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# Preliminary Molecular Characterization of cowpea (*Vigna unguiculata* (L.) Walp.) Accessions by DNA Amplification Fingerprinting (DAF)

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**Abstract**

Genetic characterisation of germplasm accessions is very important for gene bank managers, since it allows more efficient sampling of the available resources, improved identification of the genetic variation for breeders and also a better management of the available gene pool. Here we describe our findings by application of DAF (DNA Amplification Fingerprinting) methodology to discriminate *cowpea (Vigna unguiculata*) germplasm accessions. A total of 30 genotypes have been analysed, including 28 accessions of cowpea, one accession of *V. angularis* and one accession of *V. umbellata* from genebanks in Brazil (27 accessions), Germany (two accessions) and USA (one accession). Nine random (10-mers) primers were used, with an average of 7.8 bands and 5.2 polymorphisms per primer. The resulting data-matrix included 69 analysed bands with a total of 1342 characters. The dendrogram generated by the UPGMA analysis was able to distinguish cowpea from the remaining species and also to evidence diversity at the intraspecific level.

**Keywords:** Cowpea · Crop evolution · Genetic diversity · Germplasm characterisation · Molecular markers · *Vigna.*

## Introduction

Molecular studies of cultivated plants and their wild relatives bring important evidences to the establishment of breeding strategies, especially when interspecific crosses are necessary for mapping purposes or for the incorporation of new features (Benko-Iseppon, 2001).

Cowpea (Vigna unguiculata (L.) Walp) is widely cultivated throughout the world. Particularly in the developing countries of South America, Africa and Asia, where plant proteins comprise 83% of total dietary protein (Mahe et al., 1994). Cowpea represents one of the best hopes for combating shortage in food supply.

Called "feijão-macassar" or "feijão-de-corda" in northeastern Brazil, cowpea is an important component of Brazilian diet and agricultural production (May et al., 1988; Freire-Filho et al., 1999). Having superior nutritional qualities to dry beans (Phaseolus vulgaris L.); it is the main constituent of the daily diet of the economically depressed rural class in North and Northeastern Brazil (Freire-Filho et al., 1999). Especially in the semiarid regions of Northeast Brazil, where dry beans do not grow, cowpea constitutes the most important food legume (Freire-Filho, 1988; Freire-Filho et al., 1999), representing 73% of all beans consumed and nearly 10% of total agronomic production value.

DNA amplification fingerprinting (DAF) methodology is a simple and powerful method for generating molecular markers, with the additional advantage of being relatively low-priced and highly reproducible (Winter et al., 2000; Benko-Iseppon et al., 2003; Rakshit et al., 2003; Simon et al., 2007).

Since countries in development need to embrace new technologies to overcame consumer needs for better food with accessible prices, we started testing DAF methodology and its applicability to characterise 28 cowpea genotypes as compared with two other *Vigna* species (*V. angularis* and *V. umbellata*)

## Materials and Methods

### Plant Material and DNA extraction

The plants have been cultivated in pots with 5 kg soil capacity containing a mixture of two parts of soil and one part of manure. Prior to their sowing the seeds have been disinfected with 0.5% sodium hypochlorite. After germination four plants per pot have been maintained, which remained in the glasshouses until their processing. DNA was isolated from young leaflets using a modified CTAB (cetyl-trimethyl-amoniumbromide) protocol (Weising et al., 1995). Contaminating polysaccharides were selectively precipitated (Michaels et al., 1994). DNA concentrations were determined electrophoretically using known amounts of phage l-DNA as a reference.

### DNA amplification fingerprinting and electrophoresis

DAF followed the procedure of Caetano-Anollés et al. (1991) with some modifications introduced by Winter et al. (2000) as follows: PCR was carried out using random primers procured from Eurogentec (Cologne, Germany), Operon Technologies (Alameda, USA) or Roth (Karlsruhe, Germany), respectively. Each 15 ml PCR reaction contained 1.5 ml 10x PCR buffer (Eurogentec, Cologne, Germany), 2.5 mM MgCl2; 10 mM dNTP-mix; 0.4 U *"S*ilverstar" *Taq* DNA polymerase (Eurogentec Cologne, Germany), 40 pmol oligonucleotide primer, and 1 ng/ml 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, with a final elongation at the same temperature for 2 min. The reaction products were separated on 1.8% agarose gels stained with ethidium bromide and viewed under ultraviolet light.

### *Data analysis*

A data matrix was constructed from the analysis of the bands generated by DAF and visualised after agarose gel electrophoresis. For this purpose, we considered the presence (1) or absence (0) of each analysed band for each of the studied individuals.

The UPGMA analysis was carried out with aid of the software MEGA (Molecular Evolutionary Genetic Analysis) version 2.0 for Windows, kindly provided by the authors (Kumar et al., 2004). The generated tree was viewed with the program TREEVIEW32 for Windows (Page, 1996), kindly provided by Dr. Robert Page of the Glasgow University, Scotland.

**Results**

The analysed material came from four different germplasm banks (Table 1) and included 30 accessions of three different *Vigna* species (one of *V. angularis,* one of *V. umbellata,* and 28 accessions of *V. unguiculata*). .

**Table 1:** List of analysed *Vigna* genotypes including name of species, accession number or cultivar name, germplasm bank and original area of cultivation. Legend for collaboration germplasm banks: **Embrapa/CPQMN** = Empresa Brasileira de Pesquisas Agropecuárias, Centro de Pesquisas do Meio Norte, Teresina, Piauí, Brazil; **IPA** = Empresa Pernambucana de Pesquisa Agropecuária, Recife, Pernambuco, Brazil; **IPK** = Institut für Pflanzengenetik und Kulturpflanzenforschung, Gatersleben, Germany; **UCA** = University of California, Riverside, USA. Cultivar names or numbers indicated as "[A]" were studied previously by Simon et al. (2007). Remaining material (indicated as "[B]" are studied here for the first time.

| Ord. | Species | Name of cultivar or accession Number | Genebank | Original source or area of cultivation |
|---|---|---|---|---|
| 1. | *V. unguiculata* (L.) Walp. | AR-87-435[B] | Embrapa/CPQMN | Brazil |
| 2. | *V. unguiculata* (L.) Walp. | BR-17 Gurguéia[A] | Embrapa/CPQMN | Brazil |
| 3. | *V. unguiculata* (L.) Walp. | BR-2 Bragança[B] | Embrapa/CPQMN | Brazil |
| 4. | *V. unguiculata* (L.) Walp. | BR-5 Maratoã[B] | Embrapa/CPQMN | Brazil |
| 5. | *V. unguiculata* (L.) Walp. | BR-5 Rouxinol[B] | Embrapa/CPQMN | Brazil |
| 6. | *V. unguiculata* (L.) Walp. | Cacheado[B] | Embrapa/CPQMN | Brazil |
| 7. | *V. unguiculata* (L.) Walp. | Canapu 02[B] | Embrapa/CPQMN | Brazil |
| 8. | *V. unguiculata* (L.) Walp. | Capela[B] | Embrapa/CPQMN | Brazil |
| 9. | *V. unguiculata* (L.) Walp. | Corujinha[B] | Embrapa/CPQMN | Brazil |
| 10. | *V. unguiculata* (L.) Walp. | Manaus[A] | Embrapa/CPQMN | Brazil |
| 11. | *V. unguiculata* (L.) Walp. | Monteiro[B] | Embrapa/CPQMN | Brazil |
| 12. | *V. unguiculata* (L.) Walp. | Princess Ann[B] | Embrapa/CPQMN | Brazil |
| 13. | *V. unguiculata* (L.) Walp. | Vagem Roxa – Piripiri[B] | Embrapa/CPQMN | Brazil |
| 14. | *V. unguiculata* (L.) Walp. | CE-315[B] | Embrapa/CPQMN | Brazil |
| 15. | *V. unguiculata* (L.) Walp. | IT-82G-9[B] | Embrapa/CPQMN | Brazil |
| 16. | *V. unguiculata* (L.) Walp. | IT-91K-118-2[B] | Embrapa/CPQMN | Brazil |
| 17. | *V. unguiculata* (L.) Walp. | MNC-1514-1[B] | Embrapa/CPQMN | Brazil |
| 18. | *V. unguiculata* (L.) Walp. | TE97-299G-24[B] | Embrapa/CPQMN | Brazil |
| 19. | *V. unguiculata* (L.) Walp. | TE97-309G-4[B] | Embrapa/CPQMN | Brazil |

| | | | | |
|---|---|---|---|---|
| 20. | *V. unguiculata* (L.) Walp. | TE97-321G-8[B] | Embrapa/CPQMN | Brazil |
| 21. | *V. unguiculata* (L.) Walp. | TE97-323G-4[B] | Embrapa/CPQMN | Brazil |
| 22. | *V. unguiculata* (L.) Walp. | VITA-7[B] | Embrapa/CPQMN | Brazil |
| 23. | *V. unguiculata* (L.) Walp. | IT-845-2049 (UCR 430)[B] | UCR | USA |
| 24. | *V. unguiculata* (L.) Walp. | CNC-0434[A] | IPA | Brazil |
| 25. | *V. unguiculata* (L.) Walp. | IPA-204[A] | IPA | Brazil |
| 26. | *V. unguiculata* (L.) Walp. | IPA-205[A] | IPA | Brazil |
| 27. | *V. unguiculata* (L.) Walp. | IPA-206[A] | IPA | Brazil |
| 28. | *V. unguiculata* (L.) Walp. | Sempre Verde[A] | IPA | Brazil |
| 29. | *V. angularis* (Willd.) Ohwi et Ohashi | PHA 8023/79[A] | IPK | Asia |
| 30. | *V. umbellata* (Thumb.) Ohwi et Ohashi | PHA 8126/76[A] | IPK | Indonesia |

The most informative primers considering total number of bands and total number of polymorphisms were OP-C03 (12 bands, nine polymorphic) and OP-G06 (14 bands, six polymorphic). The primer OP-A14 revealed the lower number of amplicons, generating three bands and only two polymorphisms. Figure 1 shows two representative pictures of amplification products including a very informative primer (OP-G06) and a less informative one (OP-A14). Table 2 presents a list of the primers used, including their sequence, number of generated and polymorphic bands.

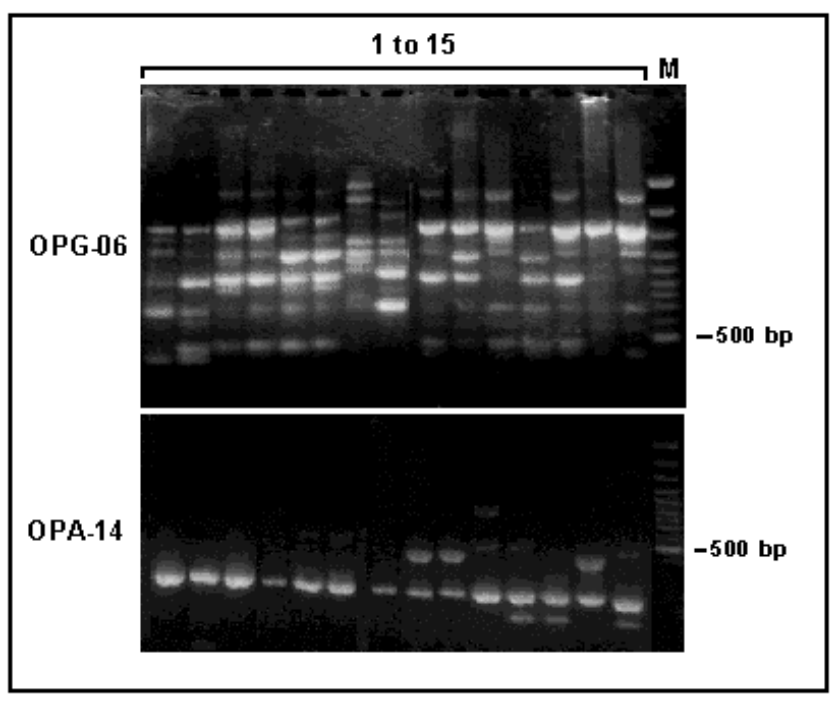


**Figure 1.** A representative picture of DAF products including a very informative primer (OPG-06) and a primer that displayed few bands (OPA-14). Order of the genotypes: Cowpea 1-6 and 9-15. *V. umbellata* (7) and *V. angularis* (8).

Accession Numbers: **1**=IT-845-2049(UCR 430); **2**=CNC-0434; **3**=Sempre Verde; **4**=IPA-206; **5**=IPA-205; **6**=IPA-204; **7**=PHA 8126/76; **8**=PHA 8023/79; **9**=BR-5 Maratoã; **10**=BR-5 Rouxinol; **11**=IT-82G-9; **12**=IT-91K-118-2; **13**=TE97-299G-24; **14**=TE97-321G-8; **15**=TE97-323G-4. Fragment sizes for polymorphic bands (in bp) are indicated on the right, as calculated from a 100 bp ladder (last lane, M).

**Table 2. Primers used in the DAF reactions including sequence composition, total number of generated bands and number of polymorphic bands.**

| Primer | | Total number of bands | Number of polymorphisms |
|---|---|---|---|
| Designation | Sequence (5'→ 3') | | |
| OP-A04 | AATCGGGCTG | 05 | 04 |
| OP-A14 | TCTGTCCTGG | 03 | 02 |
| OP-C03 | GGGGGTCTTT | 12 | 09 |
| OP-C08 | TGGACCGGTG | 05 | 05 |
| OP-C15 | GACGGATCAG | 11 | 05 |
| OP-G05 | CTGAGACGGA | 06 | 06 |
| OP-G06 | GTGCCTAACC | 14 | 06 |
| OP-G10 | AGGGCCGTCT | 08 | 06 |
| OP-G12 | GAGCTCACGA | 06 | 04 |
| Average number of bands generated per primer | | 7.8 | 5.2 |

A total of 70 bands have been generated, from which 47 beard polymorphisms. In average, the primers generated a total of 7.8 bands and 5.2 polymorphisms per primer.

The resulting data-matrix included 69 analysed bands with a total of 1342 characters. The dendrogram generated by the UPGMA analysis is presented in Figure 2. The generated tree presented two major branches with the cultivar 'Vita 7' in an isolated position.

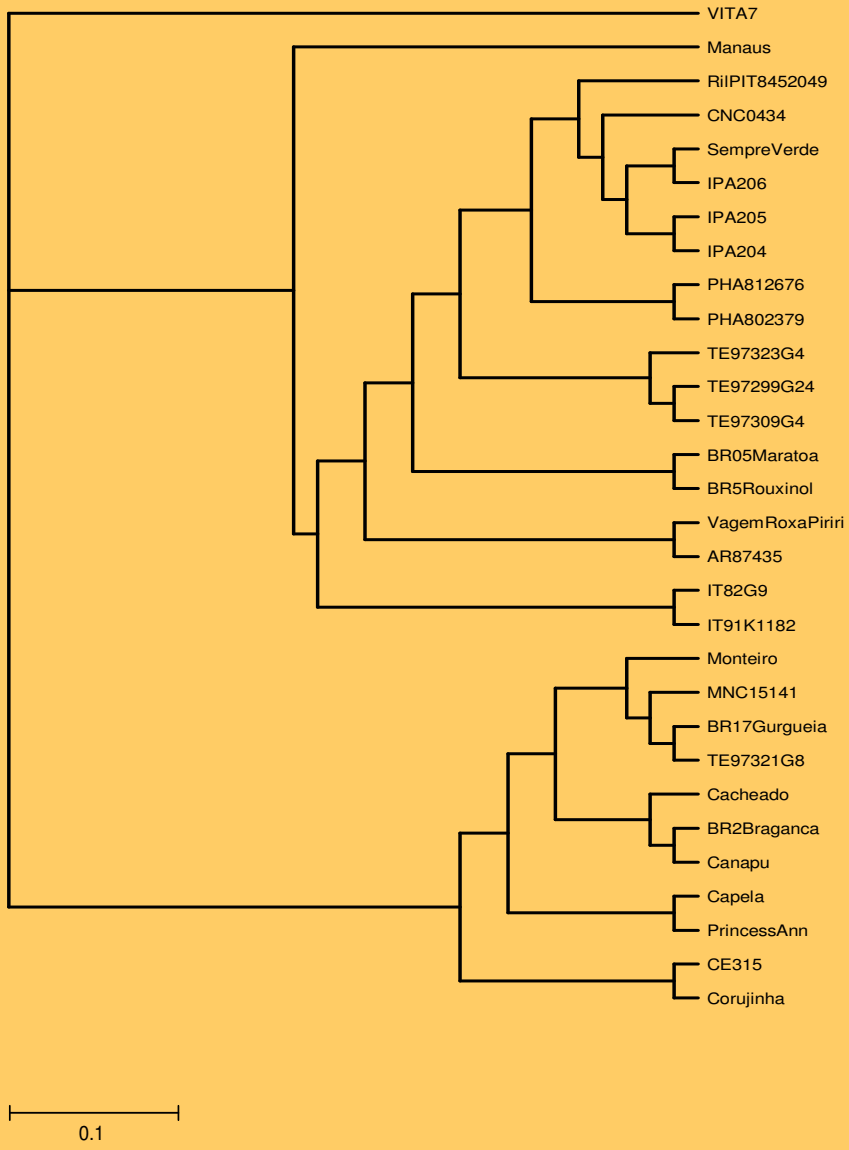


**Figure 2.** Phenogram generated from DAF data using UPGMA and method with the program MEGA V.2.0. For further information regarding the analyzed genotypes see Table 1. Bar indicates genetic distance.

## Discussion

The present work represents the first molecular evaluation regarding 21 Brazilian germplasm accessions of *V. unguiculata* (Table 1) and includes important material for current breeding programs. Despite the low number of primers used, the DAF methodology was very efficient in generating molecular markers for this first evaluation, a result that confirms previous evidences presented by Simon (2002) and Simon et al. (2007). Since Simon (2002) used a higher number of primers (26) selected from a total of 262, the average

number of polymorphism per primer was superior with 13.6 bands/primer, against 7.9 in the present work.

Santalla et al. (1998) analysed 22 *Vigna* genotypes of three different taxa (*V. mungo* L. Hepper, *V. luteola* (Jacq.) Benth. and *V. radiata* L. Wilcz ssp. *sublobata* (Roxb.) Verdc.) by RAPD (Random Amplified Polymorphism do DNA). From the 60 primers they used 32 were monomorphic and only 26 generated polymorphisms in an average of 8.2 markers per primer, a similar result to the present work. Considering the low number of primers we used and the previous results of Simon (2002) it is clear that DAF is more effective for generating markers in the genus *Vigna*, as compared with RAPD.

The same conclusion was presented in previous works with Chickpea (*Cicer arietinum* L.) a cultivated leguminous of the same family as *Vigna* (Fabaceae). In the course of mapping studies of this crop, RAPD showed most pronounced segregation distortion as compared with DAF and other five types of molecular markers. Additionally segregation of markers was not completely reproducible with RAPD, opposite to the observation of the authors regarding DAF markers (Winter et al., 2000; Benko-Iseppon et al., 2003; Rakshit et al., 2003). On the basis of our observations and previous reports, we consider DAF to be the marker of choice for future cowpea breeding programs.

Previously to the present work and the study of Simon et al. (2007), another approach to evaluate genetic diversity of cowpea accessions from genebanks was based on isoenzymatic polymorphisms carried out by Pasquet (1996a, 1996b, 2000) that analysed wild and cultivated accessions. Despite the application of many enzymatic systems, the author found few polymorphisms between cultivated accessions and only discrete variability among geographic clusters. Comparing these results with both studies carried out with DAF, it is also clear that this type of markers is advantageous, since it allowed the distinction of some accessions from their near relatives, as it is the case of the accessions IPA 204, 205 and 206 that remained in the same branch but could be distinguished from each other. The same can be affirmed regarding the cultivars ‘BR5-Maratoa’ and ‘BR5-Rouxinol’, two related cultivars that differ on the basis of selection for different agronomical features.

A most complex and laboured type of marker are microsatellite markers. Li et al. (2001) used 46 primer pairs developed to microsatellites to discriminate 90 cowpea breeding lines developed at the International Institute for Tropical Agriculture, Nigeria (IITA). They generated a total of 27 polymorphisms that, despite the low number of features, generated a phenogram able to distinguish most of the lines. Considering the time consumption of this method and the number of features generated we consider more advisable to recommend this type of marker for mapping purposes, especially considering their co-dominant segregation pattern that generate very robust markers.

Additional approaches focused on the use of different molecular markers to assess genetic diversity in cowpea such as random amplified polymorphic DNA (RAPD; Xavier et al. 2005) and amplified fragment length polymorphism (AFLP; Fang et al. 2007), revealing relatively low genetic diversity in the analyzed cultivated cowpea accessions.

Freire-Filho (1988) suggests that cowpea was introduced to Brazil from Europe and West Africa by European colonizers and African slaves during the 16$^{th}$ and 17$^{th}$ centuries. Simon et al. (2007) suggested that the considerable levels of polymorphisms found in her work for Brazilian accessions could have arisen during the last four centuries of recurrent selection and the influence of local environmental conditions. We agree with both hypothesis, but one should also consider the genotypes introduced from other countries for breeding purposes in the course of the decade of 1970 and later. Some of the most important cowpea cultivars have been obtained from crossing procedures to introduced material from IITA, contributing to an increase of diversity in the locally used gene pool.

Despite the considerations above, considering the present approach and the previous work of Simon et al. (2007), it is clear that the intraspecific diversity of *V. unguiculata* is higher than that observed in other legume crops, as for example Chickpea (Winter et al., 2000; Benko-Iseppon et al., 2003; Rakshit et al., 2003) or Soybean (*Glycine max* L.) (Caetano-Anollés and Gresshof, 1994).

Comparing the branching pattern of both dendrograms generated by approaches using DAF (i.e., present work and that of Simon et al., 2007) it is clear that the dendrogram of these last authors is most robust, especially considering the higher number of features (bands) analysed by the author. Even considering this limitation and the preliminary nature of the present work, some similarities can be recognized comparing both works, regarding the branching pattern. For example both species *V. angularis* (accession PHA 8023/79) and *V. umbellata* (PHA 8126/76) remained together in the same branch in both evaluations, even though in the evaluation of Simon et al. (2007) they occupied a basal position as compared with the remaining cowpea genotypes. Also similar grouping of genotypes can be observed for the cowpea accessions IPA-204, 205 and 206.

Considering the cowpea genotypes analysed here for the first time, some interesting observations can be done regarding the branching. Both African accessions from IITA (IT82G9 and IT91K1182) remained close together in a basal position in the main branch where most of the Brazilian cultivars and accessions grouped, confirming the influence of introduced material in Brazilian recent breeding programs. On the opposite, the Californian accession RiPIT8452049 (received from the University of California, Riverside) was positioned in a higher position in the same branch, which can be explained by the advanced stage that breeding programs on this crop have been carried out at the Univ. of California at Riverside, especially considering that the field selection of the Californian group is directed to features and environmental conditions very different from that existing in tropical countries.

It is also interesting to discuss the position of the cultivar ‘Manaus’ in a basal position of the main branch generated by the present evaluation. It has been considered that cowpea was first introduced to Brazilian coast region and that it was introduced later to the Amazonian region by migrating people from Brazilian north-eastern region (Guazelli, 1988). Meanwhile cowpea is a very important crop in that region, especially for subsistence agriculture, since *Phaseolus* beans do not grow in the environmental conditions of the

Amazonian region (EMBRAPA, 1979). The position of this cultivar in a basal position as compared with the remaining accessions and almost intermediary to both branches may suggest that its introduction occurred very early and that its original features have been maintained throughout the years.

Many cultivars obtained from small communities (e.g. 'Monteiro', 'Cacheado', 'Canapu', 'Capela' and 'Corujinha') grouped in a most basal branch together with some accessions derived from crossing procedures, as CE315, MNC15141 and TE97321G8. We have requested the pedigrees of the analysed genotypes and it is to suppose that this knowledge will support the present branching, since many crosses have been carried out using such cultivars, especially in the search for sources of resistance to pathogens.

The present work is a further step to support cowpea-breeding projects in Brazil with molecular markers. The perspectives seem very promising and shall be still more successful with the integration of morphological features to the data matrix.

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