Genetic diversity and phylogenetic relationships in *Vigna* Savi germplasm revealed by DNA amplification fingerprinting

M.V. Simon, A.-M. Benko-Iseppon, L.V. Resende, P. Winter, and G. Kahl

Abstract: The pantropical genus *Vigna* (Leguminosae) comprises 7 cultivated species that are adapted to a wide range of extreme agroclimatic conditions. Few data are available on the relationships among these cultivated species or on their importance as sources of resistance against biotic and abiotic stresses. Therefore, we optimized DNA amplification fingerprinting (DAF) to estimate the genetic diversity within, and genetic relationships among, a representative core collection of cowpea, as compared with 16 accessions representing cultivars from 6 *Vigna* species. A set of 26 primers was selected from 262 tested random primers and used for the characterization of 85 *Vigna* accessions (6 *V. angularis*, 4 each of *V. mungo* and *V. radiata*, 2 *V. umbellata*, 1 *V. aconitifolia*, and 68 *V. unguiculata*), with *Phaseolus vulgaris* subsp. *vulgaris* as outgroup. A total of 212 polymorphic bands were used for maximum parsimony analysis. Our results clearly distinguished Brazilian from African *V. unguiculata* genotypes. At the species level, *V. angularis* was the most related and *V. radiata* the most divergent species relative to *V. unguiculata*. DAF markers were also informative at the intraspecific level, detecting a large diversity between cowpea cultivars. The implications of the presented results for cowpea breeding programs are discussed.

Key words: cowpea, molecular markers, germplasm, crop evolution.

Résumé : Le genre pantropical *Vigna* (Leguminosae) comprend 7 espèces cultivées qui sont adaptées à une vaste gamme de conditions agro-climatiques extrêmes. Peu de données sont disponibles sur les relations entre ces espèces cultivées ou sur leur importance en tant que sources de résistance contre des stress biotiques et abiotiques. L'auteur a optimisé un protocole de production d'empreintes génétiques (DAF; « DNA amplification fingerprinting ») pour estimer la diversité génétique intraspécifique et les relations génétiques interspécifiques au sein d'une collection représentative du niébé et vis-à-vis 16 accessions représentant des cultivars de 6 espèces du genre *Vigna*. Un jeu de 26 amorces a été choisi parmi 262 amorces testées aléatoirement et a été employé pour la caractérisation de 85 accessions de *Vigna* (6 *V. angularis*, 4 chacune du *V. mungo* et du *V. radiata*, 2 du *V. umbellata*, 1 du *V. aconitifolia* et 68 accessions du *V. unguiculata*) en utilisant le *Phaseolus vulgaris* subsp. *vulgaris* comme groupe externe. Un total de 212 bandes a été employé dans le cadre d'une analyse de parcimonie maximale. Ces résultats ont permis de distinguer clairement les génotypes brésiliens des génotypes africains du *V. unguiculata*. Quant aux espèces, le *V. angularis* était l'espèce la plus apparentée au *V. unguiculata* alors que le *V. radiata* était la plus distante. Les marqueurs DAF se sont également avérés informatifs au niveau intraspécifique en permettant de détecter une grande diversité entre cultivars du niébé. Les implications des résultats présentés pour les programmes d'amélioration génétique du niébé sont discutées.

Mots-clés : niébé, marqueurs moléculaires, ressources génétiques, espèces cultivées, évolution.

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Introduction

Knowledge of the relatedness of important crop species and wild relatives has been a major goal of, and prerequisite for, many breeding programs, since wild species are often a repository of agronomically important traits. Additionally, the genetic characterization of different germplasm accessions is of prime importance for gene bank management, since it allows better exploitation of the existing gene pool, more efficient sampling of the available germplasm resources, and improved identification of the genetic variation for breeding.

Grain legumes are among the most important crops in many countries, since they provide nearly one-quarter of the world's dietary protein. For approximately 700 million people, grain legumes are an essential source of protein (Nagl et al. 1997). Particularly in the developing countries of South

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America, Africa, and Asia, where plants provide 83% of total protein in the average diet (Mahe et al. 1994), grain legumes are prime candidates for combating shortages in food supply.

The genus *Vigna* of the Leguminosae is pantropical and comprises 170 species, of which 120 occur in Africa (66 endemic), 22 in India, Southeast Asia (16 endemic), and a few in America and Australia (Faris 1965). These species are adapted to a wide range of extreme environmental conditions. They grow in poor soils without supplementary nitrogen and, therefore, are particularly advantageous for subsistence agriculture (Santalla et al. 1998).

For a long period of time Brazil was the second largest producer of cowpeas (*V. unguiculata* (L.) Walp.) worldwide, contributing 26% of the world's and 82% of the American continent's production (Watt et al. 1988). Nowadays, Brazil's production ranks third after Nigeria's and Niger's. According to official Brazilian estimates, cowpea is cultivated on approximately 1.5 million ha, especially in the northeastern region, with an annual production of 500 000 t nurturing approximately 25 million people (IBGE 1998).

Cowpea was probably introduced to Brazil from Europe and West Africa by European colonizers and African slaves during the 16th and 17th centuries. The traditional landraces, selected for desirable traits, have been maintained by small communities throughout the last four centuries (Freire-Filho 1988). About 3.6 million slaves were brought to Brazil, directly from Africa, most of them to the ports of Recife, Salvador, Rio de Janeiro, and São Luiz. Historical records indicate that most of the slaves who arrived directly came from Angola, Mozambique, and Congo. They brought much of their culture and also seeds from plants used in their favorite dishes or in rituals, as was the case for cowpea. A number of communities known as *mocambos* or *quilombos* were founded in the Brazilian northeastern region by escaped slaves. Several *quilombos* remain in relative isolation to this day, preserving much of their original identity (Curtin 1969).

A large active cowpea germplasm has been maintained by Embrapa (Empresa Brasileira de Pesquisa Agropecuária) in Teresina, state of Piauí, including 3618 cowpea accessions and 37 accessions of 8 other *Vigna* species (Freire-Filho et al. 1999).

Only a few studies have used molecular markers for *Vigna* germplasm characterization so far. However, linkage maps employing isoenzymes, RFLPs (restriction fragment length polymorphisms), RAPDs (random amplified polymorphic DNAs), and AFLPs (amplified fragment length polymorphisms), sometimes associated with morphological characters, are already established (Menancio-Hautea et al. 1993*a*, 1993*b*; Kaga et al. 1996; Menéndez et al. 1997; Ouédragono et al. 2001).

The genetic diversity in cowpea was estimated by morphological and physiological traits (Ehlers and Hall 1997), but the genetic relatedness of cowpea cultivars and other *Vigna* species is still hardly understood. Pasquet (1996*a*) characterized about 150 wild accessions of *V. unguiculata* with 20 isozymes and 35 morphological characters to investigate the evolutionary history of the group. He suggested northeastern Africa as the putative domestication centre of the crop and *V. unguiculata* var. *spontanea* as the most probable ancestor of cultivated cowpea. In 191 cultivated accessions of *V. unguiculata,* Pasquet (1996*b*) detected only low levels of isozyme polymorphisms, though later on he observed discrete variations in 21 isozymes on 271 cowpea accessions from different geographic regions (Pasquet 2000).

The relationships between 4 African and Asian *Vigna* species were assessed with isoenzymes by Jaaska (1999), who revealed 5 major monophyletic groups distinguishing species from the 2 continents. In the generated cladogram, *V. radiata* (L.) Wilcz. appeared as a basal clade close to *V. aconitifolia* (Jacq.) Marechal and *V. umbellata* (Thunb.) Ohwi & Ohashi, with *V. unguiculata* in a separate branch.

DNA marker variability within the genus *Vigna* has been studied by only 2 groups. Santalla et al. (1998) analyzed 19 genotypes of *V. radiata* and compared different genotypes of the 3 wild taxa *V. mungo* (L.) Hepper, *V. luteola* (Jacq.) Benth., and *V. radiata* subsp. *sublobata*. The different *V. radiata* accessions grouped together and were clearly distinguished from the remaining species positioned in different branches. More recently, Li et al. (2001) employed 46 microsatellite markers to identify relationships between 90 cowpea breeding lines developed at the International Institute of Tropical Agriculture (IITA, Ibadan, Nigeria). Many of them were polymorphic, and only 27 presented polymorphisms. The resulting dendrogram was consistent with the known pedigree of the cowpea lines.

The present study represents the first DNA marker-based evaluation of a larger set of cowpea accessions, also comparing Brazilian and African cowpea genotypes, some of them wild, representing important sources of resistance against biotic and abiotic stresses. Also, up to now, no DNA markers have been applied to detect the interspecific relatedness of cowpea with other cultivated species of the genus. We employed a modified version of the DNA amplification fingerprint technique (DAF, Caetano-Anollés et al. 1991), which is comparable to RAPD (Welsh and McClelland 1990) but requires less DNA (0.1 to 1 ng/μL) and much higher primer concentrations. We chose DAF because in a mapping project in chickpea (*Cicer arietinum* L.), DAF was superior to RAPD, especially since banding patterns were highly reproducible and polymorphic despite the extensive monotony of the chickpea genome (Winter et al. 2000; Benko-Iseppon et al. 2003; Rakshit et al. 2003).

Important accessions and traditional parental lines used in breeding programs have been selected for the present evaluation, which will assist future planning of breeding strategies, especially regarding planned crosses for mapping purposes. Thus, this study aims at evaluating Brazilian and African cultivars of cowpea, highly adapted to local climatic conditions, as compared with other cultivated species and their accessions.

Materials and methods

Plant material and DNA extraction

In this study 85 genotypes of 7 different *Vigna* species were analysed. These comprised 6 accessions of *V. angularis* (Willd.) Ohwi & Ohashi, 4 accessions of both *V. mungo* and *V. radiata*, 2 accessions of *V. umbellata,* 1 accession of *V. aconitifolia*, and 68 accessions of *V. unguiculata* (Table 1). Among these, 12 genotypes of

V. unguiculata subsp. *unguiculata* came from wild collections maintained in germplasm banks. Four accessions of near relatives of *V. unguiculata* were also used, namely 2 of subsp. *sesquipedalis* (L.) Verdc. and 2 of subsp. *cylindrica* (L.) Verdc. Additionally, 4 cultivars of *Phaseolus vulgaris* L. subsp. *vulgaris*, 'Neckarkönigin', 'Delinel', 'Odeon', and 'Jutta' (Germany), were employed as outgroup for the phylogenetic analysis.

Prior to sowing, the seeds were surface-sterilized with 4% sodium hypochlorite, and 4 plants per pod were cultivated in 5 kg of a mixture of 2 parts soil and 1 part manure. DNA was isolated from young leaflets using a modified CTAB (cetyl trimethylammonium bromide) protocol (Weising et al. 2005). Contaminating polysaccharides were selectively precipitated (Michaels et al. 1994), and DNA concentrations were determined electrophoretically using known amounts of phage λ DNA as reference.

DNA amplification fingerprinting and electrophoresis

DAF followed the procedure of Caetano-Anollés et al. (1991) with the following modifications: PCR was carried out on a PerkinElmer GeneAmp® 9700 thermal cycler using random primers procured from Eurogentec (Cologne, Germany), Operon Technologies (Alameda, California), or Roth (Karlsruhe, Germany). Each 15 μL PCR reaction contained 1.5 μL 10× PCR buffer (Eurogentec), 2.5 mmol/L $MgCl_2$, 10 mmol/L deoxynucleoside triphosphate mix; 0.4 U "Silverstar" *Taq* DNA polymerase (Eurogentec), 40 pmol oligonucleotide primer, and 1 ng/μL of template DNA. The DNA was first denatured for 2 min at 95 °C, followed by 40 cycles of 15 s denaturation at 95 °C, 1 min annealing at 35 °C, and 2 min elongation at 72 °C, and final elongation at 72 °C for 2 min. The reaction products were separated on 1.8% agarose gels, stained with ethidium bromide, and visualized under ultraviolet light.

Primer selection

For the identification of informative primers a total of 262 oligonucleotides were initially tested on 4 *Vigna* genotypes: 2 accessions of *V. unguiculata* (IPA 204 and CNC 0434), 1 accession of *V. angularis* (PHA 8023/79), and 1 accession of *V. umbellata* (PHA 8126/79) (Table 1). The oligonucleotides included 214 decamers, 10 octamers, 18 15-mers, and 20 mini-hairpin decamers.

Data analysis

Phylogenetic analysis of the DAF data was performed using the program MEGA (Molecular Evolutionary Genetic Analysis), Version 2 for Windows, kindly provided by the authors (Kumar et al. 2004), using maximum parsimony and neighbour-joining methods (bootstrap analysis, 1000 replicates). The resulting consensus tree was created with the program TreeView for Windows (Page 1996), kindly provided by Dr. Robert Page (University of Glasgow, Scotland).

Results

In the first screening, 262 different arbitrary primers were tested on 4 *Vigna* genotypes: 2 Brazilian accessions of *V. unguiculata* (IPA 204 and CNC 0434) and 1 accession each of *V. angularis* (PHA 8023/79) and *V. umbellata* (PHA 8126/79). From this initial screening, 26 primers emerged as highly informative at the intra- and interspecific level (Table 2); they amplified clear, reproducible bands in DAF reactions and were therefore used on all accessions listed in Table 1.

Amplifications with the selected primers produced an average of 14.8 amplicons with 13.6 polymorphisms between *Vigna* species and 1.2 polymorphisms at the intraspecific level between *V. unguiculata* accessions. The most informative primer was OP-K08 with 19 polymorphic bands at the interspecific level and 3 bands at the intraspecific level in *V. unguiculata*. A total of 212 bands were considered for the construction of the data matrix. Figure 1 shows a representative DAF amplification profile with polymorphisms at the intra- and interspecific levels.

Results obtained after data matrix analysis with MEGA (bootstrap analysis, 1000 replicates) are presented in Fig. 2. With the exception of *V. unguiculata* genotypes, all remaining species were grouped together in 1 clade, which was clearly divided into 3 groups. One group was represented by a single accession of *V. aconitifolia*, from which both the other clades emerged. The second group consisted of 6 *V. angularis* and 2 *V. umbellata* accessions, positioned at the base of this clade. The third clade was represented by 2 groups, 1 with 4 accessions of *V. mungo* and the other with 5 accessions of *V. radiata* (Fig. 2).

The phylogenetic tree of the *V. unguiculata* accessions was based on 2 accessions (IT86D472 and IT86D535) originally sent from IITA to Empresa Brasileira de Pesquisa Agropecuária (EMBRAPA, Brazil) (Table 1). From this clade, a clade containing Asian (China, India, Iraq, and Nepal) and African accessions (Angola, Egypt, and Ghana) received from IPK (Institut für Pflanzengenetik und Kulturpflanzenforschung Gatersleben, Germany) and a single accession cultivated in Colombia, also received from IPK, branched off (Table 2; Fig. 2). The Brazilian genotypes 'Canapu Amarelo' and 'IPA 206' are at the base of all Brazilian cultivars (Fig. 2). Only a few non-Brazilian accessions were part of these clades, including a *V. unguiculata* genotype from a street market in Istanbul, Turkey, that was grouped together with accession KEW 73260 (from the UK) and lineages of the Brazilian TE group (Table 1, Fig. 2).

Discussion

Both branching and distribution of genotypes in the cladogram tree are highly consistent with their taxonomic position (e.g., Maréchal et al. 1978) and the pedigree of some Brazilian accessions (Freire et al. 1999), confirming *Phaseolus* as an adequate outgroup for the analysis of different *Vigna* species and accessions.

For the evaluation of these characters, DAF proved to be highly efficient for the generation of informative molecular markers. The results were highly reproducible and displayed considerable levels of polymorphisms at the intra- and interspecific levels. A total of 212 bands have been considered for the construction of the data matrix, generated from only 26 previously selected primers. This represents an average of 13.6 polymorphisms per primer. Our approach was definitely more efficient for the detection of polymorphisms

Table 1. Accession numbers and origin of *Vigna* genotypes used in the present study.

Taxon	Accession No.	Germplasm bank	Original source or area of cultivation
V. aconitifolia	PHA 8150/80	IPK	India
V. angularis	PHA 8211/85	IPK	Georgia
V. angularis	PHA 8135/80	IPK	Ethiopia
V. angularis	PHA 8125/81	IPK	Korea
V. angularis	PHA 8124/79	IPK	Japan
V. angularis	PHA 8023/79	IPK	Japan
V. angularis	Carl Pabst, no No.	—	Germany
V. mungo	1619 VG	BGM	Africa
V. mungo var. *mungo*	L 1677/91	IPK	Zaire
V. mungo	PHA 8096/97	IPK	Afghanistan
V. mungo	TRS Urid.	KEW	India
V. radiata	PHA 8129/79	IPK	Egypt
V. radiata	PHA 8051/76	IPK	Greece
V. radiata	Carl Pabst, no No.	—	China
V. radiata var. *radiata*	PHA 8055/76	IPK	China
V. umbellata	PHA 8126/76	IPK	Indonesia
V. umbellata	PHA 8127/79	IPK	Indonesia
V. unguiculata subsp. *unguiculata*	821 'Vita 6'	IPA	Brazil
V. unguiculata subsp. *unguiculata*	'Balinha' 305	IPA	Brazil
V. unguiculata subsp. *unguiculata*	'BR-17 Burguês'	EMBRAPA	Brazil
V. unguiculata subsp. *unguiculata*	'BR-14 Mulato'	EMBRAPA	Brazil
V. unguiculata subsp. *unguiculata*	'BR-17 Burguês-G'	EMBRAPA	Brazil
V. unguiculata subsp. *unguiculata*	'BR-9 Longá'	EMBRAPA	Brazil
V. unguiculata subsp. *unguiculata*	'Cabeçudo'	IPA	Brazil
V. unguiculata subsp. *unguiculata*	'Canapu'	EMBRAPA	Brazil
V. unguiculata subsp. *unguiculata*	'Canapu amarelo'	IPA	Brazil
V. unguiculata subsp. *unguiculata*	'Canapu precoce'	IPA	Brazil
V. unguiculata subsp. *unguiculata*	CB-3	EMBRAPA	Brazil
V. unguiculata subsp. *unguiculata*	CNC 0434	IPA	Brazil
V. unguiculata subsp. *unguiculata*	CNC 1115-16F	IPA	Brazil
V. unguiculata subsp. *unguiculata*	CNCX 1010-4F	IPA	Brazil
V. unguiculata subsp. *unguiculata*	CNCX 1101	IPA	Brazil
V. unguiculata subsp. *unguiculata*	CNCX 1112-4F	IPA	Brazil
V. unguiculata subsp. *unguiculata*	CNCX 1114-4F	IPA	Brazil
V. unguiculata subsp. *unguiculata*	CNCX 1115-11F	IPA	Brazil
V. unguiculata subsp. *unguiculata*	CNCX 1115-18F	IPA	Brazil
V. unguiculata subsp. *unguiculata*	CNCX 1115-20F	IPA	Brazil
V. unguiculata subsp. *unguiculata*	'EPACE 1'	IPA	Brazil
V. unguiculata subsp. *unguiculata*	'EPACE 10'	IPA	Brazil
V. unguiculata subsp. *unguiculata*	'EPACE 11'	IPA	Brazil
V. unguiculata subsp. *unguiculata*	'IPA 201'	IPA	Brazil
V. unguiculata subsp. *unguiculata*	'IPA 202'	IPA	Brazil
V. unguiculata subsp. *unguiculata*	'IPA 204'	IPA	Brazil
V. unguiculata subsp. *unguiculata*	'IPA 205'	IPA	Brazil
V. unguiculata subsp. *unguiculata*	'IPA 206'	IPA	Brazil
V. unguiculata subsp. *unguiculata*	'IPA 02'	IPA	Brazil
V. unguiculata subsp. *unguiculata*	IPEAN V-69	IPA	Brazil
V. unguiculata subsp. *unguiculata*	IT 82D699	IITA	Africa
V. unguiculata subsp. *unguiculata*	IT 85D3850-2	IITA	Africa
V. unguiculata subsp. *unguiculata*	IT 86D472	IITA	Africa
V. unguiculata subsp. *unguiculata*	IT 86D535	IITA	Africa
V. unguiculata subsp. *unguiculata*	'João Paulo II'	EMBRAPA	Brazil
V. unguiculata subsp. *unguiculata*	L 382002-A	EMBRAPA	Brazil
V. unguiculata subsp. *unguiculata*	L351002A	EMBRAPA	Brazil
V. unguiculata subsp. *unguiculata*	L539001(T16)	EMBRAPA	Brazil
V. unguiculata subsp. *unguiculata*	L775011	EMBRAPA	Brazil

Table 1 (*concluded*).

Taxon	Accession No.	Germplasm bank	Original source or area of cultivation
V. unguiculata subsp. *unguiculata*	L950002	IPA	Brazil
V. unguiculata subsp. *unguiculata*	L9556002	EMBRAPA	Brazil
V. unguiculata subsp. *unguiculata*	'Manaus'	EMBRAPA	Brazil
V. unguiculata subsp. *unguiculata*	MRC11213	EMBRAPA	Brazil
V. unguiculata subsp. *unguiculata*	'Paulista'	IPA	Brazil
V. unguiculata subsp. *unguiculata*	Santa Inácia BIV411	EMBRAPA	Brazil
V. unguiculata subsp. *unguiculata*	'Sempre Verde'	EMBRAPA	Brazil
V. unguiculata subsp. *unguiculata*	TE 867517 E-2	IPA	Brazil
V. unguiculata subsp. *unguiculata*	TE 867556 E	IPA	Brazil
V. unguiculata subsp. *unguiculata*	TE 90169-4F	IPA	Brazil
V. unguiculata subsp. *unguiculata*	TE 90179-9F	IPA	Brazil
V. unguiculata subsp. *unguiculata*	TE 90180-6F	IPA	Brazil
V. unguiculata subsp. *unguiculata*	TE 90180-9F	IPA	Brazil
V. unguiculata subsp. *unguiculata*	TE 91191-8F	IPA	Brazil
V. unguiculata subsp. *unguiculata*	TE 91195-7F	IPA	Brazil
V. unguiculata subsp. *unguiculata*	VCRA31	EMBRAPA	Brazil
V. unguiculata subsp. *unguiculata*	Istanbul	Local market	Turkey
V. unguiculata subsp. *unguiculata*	73260	KEW	England
V. unguiculata subsp. *unguiculata*	VIG 66/85	IPK	Egypt
V. unguiculata subsp. *unguiculata*	VIG 69/79	IPK	Iraq
V. unguiculata subsp. *unguiculata*	L 2102/98	IPK	Nepal
V. unguiculata subsp. *unguiculata*	VIG 58/80	IPK	Angola
V. unguiculata subsp. *unguiculata*	VIG 71/82	IPK	Ghana
V. unguiculata subsp. *unguiculata*	VIG 7/76	IPK	Not available
V. unguiculata subsp. *unguiculata*	VIG 50/80	IPK	Colombia
V. unguiculata subsp. *cylindrica*	VIG 1/78	IPK	India
V. unguiculata subsp. *cylindrica*	VIG 79/82	IPK	Egypt
V. unguiculata subsp. *sesquipedalis*	VIG 42/83	IPK	Philippines
V. unguiculata subsp. *sesquipedalis*	VIG 28/76	IPK	China

Note: EMBRAPA, Empresa Brasileira de Pesquisa Agropecuária, Centro de Pesquisas do Trópico Semi-Árido (CPATSA), Petrolina, Pernambuco, Brazil; IPA, Empresa Pernambucana de Pesquisa Agropecuária, Recife, Pernambuco, Brazil; IPK, Institut für Pflanzengenetik und Kulturpflanzenforschung Gatersleben, Germany; KEW, Royal Botanic Gardens of Kew, London, UK; BGM Botanischer Garten, Mainz, Germany. Accessions with no germplasm designations were acquired at local markets.

than that of Santalla et al. (1998). The authors used 60 primers to characterize 22 *Vigna* genotypes of the 3 taxa *V. mungo*, *V. luteola*, and *V. radiata* subsp. *sublobata*. From these, 32 primers were monomorphic and the remaining 28 generated a total of 246 fragments, with an average of 8.2 polymorphic fragments per primer. These results strongly suggest that an initial primer selection is advisable prior to amplification with random primers for any analysis of *Vigna* genotypes.

Isoenzyme polymorphisms were employed by Pasquet (1996*a*, 1996*b*, 2000) to analyze wild and cultivated accessions of *V. unguiculata*. Only low levels of polymorphism between cultivated accessions and only discrete variability among geographic clusters were discovered. However, in sharp contrast, one marker type and only 26 DAF amplifications were sufficient in our experiments to distinguish each and every analyzed genotype.

Jaaska (1999) analyzed 23 African and 3 Asian *Vigna* species with 22 enzyme systems and identified 5 major monophyletic groups distinguishing genotypes of both geographic regions. It is interesting to note that the author used a program for cladistic analysis but did not report which species was defined as the outgroup before the evaluation of phylogenetic relationships. However, this is one of the most important steps in any cladistic evaluation, since a change in the outgroup can lead to a completely different tree topography. This may explain the difference between this author's data — *V. radiata* as a basal clade from which *V. aconitifolia* and *V. umbellata* were putatively derived — and our results, which show the former species in a derived position relative to both remaining taxa.

In another approach to identify relationships between 90 cowpea breeding lines developed at IITA (Li et al. 2001), 46 microsatellite markers detected a total of only 27 polymorphisms, which were used to generate a cladogram considered largely consistent with the known pedigree of the cowpea lines. The number of characters, however, is not enough for the effective generation of a genetic distance matrix. Also, a comparison between these authors' results and our data is not possible, since different accessions were evaluated in both approaches.

In a recent study using AFLP, Coulibaly et al. (2002) compared 47 cultivated cowpea accessions (subsp. *unguiculata* var. *unguiculata*) with 52 wild weedy annuals of subsp. *unguiculata* var. *spontanea* from West Africa. This method was able to detect high levels of polymorphism, revealing intensive gene flow among both groups. The authors emphasized the superiority of this method over isozymes and its

Table 2. Designation and sequence of employed DNA amplification fingerprinting primers, numbers of polymorphic bands at the inter- and intraspecific levels, and total number of generated bands.

Primer		Polymorphisms		
Designation	Sequence (5′ → 3′)	Interspecific	Intraspecific	Total no. of bands
OP-B03	CATCCCCCTG	9	1	12
OP-B06	TGCTCTGCCC	10	1	14
OP-B07	GGTGACGCAG	7	1	18
OP-C08	TGGACCGGTG	21	0	21
OP-C09	CTCACCGTCC	16	2	16
OP-C15	GACGGATCAG	12	0	12
OP-D08	GTGTGCCCCA	12	0	12
OP-G03	GAGCCCTCCA	16	0	16
OP-G05	CTGAGACGGA	17	2	17
OP-G06	GTGCCTAACC	18	3	18
OP-G10	AGGGCCGTCT	17	2	17
OP-G12	CAGCTCACGA	16	3	16
OP-J08	CATACCGTGG	10	1	12
OP-J12	GTCCCCTGG	13	3	13
OP-J13	CCACACTACC	16	2	16
OP-J15	TGTAGCAGGG	10	0	12
OP-K08	GAACACTGGG	19	3	19
OP-K14	CCCGCTACAC	18	2	18
OP-K15	CTCCTGCCAA	14	1	14
OP-M20	AGGTCTTGGG	14	0	17
OP-P06	GTGGGCTGAC	9	0	10
OP-P08	ACATCGCCCA	16	1	17
OP-P15	GGAAGCCAA	6	0	11
OP-U17	ACCTGGGGAG	13	0	13
R-1605	GTCCTCAACG	15	3	15
R-2609	GAACCTACGG	9	0	9
Average no. of bands generated		13.6	1.2	14.8

ability to uncover variation within domesticated and wild cowpea. When compared with DAF, the method certainly presents corresponding qualities in the generation of polymorphisms, with the only disadvantage of being more expensive and time-consuming.

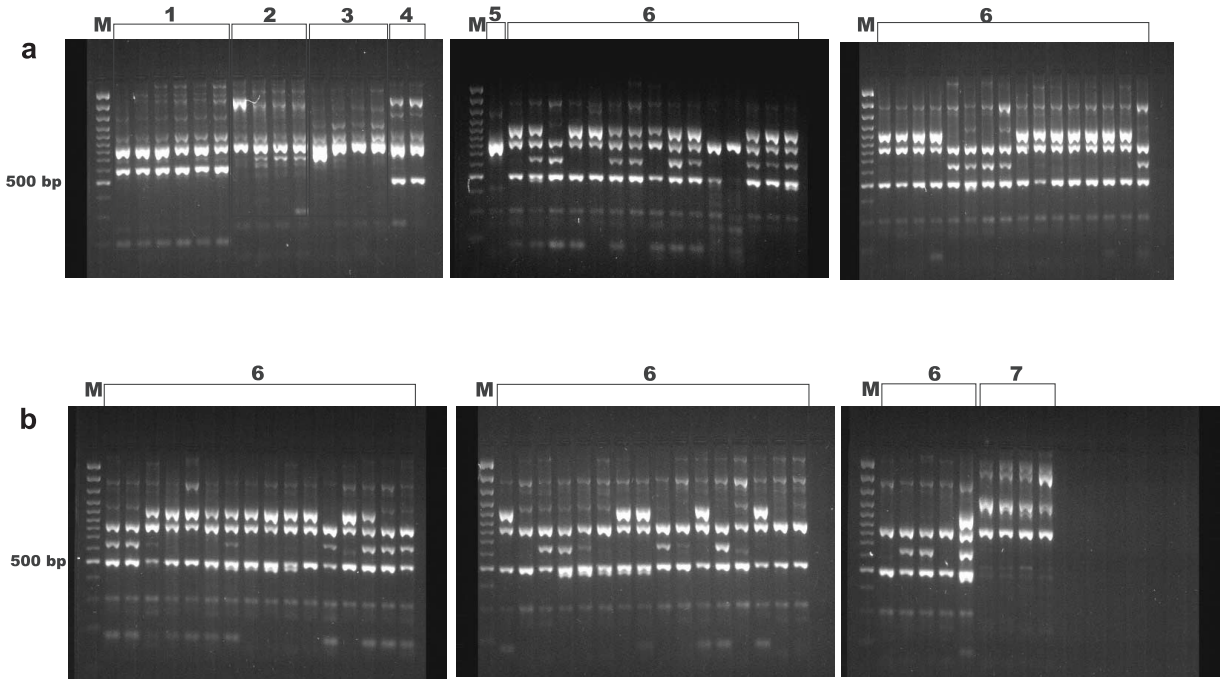
At the species level, only Santalla et al. (1998) compared *V. radiata* (19 genotypes) with the 3 wild taxa *V. mungo*, *V. luteola*, and *V. radiata* subsp. *sublobata*. Their results unified the different *V. radiata* accessions, distinguishing them clearly from the remaining species that additionally were positioned in different branches. In our approach, both *V. radiata* and *V. mungo* were also positioned in separate branches, even though they occupied adjacent positions and placed side by side within the same clade.

The wide genetic diversity among Brazilian landraces and cultivars of cowpea is remarkable, since all African/Asian genotypes and also subsp. *cylindrica* and *sesquipedalis* were grouped together, in contrast to the Brazilian accessions that were grouped in 5 different clades. It is interesting that the grouping is very consistent with the known pedigree of the studied local accessions. For example, the lines of the group CNCx were grouped together with the related CNC cultivars in the most derived upper branch. A similar result was observed for the lines of the TE group, developed by Embrapa CPMN (Centro de Pesquisa do Meio-Norte; Middle Northern Research Station), and the cultivars developed by IPA (Empresa Pernambucana de Pesquisa Agropecuária). This last group of cultivars remained close to the cultivar 'Canapu amarelo' and the non-Brazilian cowpeas, which is consistent with the fact that introduced material was used in breeding experiments (Freire et al. 1999).

Our results suggest that each cross using Brazilian cowpea accessions from different branches of the presented dendrogram may be suitable for genetic mapping using molecular markers, especially DAF. In other *Vigna* species, this is not necessarily the case. For example, using an intraspecific cross for genetic mapping was not possible in the case of *V. angularis* (adzuki bean), since the existing genetic diversity was too low for the generation of a genetic map. Consequently, only an interspecific cross between *V. angularis* and *V. nakashimae* permitted the successful construction of a linkage map with RFLP markers (Kaga et al. 1996). Also, cowpea germplasm available in other parts of the world seems to be genetically rather similar, since a dense genetic map with RAPD, AFLP, and RFLP markers required crossing of domesticated inbred lines from 2 continents (America and Africa, Menéndez et al. 1997).

Regarding the taxonomic position of the analyzed genotypes, some taxa belonging to the subgenus *Vigna*, including those studied here, *V. unguiculata* subsp. *unguiculata* (cultigroup Unguiculata), subsp. *sesquipedalis* (cultigroup Sesquipedalis), and subsp. *cylindrica* (cultigroup Biflora), all belonging to section *Catiang*, were positioned together in a clade indicated as "subspecies". This branch included some

Fig. 1. Representative patterns of DNA amplification fingerprinting products with polymorphisms at the intra- and interspecific levels, generated by primer R2609 in the species (1) *Vigna angularis*, (2) *V. mungo*, (3) *V. radiata*, (4) *V. umbellata*, (5) *V. aconitifolia*, (6) *V. unguiculata*, and (7) *Phaseolus vulgaris*. M, 100 bp ladder molecular weight marker.



members of subsp. *unguiculata* from different provenances (including Iraq, Nepal, Egypt, Angola, Ghana, and Columbia). Interestingly, some members of subsp. *cylindrica* and subsp. *sesquipedalis* were in separate positions within this clade, suggesting that all 3 cultigroups are probably genetically quite similar and may have hybridized during the domestication of cowpea. In fact, hybridization events among wild weedy and cultivated cowpea were observed by Rawal (1975) in northern Nigeria and Niger (western Africa). The same observation was made based on AFLP markers in cultivated cowpea (subsp. *unguiculata* var. *unguiculata*) and wild weedy annuals (subsp. *unguiculata* var. *spontanea*) from the same African region, indicating intensive gene flow among both groups (Coulibaly et al. 2002).

It is interesting to note that cowpea is highly self-pollinated in most environments, the result of a cleistogamous flower. Occasionally, low levels of outcrossing occur, which may be due to visitation by large bees (Ehlers and Hall 1997). This reproductive mechanism may preserve the genetic isolation of some populations within these cultigroups, since experimental crosses, including representatives of all taxa of the section *Catiang*, were successful, most of them presenting fertile hybrids (Ng 1995). This suggests that no great reproductive barriers are present when pollination occurs.

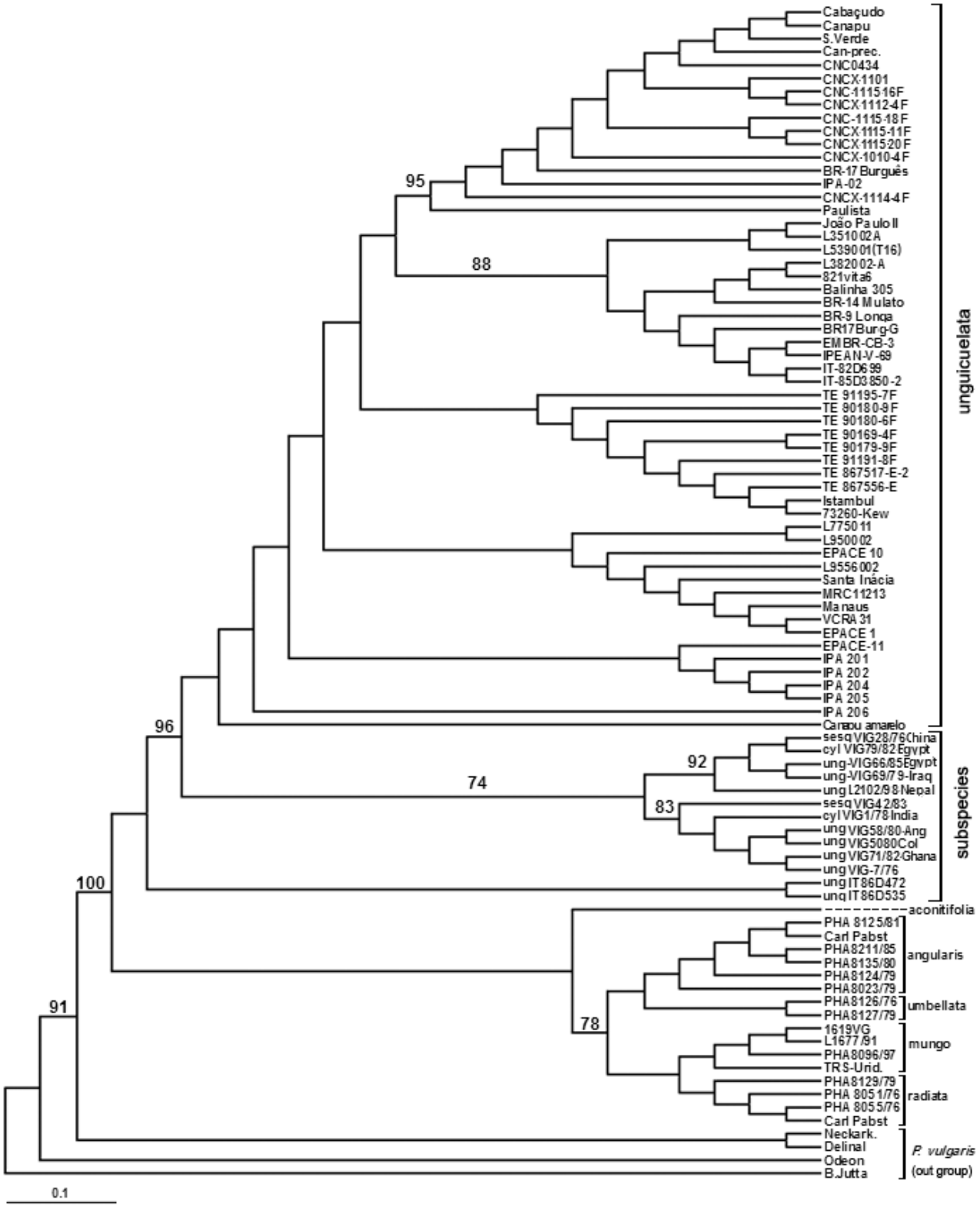
Some researchers suggest that this crop, called "phaseolus" or "phaseolo" by the Romans, was spread from West Africa to the Mediterranean region about 2000 years ago, with the presumption that seeds of this crop were also transported very early to Roman colonies (Perrino et al. 1993). This may explain the inclusion of germplasm from different geographic regions within this "subspecies" clade. It is interesting to note that no Brazilian germplasm joined this clade, suggesting a relative isolation with little germplasm introgression from other geographic sources.

The hypothesis of Freire-Filho (1988), that cowpea was introduced into Brazil from Europe and West Africa by European colonizers and African slaves, respectively, during the 16th and 17th centuries cannot be confirmed by the present work, first, because the nearest group was the "subspecies" clade, including cultivars from different geographic provenances, and second, because most divergent local varieties identified through a comparison with the African/Asian gene pool are cultivars 'Cabeçudo', 'Canapu', and 'Sempre Verde' of *V. unguiculata*, well known for their traditional use in small Brazilian communities.

The remaining *Vigna* species analyzed here include 5 Asiatic domesticated taxa (*V. aconitifolia*, *V. angularis*, *V. mungo*, *V. radiata*, and *V. umbellata*), all included in the subgenus *Ceratotropis*. This subgenus is considered genetically divergent from cowpea, a supposition also supported by our results. Often called "Asian *Vigna*" because of its geographic distribution, limited to Asia, this group is remarkably different from the other 6 *Vigna* subgenera (Tateishi 1996). Even though some evaluations are available regarding the diversity within some species, few comparative studies including all 5 species have been carried out.

Given the taxonomic classification of the 5 species in the subgenus *Ceratotropis*, 3 groupings have been recognized (Maréchal et al. 1978): Sect. *Aconitifoliae*, including *V. aconitifolia* (in our dendrogram in an isolated position separated from the remaining 4 species); Sect. *Angulares*, including *V. angularis* and *V. umbellata* (here side by side in the same subclade); and Sect. *Ceratotropis*, including *V. mungo* and *V. radiata* (also together in the same branch).

Fig. 2. Consensus tree of the analyzed *Vigna unguiculata* germplasm, wild relatives, and other *Vigna* species based on DNA amplification fingerprinting data. Phenogram generated with the program TreeView based on maximum parsimony analyses in MEGA. Bar indicates genetic distance. Bootstrap values >70% (neighbour-joining, 1000 replicates) are depicted above the branches.



This reveals that our evaluation is highly consistent with the taxonomic grouping of these species.

Similar results have also been observed in a previous molecular phylogeny based on sequencing of rDNA internal transcribed spacer and *atp*Bp-*rbc*L intergenic spacer of cpDNA sequences (Doi et al. 2002), with separation of the

species within the 3 above-mentioned sections. As in our study, *V. aconitifolia* presented an ancestral position in comparison with the remaining species of subgenus *Ceratotropis*, with the whole group also in a basal clade when compared with some members of cowpea.

The ancestral position of *V. aconitifolia* and of the section *Ceratotropis* in both evaluations is interesting. These species occur in Asia, *V. aconitifolia* being native to India, Pakistan, and Myanmar or Ceylon (Marechal et al. 1978).

The genus *Vigna* presents pantropical distribution, but most of its ca. 170 species (120) occur in Africa (66 of them endemic) with only 22 species native to India and southeastern Asia (16 endemic) and few species from America and Australia. Because of this higher diversity in Africa, most authors consider this continent as the origin of the genus (Faris 1965; Freire-Filho 1988; Ghafoor et al. 2001).

On the basis of field observations in many continents, including Africa and Asia, Vavilov (1926) indicated southwestern Asia as the origin of many legume species. In a compilation of many field notes, Vavilov (1987) described the variability within the Fabaceae (Leguminosae) and recognized differentiation trends in genera and cultivars by monitoring characters of seeds, fruits, flowers, and vegetative organs in accessions of Vicieae, Trifolieae, Loteae, Galegeae, and Phaseoleae (including *Vigna* species).

Our evidence and also the molecular data of Doi et al. (2002) do not confirm the African theory of ancient origin for this group. Therefore, further evaluations, preferably including a larger number of species (especially native species), may bring more light to this question.

Regarding the application of the present data to cowpea breeding, our results reveal that cowpea (with emphasis on the local Brazilian germplasm) comprises sufficient genetic diversity for the design of crosses for mapping purposes employing linkage analysis of quantitative trait loci and also fine-mapping to identify genes governing important traits (e.g., resistance against viral, bacterial, or fungal pathogens) with the aid of DNA markers to assist in the selection for these traits.

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