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Assessment of hybridization among wild and cultivated *Vigna unguiculata* subspecies revealed by arbitrarily primed polymerase chain reaction analysis

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Abstract

Background and aims Intra-species hybridization and incompletely homogenized ribosomal RNA repeat units have earlier been reported in 21 accessions of *Vigna unguiculata* from six subspecies using internal transcribed spacer (ITS) and 5S intergenic spacer (IGS) analyses. However, the relationships among these accessions were not clear from these analyses. We therefore assessed intra-species hybridization in the same set of accessions.

Methodology Arbitrarily primed polymerase chain reaction (AP-PCR) analysis was carried out using 12 primers. The PCR products were resolved on agarose gels and the DNA fragments were scored manually. Genetic relationships were inferred by TREECON software using unweighted paired group method with arithmetic averages (UPGMA) cluster analysis evaluated by bootstrapping and compared with previous analyses based on ITS and 5S IGS.

Principal results A total of 202 (86 %) fragments were found to be polymorphic and used for generating a genetic distance matrix. Twenty-one *V. unguiculata* accessions were grouped into three main clusters. The cultivated subspecies (var. *unguiculata*) and most of its wild progenitors (var. *spontanea*) were placed in cluster I along with ssp. *pubescens* and ssp. *stenophylla.* Whereas var. *spontanea* were grouped with ssp. *alba* and ssp. *tenuis* accessions in cluster II, ssp. *alba* and ssp. *baoulensis* were included in cluster III. Close affinities of ssp. *unguiculata,* ssp. *alba* and ssp. *tenuis* suggested inter-subspecies hybridization.

Conclusions Multi-locus AP-PCR analysis reveals that intra-species hybridization is prevalent among *V. unguiculata* subspecies and suggests that grouping of accessions from two different subspecies is not solely due to the similarity in the ITS and 5S IGS regions but also due to other regions of the genome.

Introduction

Vigna unguiculata is an important legume crop belonging to section *Catiang* in the subgenus *Vigna. Vigna* comprises a heterogeneous assemblage of 39 species of African origin that are divided into six sections, viz. *Vigna*, *Comosae*, *Macrodontae*, *Reticulatae*, *Liebrechtsia* and *Catiang* (Maxted *et al.* 2004). Disagreement still exists over the primary centres of domestication of *V. unguiculata*; however, based on the studies, different centres have been proposed such as Northeast Africa, West Africa, Ethiopia, Asia, etc. (Baudoin and Maréchal 1985; Pasquet 1996, 1999; Garba and Pasquet 1998;

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Coulibaly *et al.* 2002; Ba *et al.* 2004; Timko and Singh 2008). These include both wild and cultivated taxa of perennial and annual species, having high variation in their morphological characterization (Pasquet 1999). Cultivated species differ from wild species in having non-dehiscent pods, larger pods and non-dormant larger seeds (Lush and Evans 1981). Apart from these characters associated with domestication, some traits such as rhomboid leaves and anthocyanin pigmentation of the internodes, the length of the floral peduncle, photosensitivity and morphology of the seeds and pods are the principal variations in the cultivated forms.

Vigna unguiculata has 11 subspecies that differ from one another with respect to various morphological characteristics (Pasquet 1999; Maxted *et al.* 2004). Five of the subspecies, viz. *baoulensis*, *burundiensis*, *letouzeyi*, *aduensis* and *pawekiae*, are perennial, allogamously adapted to humid environments and are mainly recognized by their floral characteristics. Five other subspecies, viz. *alba*, *pubescens*, *tenuis*, *stenophylla* and *dekindtiana*, are wild, perennial, autogamous and are recognized by their vegetative traits showing their adaptation to drier and coastal environments. Only one subspecies is annual (ssp. *unguiculata*), comprising wild (var. *spontanea*) and cultivated (var. *unguiculata*) forms. The var. *spontanea* is a savanna taxon and often grows as a weed in and around cultivated fields. The cultivated forms of *V. unguiculata* are classified into five cultivar groups (cv.-gr.): *Unguiculata*, *Biflora, Sesquipedalis, Textilis* and *Melanophthalmus* (Pasquet 1998).

The relationship between the subspecies from subgenus *Vigna* has been analysed in the recent past by using different parameters, including morphological (Padulosi 1993) and biochemical parameters (seed storage proteins: Fotso *et al.* 1994; isozymes: Panella and Gepts 1992; Vaillancourt *et al.* 1993; Pasquet 1999, 2000). Molecular marker techniques, viz. AFLP (amplified fragment length polymorphism; Coulibaly *et al.* 2002), RAPD (randomly amplified polymorphic DNA; Ba *et al.* 2004), SSR (simple sequence repeat; Asare *et al.* 2010) and nrRNA (nuclear ribosomal RNA) spacer sequences (Saini 2005; Saini *et al.* 2008; Saini and Jawali 2009; Vijaykumar *et al.* 2010, 2011), have also been used to help understand relationships among the wild and cultivated accessions of *V. unguiculata*. Relationships among subspecies of most of the *Vigna* species could be analysed with the exception of *V. unguiculata*. The relationships among taxa belonging to *V. unguiculata* subspecies inferred by 18S-5.8S-26S rRNA ITS (internal transcribed spacer) did not show subspecies-specific clustering, but an intragenomic ITS variant was detected in an accession belonging to *V. unguiculata* ssp. *tenuis* (NI 1637; Vijaykumar *et al.* 2010). In addition, some accessions of *V. unguiculata* have very intriguing relationships among the 5S IGS (intergenic spacer) variants in relation to subspecies. This indicates extensive hybridization among the *V. unguiculata* subspecies, which is generally uncommon in rRNA gene units (Vijaykumar *et al.* 2011).

Arbitrarily amplified DNA markers are used extensively for reconstructing relationships among various species (Bussel *et al.* 2005). Randomly amplified polymorphic DNA has the ability to amplify DNA from dispersed polymorphic loci from the genome (Baral and Bosland 2002; Rasul *et al.* 2007; Wang *et al.* 2011) and has been used to assess genetic diversity among and between species/subspecies/cultivars of *Vigna* species (Kaga *et al.* 1996; Santalla *et al.* 1998; Lakhanpaul *et al.* 2000; Ba *et al.* 2004; Saini *et al.* 2004; Raturi *et al.* 2011). There are several studies where the same set of taxa has been analysed by single-locus sequence analysis as well as multi-locus techniques such as RAPD to get more insight into relationships at population, subspecies and species levels (Hess *et al.* 2000; Ortíz-Dorda *et al.* 2005; Qi *et al.* 2008; Wang *et al.* 2011).

The objective of the present study was to assess the genetic relationships among wild and cultivated *V. unguiculata* germplasm using a multi-locus marker technique, AP-PCR (arbitrarily primed polymerase chain reaction).

Materials and methods

Plant material

Twenty-one accessions of *V. unguiculata*, belonging to six subspecies (ssp. *unguiculata*, ssp. *tenuis*, ssp. *alba*, ssp. *pubescens*, ssp. *stenophylla* and ssp. *baoulensis*) obtained from the National Botanic Garden, Belgium (Meise collection), were used for the present study. The details of *Vigna* accessions used, along with the longitude and latitude coordinates are given in Table 1. The same set of accessions has also been used in previous studies (Vijaykumar *et al.* 2010, 2011).

DNA isolation, PCR amplification and agarose gel electrophoresis

Total DNA was isolated, purified and quantitated by procedures described in Vijaykumar *et al.* (2010). Arbitrarily primed PCR was carried out according to Saini *et al.* (2004) using 12 primers 18–23 bases in length (Table 2). The PCR mixture (25 μL) contained 1× reaction buffer (10 mM Tris-HCl pH 9.0, 2 mM $MgCl_2$) from Bangalore Genei Pvt Ltd (Bangalore, India), 0.2 μM primer (BRIT, Mumbai, India), 0.2 mM each dNTP (Roche Applied Science, Mannheim,

Table 1 ***Vgna unguiculata*** **accessions along with their country of origin and germplasm accession numbers.**

No.	*Vigna* accessions[a]	Section	Germplasm accession no.[b]	Country of origin[c]	Longitude and latitude
1	*V. unguiculata* (L.) Walp. ssp. *ung.* cv.-gr. *Unguiculata*	*Catiang*	NI 479	DR Congo	023 57 E, 06 45 S
2	*V. unguiculata* (L.) Walp. ssp. *ung.* cv.-gr. *Sesquipedalis*	*Catiang*	NI 269	China	NA
3	*V. unguiculata* (L.) Walp. ssp. *ung.* var. *spontanea*#	*Catiang*	NI 1405	Tanzania	039 13 E, 06 00 S
4	*V. unguiculata* (L.) Walp. ssp. *ung.* cv.-gr. *Textilis*	*Catiang*	NI 816	Togo	NA
5	*V. unguiculata* (L.) Walp. ssp. *ung.* var. *spontanea*#	*Catiang*	NI 1639	Namibia	021 40 E, 18 10 S
6	*V. unguiculata* (L.) Walp. ssp. *ung.* var. *spontanea*	*Catiang*	NI 1668	Kenya	040 54 E, 02 17 S
7	*V. unguiculata* (L.) Walp. ssp. *ung.* var. *spontanea*	*Catiang*	NI 1687	Yemen	044 00 E, 13 58 N
8	*V. unguiculata* (L.) Walp. ssp. *ung.* var. *spontanea*	*Catiang*	NI 1475	Malawi	034 07 E, 10 35 S
9	*V. unguiculata* (L.) Walp. ssp. *ung.* var. *spontanea*	*Catiang*	NI 1384	Botswana	027 25 E, 21 00 S
10	*V. unguiculata* (L.) Walp. ssp. *ung.* var. *spontanea*	*Catiang*	NI 945	Niger	003 26 E, 12 23 N
11	*V. unguiculata* (L.) Walp. ssp. *ung.* var. *spontanea*	*Catiang*	NI 319	D.R. Congo	023 57 E, 06 45 S
12	*V. unguiculata* (L.) Walp. ssp. *ung.* var. *spontanea*	*Catiang*	NI 1507	Zimbabwe	032 05 E, 19 57 S
13	*V. unguiculata* (L.) Walp. ssp. *tenuis*	*Catiang*	NI 1712	South. Africa	030 50 E, 30 10 S
14	*V. unguiculata* (L.) Walp. ssp. *tenuis*	*Catiang*	NI 1636	Zambia	027 35 E, 13 38 S
15	*V. unguiculata* (L.) Walp. ssp. *tenuis**	*Catiang*	NI 1637	Mozambique	032 50 E, 26 03 S
16	*V. unguiculata* (L.) Walp. ssp. *alba*	*Catiang*	NI 1652	Angola	013 15 E, 09 50 S
17	*V. unguiculata* (L.) Walp. *alba*	*Catiang*	NI 1388	Congo	011 51 E, 04 43 S
18	*V. unguiculata* (L.) Walp. ssp. *baoulensis*	*Catiang*	NI 1651	Ivory Coast	005 50 W, 06 43 N
19	*V. unguiculata* (L.) Walp. ssp. *stenophylla*	*Catiang*	NI 1478	Botswana	026 14 E, 23 55 S
20	*V. unguiculata* (L.) Walp. ssp. *pubescens*	*Catiang*	NI 856	Tanzania	038 23 E, 06 43 S
21	*V. unguiculata* (L.) Walp. ssp. *pubescens*	*Catiang*	NI 989	Kenya	039 46 E, 03 55 S

NA, information not available.
Hashes indicate *Vigna* accessions harbouring single 5S IGS (Vijaykumar *et al.* 2011).
An asterisk indicates *Vigna* accession harbouring intra-genomic ITS variant (Vijaykumar *et al.* 2010).
[a]List of *Vigna* accessions used. ssp., subspecies; *ung.*, *unguiculata*; var., variety; cv.-gr., cultivar-group.
[b]Accession numbers of the National Botanic Garden, Meise, Belgium.
[c]DR Congo, Democratic Republic of Congo.

Germany), 1.0 unit of *Taq* DNA polymerase (Bangalore Genei Pvt Ltd) and 100 ng of template genomic DNA. Amplified products were separated on a 2 % agarose (Sigma, Saint Louis, MO, USA) gel in 1× TBE at a constant voltage of 8 V/cm, stained with ethidium bromide according to Sambrook and Russell (2001) and photographed under UV light on a Gel-doc system from Syngene, Inc. (Cambridge, UK). Sizes of the PCR products were estimated by using GeneTools software of the Gel-doc system and comparing with the DNA size standards.

Data analysis

The AP-PCR was carried out twice and profiles were analysed manually; the amplified products were scored as present (1) or absent (0) for each primer–accession combination. Molecular data were used to compute genetic distances (Nei and Li 1979) within and between *V. unguiculata* subspecies using TREECON (version 1.3b; Van de Peer and De Watcher 1994; http://bioc-www.uia.ac.be/u/yvdp/treeconw.html) and a dendrogram was generated using the unweighted paired group method with arithmetic averages (UPGMA). Statistical analysis was carried out by the bootstrap method (Felsenstein 1985).

Results

DNA fingerprinting of *V. unguiculata* accessions

Initially, several long primers were screened for their utility in detecting polymorphism in two *V. unguiculata* accessions, viz. NI 479 and NI 1405 and 12 primers

Table 2 Characteristics of primers used for AP-PCR analysis along with the number of total and polymorphic bands obtained.

No.	Primer	Sequence	Length (bases)	T_m (°C)	G + C (%)	Total no. of bands	No. of polymorphic bands
1	SS 1.2	CTCGTCTGAGATCGGAGG	18	68	60	21	18
2	SS 5.1	GGAAGATGGTCATGGTGG	18	63	50	17	15
3	SS 9.1	GTACAGGACAAGATGCTT	18	58	45	23	18
4	SS 13.2	CAGGATGAGAGTTGGTTGGTAG	22	69	50	14	11
5	SS 19.1	GACATCTCTAGTGCACACAT	20	60	45	25	21
6	SS 24.1	TTTAATATCACCACCACACC	20	50	46	13	12
7	VM 3.2	GAGCCAGGGCACAGGTAGT	19	55	63	18	16
8	VM 5.1	AGCGACGGCAACAACGAT	18	50	56	25	23
9	VM 11.1	CGGGAATTAACGGAGTCACC	20	54	55	21	17
10	VM 13.2	GTCCCCTCCCTCCCACTG	18	57	72	22	20
11	VM 19.1	TATTCATGCGCCGTGACACTA	21	52	48	17	15
12	VM 71.1	TCGTGGCAGAGAATCAAAGACAC	23	55	48	17	16

were selected for further studies. These primers detected intra-specific variations generating scorable amplicons, reproducible patterns and generated 233 markers in the range of 100–3500 bp. Among these, 202 markers were polymorphic, amounting to 86.6 % polymorphism. Arbitrarily primed PCR amplification in the 21 *V. unguiculata* accessions using primers SS 9.1 and SS 5.1 is shown in Fig. 1A and B. The number of polymorphic bands obtained ranged from 11 (SS 13.2) to 23 (VM 5.1) (Table 2). On average, each AP-PCR primer generated ~19.4 scorable fragments and 16.8 polymorphic fragments.

Cluster analysis

The dendrogram obtained from the combined data of 12 primers delineated all the *V. unguiculata* accessions into three main clusters with low bootstrap values (Fig. 2). Cluster I was further divided into two subclusters (IA and IB). Subcluster IA comprised accessions belonging to *V. unguiculata* ssp. *unguiculata.* This subcluster included three cv.-gr. from *V. unguiculata* ssp. *unguiculata*, viz. cv.-gr. *Unguiculata*, cv.-gr. *Sesquipedalis* and cv.-gr. *Textilis*, along with six accessions of *V. unguiculata* ssp. *unguiculata* var. *spontanea*, the wild progenitors of cultivated *V. unguiculata* (*V. unguiculata* ssp. *unguiculata* var. *unguiculata*). The evolved cultivars, viz. cv.-gr. *Unguiculata,* cv.-gr. *Sesquipedalis* and cv.-gr. *Textilis*, grouped together with high (100 %) bootstrap values. Six out of the nine *V. unguiculata* ssp. *unguiculata* var. *spontanea* accessions were placed in subcluster IA and they clustered closely with the cultivated *V. unguiculata* accessions supported by high bootstrap values (84 and 59 %). In subcluster IA, the three var. *spontanea* from Eastern African countries, viz. NI 1686 (Kenya), NI 1405 (Tanzania) and NI 1475 (Malawi), were relatively divergent. Subcluster IB in cluster I included accessions belonging to two different subspecies of *V. unguiculata:* ssp. *pubescens* (NI 856, NI 989) and ssp. *stenophylla* (NI 1478). The clustering of the accessions of both these subspecies was supported by high (99 %) bootstrap values.

Cluster II comprised seven taxa belonging to three subspecies, viz. *V. unguiculata* ssp. *unguiculata, V. unguiculata* ssp. *tenuis* and *V. unguiculata* ssp. *baoulensis*. Three *V. unguiculata* ssp. *unguiculata* var. *spontanea* accessions from South African countries, viz. NI 1507 (Zimbabwe), NI 1639 (Namibia) and NI 1384 (Botswana), in cluster II were found to be divergent from the six *V. unguiculata* ssp. *unguiculata* var. *spontanea* accessions placed in cluster I (Fig. 2). Cluster III included only two accessions belonging to two different subspecies: ssp. *alba* (NI 1652, Angola) and ssp. *baoulensis* (NI 1651, Ivory Coast).

Discussion

Multi-locus marker techniques such as RAPD, AP-PCR, inter simple sequence repeat, AFLP, etc. have been extensively used for a variety of genetic analyses, including investigating genetic relationships; they can also provide valuable information on phylogenetics and systematics, if used appropriately (Bussell *et al.* 2005). These multi-locus markers have also been extensively used for analysis of species of Asian and African origin from the

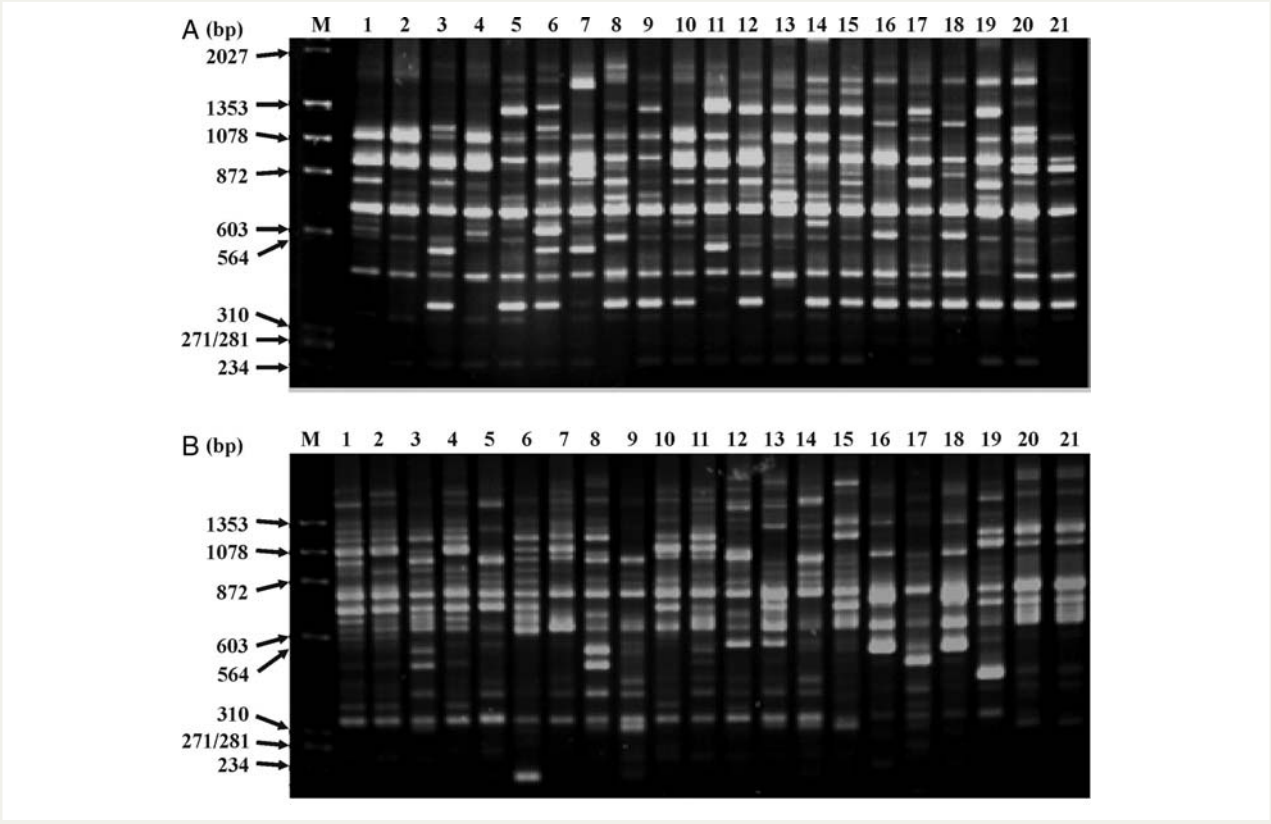

Fig. 1 AP-PCR profiles of *V. unguiculata* genotypes obtained with primers SS9.1 (A) and SS5.1 (B). Lane 1: *V. u.* ssp. *unguiculata* cv.-gr. *Unguiculata* (NI 479); Lane 2: *V. u.* ssp. *unguiculata* cv.-gr. *Sesquipedalis* (NI 269); Lane 3: *V. u.* ssp. *unguiculata* var. *spontanea* (NI 1405); Lane 4: *V. u.* ssp. *unguiculata* cv.-gr. *Textilis* (NI 816); Lane 5: *V. u.* ssp. *unguiculata* var. *spontanea* (NI 1639); Lane 6: *V. u.* ssp. *unguiculata* var. *spontanea* (NI 1668); Lane 7: *V. u.* ssp. *unguiculata* var. *spontanea* (NI 1687); Lane 8: *V. u.* ssp. *unguiculata* var. *spontanea* (NI 1475); Lane 9: *V. u.* ssp. *unguiculata* var. *spontanea* (NI 1384); Lane 10: *V. u.* ssp. *unguiculata* var. *spontanea* (NI 945); Lane 11: *V. u.* ssp. *unguiculata* var. *spontanea* (NI 319); Lane 12: *V. u.* ssp. *unguiculata* var. *spontanea* (NI 1507); Lane 13: *V. u.* ssp. *tenuis* (NI 1712); Lane 14: *V. u.* ssp. *tenuis* (NI 1636); Lane 15: *V. u.* ssp. *tenuis* (NI 1637); Lane 16: *V. u.* ssp. *alba* (NI 1652); Lane 17: *V. u.* ssp. *alba* (NI 1388); Lane 18: *V. u.* ssp. *baoulensis* (NI 1651); Lane 19: *V. u.* ssp. *stenophylla* (NI 1478); Lane 20: *V. u.* ssp. *pubescens* (NI 856); Lane 21: *V. u.* ssp. *pubescens* (NI 989); Lane M contains a mixture of ϕX174 DNA (HaeIII digest) and λ (HindIII digest).

genus *Vigna* (Li *et al.* 2001; Coulibaly *et al.* 2002; Ba *et al.* 2004; Dikshit *et al.* 2005; Ruchi *et al.* 2009; Raturi *et al.* 2011). In the present study, the AP-PCR profiles of 21 taxa belonging to different subspecies of *V. unguiculata* showed moderate divergence and were used to determine their genetic relationships. The same set of taxa was previously analysed using ITS (Vijaykumar *et al.* 2010) and 5S IGS (Vijaykumar *et al.* 2011) regions. Hence, the phylogenetic trees obtained from these analyses were compared to understand how some of the taxa have evolved.

In general, the analyses of species/subspecies using multi-locus genome-wide marker analyses show clustering at the species as well subspecies level. On the contrary, the dendrogram obtained from the AP-PCR analyses of the taxa belonging to *V. unguiculata* did not show clear-cut grouping of taxa at the subspecies level. Out of the 12 taxa belonging to *V. unguiculata* ssp. *unguiculata*, nine were placed in cluster 1A, and the other three were grouped with other subspecies, albeit at low bootstrap values. Among the other subspecies (ssp. *pubescens*, ssp. *tenuis* and ssp. *alba*) where more than one taxa was analysed, two accessions of ssp. *pubescens* were placed together (in subcluster IB) along with ssp. *stenophylla*. Accessions of both ssp. *tenuis* and ssp. *alba* also showed divergence and did not cluster together. Accession NI 1388 of ssp. *alba* was placed in cluster II along with *V. unguiculata* ssp. *tenuis* and *V. unguiculata* var. *spontanea*, while NI 1652 was placed in cluster III with ssp. *baoulensis*. The three accessions (NI 1636, NI 1637 and NI 1712) of ssp. *tenuis*, though placed in cluster II, were highly divergent.

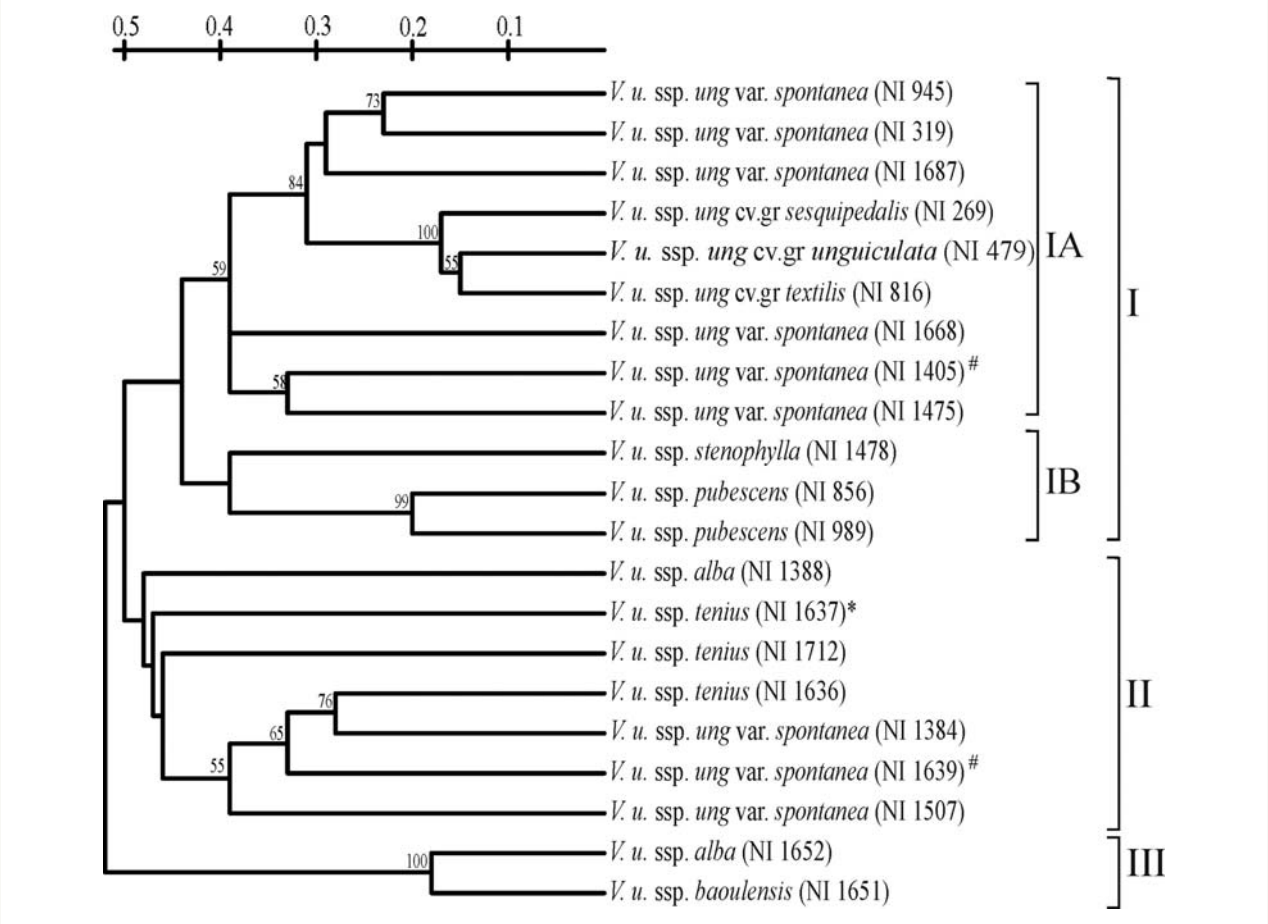

Fig. 2 UPGMA cluster analysis of 21 *V. unguiculata* genotypes based on the AP-PCR marker data. Numbers at nodes indicate bootstrap values (in %) for a 1000-replicate analysis. An asterisk indicates the *V. u.* ssp. *tenuis* accession that showed intra-genomic ITS (Vijaykumar *et al.* 2010) and hashes indicate the *V. u.* ssp. *unguiculata* var. *spontanea* accessions that showed single type 5S IGS (Vijaykumar *et al.* 2011).

Accession NI 1637, which harboured intra-genomic ITS variant (Vijaykumar *et al.* 2010) was placed in cluster II and showed more affinity towards ssp. *unguiculata* var. *spontanea* (NI 1384) compared with the other two ssp. *tenuis* accessions (NI 1637, NI 1712) (Fig. 2). Our results are also in agreement with previous reports (Pasquet 1999, 2000; Coulibaly *et al.* 2002).

The present AP-PCR analysis clearly delineated cultivated *V. unguiculata* accessions from wild types. The present analyses placed the majority of the ssp. *unguiculata* var. *spontanea* accessions with the cultivated *V. unguiculata* in cluster I. However, three ssp. *unguiculata* var. *spontanea* accessions, viz., NI 1384, NI 1507 and NI 1639, were highly divergent and grouped with ssp. *tenuis* and ssp. *alba* in cluster II. Two of these accessions, i.e. NI 1384 and NI 1639 (=SP 160), were part of the var. *spontanea* BWA group (includes accessions from Botswana) in Pasquet (1999); however, NI 1507 (=MT 76) was not, which reinforces the hybridization origin of this group of var. *spontanea* accessions from southern Africa. A close relationship between NI1639 and NI 1384 was also observed based on the ITS analysis (Vijaykumar *et al.* 2010). The clustering of var. *spontanea* with ssp. *tenuis* and ssp. *alba* may be the result of hybridization between these taxa. These results confirm that, in general, the genomes of ssp. *unguiculata* var. *spontanea*, ssp. *tenuis* and ssp. *alba* have indeed undergone hybridization, and this has led to their convergence with accessions of other subspecies. Accession NI 1637 of ssp. *tenuis* was grouped with two other accessions of ssp. *tenuis* (NI 1712, NI 1636); however, this accession harboured intra-individual ITS and 5S IGS variants (Vijaykumar *et al.* 2010, 2011). In the case of ITS analysis, the two variants were found to have originated as a result of hybridization between ssp. *tenuis* and ssp. *pubescens*. However, AP-PCR analysis revealed that the major

proportion of the genome of the NI 1637 accession is close to ssp. *tenuis*. This indicates that although rRNA genes (18S-5.8S-26S rRNA and 5S rRNA) have not been homogenized subsequent to subspecies hybridization in NI 1637 (Vijaykumar *et al.* 2010, 2011), the genome is homogenized towards ssp. *tenuis*. The present analysis grouped one accession each from ssp. *baoulensis* (NI 1651) and ssp. *alba* (NI 1652) in cluster III (Fig. 2). However, this accession of ssp. *alba*, based on ITS, was found to be close to ssp. *unguiculata* var. *spontanea* (NI 1384 and NI 1639). This is in accordance with previous studies based on AFLP (Coulibaly *et al.* 2002) and isozymes (Pasquet 1999). The three ssp. *unguiculata* var. *spontanea* accessions, viz. NI 1384, NI 1507 and NI 1639, also appear to be a product of hybridization between ssp. *unguiculata* and ssp. *tenuis*.

The accession NI 1478 belonging to ssp. *stenophylla* showed close affinity to NI 1475 from ssp. *unguiculata* on the basis of rRNA gene analyses (Vijaykumar *et al.* 2010, 2011). Under the present investigation it grouped with ssp. *pubescens*, suggesting that NI1478 may have undergone hybridization with ssp. *unguiculata* and ssp. *pubescens,* during the course of evolution. However, more detailed studies would be required to prove these aspects, as perfect assessment of positions of distant accessions may not be possible solely on the basis of AP-PCR marker analysis.

In general, different taxa of the same species/genus are analysed by different approaches and the results inferred from such studies are often not directly comparable. If done appropriately, studies on the same set of taxa using a multiple marker system help in understanding the genetic relationships at subspecies/population level, along with evolutionary/phylogenetic relationships at species level, and also allow us to decipher how different *loci vis-á-vis* genome have evolved. Furthermore, multi-locus markers are also useful when single-locus regions fail to resolve phylogenetic/genetic relationships (Després *et al.* 2003). Despite these advantages, studies on the same set of taxa using multiple markers are not often carried out and only a few molecular phylogenetic studies have been reported where the rRNA gene sequences and multi-locus marker analysis have been performed using the same set of taxa (Blattner *et al.* 2001). Arbitrarily primed-PCR techniques have also been explored to study the genetic relationships among species in combination with sequence-based analysis (Hess *et al.* 2000; Jorgensen *et al.* 2003; Ortíz-Dorda *et al.* 2005; Qi *et al.* 2008; Wang *et al.* 2011). The same set of accessions has been used for assessment of phylogenetic relationships using both multi-locus and single-locus markers in different species such as *Olea europaea* complex (Hess *et al.* 2000), *Trollius* species complex (Després *et al.* 2003), *Atriplex halimus* (Ortíz-Dorda *et al.* 2005), genus *Rehmannia* (Qi *et al.* 2008), genus *Zea* (Wang *et al.* 2011) and *Vigna* (Raturi *et al.* 2011).

In the present investigation we have used the same set of *V. unguiculata* accessions and the results were very useful in understanding the evolution of some of the species/taxa analysed. Our previous studies (Vijaykumar *et al.* 2010, 2011) suggest that intra-species hybridization has affected the rRNA gene loci during the course of evolution. Consequently, it was difficult to infer phylogenetic relationships among certain *V. unguiculata* subspecies. Hence, the present analysis was carried out to infer the relationships among the same set of taxa using information generated from multi-locus markers that can analyse many regions of the genome. The results presented in this study suggest that regions of the *V. unguiculata* genome other than ITS and 5S IGS also show evidence of inter-subspecies hybridization.

Our previous study on the analysis of *Vigna* species from subgenus *Vigna* (African *Vigna*) using the 18S-5.8S-26S nrDNA ITS region gave evidence of hybridization and slow molecular drive of the repeat units. An accession of ssp. *tenuis* harboured an additional ITS variant (more similar to ssp. *pubescens*); however, it was not active transcriptionally. Furthermore, certain accessions of ssp. *unguiculata*, ssp. *tenuis* and ssp. *alba* showed close relationships with other subspecies. This suggested that these accessions have also undergone hybridization but subsequently the ITS sequences have homogenized towards the other subspecies (Vijaykumar *et al.* 2010). Analysis of the same set of taxa by 5S IGS region also suggested hybridization among several *V. unguiculata* subspecies (Vijaykumar *et al.* 2011). The 5S IGS sequences were homogenized to a much lower extent as compared with the ITS sequences, although the number of 5S loci in *V. unguiculata* were reported to be lower than 18S-5.8S-25S genes (Galasso *et al.* 1995).

The evidence for inter-subspecies hybridization among the *V. unguiculata* accessions came from the fact that despite using a large number of markers, relationships among accessions of certain subspecies (ssp. *unguiculata* var. *spontanea*, ssp. *alba* and ssp. *tenuis*) in cluster II were not supported by high bootstrap values. The low bootstrap values have been attributed to very frequent intercrossing among the taxa analysed (Barkley *et al.* 2006; Chao *et al.* 2007; Chapuis *et al.* 2008). Our results show that subspecies relationships within *V. unguiculata* cannot be inferred and this could be due to intra-subspecies hybridization. Similar findings were recorded in our previous studies using rRNA gene analysis (Vijaykumar *et al.* 2011).

The results from the present analyses confirm that the *V. unguiculata* subspecies have undergone inter-subspecies hybridization and introgression, with most taxa maintaining the variant 5S IGS sequences while very few maintain variant ITS sequences. Hence, irrespective of the actual multi-locus marker analysis, the relationships among the subspecies could not be clearly inferred.

Conclusions and forward look

The present analysis of *V. unguiculata* subspecies using AP-PCR markers clearly shows that extensive hybridization has occurred among certain subspecies. The same set of taxa when previously analysed by ITS and 5S IGS sequences had shown evidence of hybridization and incomplete homogenization of rRNA repeat units. The present study based on multi-locus analysis further substantiates the previous findings on intra-subspecies hybridization events among certain *V. unguiculata* subspecies. The present study thus advocates further in-depth analysis of such interactions among the *V. unguiculata* subspecies, along with eco-geographical parameters.

Contributions by the authors

The project was conceived and planned by N.J. The major experimental part of the study was carried out by A.V. as part of her PhD thesis. Some parts of the experiments and data analysis were done by A.S.

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Conflict of interest statement

None declared.

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