

A RAPD study of the *Sarcostemma* group of *Cynanchum* (Apocynaceae-Asclepiadoideae-Asclepiadeae)

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Abstract One hundred and thirty-seven accessions of *Cynanchum viminale* and its relatives, formerly known as *Sarcostemma*, were studied using randomly amplified polymorphic DNA (RAPD). A fingerprinting technique was used because sequencing had failed to differentiate between morphologically separable groups. Chromosome counts were conducted to establish the ploidy level of the accessions. The banding patterns resulting from RAPD analysis were evaluated with Canonical Analysis of Principal Coordinates, Permanova and neighbour-joining. A strong geographic component was found in the structure of the group. Taxa considered species or subspecies based on morphology often formed coherent groups. The data are interpreted to reflect at least two cycles of diversification: the first one from Madagascar and the second one most likely from the East African–Arabian region, reaching Madagascar again. In mainland Africa, polyploidisation has occurred several times.

Keywords CAP · Madagascar · Old World · Neighbour-joining · Permanova · Chromosome numbers · Ploidy level · Species concept

Introduction

Leafless, succulent *Cynanchum* L. species were long considered a particularity of Madagascan flora. A molecular

phylogeny of *Cynanchum*, based on cpDNA and nuclear ITS (Internal Transcribed Spacer) data (Liede and Kunze 2002) showed, however, that all leafless, stem-succulent taxa form a well-supported clade nested inside leafy, non-succulent Madagascan *Cynanchum* species. This clade comprises not only the single leafless succulent *Cynanchum* of the African mainland, *C. gerrardii* (Harv.) Liede, but also species of the so far separate genus *Sarcostemma*. *Sarcostemma*, founded by R. Brown (1811) for species with a ‘double’ corona of an outer ring and inner staminal corona parts [$C_{(is)} + C_s$, according to Liede and Kunze (1993)], as opposed to the ‘simple’ ring-shaped corona of typical *Cynanchum* [$C_{(is)}$, according to Liede and Kunze (1993)] had been considered one of the notoriously difficult groups in Asclepiadeae, displaying highly variable vegetative and floral characters (Liede and Adams 1991; Fig. 1), as well as morphologically indistinguishable di- and polyploid populations (in *C. viminale* subsp. *odontolepis* and, exceptionally, in *C. viminale* subsp. *brunonianum* and subsp. *suberosum*; cf. Table 1).

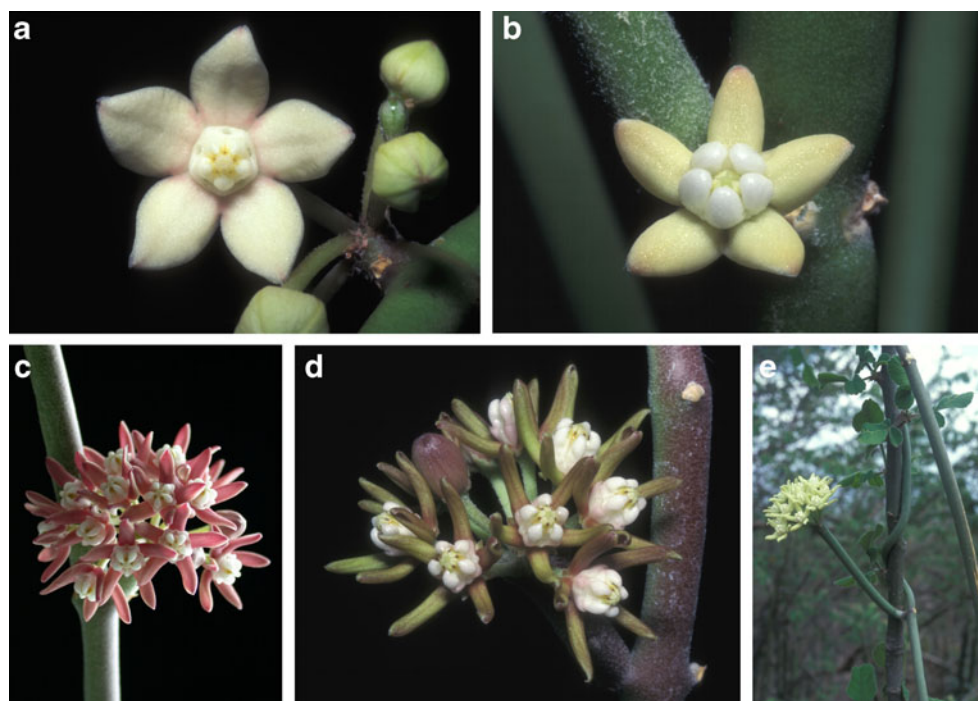
Holm (1950) had further complicated the genus concept when he included into *Sarcostemma* a wide range of leafy, non-succulent genera characterised by a ‘double’ corona. This concept was shown to be highly artificial by one of the early molecular studies in Asclepiadoideae (Liede and Täuber 2000), which showed that *Oxystelma* R. Br., *Funastrum* E. Fourn., *Pentacyphus* Schltr. and *Philibertia* Kunth are not monophyletic with core *Sarcostemma*, but even belong to different subtribes of Asclepiadeae.

The position of *Sarcostemma* inside the succulent Malagasy *Cynanchum* clade (hereafter referred to as the ‘*C. viminale* group’) made it clear that the group had originated in Madagascar (Meve and Liede 2002). However, it is not clear whether one or several colonization events have led to the large area this group inhabits outside Madagascar, ranging over frost-free, semi-arid and coastal habitats from South Africa to

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Fig. 1 Examples from the *Sarcostemma* subgroup of *Cynanchum* (a–e). (a) *Cynanchum antsiranense* (Madagascar, Lavranos 28772). (b) *Cynanchum arabicum* (Yemen, Radcliffe-Smith 4624). (c) *Cynanchum forskaolium* (Yemen, Noltee 864). (d) *Cynanchum stoloniferum* (Tanzania, Noltee 199). (e) *Cynanchum viminalis* subsp. *viminalis* in habitat (Tanzania, Mkomasi; Liede & Meve 3389). Photos by U. Meve



Australia. It is also not yet clear whether the areas of highest morphological diversity in the *C. viminalis* group outside Madagascar (north-east Africa and Arabia) are the places of original colonization with subsequent radiation or whether radiation has occurred more rapidly in these areas than elsewhere, possibly because of a higher variability of available habitats. Finally, the possibility that Madagascar has been recolonized by lineages from the mainland cannot be ruled out. Therefore, original lineages and re-immigrants may now live side by side.

These interesting questions have spurred several attempts to get a molecular handle on the *C. viminalis* group (core *Sarcostemma*, as defined above). A wide range of molecular markers commonly used in species-level studies has been tested (*trnT*-L, *trnL*-F, *rpl16-trnL*, 3'*rps16*-5'*trnK*^(UUU) and *trnD*^{GUC}-*trnT*^{GGU} intergenic spacers; *trnK* and *trnL* intron, *accD*, *rpl16*, *rpoB*, *rpoC*; ITS), but none yielded any differences between morphologically distinguishable groups (Liede-Schumann, unpublished data). This indicates a putatively very recent and rapid radiation that is probably still underway. Nevertheless, the need for taxonomic identification, for example for various flora projects, requires a method of distinguishing taxa independently of the availability of a phylogeny. Therefore, the traditional morphological and karyological concept of delimiting taxa within the *C. viminalis* group has been consistently applied, defining groups with floral differences as species and vegetatively diverging ones as subspecies of *C. viminalis* (cf. Liede and Meve 1989, 1992, 1993, 1995, 2001; Meve and Liede 1996, 1997).

In addition to sequencing attempts, several fingerprinting methods were taken into consideration using the large living

collection accumulated over the years in the greenhouse of Bayreuth University. An isozyme study was attempted first, but no usable data could be gathered—most likely because the latex disturbed the reactions (interpretable data were obtained for taxa with little latex, such as *Ceropegia* L.; Liede-Schumann, unpublished data). Therefore, a randomly amplified polymorphic DNA (RAPD) analysis was carried out for 137 different accessions as a method of creating a large number of characters for a rather large dataset with little genetic variation at low cost.

RAPD has been used in Asclepiadoideae several times with different goals. Genetic diversification was studied in *Tylophora indica* (Burm.) Merrill, a rare and possibly endangered Indian species (Parani and Parida 1997), and in *Gymnema sylvestre* R. Br., a species with some economic potential (Nair and Keshavachandran 2006). With the aim of solving a taxonomic problem, Meve et al. (2001) differentiated between two morphologically recognisable subspecies of *Ceropegia aristolochioides* Decne., showing that at this taxonomic level a RAPD study may lend additional support to a hypothesis derived from morphology. Because Mahmood et al. (2010) tried to use RAPDs to distinguish between rather distantly related taxa (*Caralluma tuberculata* N.E.Br. and *Caralluma edulis* (Edgew.) Hook.) they were unable to differentiate between the two species. These two species, although treated as *Caralluma* for a long time, are not closely related and belong to two different genera as *Caudanthera edulis* (Edgew.) Meve & Liede and *Apteranthes tuberculata* (N.E.Br.) Meve & Liede (Meve and Liede 2004).

Table 1 Taxa, voucher information and abbreviations used in this study. Information on chromosome numbers and failed markers is included where applicable

Taxon	Voucher	Locality information	Abbreviation	Chromosome number 2n=	Chromosome number information	Markers failed	Dataset in which sample is used
<i>C. antisranense</i> (Meve & Liede)	Lavranos et al. 28772 (UBT)	Madagascar: Antsiranana	Mada;antsira;28772	22	Meve and Liede (1997)		1,2
<i>C. antisranense</i> (Meve & Liede)	Mangelsdorff 47 (UBT)	Madagascar: Antsiranana	Mada;antsira;RMM47	22	Albers and Meve (2001)		1,2
<i>C. antisranense</i> (Meve & Liede)	Supthut 96653 (=ZSS 96-2248c) (ex hort. UBT, ZSS)	Madagascar: Mahajanga	Mada;antsira;96653	22	New record		1,2
<i>C. arabicum</i> (Bruyns & P.I.Forst.)	Radcliffe-Smith & Henchie 4624 (UBT)	Yemen: North	Arab;arabicum;RS4624	22	Albers and Meve (2001)		1,2
<i>C. arabicum</i> (Bruyns & P.I.Forst.)	Radcliffe-Smith 4592 (UBT)	Yemen: North	Arab;arabicum;RS4592	22	New record		1,2
<i>C. arabicum</i> (Bruyns & P.I.Forst.)	Ricanek & Hanacek 211 (in cult. UBT)	Yemen: North	Arab;arabicum;RH211	–	–	P212	Excl.
<i>C. brevipedicellatum</i> (P. I. Forst.)	Carter s.n. sub Forster 2390 (UBT)	Australia: Queensland	Aust;breviped;PIF2390	22	Albers and Meve (2001)		1,2
<i>C. brevipedicellatum</i> (P. I. Forst.)	Muir s.n. sub Forster 1653 (UBT)	Australia: Queensland	Aust;breviped;PIF1653	22	Albers and Meve (2001)		1,2
<i>C. daltonii</i> (Decne. ex Webb)	Kilian sub BG Berlin 116-24-86-10 (B, UBT)	Cape Verde Islands	Cver;daltonii;116_24_86_10	44	New record		1,2
<i>C. forskalianum</i> (Schult.) Meve & Liede	Noltee 864 (UBT)	Yemen: North	Arab;forsk;FN864	22	Albers and Meve (2001)		1,2
<i>C. forskalianum</i> (Schult.) Meve & Liede	Noltee 964 (UBT)	Yemen: North	Arab;forsk;FN964	22	Meve and Liede (1996)		1,2
<i>C. membranaceum</i> (Liede & Meve)	Liede & Conrad 2765 (ULM, TAN)	Madagascar: Tuléar	Mada;membran;LC2765	22	Liede and Meve (1995)		1,2
<i>C. mulanjense</i> (Liede & Meve)	Liede 2899 (in cult. UBT)	Malawi: Southern region	E Afr;mulanjense;L2899	–	–		1,2
<i>C. mulanjense</i> (Liede & Meve)	Liede 2900 (UBT)	Malawi: Southern region	E Afr;mulanjense;L2900	22	Liede and Meve (1992)		1,2
<i>C. mulanjense</i> (Liede & Meve)	Liede 2902 (UBT)	Malawi: Southern region	E Afr;mulanjense;L2902	22	New record		1,2
<i>C. pearsonianum</i> Liede & Meve	Liede & Hammer 2523 (UBT)	South Africa: Northern Cape	SAfr;pearsonianum;L2523	22	Albers and Meve (2001)		1,2
<i>C. pearsonianum</i> Liede & Meve	Liede & Meve 582 (K, MSUN)	South Africa: Northern Cape	SAfr;pearsonianum;LM582	22	Liede and Meve (1989)		1,2
<i>C. resiliens</i> (B.R.Adams & R.W.K.Holland) Goyder	Albers et al. 503 (in cult. UBT)	Zimbabwe: Mashonaland East	E Afr;resiliens;ALM503	–	–		1,2
<i>C. resiliens</i> (B.R.Adams & R.W.K.Holland) Goyder	Albers et al. 515 (UBT)	Zimbabwe: Manicaland	E Afr;resiliens;ALM515	22	Albers and Meve (2001)		1,2
<i>C. resiliens</i> (B.R.Adams & R.W.K.Holland) Goyder	Lavranos s.n. sub JHB 16 (in cult. UBT)	Zimbabwe: Manicaland	E Afr;resiliens;JHB16	22	New record		1,2
<i>C. sarcomedum</i> Meve & Liede	Ricanek & Hanacek 084 (in cult. UBT)	India: Tamil Nadu	Indi;sarcomed;RH084	22	New record		1,2
<i>C. sarcomedum</i> Meve & Liede	Ricanek & Hanacek 085 (in cult. UBT)	India: Tamil Nadu	Indi;sarcomed;RH085	22	New record		1,2
<i>C. socotranum</i> (Lavranos) Meve & Liede	Smith & Lavranos 309 (UBT)	Yemen: Socotra	Arab;socotranum;SM309	22	Meve and Liede (1996)		1,2
<i>C. stoloniferum</i> (B.R.Adams & R.W.K.Holland) Goyder	Brandham 2406 (= Kew 437-77-3866) (UBT)	Kenya: Rift Valley	E Afr;stolo;Brand2406	–	–		1,2

Table 1 (continued)

Taxon	Voucher	Locality information	Abbreviation	Chromosome number 2n=	Chromosome number information	Markers failed	Dataset in which sample is used
<i>C. stoloniferum</i> (B.R.Adams & R.W.K.Holland) Goyder	Field & Powys 214 (UBT)	Kenya: Coast	EAfr;stolo;FP214	44	New record		1,2
<i>C. stoloniferum</i> (B.R.Adams & R.W.K.Holland) Goyder	Kew 443-77-4049 (=Field s.n.) (in cult. UBT)	Kenya: Coast	EAfr;stolo;443_77_4049	–	–		1,2
<i>C. stoloniferum</i> (B.R.Adams & R.W.K.Holland) Goyder	Kew 443-77-6779 (=Field & Powys 214) (UBT)	Kenya: Coast	EAfr;stolo;443_77_6779	44	New record		1,2
<i>C. stoloniferum</i> (B.R.Adams & R.W.K.Holland) Goyder	Liede & Newton 3174 (MSUN, ULM, UBT)	Kenya: Rift Valley	EAfr;stolo;LN3174	44	Albers and Meve (2001)		1,2
<i>C. stoloniferum</i> (B.R.Adams & R.W.K.Holland) Goyder	Liede & Newton 3220 (in cult. UBT)	Kenya: Rift Valley	EAfr;stolo;LN3220	44	New record		1,2
<i>C. stoloniferum</i> (B.R.Adams & R.W.K.Holland) Goyder	Noltee 199 (UBT)	Tanzania: Arusha	EAfr;stolo;FN199	44	Liede and Meve (1993)		1,2
<i>C. vanlessenii</i> (Lavrano) Goyder	Noltee 870 (MSUN, UBT)	Yemen: Northern	Arab;vanlessenii;FN870	22	Meve and Liede (1996)		1,2
<i>C. vanlessenii</i> (Lavrano) Goyder	Gilbert & Vollesen s.n. (UBT)	Ethiopia: Arussi	EAfr;vanlessenii;Gilbert	22	Meve and Liede (1996)		1,2
<i>C. vanlessenii</i> (Lavrano) Goyder	ex Saxena s.n. (in cult. UBT)	India	Indi;vanlessenii;Sax	–	–		1,2
<i>C. vanlessenii</i> (Lavrano) Goyder	Saxena sub 20 (UBT)	India: Tamil Nadu	Indi;vanlessenii;Sax20	22	New record	P05	2
<i>C. viminalis</i> (L.) L. (subsp. indet.)	Mangelsdorff Y21 (in cult. UBT)	Yemen: North	Arab;vim;RMY21	22	New record		1,2
<i>C. viminalis</i> (L.) L. (subsp. indet.)	Foresti 1 (in cult. UBT)	Kenya: Coast	EAfr;vim;Foresti	22	New record		1,2
<i>C. viminalis</i> (L.) L. (subsp. indet.)	Liede 2880 (in cult. UBT)	Malawi: Southern region	EAfr;vim;L2880	44	New record		1,2
<i>C. viminalis</i> (L.) L. (subsp. indet.)	Hanacek & Rikanek 083 (in cult. UBT)	India: Tamil Nadu	Indi;vim;HR083	–	–	P106	Excl.
<i>C. viminalis</i> (L.) L. (subsp. indet.)	Liede & Conrad 2701 (in cult. UBT)	Madagascar: Tuléar	Mada;vim;LC2701	22	Albers and Meve (2001)	P09 P106 P209	Excl.
<i>C. viminalis</i> (L.) L. (subsp. indet.)	Albers et al. 3577 (in cult. UBT)	South Africa: Eastern Cape	SAfr;vim;Alb3577	22	New record		1,2
<i>C. viminalis</i> (L.) L. (subsp. indet.)	Cumming 1803 (UBT)	South Africa: Mpumalanga	SAfr;vim;Cum1803	22	New record	P05 P09 P209	Excl.
<i>C. viminalis</i> (L.) L. (subsp. indet.)	Cumming 2194 (in cult. UBT)	South Africa: Western Cape	SAfr;vim;Cum2194	–	–		1,2
<i>C. viminalis</i> (L.) L. (subsp. indet.)	Cumming 2383 (in cult. UBT)	South Africa: Eastern Cape	SAfr;vim;Cum2383	44	New record		1,2
<i>C. viminalis</i> (L.) L. (subsp. indet.)	Cumming 2495 (in cult. UBT)	South Africa: Northern Cape	SAfr;vim;Cum2495	22	New record		1,2
<i>C. viminalis</i> (L.) L. (subsp. indet.)	Cumming 2507 (in cult. UBT)	South Africa: Eastern Cape	SAfr;vim;Cum2507	22	New record		1,2
<i>C. viminalis</i> (L.) L. (subsp. indet.)	Cumming 2856 (in cult. UBT)	South Africa: Eastern Cape	SAfr;vim;Cum2856	22	New record		1,2
<i>C. viminalis</i> (L.) L. (subsp. indet.)	Cumming 2882 (in cult. UBT)	South Africa: Western Cape	SAfr;vim;Cum2882	44	New record		1,2
<i>C. viminalis</i> (L.) L. (subsp. indet.)	Cumming 2910 (UBT)	South Africa: Eastern Cape	SAfr;vim;Cum2910	44	New record		1,2
<i>C. viminalis</i> (L.) L. (subsp. indet.)	Cumming 3158 (in cult. UBT)	South Africa: Northern Cape	SAfr;vim;Cum3158	22	New record		1,2
<i>C. viminalis</i> (L.) L. (subsp. indet.)	Liede & Meve 598 (in cult. UBT)	Namibia: Gobabis	SAfr;vim;LM598	–	–		1,2

Table 1 (continued)

Taxon	Voucher	Locality information	Abbreviation	Chromosome number 2n=	Chromosome number information	Markers failed	Dataset in which sample is used
<i>C. viminale</i> (L.) L. (subsp. indet.)	McMurtey & Bleck s.n. sub JHB 10 (in cult. UBT)	Botswana: Gaborone	SAfr;vim;JHB10	22	New record	P04	2
<i>C. viminale</i> (L.) L. (subsp. indet.)	Van Jaarsveld 7915 (UBT)	South Africa: Eastern Cape	SAfr;vim;Jaar7915	22	New record		1,2
<i>C. viminale</i> (L.) L. (subsp. indet.)	Ehrich s.n. sub BG Berlin 285-08-93-10 (UBT)	Mali	WAfr;vim;285_08_93_10	44	New record	P04 P05	2
<i>C. viminale</i> (L.) L. subsp. <i>australe</i> (P. I. Forst.) Meve & Liede	Ballingall 1868 (UBT)	Australia: Western Australia	Aust;vim_aust;Ball1868	22	New record		1,2
<i>C. viminale</i> (L.) L. subsp. <i>australe</i> (P. I. Forst.) Meve & Liede	Kenneally 9410b (in cult. UBT)	Australia: Western Australia	Aust;vim_aust;Ken9410b	–	–		1,2
<i>C. viminale</i> (L.) L. subsp. <i>australe</i> (P. I. Forst.) Meve & Liede	Kenneally 9478 (UBT)	Australia: Western Australia	Aust;vim_aust;Ken9478	22	New record		1,2
<i>C. viminale</i> (L.) L. subsp. <i>australe</i> (P. I. Forst.) Meve & Liede	Olsen s.n. sub Forster 2887 (in cult. UBT)	Australia: Queensland	Aust;vim_aust;Olsen2887	22	New record		1,2
<i>C. viminale</i> (L.) L. subsp. <i>australe</i> (P. I. Forst.) Meve & Liede	Symon 13210 (in cult. UBT)	Australia: South Australia	Aust;vim_aust;Symon13210	–	–		1,2
<i>C. viminale</i> (L.) L. subsp. <i>brunonianum</i> (Wight & Arn.) Meve & Liede	Wood 3431 (UBT)	Yemen: North	Arab;vim_bruno;Wood3431	22	Albers and Meve (2001)		1,2
<i>C. viminale</i> (L.) L. subsp. <i>brunonianum</i> (Wight & Arn.) Meve & Liede	Labhart s.n. (ZSS)	Yemen: North	Arab;vim_bruno;Labhart	–	–		1,2
<i>C. viminale</i> (L.) L. subsp. <i>brunonianum</i> (Wight & Arn.) Meve & Liede	Barthlott & Porembski 13676 (BG Bonn)	Seychelles	Asia;vim_bruno;13676	22	New record		1,2
<i>C. viminale</i> (L.) L. subsp. <i>brunonianum</i> (Wight & Arn.) Meve & Liede	Bonn s.n. (UBT)	Thailand: Ayuthaya	Asia;vim_bruno;Bonn	22	New record		1,2
<i>C. viminale</i> (L.) L. subsp. <i>brunonianum</i> (Wight & Arn.) Meve & Liede	Liede 3272 (UBT)	Philippines: Mindoro	Asia;vim_bruno;L3272	22	New record	P204	Excl.
<i>C. viminale</i> (L.) L. subsp. <i>brunonianum</i> (Wight & Arn.) Meve & Liede	Liede 3299 (UBT)	Philippines: Oriental Zamboanga	Asia;vim_bruno;L3299	22	New record		1,2
<i>C. viminale</i> (L.) L. subsp. <i>brunonianum</i> (Wight & Arn.) Meve & Liede	Thorut s.n. (UBT)	Thailand: Chaiyaphum	Asia;vim_bruno;Thorut	22	New record		1,2
<i>C. viminale</i> (L.) L. subsp. <i>brunonianum</i> (Wight & Arn.) Meve & Liede	Bolton 700 (in cult. UBT)	Australia: Queensland	Aust;vim_bruno;Bol700	22	Albers and Meve (2001)		1,2
<i>C. viminale</i> (L.) L. subsp. <i>brunonianum</i> (Wight & Arn.) Meve & Liede	Cribb s.n. sub Forster 2170 (ex hort. P.I. Forster)	Australia: Queensland	Aust;vim_bruno;PIF2170	22	New record	P106	Excl.
<i>C. viminale</i> (L.) L. subsp. <i>brunonianum</i> (Wight & Arn.) Meve & Liede	Forster 1644 (in cult. UBT)	Australia: Queensland	Aust;vim_bruno;PIF1644	22	New record		1,2
<i>C. viminale</i> (L.) L. subsp. <i>brunonianum</i> (Wight & Arn.) Meve & Liede	Wetz s.n. sub BG Kopenhagen 19731 (= BG Hamburg 3251/88) (C, UBT)	Zaire	EAfr;vim_bruno;19731	44	New record	P05	2

Table 1 (continued)

Taxon	Voucher	Locality information	Abbreviation	Chromosome number 2n=	Chromosome number information	Markers failed	Dataset in which sample is used
<i>C. viminalis</i> (L.) L. subsp. <i>brunonianum</i> (Wight & Arn.) Meve & Liede	Cook s.n. (in cult. UBT, Z)	India: Tamil Nadu	Indi; vim_bruno; Cook	22	New record		1,2
<i>C. viminalis</i> (L.) L. subsp. <i>crassicaule</i> Liede & Meve	Liede 3173 (ULM, UBT)	Kenya: Rift Valley	EAfr; vim_crassi; LN3173	22	Liede-Schumann and Meve (2005)		1,2
<i>C. viminalis</i> (L.) L. subsp. <i>crassicaule</i> Liede & Meve	Meve 938 (=Goyder et al. 4029) (EA, K, UBT)	Kenya: Coast	EAfr; vim_crassi; UM938	22	Liede-Schumann and Meve (2005)		1,2
<i>C. viminalis</i> (L.) L. subsp. <i>crassicaule</i> Liede & Meve	Powys s.n. (in cult. UBT)	Kenya: Rift Valley	EAfr; vim_crassi; Powys	22	New record	P20 P110b	Excl.
<i>C. viminalis</i> (L.) L. subsp. <i>odontolepis</i> (Balf.f.) Goyder	Liede 3197 (UBT)	Kenya: Coast	EAfr; vim_odonto; LN3197	22	Meve and Liede (1996)		1,2
<i>C. viminalis</i> (L.) L. subsp. <i>odontolepis</i> (Balf.f.) Goyder	Field & Powys 186 (= Kew 443-1977-4037) (UBT)	Kenya: Coast	EAfr; vim_odonto; FP186	22	Albers and Meve (2011)		1,2
<i>C. viminalis</i> (L.) L. subsp. <i>odontolepis</i> (Balf.f.) Goyder	Liede & Meve 3354 (in cult. UBT)	Tanzania: Arusha	EAfr; vim_odonto; LM3354 (3354a, b, c)	22	New record		1,2
<i>C. viminalis</i> (L.) L. subsp. <i>odontolepis</i> (Balf.f.) Goyder	Meve 935 (in cult. UBT)	Kenya: Coast	EAfr; vim_odonto; UM935	22	New record		1,2
<i>C. viminalis</i> (L.) L. subsp. <i>odontolepis</i> (Balf.f.) Goyder	Meve et al. 947 (UBT)	Kenya: Coast	EAfr; vim_odonto; UM947	44	New record		1,2
<i>C. viminalis</i> (L.) L. subsp. <i>odontolepis</i> (Balf.f.) Goyder	Liede & Newton 3159 (in cult. UBT)	Kenya: Rift Valley	EAfr; vim_odonto; LN3159	44	New record		1,2
<i>C. viminalis</i> (L.) L. subsp. <i>odontolepis</i> (Balf.f.) Goyder	Liede & Hammer 2541 (UBT)	South Africa: Northern Cape	SAfr; vim_odonto; LH2541	44	New record		1,2
<i>C. viminalis</i> (L.) L. subsp. <i>odontolepis</i> (Balf.f.) Goyder	Liede 2905 (UBT)	South Africa: Eastern Cape	SAfr; vim_odonto; L2905	44	New record		1,2
<i>C. viminalis</i> (L.) L. subsp. <i>odontolepis</i> (Balf.f.) Goyder	Liede 2910 (UBT)	South Africa: Eastern Cape	SAfr; vim_odonto; L2910	44	New record		1,2
<i>C. viminalis</i> (L.) L. subsp. <i>odontolepis</i> (Balf.f.) Goyder	Liede 2920 (UBT)	South Africa: Eastern Cape	SAfr; vim_odonto; L2920	44	New record		1,2
<i>C. viminalis</i> (L.) L. subsp. <i>orangeanum</i> (Liede & Meve)	Van Jaarsveld 18715 (in cult. UBT)	Namibia: Nauchas	SAfr; vim_odonto; 18715	44	New record		1,2
<i>C. viminalis</i> (L.) L. subsp. <i>orangeanum</i> (Liede & Meve)	Lavranos & Bleck s.n. sub JHB 9 (UBT)	South Africa: Northern Cape	SAfr; vim_orange; JHB9	–	–		1,2
<i>C. viminalis</i> (L.) L. subsp. <i>orangeanum</i> (Liede & Meve)	Liede & Meve 579 (K, MSUN)	South Africa: Northern Cape	SAfr; vim_orange; LM579	22	Liede and Meve (1993)		1,2
<i>C. viminalis</i> (L.) L. subsp. <i>orangeanum</i> (Liede & Meve)	Liede & Meve 587a (in cult. UBT)	Namibia: Steinhausen	SAfr; vim_orange; LM587a	22	New record		1,2
<i>C. viminalis</i> (L.) L. subsp. <i>orangeanum</i> (Liede & Meve)	Liede & Meve 587b (UBT)	Namibia: Steinhausen	SAfr; vim_orange; LM587b	22	New record		1,2
<i>C. viminalis</i> (L.) L. subsp. <i>orangeanum</i> (Liede & Meve)	Liede & Meve 587c (UBT)	Namibia: Steinhausen	SAfr; vim_orange; LM587c	44	New record		1,2
<i>C. viminalis</i> (L.) L. subsp. <i>stipitaceum</i> (Forssk.) Meve & Liede	Collenette 8452 (UBT)	Oman	Arab; vim_stipi; Coll8452	22	New record		1,2

Table 1 (continued)

Taxon	Voucher	Locality information	Abbreviation	Chromosome number 2n=	Chromosome number information	Markers failed	Dataset in which sample is used
<i>C. viminalis</i> (L.) L. subsp. <i>stiptaceum</i> (Forssk.) Meve & Liede	Jonkers 80=IPPS 3888 (UBT)	Oman	Arab;vim_stipi;IPPS3888	22	Meve and Liede (1996)		1,2
<i>C. viminalis</i> (L.) L. subsp. <i>stiptaceum</i> (Forssk.) Meve & Liede	Mies s.n.2 (in cult. DUS, UBT)	Yemen: South	Arab;vim_stipi;Mies	22	New record		1,2
<i>C. viminalis</i> (L.) L. subsp. <i>stiptaceum</i> (Forssk.) Meve & Liede	Ricanek & Hanacek 204 (in cult. UBT)	Yemen: North	Arab;vim_stipi;RH204	–	–		1,2
<i>C. viminalis</i> (L.) L. subsp. <i>stiptaceum</i> (Forssk.) Meve & Liede	Zimmer & Mies s.n. (UBT)	Yemen: North	Arab;vim_stipi;Zim_Mies	22	New record		1,2
<i>C. viminalis</i> (L.) L. subsp. <i>stiptaceum</i> (Forssk.) Meve & Liede	Liede & Newton 3191 (in cult. UBT)	Tanzania: Central Prov.	EAfr;vim_stipi;LN3191	22	New record		1,2
<i>C. viminalis</i> (L.) L. subsp. <i>stiptaceum</i> (Forssk.) Meve & Liede	IPPS 603 (in cult. UBT)	Somalia	EAfr;vim_stipi;IPPS603	22	New record		1,2
<i>C. viminalis</i> (L.) L. subsp. <i>stiptaceum</i> (Forssk.) Meve & Liede	Liede 2515 (UBT)	South Africa: Northern Cape	SAfr;vim_stipi;LH2515	22	New record		1,2
<i>C. viminalis</i> (L.) L. subsp. <i>suberosum</i> (Meve & Liede) Goyder	Albers et al. 517 (in cult. UBT)	Zimbabwe: Matabeleland North	EAfr;vim_suber;ALM517	22	New record		1,2
<i>C. viminalis</i> (L.) L. subsp. <i>suberosum</i> (Meve & Liede) Goyder	Albers et al. 540 (UBT)	Zimbabwe: Bulawayo Prov.	EAfr;vim_suber;ALM540	22	New record		1,2
<i>C. viminalis</i> (L.) L. subsp. <i>suberosum</i> (Meve & Liede) Goyder	Erwee et al. 562 (UBT)	Zimbabwe: Bulawayo Prov.	EAfr;vim_suber;ELM562	22	New record		1,2
<i>C. viminalis</i> (L.) L. subsp. <i>suberosum</i> (Meve & Liede) Goyder	Liede & Meve 599 (UBT)	Namibia: Steinhilshausen	EAfr;vim_suber;LM599	22	New record		1,2
<i>C. viminalis</i> (L.) L. subsp. <i>suberosum</i> (Meve & Liede) Goyder	Liede & Meve 3370 (UBT)	Tanzania: Arusha	EAfr;vim_suber;LM3370	44	New record		1,2
<i>C. viminalis</i> (L.) L. subsp. <i>suberosum</i> (Meve & Liede) Goyder	Albers et al. 575 (in cult. UBT)	Zimbabwe: Mavingo Prov. East Africa	EAfr;vim_suber;ALM575	22	New record	P05	2
<i>C. viminalis</i> (L.) L. subsp. <i>suberosum</i> (Meve & Liede) Goyder	Cumming 792 (UBT)	East Africa	EAfr;vim_suber;Cum792	22	New record	P209	Excl.
<i>C. viminalis</i> (L.) L. subsp. <i>suberosum</i> (Meve & Liede) Goyder	Erwee et al. 545 (UBT)	Zimbabwe: Matabeleland	EAfr;vim_suber;ELM545	22	Meve and Liede (1996)	P210	Excl.
<i>C. viminalis</i> (L.) L. subsp. <i>suberosum</i> (Meve & Liede) Goyder	Field & Powys 200 (=Kew 443-1977-4040) (in cult. UBT)	Kenya: Coast	EAfr;vim_suber;FP200	22	New record	P101b	Excl.
<i>C. viminalis</i> (L.) L. subsp. <i>suberosum</i> (Meve & Liede) Goyder	Liede 631 (in cult. UBT)	South Africa: Mpumalanga	SAfr;vim_suber;L631	22	New record		1,2
<i>C. viminalis</i> (L.) L. subsp. <i>suberosum</i> (Meve & Liede) Goyder	Liede 2936 (UBT)	South Africa: Free State	SAfr;vim_suber;L2936	22	New record		1,2
<i>C. viminalis</i> (L.) L. subsp. <i>suberosum</i> (Meve & Liede) Goyder	Mc Mentrey s.n. sub JHB 3 (UBT)	South Africa: Mpumalanga	SAfr;vim_suber;JHB3	22	New record		1,2
<i>C. viminalis</i> (L.) L. subsp. <i>thunbergii</i> (G. Don) Liede & Meve	Albers & Meve 42 (in cult. UBT)	South Africa: Northern Cape	SAfr;vim_thunb;AM42	44	Liede and Meve (1993)		1,2
<i>C. viminalis</i> (L.) L. subsp. <i>thunbergii</i> (G. Don) Liede & Meve	Cumming 2121 (in cult. UBT)	South Africa: Eastern Cape	SAfr;vim_thunb;Cum2121	44	New record	P02	Excl.

Table 1 (continued)

Taxon	Voucher	Locality information	Abbreviation	Chromosome number 2n=	Chromosome number information	Markers failed	Dataset in which sample is used
<i>C. viminalis</i> (L.) L. subsp. <i>thunbergii</i> (G. Don) Liede & Meve	Cumming 2166 (in cult. UBT)	South Africa: Eastern Cape	SAfr;vim_thunb;Cum2166	44	New record		1,2
<i>C. viminalis</i> (L.) L. subsp. <i>thunbergii</i> (G. Don) Liede & Meve	Cumming 2301 (in cult. UBT)	South Africa: Eastern Cape	SAfr;vim_thunb;Cum2301	44	New record		1,2
<i>C. viminalis</i> (L.) L. subsp. <i>thunbergii</i> (G. Don) Liede & Meve	Cumming 2382 (in cult. UBT)	South Africa: Eastern Cape	SAfr;vim_thunb;Cum2382	44	New record		1,2
<i>C. viminalis</i> (L.) L. subsp. <i>thunbergii</i> (G. Don) Liede & Meve	Cumming 2566 (in cult. UBT)	South Africa: Western Cape	SAfr;vim_thunb;Cum2566	44	New record	P105	Excl.
<i>C. viminalis</i> (L.) L. subsp. <i>thunbergii</i> (G. Don) Liede & Meve	Hammer s.n. sub Liede 2869 (in cult. UBT)	South Africa: Western Cape	SAfr;vim_thunb;LH2869	44	New record		1,2
<i>C. viminalis</i> (L.) L. subsp. <i>thunbergii</i> (G. Don) Liede & Meve	Lavranos & Bleck s.n. in JHB 1 (in cult. UBT)	South Africa: Western Cape	SAfr;vim_thunb;JHB1	44	Liede and Meve (1993)		1,2
<i>C. viminalis</i> (L.) L. subsp. <i>thunbergii</i> (G. Don) Liede & Meve	Lavranos 17940 (in cult. UBT)	Namibia: Rosh Pinah	SAfr;vim_thunb;Lav17940	44	New record		1,2
<i>C. viminalis</i> (L.) L. subsp. <i>thunbergii</i> (G. Don) Liede & Meve	Liede & Hammer 2507 (in cult. UBT)	South Africa: Western Cape	SAfr;vim_thunb;LH2507	44	Albers and Meve (2001)	P02	Excl.
<i>C. viminalis</i> (L.) L. subsp. <i>thunbergii</i> (G. Don) Liede & Meve	Liede & Hammer 2514 (UBT)	South Africa: Northern Cape	SAfr;vim_thunb;LH2514	44	New record		1,2
<i>C. viminalis</i> (L.) L. subsp. <i>thunbergii</i> (G. Don) Liede & Meve	Liede & Hammer 2521 (in cult. UBT)	South Africa: Northern Cape	SAfr;vim_thunb;LH2521	44	New record		1,2
<i>C. viminalis</i> (L.) L. subsp. <i>thunbergii</i> (G. Don) Liede & Meve	Liede & Hammer 2522 (UBT)	South Africa: Northern Cape	SAfr;vim_thunb;LH2522	44	New record	P05	2
<i>C. viminalis</i> (L.) L. subsp. <i>thunbergii</i> (G. Don) Liede & Meve	Liede & Hammer 2545 (UBT)	South Africa: Western Cape	SAfr;vim_thunb;LH2545	44	New record	P05	2
<i>C. viminalis</i> (L.) L. subsp. <i>thunbergii</i> (G. Don) Liede & Meve	Liede & Hammer 2546 (in cult. UBT)	South Africa: Western Cape	SAfr;vim_thunb;LH2546	44	New record		1,2
<i>C. viminalis</i> (L.) L. subsp. <i>thunbergii</i> (G. Don) Liede & Meve	Liede & Meve 608 (UBT)	South Africa: Northern Cape	SAfr;vim_thunb;LM608	44	Liede and Meve (1993)		1,2
<i>C. viminalis</i> (L.) L. subsp. <i>thunbergii</i> (G. Don) Liede & Meve	Liede 2551 (in cult. UBT)	South Africa: Western Cape	SAfr;vim_thunb;LH2551	44	New record	P09 P106 P206	Excl.
<i>C. viminalis</i> (L.) L. subsp. <i>thunbergii</i> (G. Don) Liede & Meve	Liede 2921 (UBT)	South Africa: Eastern Cape	SAfr;vim_thunb;L2921	44	New record		1,2
<i>C. viminalis</i> (L.) L. subsp. <i>thunbergii</i> (G. Don) Liede & Meve	Wiesse s.n. sub Liede 2549 (UBT)	South Africa: Western Cape	SAfr;vim_thunb;LH2549	44	Liede and Meve (1993)		1,2
<i>C. viminalis</i> (L.) L. subsp. <i>thunbergii</i> aff.	Liede 2912 (UBT)	South Africa: Eastern Cape	SAfr;vim_thunb;L2912	22	New record	P05	2
<i>C. viminalis</i> (L.) L. subsp. <i>viminalis</i>	Liede & Meve 3389 (UBT)	Tanzania: Arusha	EAfr;vim_vim;LM3389	22	New record	P05	2
<i>C. viminalis</i> (L.) L. subsp. <i>viminalis</i>	ex Saxena s.n. (in cult. UBT)	India: Rajasthan	Indi;vim_vim;Sax_sn	–	–		1,2
<i>C. viminalis</i> (L.) L. subsp. <i>viminalis</i>	Hanacek & Rikanek 112	India: Tamil Nadu	Indi;vim_vim;HR112	22	New record		1,2
<i>C. viminalis</i> (L.) L. subsp. <i>viminalis</i>	Hanacek & Rikanek 129 (in cult. UBT)	India: Tamil Nadu	Indi;vim_vim;HR129	22	New record		1,2
<i>C. viminalis</i> (L.) L. subsp. <i>viminalis</i>	Rikanek & Hanacek 017 (UBT)	India: Maharashtra	Indi;vim_vim;RH017	22	New record		1,2
<i>C. viminalis</i> (L.) L. subsp. <i>viminalis</i>	Saxena 6 (ex hort. UBT)	India: Tamil Nadu	Indi;vim_vim;Sax6	22	New record		1,2
<i>C. viminalis</i> (L.) L. subsp. <i>viminalis</i>	Saxena 21 (in cult. UBT)	India: Rajasthan	Indi;vim_vim;Sax21	22	New record		1,2
<i>C. viminalis</i> (L.) L. subsp. <i>viminalis</i>	Liede & Conrad 2704 (UBT)	Madagascar: Toliana	Mada;vim_vim;LC2704	22	Liede and Meve (1995)		1,2

The present study aimed to elucidate variability in the morphologically diverse, yet seemingly genetically uniform, *C. viminalis* complex. In particular, we set to discover whether the distinction of species and subspecies in the *C. viminalis* complex, depending on floral or vegetative differences (acc. to Liede and Meve 1989), is mirrored by genetic difference. If so, ‘species’ should be more different from each other than ‘subspecies’. Secondly, the role of geographical variation is explored, because, unlike in the *Ceropegia* example previously studied (Meve et al. 2001), morphological and geographical variation are often not parallel in the *C. viminalis* complex. Finally, we aimed to find out whether frequently observed tetraploidy is restricted to certain lineages or whether it has arisen several times.

Material and methods

Taxon sampling

The present work is based on the living collection maintained at the University of Bayreuth (UBT). Sampling comprises 137 accessions representing almost all published taxa, 14 different species and 9 subspecies of *C. viminalis*, and covers most of the area of the group. For one accession, *C. viminalis* subsp. *orangeanum* (Liede & Meve 587), three different plants (a, b, c) of the same population were available. One collection of *C. stoloniferum* (Powys 214) was present in two accessions which had arrived at UBT from different sources. A randomly picked sample of *C. viminalis* subsp. *odontolepis* (Liede & Meve 3354) was used as a positive control, cutting four samples from the same plant and treating these samples independently. The complete dataset, therefore, comprised 140 samples.

Missing from the dataset is *Cynanchum oresbium* (Bruyns) Goyder from Mozambique, a species described by Bruyns (2003) after work for the present article was completed. A recently recognised species from southern Yemen, *C. areysianum* (Bruyns) Meve & Liede (Bruyns 2011; Meve and Liede-Schumann, *in press*) is also not included here. Of the areas with a fair diversity of the *C. viminalis* group, no material could be secured from Angola or from the Mascarenes. For the latter, Bosser and Marais (2005) published only ‘*Sarcostemma viminalis*’, but their illustration (p. 27: plate 9) depicts an extreme variability of floral and corona shapes, so that, according to the concept of the present authors, at least two more species probably occur. One sample from the Seychelles, Barthlott & Porembski 13676, was available. Voucher information is given in Table 1.

Molecular methods

From 137 plants growing in the greenhouses at UBT, 200–500 mg fresh plant material was removed with sterilised tweezers

and razor blades, and immediately put into aluminium foil bags on ice. DNA was extracted immediately after sampling following the CTAB (cetyltrimethylammonium bromide) protocol (Doyle and Doyle 1987); however, the DNA was precipitated with isopropanol for 1 h and the dried DNA pellets were resuspended in 200 µl of purified water (LiChrosolv Fa. Merck).

Laboratory work was conducted at UBT between 1999 and 2003. One hundred and forty samples were divided into three batches because of the limited capacity of the thermocycler. Each batch included a negative control (distilled water instead of DNA solution) and a positive control (Liede & Meve 3354). For each batch, a separate master mix was prepared. All batches were run subsequently in the same Thermocycler, a Uno-II (Biometra, Göttingen, Germany). The annealing temperature was set to 33 °C or 34 °C depending on the primer and its melting temperature, as indicated by the manufacturer. Primers of the random-primer kits A, B and C (Roth, Karlsruhe, Germany) were used and suitable primers were determined in a preliminary experiment with 25 samples. Only primers that showed a reaction and did not produce a uniform pattern over all 25 samples were used for the final analysis, resulting in 25 suitable primers (Table 2).

Each sample had a volume of 25 µl and contained 0.02 mMol=800 µM MgCl₂ (Qiagen, Hilden, Germany); 5 pMol=200 nM Primer (Roth); 4 dNTPs, each 2.5 nMol=100 µM (Roth); 1 U Taq-Polymerase (Qiagen); and filled to volume with Reaction Buffer pH 8.7 (Qiagen) consisting of Tris-HCl, KCl, NH₄SO₄ and MgCl₂ (15 mM).

For each primer, samples had to be distributed onto six gels run parallel under identical conditions as one gel could only load 28 samples. For the run, the whole polymerase chain reaction product of each sample was used. All gels were run at 100 V under identical conditions in a climate chamber set to 15 °C. The run was stopped after 6–8 h depending on the primer and the DNA fragments were visualised with ethidium bromide under ultraviolet light. The images were taken and stored with a gel documentation system (MWG Biotech, Ebersberg, Germany). Images for one primer were then combined for the computerised analysis (RFLPscan, Scanalytics Inc., Billerica, USA) with the aid of the standard 100 bp DNA ladder (Gibco, Darmstadt, Germany) which had been run in both the right and the left outermost pockets of each gel.

Data analysis

Strong, clear bands were scored as present or absent. Despite the preliminary experiment, some samples did not show a reaction with all primers. Those data cells were scored as ‘?’.

Three matrices were constructed. The first matrix contained all taxa which had produced bands with all primers—116 individuals and 522 characters (479 variable ones; Dataset 1). For the second matrix, the individuals for

Table 2 Polymorphism of the 25 RAPD primers used for analysing the 140 samples of the *Cynanchum viminalis* group

Primer	Primer number of amplified bands		number of samples failed
	total	polymorphic	
P02	20	20	2
P03	13	13	0
P04	15	14	2
P05	20	20	9
P09	28	27	3
P10	21	20	0
P12	24	24	0
P13	24	22	0
P18	21	20	0
P20	24	24	1
P101	23	22	1
P105	26	26	1
P106	23	21	4
P110b	18	16	1
P115	22	21	0
P202	31	29	0
P204	19	19	2
P206	29	28	1
P208	27	25	0
P209	16	14	3
P210	12	11	1
P211	15	11	1
P212	15	14	0
P216	27	13	1
P218	9	27	0
Total	522	498	—

which primers had failed were considered. Thirteen markers did not produce results with one or more samples (Tables 1 and 2)—the most extreme case being marker P05, which failed in nine samples, followed by P106 (four samples). In three samples, three primers failed, in two samples, two primers failed; the rest are single failures (Table 1). Increasing the number of individuals to 140, but reducing the number of variable characters to 234 (223 variable), which would be the consequence of removing the 14 incomplete markers (as distance analyses cannot deal with missing data) greatly reduces the signal in the data (Liede-Schumann, unpublished data). Therefore, samples with missing primers were checked for whether they constituted groups not represented in the complete matrix from both the taxonomical and the geographical points of view. The only sample that seemed indispensable under these considerations was *Ehrlich s.n. sub BG Berlin 285-08-93-10* because it was the only sample from West Africa available. As this sample is deficient for markers P04 and P05, a second

matrix was constructed without these two primers and without all samples deficient for other primers than P04 and P05 (Table 1), resulting in a matrix of 125 individuals and 487 characters (450 variable ones; Dataset 2).

Finally, we used a subset of Dataset 1 reduced to include only accessions belonging to *C. viminalis*, but labelled to show their sub-specific identification. This dataset (Dataset 3) comprised 84 accessions and 522 characters.

The error rate (Bonin et al. 2007) was calculated as follows:

error rate = [number of bands not scored the same/ total number of bands scored (equal to the number of bands × compared individuals)] × 100 .

As controls, the replicate samples were compared. The three accessions prepared for this purpose (Liede & Meve 3354) resulted in 4.3 % for Dataset 1 and 4.0 % for Dataset 2; the three accessions from one population (Liede & Meve 587a, b, c) resulted in 4.9 % for Dataset 1 and 4.6 % for Dataset 2; and the two accessions of *Powys 214* resulted in 2.9 % for both datasets. However, as the influence of errors on the conclusions of population genetic studies are considered low, provided that the number of loci scored is high enough (Pompanon et al. 2005), no correction has been applied to the data. P12 and P101 were the primers with the highest number of mismatches. However, removal of these two primers did not have an effect on the results of the analyses described in the following (not shown).

To test whether the patterns of bands differ among different geographic regions (125 accessions), species (115 accessions) and subspecies (83 accessions) of *C. viminalis* we calculated Jaccard similarity matrices based on the presence and absence of bands, and performed Canonical Analysis of Principal Coordinates (CAP) discriminant, as well as PERMANOVA (10,000 permutations) analyses—both provided in PRIMER (Anderson et al. 2008; Clarke and Gorley 2006). Thirteen of the accessions used for the subspecies dataset could not be assigned to a subspecies based on morphological data. After calculating a CAP analysis without these accessions, they were added to the dataset in order to place them onto the axes of the existing CAP model and to classify them into one of the existing subspecies (Anderson et al. 2008).

Neighbour-joining analyses were conducted on Datasets 1 and 2 using the software TREECON for Windows, v. 1.3b (Van de Peer and De Wachter 1997). Distances were calculated using the settings ‘RFLP/AFLP/RAPD data’ and ‘Link et al. 1995’, which uses the Jaccard (1908) coefficient of community (Jaccard 1908; Link et al. 1995). One thousand bootstrap pseudo-replicates were created for each dataset. As we have no information on the correct outgroup, midpoint rooting (the root is set at the midpoint between the two most divergent accessions) was used (Hess and De Moraes Russo 2007). To check for the influence of addition sequences, ten permuted datasets were constructed and subjected to the same analysis.

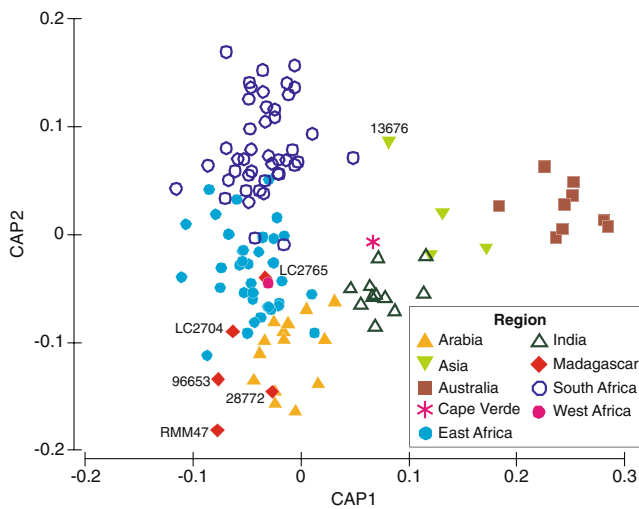


Fig. 2 CAP Plot analysing geographical clustering of accessions (Dataset 2; 125 taxa, 487 characters, resemblance: Jaccard, PERMANOVA_Pseudo-F $df=8,116=3.27$, $p < 0.001$)

Results

Each primer yielded between 9 and 31 characters (20.9 on average). There were clear differences in the band patterns among the different regions (Dataset 2; PERMANOVA_Pseudo-F $df=8,116=3.27$, $p < 0.001$) and 79 % of the accessions were correctly assigned to our pre-defined regions in the CAP discriminant analysis (Fig. 2). The Arabian

accessions take a central position on the CAP1 axis, with the Indian, Asian and Australian accessions to the one side (Fig. 2). On the other side, the Arabian and South African accessions are linked by the East African accessions with overlaps on both sides. The unclear delimitation between these regions is reflected by the fact that the majority of the 26 (of 125) samples labelled as ‘misclassified’ in the CAP analysis represent mix-ups between East and South Africa (10 samples), and East Africa and Arabia (6 samples). The single mainland West African accession is placed amidst the East African accessions in the CAP plot, while the single accession from the Cape Verde Islands appears isolated from all other accessions, but very close to the Indian ones. The Madagascan samples do not form a coherent group. The three accessions of *C. antsiranense* (28772, RMM 47, 96653 in Fig. 2) form a group close to the Arabian taxa, but the other two Madagascan samples are among the ‘misclassified’ ones, attributed to the Indian (LC2765) or East African (LC2704) group. The single sample from the Seychelles (13676) appears isolated and is closest to the South African samples. Essentially the same geographic pattern is found in the matrix considering all primers, but fewer samples (Dataset 1; PERMANOVA_Pseudo-F $df=7,108=3.44$, $p < 0.001$). Here, 82 % of the accessions are correctly attributed in the CAP analysis (results not shown). Of the 21 misclassified samples, 12 are interchanged between South Africa, East Africa and Arabia.

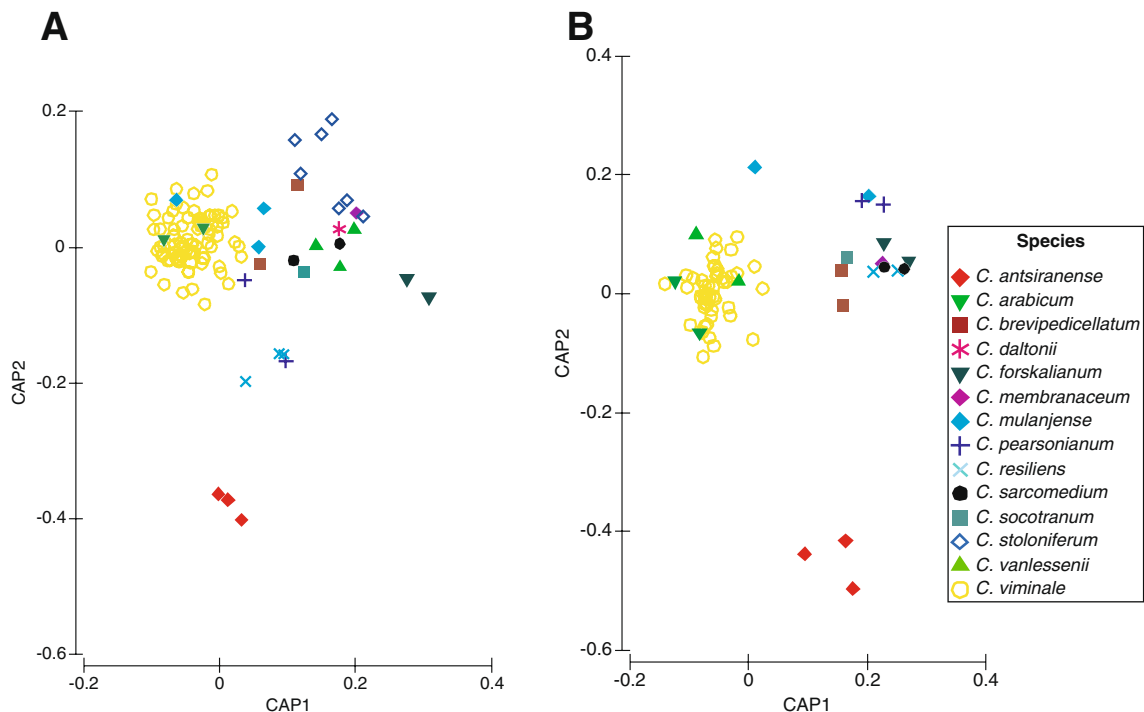
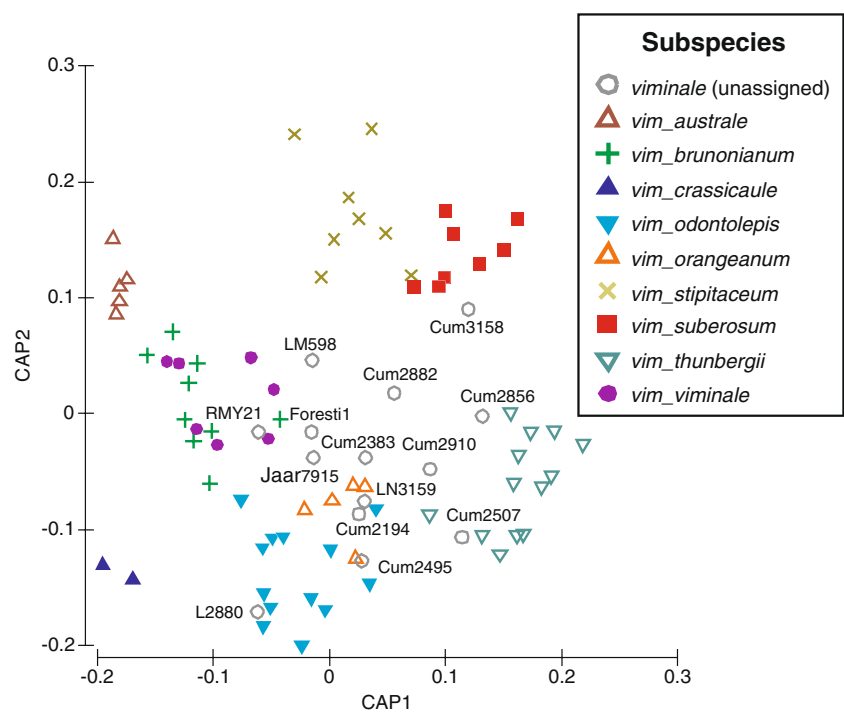


Fig. 3 (a) CAP plot analysing species of the *Sarcostemma* group of *Cynanchum* (Dataset 1; 115 accessions, 522 characters, resemblance: Jaccard, PERMANOVA_Pseudo-F $df=13,102=1.98$, $p < 0.001$). (b) CAP

Plot analysing species of the *Sarcostemma* group of *Cynanchum*, diploid accessions only (71 accessions, 522 characters, resemblance: Jaccard, PERMANOVA_Pseudo-F $df=11,59=1.77$, $p < 0.001$)

Fig. 4 CAP plot analyzing subspecies of *C. viminalis* (70 accessions, 522 characters; 13 unassigned samples added as 'new samples'); resemblance: Jaccard, PERMANOVA_Pseudo-F_{df=8,61}=4.02, $p < 0.001$)



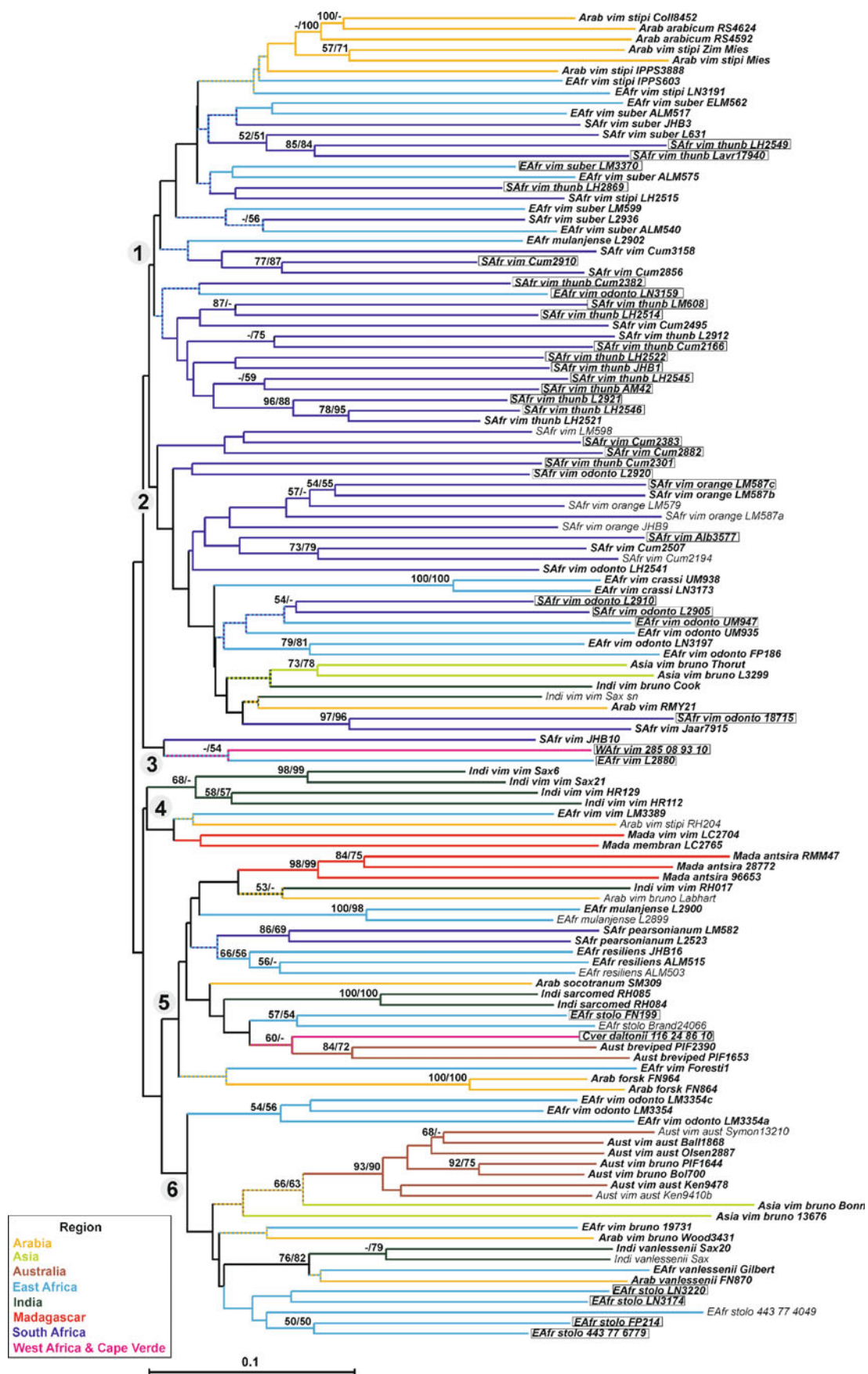
There were also differences in the band patterns among accessions assigned to different species (Dataset 1; PERMANOVA_Pseudo-F_{df=13,102}=1.98, $p < 0.001$), with 96 % correctly classified accessions in the CAP discriminant analysis (Fig. 3a). Five accessions (of 116) were not correctly assigned to our morphologically-defined species (see discussion). The vast distance between the Madagascan *C. antsiranense* and the remainder of the species, Madagascan or not, is particularly noticeable. Restricting analysis to the 71 known diploid samples [Dataset 1 (diploids only)]; PERMANOVA_Pseudo-F_{df=11,59}=1.77, $p < 0.001$; Fig. 3b) does not change the general picture, with 93 % of the samples correctly classified.

Within *C. viminalis* we also retrieved specific band patterns (Dataset 3; PERMANOVA_Pseudo-F_{df=8,61}=4.02, $p < 0.001$) with 89 % of the samples correctly assigned to the morphologically-identified subspecies in the CAP discriminant analysis. Half of the eight samples incorrectly assigned involve subsp. *brunonianum*. The morphologically easily to distinguish twining subspecies, subspp. *odontolepis*, *brunonianum* and *suberosum*, separated well in this analysis (Fig. 4). On the other hand, the morphologically difficult to separate subsp. *brunonianum* and subsp. *viminalis* did not show clear molecular separation either. One of the 13 samples that could not be assigned to any subspecies for morphological reasons was clearly attributed to subsp. *suberosum* (Cumming 3158). The other specimens were assigned to *C. viminalis* subsp. *brunonianum* (1), *viminalis* (2), *odontolepis* (4) and *thunbergii* (6), but most take intermediate positions between the morphologically-defined subspecies (Fig. 4).

Neighbour-joining analysis confirms the picture of a geographical separation overlying morphological separation

(Fig. 5). The effects of addition sequences on topology are small and have no influence on the groups discussed below. Clusters 1–3 comprise mainly taxa that have been considered subspecies of *C. viminalis* (68 of 71 accessions), while clusters 4–6 comprise mostly taxa that have been regarded as independent species in the *C. viminalis* complex (30 of 54 accessions). All clusters contain East African accessions. Cluster 1 comprises mainly East and South African taxa, but also one group of Arabian taxa. Among the latter, two accessions are embedded that have been considered as an independent species, *C. arabicum*, because of their strongly reduced corona. Likewise, cluster 2 consists predominantly of East and South African taxa, but also comprises an Asian-Indian group and one Arabian accession from Yemen. The small cluster 3 comprises only three accessions, two of them tetraploid (one unknown), which geographically span the whole African continent. In the second half of the tree most, but not all, defined species form distinct groups, such as *C. antsiranense* from Madagascar, are always associated with an Arabian and an Indian accession. Other morphologically-defined species of which the included samples form monophyletic clusters include *C. pearsonianum* from South Africa, *C. mulanjense* (even though one accession identified as *C. mulanjense* is found in cluster 1), *C. resiliens* from

Fig. 5 Neighbour-joining tree of 125 taxa and 487 characters (Dataset 2), based on Jaccard distances. Midpoint rooting has been applied. Figures above branches indicate bootstrap support > 50 % (Dataset 1 / Dataset 2). Taxa in bold have been counted with 2n=22 chromosomes, those in bold and in a box with 2n=44 chromosomes. Chromosome number of unmarked taxa is unknown. Numbers 1–6 denote clusters to facilitate discussion. Colours correspond with Fig. 2



East Africa, *C. sarcomedium* from India and *C. forskaolia-num* from Arabia (for names of species recently transferred from *Sarcostemma* to *Cynanchum* see Goyder 2008, Meve and Liede in press). Of *C. socotranum* and *C. daltonii* only one accession could be included in the analysis. The East African *C. stoloniferum* forms two branches in clusters 5 and 6. Of particular interest is the case of *C. vanlessenii*, of which the four samples included form a well-supported branch of cluster 6, regardless of the origin of the samples (India, Arabia and East Africa). The Australian samples fall into two branches, with *C. brevipedicellatum* connecting with the Cape Verde *C. daltonii* and all other samples well supported in a sister-group position to two Asian accessions, regardless of whether they were identified as subsp. *australe* or subsp. *brunonianum* of *C. viminalis*. Also supported is a group of Indian accessions classified as ‘typical’ *C. viminalis* (cluster 4).

Tetraploidy occurs in various branches of the tree, but, with exception of *C. stoloniferum* and *C. daltonii*, the recognised species in clusters 4–6 are diploid. In the subspecies, accessions attributed to subsp. *thunbergii* from South Africa are mostly tetraploid (one exception). Most accessions classified as subsp. *thunbergii* form a large branch in cluster 1, but others are distributed over several branches of clusters 1 and 2. Tetraploids have been found only in Africa (including the Cape Verde Islands), but are absent from all other areas. The African tetraploids are not monophyletic and some form supported groups with diploid taxa (e.g. Cumming 2910 and Cumming 2856 in cluster 1, Van Jaarsveld 18715 and Van Jaarsveld 7915 in cluster 2).

Discussion

While the use of RAPD or other dominant markers in phylogeny reconstruction has raised a lot of discussion (e.g. Harris 1995), their utility at the population and species level has been demonstrated repeatedly, as discussed recently by Weising et al. (2005). Wolfe and Liston (1998) pointed out that two categories of errors might limit the utility of RAPD markers. Firstly, deviations from the expectations of strict Mendelian inheritance might be caused by artifactual, non-genetic variation, organellar bands or epigenetic interactions. Secondly, problems of homology assessment might occur if bands are either erroneously judged as co-migrating or if fragments of the same length are not homologous. We kept artifactual variation at a minimum by adhering to a strictly standardised RAPD protocol (Dowling et al. 1996), with the same person (M. Gebauer) performing all the laboratory work, using chemicals from the same company and the same thermocycler, and last, but not least, running all gels at the same outer temperature in a cool room. However, little could be done to eliminate effects potentially caused by organellar bands or epigenetic interactions. Equal length of co-

migrating bands was assured by accurately resolving fragment size using a high concentration of agarose in the gel and letting the gel run for several hours; however, no tests for homology of fragments of the same size were conducted. Scoring errors were minimised by scoring only strong, clear bands and by always entrusting the same person with the scoring procedure (M. Gebauer).

Our results show that the *Sarcostemma* group of *Cynanchum* follows a complicated evolutionary pattern in which a strong geographical component overlies morphological and karyological differentiation (Figs 2 and 5). This favours the hypothesis that most areas have been colonised more than once by *Sarcostemma*-like plants.

Taking the results of Liede and Kunze (2002) and Meve and Liede (2002), that place the origin of the group firmly in Madagascar, the strong divergence of *C. antsiranense* (Fig. 1a) from all other taxa (Fig. 3) is remarkable and suggestive of a long isolated history in the *C. viminalis* complex. In their prologue, Meve and Liede (1997) state that, albeit easily recognisable, the species is by no means extraordinarily different morphologically. The other two Madagascan accessions included do not group with *C. antsiranense*. CAP analysis supports their identification as *C. membranaceum* and *C. viminalis* subsp. *viminalis* (Figs 3 and 4) but neighbour-joining analysis retrieves them as one, albeit unsupported, branch (Fig. 5, cluster 4). Thus, at least one second round of dispersal between Madagascar and the mainland is suggested by our data.

Our single sample from the Seychelles is morphologically identified as subsp. *brunonianum*, a coastal alliance; this identification is supported by all analyses. It is, however, remarkable that this sample joins a group in cluster 6 that includes a sample from Thailand and the larger of the two Australian branches, suggesting another exchange between Arabia and East Africa, and India and Australia via the Seychelles.

Our data also suggest that Australia has been settled twice by the *C. viminalis* group. The three taxa recognised morphologically for the Australian flora are distributed onto two well-supported branches (in clusters 5 and 6). The two accessions of the twining subsp. *brunonianum* form a well-supported sub-branch inside the samples of the shrubby, mostly non-twining subsp. *australe* (Fig. 5, cluster 6). The two taxa differ in habitat, with subsp. *australe* found either inland or along the subtropical west coast of the continent, and subsp. *brunonianum* with a tropical coastal distribution (Forster et al. 1996). The equally shrubby *C. brevipedicellatum* has only recently been described (Forster 1992) from the arid lands north of the area of subsp. *australe*, but, apparently, it has reached the continent independently of the latter (Fig. 5, cluster 5). In the neighbour-joining tree the two *C. brevipedicellatum* accessions are weakly joined to the tetraploid *C. daltonii* from Cape Verde (Fig. 5, cluster 5). This rather odd association is supported by the CAP

analysis of all species. Here, *C. daltonii* is among the samples considered ‘misclassified’ and it is attributed to *C. brevipedicellatum*, even though the plot of the first two dimensions does not reflect this classification (Fig. 3a). In the regional analysis, *C. daltonii* remains isolated (Fig. 2) and it is certainly among the taxa requiring additional study.

The sample geographically closest to Cape Verdes, *Ehrlich s.n.* sub BG Berlin 285-08-93-10 from Mali (also a tetraploid and of the general ‘viminale’ morph), that cannot be attributed to a particular subspecies has no association with *C. daltonii*, but with an East African and South African sample (Figs 2 and 5; cluster 3). It is morphologically indistinct and can therefore be interpreted as an extension of the East African ‘*C. viminale*’ alliance.

Most taxa considered ‘species’ seem to be monophyletic (Fig. 5); however, of several ‘species’, only two samples were available because they occur only in rather restricted areas and are known from few populations. Not monophyletic is *C. mulanjense*, where accession L2902 collected at the base of Mt. Mulanje never groups with the two samples collected from the top (clusters 1 and 5). This sample is also considered misclassified by the CAP analysis (Fig. 3a) and morphological misidentification cannot be ruled out in this case.

The tetraploid vegetatively and florally distinct *C. stoloniferum* from East Africa appears in two separate places of the neighbour-joining tree (clusters 5 and 6), but is homogeneous in the CAP analysis, even though two subgroups can be distinguished (Fig. 3a). This result possibly reflects an allopolyploid origin of this species—a hypothesis needing more intensive study.

The widespread *C. vanlessenii* always forms a supported group in neighbour-joining analysis (Fig. 5), while one sample, *Gilbert & Vollesen s.n.* from Ethiopia, is attributed to *C. viminale* by CAP analysis. The first two dimensions of CAP analysis place this sample firmly into *C. vanlessenii* (Fig. 3a), but there are differences in ordination on higher axes (results not shown).

The position of the two included accessions of the florally well-defined *C. arabicum*, which both fall into the range of *C. viminale* in the CAP plot (Fig. 3) is peculiar, and *arabicum-viminale* confusions account for two of the five misclassified taxa. This placement is supported by the neighbour-joining tree (Fig. 5, cluster 1), where the samples of *C. arabicum* form a distinct branch together with other Arabian taxa. The latter are morphologically classified as subsp. *stipitaceum* and are retrieved as sister to two East African subsp. *stipitaceum* accessions. Thus, it seems that the loss of the outer corona ring ($C_{(is)}$), making *C. arabicum* (Fig. 1b) a very easily recognisable species, is a rather recent specialisation from *C. viminale*-type flowers out of the large East–South African group. The other taxon lacking the $C_{(is)}$,

C. socotranum, also from Arabia, is placed in cluster 5, suggesting that the loss of the outer corona has occurred (at least) twice independently. CAP analysis also keeps *C. socotranum* well separate (Fig. 3).

With the exception of *C. arabicum*, the well-defined species are all found in clusters 4–6 of the neighbour-joining tree, while the subspecies of *C. viminale* are mostly, but not completely, gathered in clusters 1–3. It might be considered to reconstitute specific rank to subsp. *australe*, which stands isolated among the subspecies of *C. viminale* (Fig. 4), even though floral differences are minimal.

Within *C. viminale*, most samples are attributable to morphologically separable subspecies (Fig. 4), but morphologically intermediate samples also took intermediate positions in the CAP analysis (independent of their ploidy level; Fig. 4) so that ongoing genetic exchange between the subspecies is likely.

Ploidy level is constant within most of the groups considered species with *C. daltonii* and *C. stoloniferum* being tetraploid ($2n=44$) and all others diploid ($2n=22$). In *C. viminale*, in contrast, ploidy level changes. As far as known, subsp. *australe*, *crassicaule*, *stipitaceum* and *viminale* are always diploid, while ploidy level can vary inside the other subspecies recognised. In one case, *C. viminale* subsp. *orangeanum*, ploidy level even shifts within a population (*Liede & Meve 587a* and *b* are diploid, *587c* is tetraploid). This difference in ploidy level possibly explains the relatively high error rate between the samples of this population. Geographically, tetraploidisation is restricted to Africa (including the Cape Verde Islands), while taxa from Madagascar, Arabia, India, Asia and Australia are constantly diploid (Fig. 5, Table 1).

Despite the limitations of the RAPD technique pointed out earlier, our study sheds new light on the complexity of the *C. viminale* group. From our results we suggest that, starting in Madagascar, a first round of area expansion has occurred, followed by differentiation caused mainly by adaptation to different habitats and pollinators. This first round of differentiation has probably led to such clearly divergent taxa as *C. pearsonianum* or *C. stoloniferum*. On top of these, a second round of divergence within the same larger area (the Old World tropics and subtropics) has led to those forms resembling ‘*C. viminale*’ closely, as typically expressed by subsp. *orangeanum*, or subsp. *suberosum*. In both rounds a strong geographical pattern is found, explaining the good resolution in the geographical CAP analysis (Fig. 2). Particularly in the second radiation, differentiation is still in full swing, including rather abrupt changes in ploidy level. This pattern is probably promoted by the highly generalised floral structure within *C. viminale*, allowing access for a wide variety of insects (*Liede and Whitehead 1991*) and offering rich nectar rewards. Differences in fine floral structure and floral scent (*Jürgens et al. 2008*) support differentiation, but occasional genetic

exchange between neighbouring populations of different subspecies seems very likely, although are not documented with certainty. The role of possible vegetative reproduction is not yet explored, even though most taxa root very easily from broken off stems, hinting at the possibility of a certain degree of clonal structure. *Cynanchum viminalis* seeds are good flyers for asclepiads, but, as in most other taxa, their coma breaks off fairly easily. Still, storms may well carry some seeds to remote places. In Asclepiadoideae, a pattern with similar strong geographic component was found in *Ceropegia aristolochioides* (Meve et al. 2001), but not in *Gymnema sylvestre* (Nair and Keshavachandran 2006).

From the present study, there are a number of directions that future studies can take. A considerable diversity of species and subspecies is found in East Africa, South Africa and Arabia, but this diversity is most likely a secondary effect of diversification in the two rounds of adaptation. Here, population-based studies would be desirable. In mainland Africa, a focus on ploidy levels within and between populations would be necessary, and so would be crossing experiments to determine the degree of integrity of the species and subspecies recognised. In Arabia, furthermore, pollination would need to be studied to understand the tendency for a reduced corona ring ($C_{(is)}$), which occurs only in this area. For the bigger picture of the biogeography of the *C. viminalis* complex, however, it is crucial to increase sampling in Madagascar and the Mascarenes as we expect to find the largest genetic diversity in this rather small geographical area. Last, but not least, the very recently developed methods for whole genome sequencing (Straub et al. 2011) raise hope that the hypotheses developed here can be tested with unambiguous markers in the future.

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