groups of the genus with its greatest diversity in the New World tropics (Edmonds and Chweya 1997). There are diploid, tetraploid, and hexaploid species. The present study concentrates on the diploid species.

Solanum americanum Mill. and *S. nodiflorum* Jacq., which have shown to have both local and scientific importance, are considered by some authors as two separate species and by others as one species. The two taxa show much resemblance in their general morphology. Philip Miller in 1768 described *S. americanum* based on a specimen (*Miller s.n.*) cultivated at Chelsea Physic Garden, originally from Virginia, North America. Nicolaus Jacquin, based on type material from the African island of Mauritius (*Jacquin s.n.*), described *S. nodiflo-*

rum in 1789. The dispute about the identity of the two species emerged after Edmonds (1971) combined them into one species: *Solanum americanum*. One group of taxonomists agreed with Edmonds reduction (e.g. Gentry and Standley 1974; D'Arcy 1974a, b; Symon 1981, 1985; Howard 1989; D' Arcy and Rakotozafy 1994; Bosser et al. 2000), while others continued handling them as different species (Henderson 1974, Morton 1976, Heiser et al. 1979, Wiggins 1980). Even when they have been considered conspecific, different authors based on different morphological characteristics recognized different varieties. For example, based on hair characteristics Edmonds (1971) recognized two varieties under *S. americanum*: *S. americanum* var. *nodiflorum* (Jacq.) Edmonds and *S. americanum* var. *americanum* whereas D'Arcy (1974b), using pedicel position, distance between internodes and flower characteristics combined Edmonds varieties under *S. americanum* var. *americanum*, describing a new variety *S. americanum* var. *baylisii* D'Arcy. In Australia, Henderson, (1974), based on pedicel position at fruiting, presence or absence of stone cells, margins of adult leaves and number of fruits per peduncle recognized two subspecies under *S. nodiflorum*, i.e. *S. nodiflorum* subsp. *nodiflorum* and *S. nodiflorum* subsp. *nutans* H. J. Henderson. These observations indicate that the taxonomic situation of *S. americanum* and *S. nodiflorum* is still unclear and this fact may hamper the utilization of the available knowledge about the two taxa.

Our current study was therefore designed to address the taxonomic question whether or not material of *S. americanum* (also designated as *S. nodiflorum*) from Africa and other geographical areas was synonymous with material of *S. americanum* from America. *Solanum chenopodioides* and *S. physalifolium* were added to this study because, based on the list provided by Edmonds and Chweya (1997), they are the only other diploid species found in Africa. Furthermore, the former was what D'Arcy (1994b) considered to be *S. americanum* var. *baylisii*.

We generated AFLP markers from material of *S. americanum* and related diploid species collected from the widest possible geographical range of the target species. According to Becker et al. (1995), AFLPs are arbitrarily spread over the whole genome and co-migrating bands are predominantly homologous in closely related groups (Waugh et al. 1997, Rademaker et al. 2000). AFLP markers have previously been successful is resolving taxonomic problems and elucidating relationships among species in the genus *Solanum* (Kardolus et al. 1998; Mace et al. 1999a, b; Coulibaly et al. 2002; Jacoby et al. 2003; Dehmer and Hammer 2004; Olet 2004).

Materials and methods

Plant material. We acquired seeds of *Solanum* section *Solanum* accessions and grew them in the greenhouse. One individual per accession was taken to count chromosome numbers in the root tip cells following standard procedures. Results confirmed that accessions used in this study were all diploid. Identification of species followed Edmonds and Chweya (1997). A total of 96 individuals representing 39 accessions of *S. americanum*, related diploid species, and *Solanum dulcamara* L. (as outgroup) were used in this study. (Table 1). Except for 95160phy and A3455amer, 2 or 3 individuals represented each accession.

Collection of leaf materials and DNA isolation. 40 mg of leaf material from each of the 96 individuals was collected in a 1.5 ml Eppendorf tube and immersed in liquid nitrogen. Isolation of DNA followed the Promega genomic DNA purification kit procedure. After isolation, DNA was dissolved in 100 µl DNA hydration liquid and stored at −20°C. DNA concentration was measured using a spectrophotometer and the quality was checked by electrophoresis in a 1% agarose gel. 0.5 µg of DNA was used for AFLP analysis.

AFLP analysis. AFLP analysis followed a modified version of the protocol of Vos et al. (1995). *Eco*RI and *Mse*I restriction enzymes were used for digestion of genomic DNA. Pre-amplification was achieved using *Eco*RI + A and *Mse*I + C primers and products diluted 50 times in 10 mM Tris (pH8.0). Selective amplification was done using a D4 dye (Beckman Coulter) labeled *Eco*RI primer and an unlabeled MseI primer. Two primer combinations were used: *Eco*RI + AAC/*Mse*I +

Table 1. Accessions of diploid species and their country of origin

RU Accession No.	Accession code	Received as:	Identified as	Country of origin
904750026	90026amer	*S. photeinocarpum*	*S. americanum*	China
944750234	94234amer	*Nothocestrum latifolium*	*S. americanum*	USA (Hawaii)
954750186	95186amer	*S. americanum*	*S. americanum*	Brazil
954750354	95354amer	*S. americanum*	*S. americanum*	Mexico
954750356	95356amer	*S. americanum*	*S. americanum*	Venezuela
984750118	98118amer	*S. americanum*	*S. americanum*	Australia
994750056	99056amer	*S. nigrum*	*S. americanum*	India
A04750035	A0035amer	*Solanum* sp.	*S. americanum*	Tanzania
A14750028	A1028amer	*S. americanum?*	*S. americanum*	Uganda
A14750066	A1066amer	*S. nigrum*	*S. americanum*	Germany?
A14750092	A1092amer	*S. nigrum?*	*S. americanum*	Mexico
A14750099	A1099amer	*S. americanum*	*S. americanum*	Brazil
A14750130	A1130amer	*S. photeinocarpum*	*S. americanum*	China
A14750414	A1414amer	*S. retroflexum?*	*S. americanum*	Zimbabwe
A14750415	A1415amer	*S. retroflexum?*	*S. americanum*	Zimbabwe
A14750424	A1424amer	*S. nigrum*	*S. americanum*	Mauritius
A14750425	A1425amer	*S. nigrum*	*S. americanum*	Mauritius
A14750426	A1426amer	*S. nigrum*	*S. americanum*	Mauritius
A14750427	A1427amer	*S. nigrum*	*S. americanum*	Mauritius
A34750450	A3450amer	*S. americanum*	*S. americanum*	USA
A34750451	A3451amer	*S. americanum*	*S. americanum*	Cuba
A34750453	A3453amer	*S. americanum*	*S. americanum*	Cuba
A34750454	A3454amer	*S. americanum*	*S. americanum*	Cuba
A34750455	A3455amer	*S. americanum*	*S. americanum*	Cuba
A34750457	A3457amer	*S. americanum*	*S. americanum*	USA
A34750458	A3458amer	*S. americanum*	*S. americanum*	USA
A34750459	A3459amer	*S. americanum*	*S. americanum*	USA
A34750460	A3460amer	*S. americanum*	*S. americanum*	USA
A34750461	A3461amer	*S. americanum*	*S. americanum*	USA
A34750463	A3463amer	*S. americanum*	*S. americanum*	USA
A34750464	A3464amer	*S. americanum*	*S. americanum*	USA
884750042	88042chen	*S. chenopodioides*	*S. chenopodioides*	Switzerland
904750124	90124chen	*S. chenopodioides*	*S. chenopodioides*	Australia
914750076	91076chen	*S. chenopodioides*	*S. chenopodioides*	France?
944750185	94185chen	*S. sinaicum* subsp. *sublobatum*	*S. chenopodioides*	Romania?
954750185	95185chen	*S. ottonis*/*S. gracilius*	*S. chenopodioides*	Australia
964750073	96073phy	*S. sarrachoides*	*S. physalifolium*	Canada
954750160	95160phy	*S. nitidibaccatum*	*S. physalifolium*	France?
954750170	95170phy	*S. sarrachoides*	*S. physalifolium*	UK?
904750062	90062dulca	*S. depilatum*	*S. dulcamara*	Poland

Column 1: accession number of Radboud University Botanical and Experimental Garden seed collection; column 2: code used in this study, which was derived from the accession number and the first few letters of the specific name (column 4); column 3: name provided by the seed donor; column 4: name given after identification of the material; column 5: country of origin

CAC and *Eco*RI + ACC/*Mse*I + CAT. Selective amplification products were diluted 10 times in Sample Loading Solution (SLS, Beckman Coulter). Two microliters of this dilution were added to 33 µl of SLS buffer containing 0.2 µl of CEQ DNA size standard 600 (Beckman Coulter). Resulting fragments were analysed using Beckman Coulter 8000™ fragment analysis system with default values of study parameter with exception of size standard and model of study. In this study size standard 600 and cubic model were used.

Data analysis. The AFLP data from each primer combination separately and combined, were analysed using both phenetic and cladistic approaches, and NJ and MP trees were generated. During MP analysis, for each data set, two heuristic searches were performed and trees from the first heuristic search were used as starting trees in the second search. Afterwards, the tree topology from all methods was compared. Jackknife analyses (10,000 replicates) were run with both NJ and MP settings. All analyses were performed using PAUP version 4.0 b10 (Swofford 2001).

Morphological comparison. On the basis of our AFLP results, we compared the three clusters of *S. americanum* accessions for a number of morphological characteristics that Knapp (2001) considered important in identifying monophyletic groups and distinguishing species in the genus *Solanum*. To this end, we examined inflorescence, flower, and fruit characteristics of most of the accessions that were also used for AFLP analysis (Table 2).

Results

AFLP fragments. The *Eco*RI + AAC/*Mse*I + CAC primer combination produced 248 bands in total, of which 224 (90.3%) were polymorphic. The *Eco*RI + ACC/*Mse*I + CAT primer combination produced 225 bands, all being polymorphic. On average, the *Eco*RI + ACC/*Mse*I + CAT primer combination produced 40–50 bands per individual whereas *Eco*RI + AAC/ *Mse*I + CAC produced 30–40 bands per individual.

Clustering pattern and species recognition. Figure 1 shows the NJ tree based on all fragments generated by the two primer combinations. Five clearly distinct and well supported clusters were obtained that could be separated into two groups, A and B. Group A contained three relatively closely related clusters (I–III), all made up of individuals that were received under many different names and identified by us as *S. americanum* sensu lato. These were clearly separated from group B that consisted of two clusters (IV and V) of *S. chenopodioides* Lam. and *S. physalifolium* Rusby accessions, respectively, which were also previously identified as such. The three clusters I, II and III of *S. americanum* were separated from each other by substantial genetic distances comparable to that between *S. physalifolium* and *S. chenopodioides*. This suggested that individuals of group A were not all *S. americanum* as they were received or identified based on morphology.

Cluster I (NJ Jackknife support 100% in Fig. 1) is composed of accessions from different geographical areas, i.e. Africa, Australia, India, China Venezuela, Mexico, Cuba and Hawaii, but the AFLP-based subclusters that can be distinguished within cluster I do not reflect these geographical origins. There is also no clustering of accessions according to pedicel orientation (erect or deflexed), one of the characters used by Henderson (1974) to distinguish subspecies in *S. nodiflorum*. Individuals from cluster I conformed to Jacquin's illustration of the type specimen of *S. nodiflorum*, No. 326 in *Icones Plantarum Rariorum/Editae N. J. Jacquin* and also with *S. nodiflorum* subsp. *nodiflorum* sensu Henderson's (1974) plate 1. A number of individuals agreed in all respects with *Solanum nodiflorum* Jacq. subsp. *nutans* (type specimen *Henderson 518*), illustrated by plate 2 in Henderson (1974).

Cluster II contained solely accessions from the USA, which were also used by Dehmer (2001), and Dehmer and Hammer (2004). This cluster was 100% supported with NJ Jackknife value (Fig. 1). Individuals in this cluster compared with the type specimen of *S. americanum* at BM (*Miller s.n.*), illustrated by plate 3 in Henderson (1974). According to Henderson

Table 2. Morphological comparison of *S. nodiflorum*, Brazilian *Solanum* sp. and *S. americanum* sensu stricto

Characteristics	*S. nodiflorum*	*Solanum* sp. (Brazil)	*S. americanum*
Shape of calyx lobes	Lanceolate	Obovate	Ovate lanceolate
Calyx lobe fusion from the base	Lobes fused at the base	Often 2 or 3 lobes fused clearly above the base	Lobes fused at the base
Petal length (mm)	(4) 4.5–5 (6)	4–6	(7) 7.5–8 (9)
Petal width (mm)	(1.5) 1.9–2	(1.5) 2	2.5–3
Extent of corolla fusion from the base (mm)	(0.5) 1–1.5	0–0.5	Up to 2
Style length (mm)	(1.5) 2–2.5 (3)	(2) 3	4.3–5
Style exsertion beyond anthers	Equal or below the anthers, if exserted only up to 0.5 mm	Rarely exserted, if exserted 0.5–1.5 mm.	Clearly exserted up to 2.5–3 mm
Fruiting pedicel orientation	Deflexed or erect	Erect and spreading	Erect and spreading
Inflorescence type	Umbellate cyme or nearly so	Umbellate cyme	Extended umbellate cyme
Fruit colour	Shiny black	Shiny black	Dull black

Note: Figures in parentheses refer to infrequent values below or above the regular range or value that was recorded

(1974) and Heiser et al. (1979) this is the taxon that accurately fits the protologue of *S. americanum* Mill.

Cluster III (100% Jackknife support in the NJ tree) was exclusively composed of the Brazilian *Solanum* sp., for which no comparable type specimen was found.

Phylogenetic analyses. AFLP data generated by the two primer combinations gave similar MP trees in all heuristic searches performed (results not shown). Five clades representing the species recognized above (Fig. 1) where produced. Each clade was supported by MP consensus and Jackknife values higher than 90% except clade I representing *S. nodiflorum* that had a NJ Jackknife support of only 73%. Sub-clades within *S. nodiflorum*, corresponding in part to the subclusters within cluster I in Fig. 1, were supported with consensus value of 97–100% but they were not supported with Jackknife values. At the species level, the MP tree topology was identical to the topology observed in the NJ tree.

Discussion

Species delimitation. The difficulty of distinguishing genetically controlled characteristics from phenotypic plasticity has long been known to impede species level taxonomy in section *Solanum* (Edmonds and Chweya 1997). Confusion has also emerged from having to use mostly herbarium material that often lacks the necessary diagnostic characteristics to make an objective judgement (Heiser et al. 1979). The present study shows that although material received or identified as *S. americanum* shows a general morphological resemblance, this taxon should be split into three genetically different species, namely (1) *S. nodiflorum* (cluster I) that is a widely distributed species, (2) *S. americanum* sensu stricto represented by central American material in cluster II, and (3) a different species represented by the Brazilian accessions grouping in cluster III. This conclusion is not only supported by the phenetic results as depicted in Fig. 1, but each of the three species is an

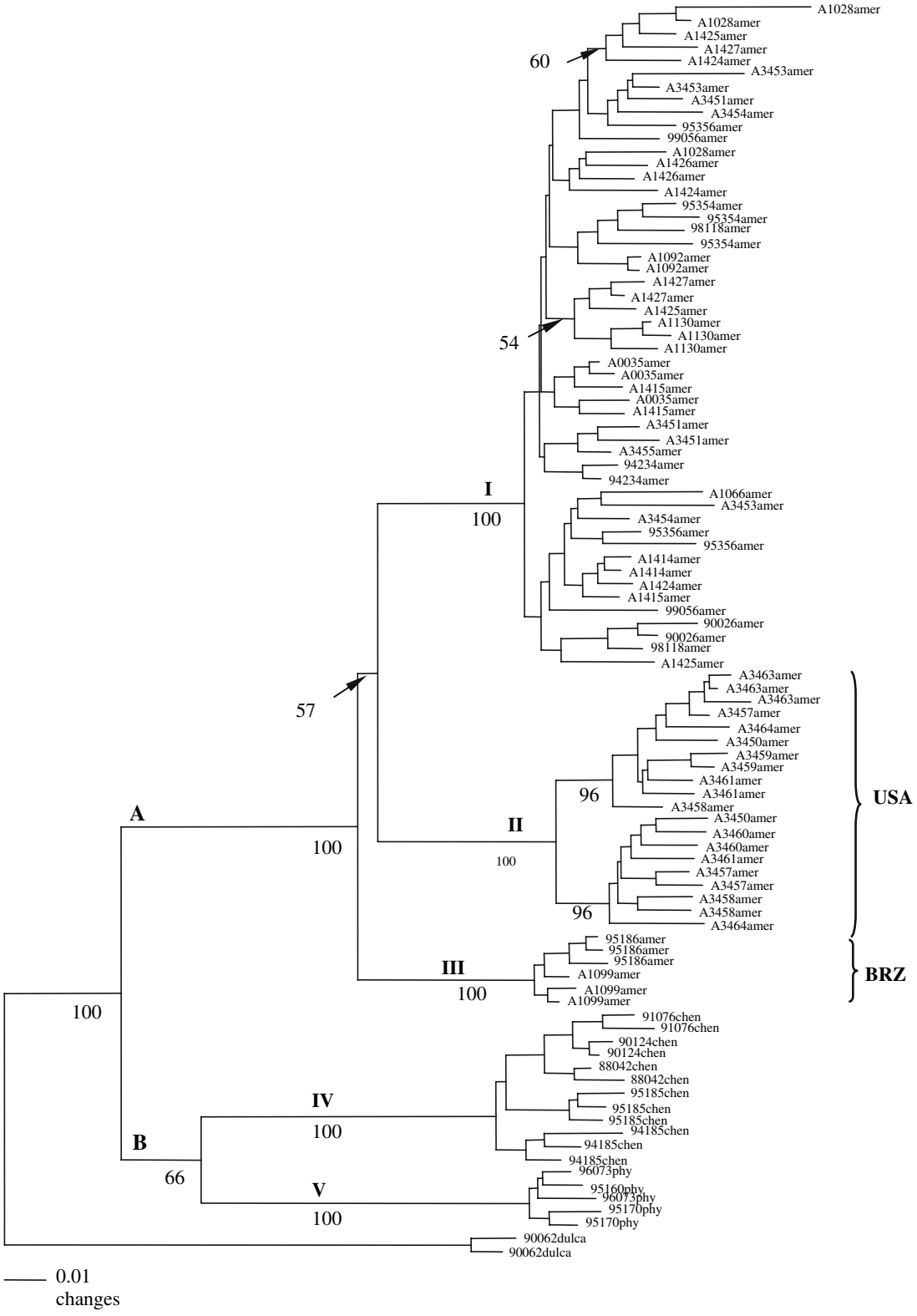

Fig. 1. NJ phenetic phylogram based on 435 polymorphic AFLP markers generated by the *Eco*RI + AAC/*Mse*I + CAC and *Eco*RI + ACC/*Mse*I + CAT primer combinations from 96 OTUs. Numbers below branches are NJ Jackknife support values

independent lineage supported with consensus support values and Jackknife values in the MP tree (not shown).

Concerning the species status of *S. nodiflorum* and *S. americanum* sensu stricto therefore, this study does not support Edmonds (1971) and Edmonds (1972) combination of these taxa but rather agrees with previous numerical taxonomic studies which concluded that the two taxa were different species (Soria and Heiser 1961, Heiser et al. 1965, Heiser et al. 1979). Dehmer (2001) and Dehmer and Hammer (2004) showed that the Cuban and the USA accessions (the same ones as shown in Fig. 1) had a considerable genetic distance. Still, these authors placed the two groups under the same species: *S. americanum* and attributed the differences to geographical provenance. Our results disagree with this conclusion, indicating that these two groups fall into two different species, one of which (including the Cuban accessions) being a world wide species. The accessions that constitute *S. nodiflorum* occur in cluster I regardless of their geographical origin. Equally, our results do not support the placement of Ugandan material with Brazilian accession 95186amer under *S. americanum* as was done by Olet (2004).

The split between *S. nodiflorum* and *S. americanum* sensu stricto is also supported by previous studies on crossing behavior, often used to discriminate different biological species. In these studies it was found that the hybrids resulting from crossing the two species were abnormal, weak, with malformed abortive flowers and difficult to keep alive (Baylis 1958, Gray 1968, Henderson 1974, Heiser et al. 1979). We have also observed that *S. nodiflorum* starts to flower much earlier than *S. americanum* and that the former set fruits without any problem in the greenhouse, but not the latter.

Although these species show general morphological resemblance, it was observed during the present study that they could still be separated based on a combination of inflorescence and flower characteristics, especially the style exsertion (Table 2 and Fig. 2). The comparison of floral size between *S. americanum* sensu stricto on one end, and *S. nodiflorum* and the Brazilian *Solanum* sp. on the other, seems to indicate that *S. americanum* sensu stricto is superficially an enlarged version of the latter species. A similar pattern has been observed between *S. sarrachoides* Sendtn. and *S. tweedianum* Hook. where the latter is superficially an enlarged version of the former (Edmonds 1986). In both cases, individuals of these species have other characteristics in common such as pedicel posture, berry colour and shape, pubescence type, leaf shape, and other vegetative characteristics.

On the other hand, characteristics that have been used in some studies to differentiate *S. americanum* from *S. nodiflorum*, e.g. presence and absences of stone cells (Morton 1976, Heiser et al. 1979) or angle of pedicel inclination used by Morton (1976), showed no pattern in the present study when plotted on Fig. 1. Accessions with one or both of these characteristics were found in both *S. nodiflorum*, in *S. americanum* sensu stricto and the Brazilian *Solanum* sp. accessions. In Australian materials Henderson (1974), found stone cells in *S. nodiflorum* subsp. *nutans* but not in subsp. *nodiflorum*. Similarly, Olet (2004) recorded stone cells in one of the two forms of *S. nodiflorum* (there called *S. americanum*) in Uganda. In *S. nodiflorum* stone cells have hardly been a useful characteristic except for materials from North America (Heiser et al. 1979). Unpredictability of stone cells has also been demonstrated by Edmonds (1986) in *S. sarrachoides* and *S. physalifolium* Rusby. var. *nitidibaccatum* (Bitter) Edmonds.

It seems likely that this group of species has its origin in South America, the centre of genetic diversity of section *Solanum* species according to Edmonds and Chweya (1997). From here they spread to the USA probably through long distance dispersal of the seeds. The resulting widened range may have resulted into founder populations in the USA which through rapid speciation resulted into

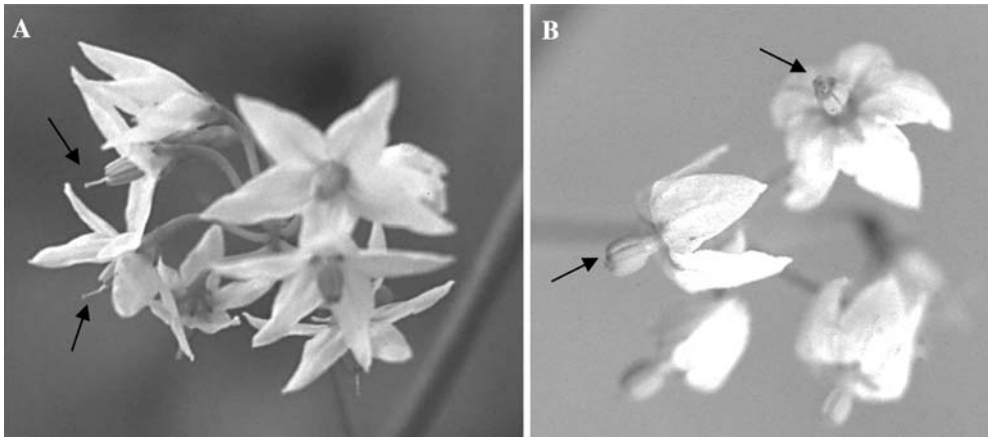

Fig. 2. Floral characteristics of *S. americanum* Mill. (**A**) and *S. nodiflorum* Jacq. (**B**). Arrows indicate the presence (**A**) and absence (**B**) of exserted styles

S. americanum sensu stricto, with large genetic differences – as shown by the NJ distances in Fig. 1 – not accompanied by large morphological differences. The wider distribution of *S. nodiflorum* can be explained based on the results of crossing studies by Soria and Heiser (1961) and Henderson (1974) that suggest that *S. nodiflorum* is an autogamous species, a mode of reproduction associated with colonisation ability, local adaptation and reproductive economy (Jain 1976). The fact that the three species still show general morphological resemblance suggests that the split is probably recent or that the new habitats the new taxa have evolved in did not impose selection pressure enough to bring about large physiological and morphological differences.

Infraspecific taxa. Earlier authors (Edmonds 1971, 1972; D'Arcy 1974b) recognized varieties within *S. americanum* sensu lato, but none of these are evident in our study. Actually, D'Arcy's *S. americanum* var. *baylisii* is, according to Edmonds and Chweya (1997), synonymous to *S. chenopodioides*, a different diploid species that constitutes cluster IV in the present study. Henderson (1974) recognized two subspecies in *S. nodiflorum* i.e. subsp. *nutans* and subsp. *nodiflorum*, but these cannot be recognized in the present results. The accessions that correspond to Henderson's subspecies *nodiflorum* are scattered in Fig. 1, and the subspecies is paraphyletic in the MP tree (not shown). Therefore, although there could be infraspecific structure within *S. nodiflorum*, based on the present study those subgroups cannot confidently be equated with Henderson's subspecies. Olet (2004) divided Ugandan materials into two morphological forms; A and B that were similar to *S. nodiflorum* subsp. *nutans* and *S. nodiflorum* subsp. *nodiflorum*, respectively, but her division was also not supported with AFLP data.

Nomenclatural considerations. Nomenclature changes, including the synonymy related to *S. americanum*, *S. nodiflorum* and the Brazilian *Solanum* sp. are beyond the scope of this study. However, the present study does have nomenclature implications and it recommends the use of the name *S. americanum* sensu stricto as used earlier (Soria and Heiser 1961, Heiser et al. 1965, Gray 1968, Henderson 1974, Heiser et al. 1979). This proposition is in conflict with Schilling (1981) who proposed to use the name *S. ptycanthum* Dunal in place of *S. americanum*. Actually, the latter name is also used for another diploid species commonly known as "eastern black nightshade", e.g. Bassett and Munro (1995), which is different from *S. nodiflorum* and *S. americanum*.

It is not possible to draw any conclusion about the nomenclature of the Brazilian *Solanum* sp. It is, however, known that Bitter

(1912) described two taxa in Brazil that were close relatives of *S. nodiflorum*, namely *S. tenellum* and *S. sciaphilum*. While revising section *Solanum* in South America, Gray (1968) synonymised the two species under *S. nodiflorum*. Although no comparable type specimen was seen, it is possible that the Brazilian *Solanum* sp. in our study corresponds to one of these taxa, most likely *S. tenellum* which according to Bitter (1912) had the smallest flower in the whole genus.

On the other hand, our study has revealed the misapplication of some names. For example *S. nigrum* L., a name referring to a hexaploid species, is still being used for diploid *S. nodiflorum* in Mauritius, Mexico and India (Table 1). In Zimbabwe the name *S. retroflexum* (a tetraploid species) is also used for *S. nodiflorum*. We assigned accessions received as *S. photeinocarpum* Nakam. & Odash. from China (Table 1) to *S. nodiflorum*. Both Gray (1968) and Henderson (1974) considered the former to be a synonym of the latter. Accession 95160phy was received as *S. nitidibaccatum* but it proved to be *S. physalifolium*. Edmonds (1986) reduced *S. nitidibaccatum* Bitt. to a synonym of *S. physalifolium*. Accession 95185chen received as *S. ottonis* Hylander /*S. gracilius* Hert. was certainly *S. chenopodioides*. Edmonds and Chweya (1997) considered both these names to be synonyms of *S. chenopodioides*. *Solanum nodiflorum* accession 94234amer from Hawaii was received as *Nothocestrum latifolium*. This is probably a labelling mistake, as this material certainly does not belong to this different genus.

At this point the following conclusions can be drawn: Cluster I represents a taxon that is distributed worldwide and should be known as *S. nodiflorum*. This is a tropical/subtropical taxon extending from the eastern coast of Africa, spreading over the Indian subcontinent and China to Australia and New Zealand. From the west coast of Africa *S. nodiflorum* extends westward into South America, the Caribbean Islands and Hawaii. In America this taxon extends from Georgia in the Southeast to California in the Southwest (Schilling 1981). *Solanum americanum* sensu stricto from the USA is clearly different from *S. nodiflorum*. There is no support in the AFLP data for the existence of subspecies within *S. nodiflorum*.

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