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Development of PCR-based chloroplast DNA markers that characterize domesticated cowpea (*Vigna unguiculata* ssp. *unguiculata* var. *unguiculata*) and highlight its crop-weed complex

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Abstract. In 1992, Vaillancourt and Weeden discovered a very important mutation for studying cowpea evolution and domestication. A loss of a *Bam*HI restriction site in chloroplast DNA characterized all domesticated accessions and a few wild (*Vigna unguiculata* ssp. *unguiculata* var. *spontanea*) accessions. In order to screen a larger number of accessions, primers were designed to check this mutation using PCR RFLP or direct PCR methods. Using these new primers, 54 domesticated cowpea accessions and 130 accessions from the wild progenitor were screened. The absence of haplotype 0 was confirmed within domesticated accessions, including primitive landraces from cultivar-groups Biflora and Textilis, suggesting that this mutation occurred prior to domestication. However, 40 var. *spontanea* accessions distributed from Senegal to Tanzania and South Africa showed haplotype 1. Whereas this marker could not be used to identify a precise center of origin, it did highlight the widely distributed cowpea crop-weed complex. Its very high frequency in West Africa could be interpreted as a result of either genetic swamping of the wild/weedy gene pool by the domesticated cowpea gene pool or as the result of domestication by ethnic groups focusing primarily on cowpea as fodder.

Key words: cowpea, *Vigna unguiculata*, chloroplast DNA, long-range PCR, gene flow, crop-weedy-wild complex.

Introduction

Cowpea [*Vigna unguiculata* (L.) Walp.] is one of the most important legume crops for human consumption. It is cultivated in all tropical areas on at least 12.5 million hectares, with an annual production of over 3 million tons world-wide. Africa is the main area of production: Central and West Africa account for more than half of the cultivated area. Cowpea is very important for low-input agriculture, which characterizes most of the African continent. It is grown predominantly for its dry seeds, which are cooked in various ways. In many regions, young leaves, fresh or dried, and unripe pods are also consumed. Cowpea is also cultivated as fodder, in the Sahelian area of West Africa as well as in the dry areas of Asia. In earlier times, cowpea from cultivar-group Textilis was cultivated for the fiber of its floral

peduncles; its seeds were generally not consumed (Pasquet and Baudoin 2001).

In addition to domesticated forms, classified as *V. unguiculata* ssp. *unguiculata* var. *unguiculata*, the species *V. unguiculata* includes also wild/weedy annual forms [ssp. unguiculata var. *spontanea* (Schweinf.) Pasquet] and 10 wild perennial subspecies (Pasquet and Baudoin 2001). Domesticated cowpea includes five cultivar-groups (Westphal 1974, Pasquet 1998). The cowpea gene-pool has been the focus of numerous studies using morphological characters (Pasquet 1993, Padulosi 1993), genetic barriers (Echikh 2000), storage proteins (Panella et al. 1993, Fotso et al. 1994), allozymes (Panella and Gepts 1992; Vaillancourt et al. 1993; Pasquet 1999, 2000), chloroplast DNA (cpDNA) RFLP (Vaillancourt and Weeden 1992), AFLP (Coulibaly et al. 2002), and RAPD markers (Mignouna et al. 1998, Ba et al. 2004).

The African origin of cowpea is now well established (Steele 1976). *V. unguiculata* ssp. *unguiculata* var. *spontanea* (also known as ssp. *dekindtiana sensu* Verdc.) is the likely progenitor of the domesticated cowpea (Pasquet 1999). However, the actual location of cowpea domestication in Africa is still uncertain. Different centers of origin and diversity have been proposed (reviewed in Ba et al. 2004). Vaillancourt and Weeden (1992) proposed a West African domestication but their study was hampered by the low number of entries in their plant sample. Their study included only 32 *V. unguiculata* var. *unguiculata* and 13 *V. unguiculata* var. *spontanea* accessions. Their argument on the origin of domesticated cowpea was based on a cpDNA RFLP marker, i.e., the presence or absence of a *Bam*HI restriction site uncovered with the cpDNA probe s13.3 (s13.3/*Bam*HI; Palmer et al. 1988). Domesticated accessions had lost the *Bam*HI site to yield a 1500 bp fragment (haplotype 1) instead of a combination of a 1300 bp and a 200 bp fragment (haplotype 0) as observed in most wild/weedy accessions. Of the 13 var. *spontanea* accessions, only the three accessions from Niger-Nigeria in their sample had haplotype 1, which led them to suggest that Nigeria was the center of domestication of cowpea (Vaillancourt and Weeden 1992). The present study was aimed at analyzing the geographic and taxonomic distribution of this potentially informative mutation in a larger number of accessions. However, the RFLP methodology is limited by several factors. It requires a relatively large amount of good quality DNA for assay; it is also time-consuming and labor-intensive. Therefore, part or our objective was to convert this RFLP marker into a more convenient PCR-based marker (Drenkard et al. 2000).

Materials and methods

Plant materials. The plant material consisted of domesticated var. *unguiculata* (54 accessions) and wild/weedy var. *spontanea* from West, East and Southern Africa (130 accessions). The domesticated accessions belonged to the five cultivar-groups, i.e., cv.-gr. Melanophthalmus (AG 1 from Algeria, EX 28 from USA, NO 574 and NO 1387 from Cameroon, IT84S2049, a breeding line from the International Institute of Tropical Agriculture, and 524B, a breeding line from the University of California, Riverside), cv.-gr. Biflora (EG 1 from Egypt, ET 1, ET 2, ET 5, ET 14, ET 25, ET 28, ET 31, ET 33, and ET 39 from Ethiopia, EX 35 from India, EX 37 from Laos, EX 51 from Pakistan, HV 1 from Burkina Faso, NO 89, NO 106, NO 1878, and NO 3113 from Cameroon, and ZR 7 from RD Congo), cv.-gr. Sesquipedalis (EX 38 from New Caledonia and EX 43 from the Philippines), and cv.-gr. Textilis (NO 27, NO 91, NO 198, NO 274, NO 275, NO 577, NO 1769, NO 2292, NO 2297, NO 2300, NO 2418, and NO 3076 from Cameroon), cv.-gr. Unguiculata (AS 3E, AS 10C, and AS 10F from South Africa, ET 3 and ET 35 from Ethiopia, EX 19 from Colombia, CS 5, CS 15, CS 53B, CS 56B, NO 74, NO 90, OU 65, and OU 100 from Cameroon, and UG 8 from Uganda). Half of the accessions studied (CS, NO, and OU accessions) were collected during an ethnobotanical survey of Cameroon and are pictured in Pasquet and Fotso (1994). With few exceptions, all the accessions studied have different isozyme profiles, and all the main clusters from the isozyme analysis (Pasquet 2000) are represented here by at least one

accession. In addition, we tried to include the largest range of morphological diversity: for example, NO 3076 is morphologically not distinguishable from a var. *spontanea* plant, but it is a landrace cultivated for its fibers like the small-seeded NO 27 and NO 91, while NO 90 and NO 574 are among the accessions with the largest seeds in their respective cultivar groups (all pictured in Pasquet and Fotso 1994). Domesticated accessions were provided by various institutions listed in Pasquet (2002).

The var. *spontanea* accessions (Table 1) were obtained from the World Phaseolinae collection maintained in the Jardin Botanique National de Belgique, Meise, Belgium (NI and SP accessions); the International Institute of Tropical Agriculture, Ibadan, Nigeria (TVNU accessions); and the International Plant Genetic Resources Institute, Harare, Zimbabwe (MT accessions). Fifty-five of them were previously studied for isozyme analysis (Pasquet 1999); the isozyme analysis of the other 75 accessions is underway. Additional information about the accessions studied (both domesticated and var. *spontanea*) can be obtained directly from the authors. All theses accessions are available from public gene banks (JBNB or IITA) or will soon be as some recently collected material is currently under multiplication.

The habitat of the collecting location is usually unknown for gene bank accessions (MT, NI, TVNU, and few SP accessions) or accessions originating from herbarium specimens (some SP accessions). For the SP accessions collected by R.S. Pasquet or K. Thoen, there is a continuum between plants collected within cultivated fields and tolerated or favoured by farmers, on the one hand, and plants collected in a National Reserve like SP 259, on the other hand. Many accessions were collected on roadsides and have an ambiguous status. For example, roadside plants can belong to a wild population in a wild habitat or to a weedy population that invaded the roadside. Based on this type of observation, we consider as ecologically weedy SP 9, SP 32, SP 37, SP 43, SP 57, SP 116, and SP 122 from Cameroon, SP 808, SP 818, SP 820, SP 821, SP 826, SP 827, and SP 829 from Ghana, SP 87, SP 204, SP 207, SP 208, SP 210, SP 211, SP 219, SP 221, SP 222, SP 248, SP 260, SP 272, SP 291, SP 292, and SP 295 from Kenya, and SP 300 from Niger. We consider as ecologically wild SP 46, SP 52, SP 93, and SP 96 from Cameroon, SP 215, SP 236, SP 244, SP 259, SP 263, SP 276, SP 278, and SP 296 from Kenya, SP 199 from Niger. However, some weedy populations from Kenya, like SP 211, SP 219, and SP 222, could have been initially wild populations driven to roadsides by cultivation in their natural habitat.

Healthy young leaf tissues were harvested from two-weeks-old cowpea seedlings grown in the greenhouse. Genomic DNA was extracted from freshly harvested leaves frozen in liquid nitrogen, following the CTAB procedure described by Doyle and Doyle (1987).

Long-range PCR. The first step for converting this cpDNA RFLP marker into a PCR-based marker was to identify the specific DNA sequence change observed by the *Bam*HI/s13.3 enzyme probe combination using a PCR-based method. Based on the location of the s13.3 fragment on the mung bean chloroplast map (Palmer et al. 1988), the s13.3 fragment contains three chloroplast genes: *atpA*, *psbB* (flanking the ends of the s13.3 fragment) and *petA* (middle of the fragment). These genes were targeted to design primers that amplify the region corresponding to the s13.3 probe. Available sequences from both dicotyledonous and monocotyledonous species were aligned using Clustal X 1.8 (*Thompson et al. 1997*). Since the expected fragment size was more than 3 kb, we employed a long-range PCR method. The following primers for long-range PCR were then designed from conserved coding sequences: atpAF: 5′-GCT TTA CCA ATA GTT GAG AC-3′; petAR: 5′-CTG CTT CAA ATA CAG TAT C-3′; petAF: 5′-GAT ACT GTA TTT GAA GCA G-3′; and psbBR: 5′-GTA TGA ACA CGA TAC CAA GG-3′. Primer melting temperatures and complementarities were checked using the Operon toolkit available at http://www.operon.com/oligos/toolkit.php. Long-range PCR was performed with the Roche Diagnostic GmbH (Indianapolis, IN) Expand Long Template PCR System, according to the manufacturer's instructions. The 50 µl PCR reactions contained 250 ng total DNA, 0.3 µM of each primer, 10 × Expand Long Template PCR buffer 1 with 1.75 mM $MgCl_2$, 350 µM of each dNTPs and 2.5 units *Taq*-DNA polymerase mix. The reaction mixtures were then subjected to an initial denaturation of 2 min at 92°C, followed by 10 cycles of 10 s at 92°C, 30 s at 55°C, and 8 min at 68°C. This was followed by 20 cycles of 10 s at 92°C, 30 s at 55°C, 8 min at 68°C plus 20 s increment per cycle.

Table 1. *V. unguculata* var. *spontanea* accessions studied and their cpDNA s13.3/*Bam*HI haplotype

Accession (geographic coordinates)	cpDNA haplotype
Angola	
SP 143 (16°27 S 15°14 E)	0
Botswana	
MT498 (22°00S 27°23 E)	0
MT612 (19°32 S 23°51 E)	0
MT621 (22°22 S 26°52 E)	0
MT623 (22°15 S 20°40 E)	0
MT1379 (18°15 S 24°20 E)	0
NI1380 (20°00 S 23°25 E)	0
NI1381 (20°10 S 23°10 E)	0
NI1382 (20°25 S 23°00 E)	0
NI1383 (19°30 S 22°15 E)	1
NI 1384 (21°00 S 27°25 E)	0
Burundi	
NI1232 (4°07 S 29°30 E)	0
Cameroon	
SP 9 (10°53 N 13°47 E)	1
SP 32 (10°07 N 14°08 E)	1
SP 37 (8°14 N 14°56 E)	0
SP 43 (9°34 N 13°26 E)	1
SP 46 (11°24 N 14°34 E)	1
SP 52 (5°04 N 14°02 E)	0
SP 57 (10°28 N 13°41 E)	1
SP 93 (5°40 N 14°08 E)	0
SP 96 (5°34 N 14°05 E)	0
SP 116 (10°46 N 14°39 E)	1
SP 122 (8°33 N 12°38 E)	1
Central African Republic	
TVNU 237 (7°20 N 18°22 E)	0
TVNU 242 (5°02 N 18°26 E)	0
TVNU 249 (6°40 N 19°10 E)	1
TVNU 257 (5°46 N 20°55 E)	1
TVNU 1151 (6°13 N 18°24 E)	0
Chad	
SP 64 (no coordinates)	0
TVNU 304 (11°26 N 15°21 E)	0
Congo (Republic)	
NI 1390 (2°52 S 15°29 E)	0
NI 1391 (2°23 S 15°44 E)	1
SP 148 (2°20 S 15°48 E)	0
SP 149 (1°12 S 15°57 E)	1
SP 151 (3°59 S 15°24 E)	1
Congo (Democratic Republic)	
NI 437 (6°45 S 23°57 E)	0
Ghana	
SP 808 (10°44 N 0°47 W)	1
SP 818 (11°04 N 0°08 W)	1
SP 820 (10°58 N 0°05 W)	1
SP 821 (11°03 N 0°03 W)	1
SP 826 (10°52 N 0°45 W)	1
SP 827 (10°52 N 0°45 W)	1
SP 829 (10°48 N 0°55 W)	1
Guinea Bissau	
SP 140 (no coordinates)	1
Kenya	
NI 1228 (3°57 S 39°32 E)	0
NI 1734 (2°17 S 40°54 E)	0
SP 87 (1°30 N 35°30 E)	0
SP 204 (1°28 N 35°02 E)	0
SP 207 (2°44 S 38°00 E)	0
SP 208 (2°48 S 37°57 E)	0
SP 210 (3°22 S 38°25 E)	0
SP 211 (4°19 S 39°32 E)	0
SP 215 (4°20 S 39°30 E)	0
SP 219 (4°16 S 39°14 E)	0
SP 221 (4°27 S 39°26 E)	1
SP 222 (4°27 S 39°26 E)	0
SP 236 (3°14 S 40°00 E)	0
SP 244 (3°19 S 39°57 E)	0
SP 248 (4°13 S 39°32 E)	0
SP 259 (4°14 S 39°24 E)	0
SP 260 (4°00 S 39°29 E)	
SP 263 (4°30 S 39°14 E)	0
SP 272 (3°56 S 39°32 E)	0
SP 276 (3°52 S 39°46 E)	0
SP 278 (2°20 S 40°20 E)	0
SP 291 (0°49 S 34°34 E)	0
SP 292 (0°38 S 34°20 E)	0
SP 295 (2°32 S 37°53 E)	0
SP 296 (2°28 S 40°27 E)	0
Malawi	
NI 1392 (11°22 S 33°56 E)	0
SP 84 (10°30 S 34°10 E)	0
SP 88 (16°30 S 34°50 E)	0
TVNU 363 (14°05 S 33°53 E)	0
TVNU 435 (15°35 S 35°10 E)	1
TVNU 437 (11°52 S 33°44 E)	0
TVNU 442 (9°51 S 33°47 E)	1
Namibia	
SP 154 (18°10 S 21°40 E)	0
SP 160 (18°10 S 21°40 E)	0
Niger	
NI 991 (13°29 N 1°57 E)	1

Table 1. (Continued)

Accession (geographic coordinates)	cpDNA haplotype
SP 66 (11°53 S 3°36 E)	0
SP 78 (11°59 N 3°19 E)	1
SP 80 (11°52 N 3°37 E)	0
SP 199 (12°22 N 3°25 E)	0
SP 300 (13°05 N 1°44 E)	1
TVNU 1148 (14°00 N 0°35 E)	1
Nigeria	
NI 951 (12°00 N 8°30 E)	1
TVNU 403 (no coordinates)	1
TVNU 417 (no coordinates)	1
TVNU 420 (no coordinates)	1
TVNU 1422 (no coordinates)	1
Senegal	
NI 963 (12°32 N 16°45 W)	1
South Africa	
NI 1167 (no coordinates)	0
SP 159 (24°10 S 31°10 E)	0
SP 192 (22°35 S 31°15 E)	0
SP 195 (26°53 S32°53 E)	0
SP 195 (26°53 S32°53 E)	0
TVNU 1343 (28°30 S 32°08 E)	1
TVNU 1351 (30°25 S 30°35 E)	0
Sudan	
SP 86 (4°01 N 32°45 E)	0
Tanzania	
MT 131 (6°20 S 29°55 E)	0
NI 1385 (2°33 S 32°55 E)	0
NI 1386 (6°08 S 39°17 E)	1
NI 1405 (6°00 S 39°13 E)	1
SP 83 (5°09 S 38°28 E)	0
SP 181 (6°50 S38°00 E)	0
SP 182 (6°50 S 38°10 E)	0
SP 183 (6°40 S 38°10 E)	0
SP 184 (6°20 S 37°50 E)	0
SP 185 (6°00 S 38°00 E)	1
SP 186 (6°10 S 37°50 E)	0
TVNU 297 (4°54 S 29°40 E)	0
TVNU 298 (6°11 S 39°11 E)	1
TVNU 301 (6°10 S 39°26 E)	1
TVNU 503 (no coordinates)	0
TVNU 531 (6°16 S 39°24 E)	0
TVNU 1248 (6°08 S 39°17 E)	0
Yemen	
SP 171 (13°58 N 44°00 E)	0
Zambia	
MT 651 (15°10 S 28°30 E)	0
NI 1171 (11°23 S 39°31 E)	0
NI 1387 (14°59 S 26°44 E)	0
TVNU 352 (14°58 S 26°37 E)	0
Zimbabwe	
MT 25 (19°50 S 28°25 E)	0
MT 55 (19°03 S 32°44 E)	0
MT 62 (20°14 S 32°50 E)	0
MT 65 (20°15 S 32°55 E)	0
MT 76 (19°57 S 32°05 E)	0
MT 99 (18°25 S 32°55 E)	0
MT 102 (18°24 S 32°55 E)	0
MT 319 (19°45 S 32°55 E)	0
NI 817 (18°12 S 31°34 E)	0
NI 1400 (18°29 S 25°45 E)	0

Final extension was done for 7 min at 68°C. The PCR reaction was carried out with a PTC-220 thermocycler (MJ Research). An aliquot of the long-range PCR product was subjected to restriction digestion with the *Bam*HI enzyme (NEBiolabs). The overnight digestion product was electrophoresed in 1.2% agarose (GibcoBRL) gel in 1 × TAE buffer and stained with 1μg/ml ethidium bromide. The electrophoresed *Bam*HI digest was transferred to Zetabind nylon membranes (CUNO) following the method described in Nodari et al. (1992). The membranes were hybridized with a ^{32}P-labeled s13.3 probe. Prehybridization, hybridization, washing and detection of DNA were performed as described by Menéndez et al. (1997). Once the fragments corresponding to the previously detected RFLP markers were identified, they were extracted from the agarose gel using GENECLEAN II (BIO101) and cloned into the pUC19 vector. The nucleotide sequence of the cloned fragment was determined using Big Dye Terminator sequencing chemistry and an automated ABI Prism® sequencing system at Davis Sequencing (Davis, CA).

PCR-RFLP and SNP primers design. The two fragments obtained after digestion, namely D1700 (haplotype 1) and W1530 (haplotype 0), were chosen for primer design for PCR-RFLP and SNP analyses (Fig. 1). For PCR-RFLP detection of the different haplotypes, the PCR reaction was carried out using CAPF designed at the 5′ end of the 1700bp RFLP fragment as forward primer and DWR as reverse primer: CAPF: 5′-ATG CTA

CCT CCC ATA AAC TTA-3′, DWR: 5′-GAA CTT AGC TCG AAT CAA C-3′. The primers were used in a 50 µl reaction containing 100 ng genomic DNA, 0.3 µM of each primers, 1.5 units of Taq DNA polymerase (Promega), 10 mM Tris-HCl (pH 9.0), 50 mM KCl, 0.1% Triton® X-100, 2.5 mM $MgCl_2$ and 200 µM dNTPs. The reaction mixture was incubated for 2 min at 94°C followed by 30 cycles of 30 s denaturation (94°C), 30 s annealing (55°C) and 90 s extension (72°C) and an additional 10 min extension at 72°C at the end of the 35 cycles. Finally, an aliquot of the PCR reaction was digested with *Bam*HI and checked on a 1% (w/v) agarose gel. For SNP detection, a SNAP (Single Nucleotide Amplified Polymorphism) analysis was performed (Drenkard et al. 2000). Haplotype-specific primers were designed at the SNP site distinguishing var. *spontanea* and domesticated cowpea. According to this strategy, primers with a mismatch at the 3′ end will allow preferential amplification of one haplotype relative to the other provided that the primers are complementary at the site of DNA sequence variation (Ugozzoli and Wallace 1991). Thus, the haplotype-specific primer matches perfectly for one haplotype (the specific haplotype) and has a 3′ mismatch for the nonspecific haplotype. However, in some cases a single base pair change at 3′ end may not be robust enough to differentiate between specific and nonspecific alleles. Thus, a deliberate mismatch from G/C at position -2 from the 3′end was introduced to increase the specificity of haplotype-specific PCR procedure (Drenkard et al. 2000). Primers D1700F and W1530F were designed to end with a 3′ position at site 166 (Fig. 2): D1700F: 5′-TCT TCT GAA TGA AAT TCA ATC A-3′, W1530F: 5′-CTT CTG AAT GAA ATT CAA TCG-3′. The primers were applied in combination, D1700/DWR and W1530/DWR. The PCR conditions were as described above. For further optimization, annealing temperatures were raised in successive experiments until only strong bands were observed in one of the two primer combinations. Then, a 6 µl aliquot of the PCR product was separated by 1% agarose gel (w/v) electrophoresis.

Results

Long-range PCR. Both primer pairs (atpAF/petAR and petAF/psbBR) amplified a fragment with a size of 10 kb in both var. *spontanea* and domesticated accessions. Once subjected to digestion with the *Bam*HI restriction enzyme, only the *atp*A-*pet*A region produced the type of polymorphism obtained in the RFLP study. The domesticated accessions showed a 1700 bp fragment (haplotype 1) while the var. *spontanea* accessions showed fragments with a size of 1530 bp and 170 bp (haplotype 0), the latter not seen in an agarose gel (Fig. 1). These sizes are slightly larger that the ones given by Vaillancourt and Weeden (1992), i.e., a 1500 bp fragment for haplotype 1 and a combination of 1300 bp and 200 bp fragments for haplotype 0. Southern hybridization of the *Bam*HI digest with the s13.3 probe (not shown) revealed a similar polymorphism pattern confirming that the marker obtained using PCR-RFLP was the same as the one obtained using Southern-based RFLP analysis. The sequence of both fragments D1700 (haplotype 1) and W1530 (haplotype 0) demonstrated that haplotype 0 possesses a *Bam*HI site from base positions 166 to 171, while haplotype 1 does not. The absence of a *Bam*HI recognition sequence in

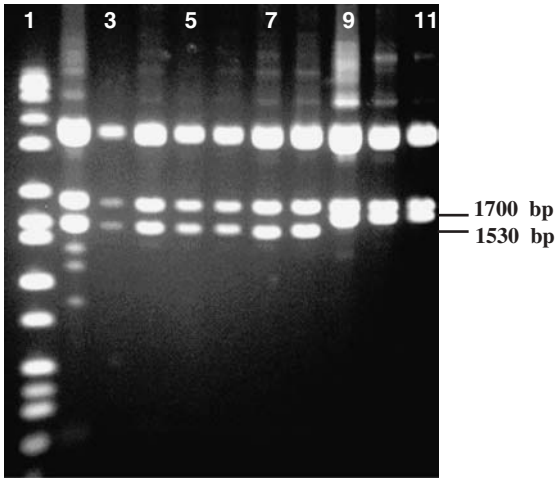

Fig. 1. CpDNA polymorphism observed in cowpea. Long-range PCR product obtained with primers atpAF and petAR were digested with *Bam*HI. Lane 1: Hi-Lo all purpose molecular marker (Bionexus). Lanes 2-8: wild accessions (MT 574, NI 794, NI 456, SP 181, MT 612, TVNU 297, SP 52,); lanes 9-11: domesticated accessions (CS 5, EX 43, ET 15)

domesticated accessions is due to a nucleotide change from G to A at site 166 (Fig. 2).

PCR-RFLP analysis. The chloroplast haplotype was checked with PCR-RFLP using primers CAPF and DWR and restriction enzyme *Bam*HI. This pair of primers amplified an approximately 1560 bp product. Subsequent digestion of the amplicon with *Bam*HI enzyme resulted in 1400 and 160 bp fragments with haplotype 0. The PCR product was not digested in genotypes with haplotype 1 (Fig. 3). After digestion of the amplification product with *Bam*HI, the presence or absence of the 160 bp fragment provided a reliable procedure for the identification of haplotypes 0 and 1, respectively.

SNP analysis. Primer combination D1700F/DWR specifically amplifies an amplicon in haplotype 1 accessions whereas an amplicon was obtained in haplotype 0 accessions only when primers W1530F and DWR were used. Therefore, two independent PCR reactions are required to characterize the cpDNA haplotypes of cowpea accessions. The presence of a fragment in the D1700F/DWR primer combination should always be counterchecked by the absence of an amplicon in the W1530F/DWR primer combination, which is indicative of haplotype 1 (Fig. 4), and vice-versa for haplotype 0. We designated, therefore, a chloroplast haplotype as 0 when the fragment was present with W1530F/DWR primers and as 1 when the fragment was present with D1700/DWR primers. In some cases, the primer combination D1700F/DWR, which is specific for domesticated accessions, generated a PCR product both in domesticated and var. *spontanea* accessions raising questions about the specificity of the primers. Similar observations were also noted with primer pairs W1530F/DWR. The most likely explanation for this is the presence of a range of template DNA concentration in the reaction mixture. This situation can be avoided by optimizing the reaction for instance, by running two identical sets of PCR reaction per primer pair with different numbers of cycles (Drenkard et al. 2000). In conclusion, either one of the above methods (SNP assay or PCR-RFLP) can be used to differentiate haplotypes 0 and 1 in cowpea. Both methods are reliable and the method of choice thus depends on cost and time required to run the analysis. To be

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                          CAPF
ET 15      GATCCCATGC TACCTCCCAT AAACTTAAAA TCCATAATAC CAATTGCTAC AGGAATACCA TTTACTTGAC  70
TV 297     GATCCCATGC TACCTCCCAT AAACTTAAAA TCCATAATAC CAATTGCTAC AGGAATACCA TTTACTTGAC  70

ET 15      CTGTCCCTGT TTGAACAGCC TCAGTTAATC CTGTTTTTCT TTGATAAGAA TCAATACGAT CTTTATAAGG  140
TV 297     CTGTCCCTGT TTGAACAGCC TCAGTTAATC CTGTTTTTCT TTGATAAGAA TCAATACGAT CTTTATAAGG  140
                    D1700F/W1530F
ET 15      TTCTTCTTCT GAATGAAATT CAATGAGATC CAGAGAAACC ATGTCTTCAT CCATAGGATT CCAAGTACCC  210
TV 297     TTCTTCTTCT GAATGAAATT CAATGGGATC CAGAGAAACC ATGTCTTCAT CCATAGGATT CCAAGTACCC  210

ET 15      GAATCAATCG AAAATTCGAT TCTATCTGAA CTGTCCATTT TCAAATGATA TCCACAGTAT TCACAAATAT  280
TV 297     GAATCAATCG AAAATTCGAT TCTATCTGAA CTGTCCATTT TCAAATGATA TCCACAGTAT TCACAAATAT  115

                                                                    DWR
ET 15      ATACAAATAA TCTACACCCC CTTTTTGATT TCATTAATTG AATTTCGTTT GTTGATTCGA GCTAAGTTCT  1533
TV 297     ATACAAATAA TCTACACCCC CTTTTTGATT TCATTAATTG AATTTCGTTT GTTGATTCGA GCTAAGTTCT  1362

ET 15      ATAAAAACAC CTGCCTTTTT TGAAATATGC TGAACAGTTC CTGTAGGTTA AGCGCCCCTT GTCAAGGAAA  1603
TV 297     ATAAAAACAC CTGCCTTTTT TGAAATATGC TGAACAGTTC CTGTAGGTTA AGCGCCCCTT GTCAAGGAAA  1432

ET 15      TGAAAGAATA ACAGGAATAT TGAAATAGGT TTAATTCTTT ATAGGATC 1651
TV 297     TGAAAGAATA ACAGGGATAT TGAAATAGGT TTAATTCTTT ATAGGATC 1480
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Fig. 2. Comparison of nucleotide sequences from domesticated (ET 15) and wild (TVNU 297) accessions. The *Bam*HI site is underlined and differences between ET 15 and TVNU 297 at the *Bam*HI site are boxed. The haplotype-specific primer sites are noted with arrows. Nucleotide sequences from 281-1453 are omitted

specific, the SNP assay requires two PCR reactions per accession, which means more gel runs, while PCR-RFLP requires one PCR reaction followed by digestion with a restriction enzyme and eventually fewer gel runs.

Accession screening. All 54 domesticated accessions had chloroplast haplotype 1. Out of 130 var. spontanea accessions investigated, 40 showed haplotype 1: NI 1383 from Botswana, SP 9, SP 32, SP 43, SP 46, SP 57, SP 116, and SP 122 from Cameroon, TVNU 249 and TVNU 257 from Central African Republic, NI 1391, SP 149, and SP 151 from Congo, all accessions from Ghana, SP 140 from Guinea Bissau, SP 221 from Kenya, TVNU 435 and TVNU 442 from Malawi, NI 991, SP 78, and SP 300 from Niger, TVNU 1148 from Niger, all accessions from Nigeria, NI 963 from Senegal, TVNU 1343 from South Africa, and NI 1386, NI 1405, SP 185, TVNU 298, and TVNU 301 from Tanzania.

The geographic distribution of this haplotype was uneven (Fig. 5). In West Africa, i.e., northern Cameroon and countries westward, 25 out of the 29 accessions had haplotype 1. North of the Equator but from Central Cameroon eastward, 2 out of 14 had haplotype 1. South of the Equator (but outside the East African coastal plains), 7 out of 52 showed haplotype 1. In the East African coastal plains, 6 out of 34 had haplotype 1 (TVNU 298 from Tanzania is without geographic coordinates). In this latter area, plants from accession SP 221 with a wild-domesticated intermediate morphology had haplotype 1. The correlation between haplotype 1 and habitat was not obvious. All accessions from Ghana had a weedy habitat and haplotype 1. In Cameroon, ecologically weedy accessions showed six haplotype 1 and 1 haplotype 0 while ecologically wild accessions showed three haplotype 0 and one haplotype 1. In Niger, the ecologically wild accession had haplotype 0 while the ecologically weedy accession had haplotype 1. In Cameroon, cpDNA haplotypes seemed linked to latitude. Accessions north of 8°33 N showed haplotype 1 while accessions south of 8°14 N had a haplotype 0. In Niger, the northern accession had a haplotype 1 while

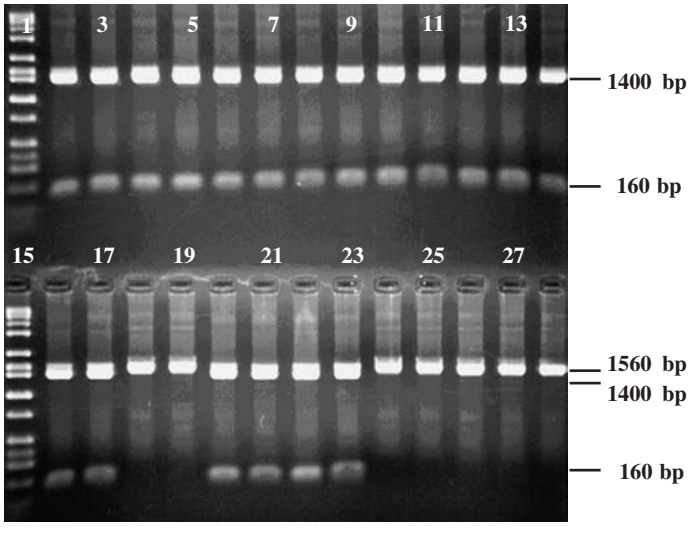

Fig. 3. PCR-RFLP analysis showing different digestion profile in domesticated and wild accessions. Presence of a 161bp band characterizes some wild haplotypes. Lane 1: Hi-Lo all purpose molecular marker (Bionexus). Lanes 2-22: wild accessions (SP 45, NI 933, NI 794, SP 39, SP 48, SP 150, NI 456, MT 449, MT 53, MT 320, SP 200, SP 189, MT 564, NI 1382, NI 1384, NI 1391, SP 185, SP 52, TVNU 297, SP 86, MT 612). Lanes 23-27: domesticated accessions (CS 5, ET 15, EX 43, NO 275, NO 574)

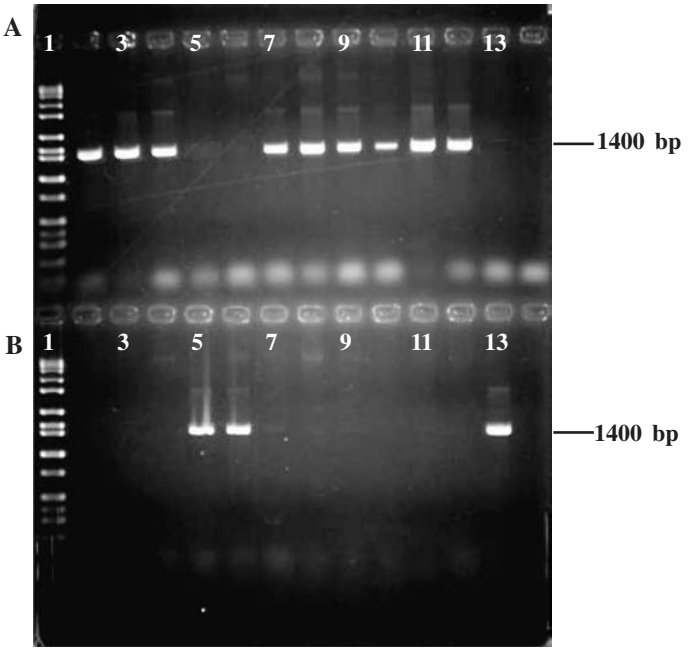

Fig. 4. SNP assay using (a) wild specific primers W1530F and DWR and (b) domesticated specific primers D1700F and DWR. Presence of a product represents either a wild or domesticated haplotype depending on the primer used. Lane 1: Hi-Lo all purpose molecular marker (Bionexus). Lanes 2-12 and 14: wild accessions (NI 1382, NI 1384, SP 143, NI 1391, SP 185, SP 52, TVNU 297, SP 86, SP 181, MT 612, NI 1392). Lane 13: domesticated accession (NO 574). Lane 14: V. vexillata (MT 327)

the southern accession had a haplotype 0. In Kenya, all accessions, except the wild-domesticated hybrids from SP 221, had a haplotype 0. In contrast with West Africa, not a single var. *spontanea* plant was found within fields in the latter country.

Discussion

Haplotype 1 in West Africa: Center of domestication or consequence of genetic swamping? Our results on the s13.3/*Bam*HI marker provide an additional example of the reduced genetic diversity and – in some cases – the monomorphism of the cowpea domesticated gene pool (Panella and Gepts 1992; Panella et al. 1993; Pasquet 1999, 2000; Coulibaly et al. 2002). All 54 domesticated accessions had chloroplast haplotype 1, including primitive accessions from cultivar-groups Textilis and Biflora (Pasquet 2000). By primitive, we mean accessions with small seeds colored like wild seeds and pods showing a tendency to shatter under very dry weather conditions. The presence of haplotype 1 in several accessions from cv.-gr. Textilis and a very primitive accession like NO 3113 suggests that the mutation studied occurred prior to domestication. The sum of observations in the current and previous research supports a single domestication for cowpea in Africa although it does not allow a more specific identification of the actual region or center of domestication within this continent. Haplotype 1 is very frequent in West Africa (from Northern Cameroon westward). This distribution correlates with similar observations about the $Amp2^{102}$ (Pasquet 1999) and AFLP markers (Coulibaly et al. 2002). One could conclude that cowpea was domesticated in the area where domestication-specific markers (including cpDNA haplotype 1) are frequent in the var. *spontanea* populations, i.e. West Africa, as Vaillancourt

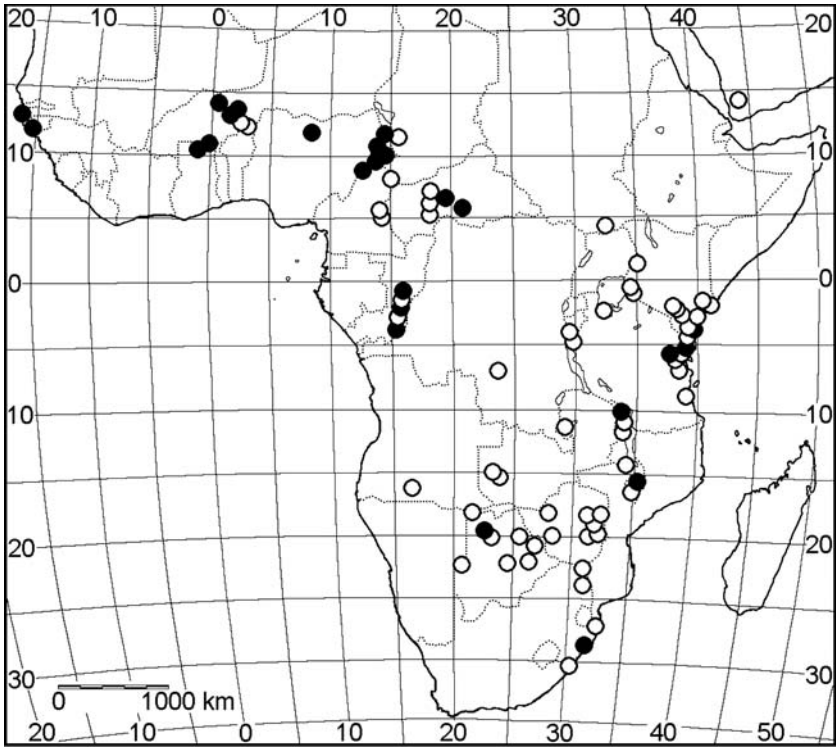

Fig. 5. Geographic distribution map of var. *spontanea* accessions with haplotype 0 (open circle) and with haplotype 1 (closed circle). When two or more accessions from same region show similar haplotype, then only one accession is shown on the map to avoid overlapping of points

and Weeden (1992) did. This hypothesis is further supported by the suggestion by Ng (1995) of a domestication by ethnic groups in western Africa, who use cowpea primarily for fodder. Nevertheless, the presence of haplotype 1 in wild cowpea outside western Africa raises the possibility that domestication could have taken place elsewhere on the continent. In this regard, Pasquet (1999) already noted that the lack of var. *spontanea* material from North-East Africa prevents an unequivocal identification of the center of domestication of cowpea.

Another possible explanation exists for the high frequency of domesticated markers in West African var. *spontanea* populations. Careful examination of herbarium data as well as collector's observations north of the Equator show that in the western part of its range, var. *spontanea* occurs mainly within a range of 5 degrees of latitude (broadly between 10°N and 15°N), with virtually no record south of 7°N. However, in the eastern part of this area, the wild progenitor of cowpea extends southward down to the forest margins, for example, in South-East Cameroon or the Central African Republic, or down to the Equator and beyond, as in Uganda and in Kenya west of the Rift valley (Pasquet, unpubl. data). Although there may be a gradient between the two areas just described, these two areas are also split ecologically. Westward, cowpea is more often a weed within the fields while eastward it appears in more natural habitats. This is easily explained by the fact that cowpea is often cultivated for fodder in West Africa. Weedy cowpea fodder is as good as domesticated cowpea fodder and farmers do not seem to destroy weedy cowpea plants from their field. Hence, wild-domesticated hybrids and their progenies stand a better chance of

surviving in West Africa than elsewhere in Africa.

In addition, because the fitness of F_1 wild-domesticated hybrids is much higher when the hybrids have a domesticated maternal parent (Pasquet, unpubl. data), domesticated genes and especially domesticated cytoplasm may be introduced into West-African and other var. *spontanea* populations through repeated backcrossing of the F_1 hybrids (with domesticated cytoplasm) with var. *spontanea* pollen. In common with other markers, including AFLP and RAPD markers (Coulibaly et al. 2002, Ba et al. 2004) and the aminopeptidase ($Amp2^{102}$) allozyme allele (Pasquet 1999), haplotype 1 has a very wide geographic distribution as it is encountered from Senegal to Tanzania and South Africa. Thus, these markers may actually illustrate the wide geographic distribution of the cowpea crop-weed complex. If gene flow from domesticated to wild populations takes place repeatedly (Haygood et al. 2003), domesticated alleles may eventually replace the native alleles in wild populations and cause genetic assimilation or swamping.

Cowpea would not be the only crop showing genetic assimilation of its wild relative populations. With common bean (*Phaseolus vulgaris*) in Mexico, a displacement of genetic diversity in wild populations due to gene flow from the domesticated populations seems to occur (Beebe et al. 1997, Papa and Gepts 2003, Gonzalez et al. 2005, Papa et al. 2005). The parallel is especially striking if we consider that in some areas of Mexico, farmers may not remove weedy *P. vulgaris* from their fields (Zizumbo-Villarreal et al. 2005). Cowpea in West Africa can also be compared with sunflower in the USA. Self-incompatible wild sunflower (*Helianthus annuus*) is an agricultural weed that hybridizes easily with domesticated sunflower (also *H. annuus*). It is common along field and road margins near domesticated plants, and studies have demonstrated crop-wild hybridization rates as high as 40% on wild plants that occur adjacent to the crop, with small but detectable levels of hybridization as far as 1000 m away (Arias and Rieseberg 1994). Crop genes are easily backcrossing into wild sunflower populations and in some areas, every wild plant sampled from areas where sunflower was cultivated had one or more crop- specific DNA markers (Whitton et al. 1997, Linder et al. 1998).

Finally, haplotype 0 has not been introduced so far into the domesticated gene-pool through cytoplasm flow. This suggests that the progeny of a var. *spontanea* plant fertilized by domesticated pollen may generally not become integrated into the domesticated gene-pool. Such an event would require repeated fertilization of the hybrid progenies by domesticated pollen (i.e. backcrossing to the domesticated type). Such progenies with increasing seed size and domesticated colors or color patterns may be less likely to escape predators before farmers choose them as seeds for their next sowing.

Further use of s13.3/*Bam*HI haplotype-specific primers. The specific primers developed in this study can also be used for further in-depth studies of cowpea populations. Once var. *spontanea* material from North-east Africa will become available, this chloroplast marker will be a key element to further evaluate the various domestication hypotheses.

Given the maternal inheritance of chloroplast genome in cowpea (Corriveau and Coleman 1988), these markers can also be used to detect the female parent in hybrid populations. Rieseberg and Soltis (1991) reviewed several cases of "chloroplast capture" through hybridization/introgression in many plant groups. In some cases, cpDNA capture was unexpected and occurred in groups not noted for hybridization. Therefore, having a diagnostic marker is desirable to detect introgressive hybridization events. Monitoring both nuclear and cytoplasmic gene flow may reflect different histories of gene exchange. The present results show that gene flow seems to occur in both directions. If we consider the nuclear allele $Amp2^{102}$, which is frequent in domesticated accessions and rare in var. *spontanea* accessions (Pasquet 1999), there are accessions with chloroplast haplotype 0 and nuclear allele $Amp2^{102}$ (SP 37 from Cameroon, SP 80 from Niger)

suggesting a var. *spontanea* flower fertilized by domesticated pollen. There are also accessions with chloroplast haplotype 1 and nuclear allele $Amp2^{100}$ (NI 1383 from Botswana, NI 1386 from Tanzania, NI 1391 from Congo, SP 140 from Guinea Bissau) suggesting a domesticated flower fertilized by var. *spontanea* pollen. Therefore, this marker is of interest in studies of gene flow between wild and domesticated cowpea populations, especially since risk assessment studies will have to be undertaken before the possible release of genetically engineered cowpea cultivars in Africa.

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References

Arias D. M., Rieseberg L. H. (1994) Gene flow between cultivated and wild sunflowers. Theor. Appl. Genet. 89-6: 655–660.

Ba F. S., Pasquet R. S., Gepts P. (2004) Genetic diversity in cowpea [*Vigna unguiculata* (L.) Walp.] as revealed by RAPD markers. Genet. Resour. Crop Evol. 51-5: 539–550.

Beebe S., Toro O. C., Gonzalez A. V., Chacon M. I., Debouck D. G. (1997) Wild-weed–crop complexes of common bean (*Phaseolus vulgaris* L. Fabaceae) in the Andes of Peru and Colombia, and their implications for conservation and breeding. Genet. Resour. Crop Evol. 44: 73–91.

Corriveau J. L., Coleman A. W. (1988) Rapid screening method to detect biparental inheritance of plastid DNA and results for over 200 angiosperm species. Amer. J. Bot. 75-10: 1443–1458.

Coulibaly S., Pasquet R. S., Papa R., Gepts P. (2002) AFLP analysis of the phenetic organization and genetic diversity of *Vigna unguiculata* L. Walp. reveals extensive gene flow between wild and domesticated types. Theor. Appl. Genet 104: 358–366.

Doyle J. J., Doyle J. L. (1987) A rapid DNA isolation procedure for small quantities of fresh leaf tissue. Phytochem. Bull. 19: 11–15.

Drenkard E., Richter B. G., Rozen S., et al. (2000) A simple procedure for the analysis of single nucleotide polymorphisms facilitates map-based cloning in Arabidopsis. Pl. Physiol. 124: 1483–1492.

Echikh N. (2000) Organisation du pool génique de formes sauvages et cultivées d'une légumineuse alimentaire, *Vigna unguiculata* (L.) Walp. Thèse de doctorat, Fac. Univ. Sci. Agron. Gembloux, Belgique.

Fotso M., Azanza J. L., Pasquet R. S., Raymond J. (1994) Molecular heterogeneity of cowpea (*Vigna unguiculata* Fabaceae) seed storage proteins. Pl. Syst. Evol. 191-1/2: 39–56.

Gonzalez A., Wong A., Delgado-Salinas A., Papa R., Gepts P. (2005) Assessment of inter simple sequence repeat markers to differentiate sympatric wild and domesticated populations of common bean. Crop Sci. 45-2: 606–615.

Haygood R., Ives A. R., Andow D. A. (2003) Consequences of recurrent gene flow from crops to wild relatives. Proc. Roy. Soc. London - Series B: Biol. Sci. 270: 1879–1886

Linder C. R., Taha I., Seiler G. J., Snow A. A., Rieseberg L. H. (1998) Long-term introgression of crop genes into wild sunflower populations. Theor. Appl. Genet. 96-3/4: 339–347.

Menéndez, C. M., Hall A. E., Gepts P. (1997) A genetic linkage map of cowpea (*Vigna unguiculata*) developed from cross between two inbred, domesticated lines. Theor. Appl. Genet 95: 1210–1217.

Mignouna H. D., Ng N. Q., Ikea J., Thottappilly G. (1998) Genetic diversity in cowpea as revealed by random amplified polymorphic DNA. J. Genet. Breed. 52: 151–159.

Ng N. Q. (1995) Cowpea, *Vigna unguiculata* (Leguminosae-Papilionoideae). In: Smartt J., Simmonds N. W. (eds.) Evolution of crop plants. 2nd ed. Longmans, New York, pp. 326–332.

Nodari R. O., Koinange E. M. K., Kelly J. D., Gepts P. (1992) Towards an integrated linkage map of common bean. II. Development of genomic DNA probes and levels of restriction fragment length polymorphism. Theor. Appl. Genet. 84: 186–192.

Padulosi S. (1993) Genetic diversity, taxonomy and ecogeographic survey of the wild relatives of cowpea (*Vigna unguiculata* (L.) Walpers). PhD Thesis, Université Catholique de Louvain, Louvain-la-Neuve, Belgium.

Palmer J. D., Osorio B., Thompson W. F. (1988) Evolutionary significance of inversions in legume chloroplast DNAs. Curr. Genet. 14: 65–74.

Panella L., Gepts P. (1992) Genetic relationships within *Vigna unguiculata* (L.) Walp. based on isozyme analyses. Genet. Resources Crop Evol. 39: 71–88.

Panella L., Kami J., Gepts P. (1993) Vignin diversity in wild and cultivated taxa of *Vigna unguiculata* (L.) Walp. (Fabaceae). Econ. Bot. 47-4: 371–386.

Papa R., Gepts P. (2003) Asymmetry of gene flow and differential geographical structure of molecular diversity in wild and domesticated common bean (*Phaseolus vulgaris* L.) from Mesoamerica. Theor. Appl. Genet. 106-2: 239–250.

Papa R., Acosta J., Delgado-Salinas A., Gepts P. (2005) A genome-wide analysis of differentiation between wild and domesticated *Phaseolus vulgaris* from Mesoamerica. Theor. Appl. Genet. 111: 1147–1158.

Pasquet R. S. (1993) Classification infraspécifique des formes spontanées de *Vigna unguiculata* (L.) Walp. à partir de données morphologiques. Bull. Jard. Bot. Nat. Belg. 62-1/4: 127–173.

Pasquet R. S., Fotso M. (1994) Répartition des cultivars de niébé (*Vigna unguiculata* (L.) Walp.) du Cameroun: influence du milieu et des facteurs humains. J. Agric. Trad. Bot. Appl. 36: 93–143.

Pasquet R. S. (1998) Morphological study of cultivated cowpea *Vigna unguiculata* (L.) Walp. Importance of ovule number and definition of cv gr Melanophthalmus. Agronomie 18-1: 61–70.

Pasquet R. S. (1999) Genetic relationships among subspecies of *Vigna unguiculata* (L.) Walp. based on allozyme variation. Theor. Appl. Genet. 98: 1104–1119.

Pasquet R. S. (2000) Allozyme diversity of cultivated cowpea *Vigna unguiculata* (L.) Walp. Theor. Appl. Genet. 101: 211–219.

Pasquet R. S., Baudoin J. P. (2001) Cowpea. In: Charrier A., Jacquot M., Hamon S., Nicolas D., (eds.) Tropical plant breeding: 177-198. Science publishers, Enfield, USA - CIRAD, Montpellier, France.

Rieseberg L. H., Soltis D. E. (1991) Phylogenetic consequences of cytoplasmic gene flow in plants. Evol. Trends Plants 5: 65–84.

Steele W. M. (1976) Cowpeas, *Vigna unguiculata* (Leguminosae-Papilionatae). In: Simmonds N. W. (ed.) Evolution of crop plants: 183–185. Longmans, London.

Thompson J. D., Gibson T. J., Plewniak F., Jeanmougin F., Higgins D. G. (1997) The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucl. Ac. Res. 25: 4876–4882.

Ugozzoli L., Wallace R. B. (1991) Allele-specific polymerase chain reaction. Methods Enzymol 2: 42–48.

Vaillancourt R. E., Weeden N. F. (1992) Chloroplast DNA polymorphism suggests Nigerian center of domestication for the cowpea, *Vigna unguiculata*, Leguminosae. Amer. J. Bot. 79: 1194–1199.

Vaillancourt R. E., Weeden N. F., Barnard J. (1993) Isozyme diversity in the cowpea species complex. Crop Sci. 33-3: 606–613.

Westphal E. (1974) Pulses in Ethiopia, their taxonomy and agricultural significance. Agricultural Research Reports 815. Centre for Agricultural Publishing and Documentation, Wageningen.

Whitton J., Wolf D. E., Arias D. M., Snow A. A., Rieseberg L. H. (1997) The persistence of cultivar alleles in wild populations of sunflowers five generations after hybridization. Theor. Appl. Genet. 95-1/2: 33–40.

Zizumbo-Villarreal D., Colunga-Garcia Marin P., Payro de la Cruz E., Delgado-Valerio P., Gepts P. (2005) Population structure and evolutionary dynamics of wild-weedy-domesticated complexes of common bean in a Mesoamerican region. Crop Sci. 45–3: 1073–1083.

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