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RESEARCH ARTICLE

Molecular characterization of cowpea [*Vigna unguiculata* (L.) Walp.] subspecies with SSR markers

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Abstract Cowpea, *Vigna unguiculata*, is an important food legume in the tropics and subtropics. However, cowpea is a complex species with more than 10 subspecies that can hybridize and produce intermediate offspring. Partly because of the complex organization of the cowpea gene pool and the lack of adequate markers for these infraspecific units, cowpea breeders are not using the wild part of the cowpea gene pool. Here, we report the molecular characterization of 34 representative accessions with 18 polymorphic simple sequence repeat (SSR) markers from coding regions. Although the SSRs failed to separate the closest groups, i.e., subsp. *alba*, subsp. *tenuis* and the perennial groups from subsp. *unguiculata*, a combination of 11 SSR markers could properly identify the main cowpea subspecies. Regarding the infraspecific phylogeny of cowpea, the SSR markers confirmed the special status of the annual subsp. *unguiculata* versus the different perennial subspecies. They suggested that subsp. *protracta* is the oldest subspecies, making the origin of the species in southern Africa likely. All the taxa of hybrid origin, i.e., subsp. *alba*, subsp. *tenuis*, subsp. *pubescens*, and the BWA group of subsp. *unguiculata*, are in a single clade clearly separated from subsp. *unguiculata*. Although a limited number of markers were tested, considering that several hundred cowpea SSRs are available, the present work shows that SSR markers can be used for the molecular characterization of cowpea subspecies and can be very helpful for understanding the complex evolutionary history of cowpea.

Keywords Cowpea · *Vigna unguiculata* · Wild relatives · SSR · Phylogenetics

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Introduction

Cowpea, *Vigna unguiculata* (L.) Walp., is an important food legume in the tropics and subtropics, especially in sub-Saharan Africa, where it is grown for its seeds as well as for fodder (Ehlers and Hall 1997; Pasquet and Baudoin 2001; Timko et al. 2007; Xu et al. 2010; Boukar et al. 2016).

Cowpea gene pool organization is fairly complex, with numerous subspecies, including some of hybrid origin (Pasquet et al. 2021). This complexity may explain why genetic resources from the wild gene pool have never been used in breeding (Boukar et al. 2016). Indeed, although the different subspecies are morphologically well identified, there are numerous accessions that are intermediate or introgressed in various ways (Pasquet et al. 2021). Therefore, it would be helpful to identify molecular markers that can characterize subspecies, confirm morphological identifications or detect, qualify, and eventually quantify introgressions in some plants or accessions. Such molecular markers that could characterize the different subspecies are definitely needed to support the cowpea research community.

However, at a low taxonomic level, ancestral polymorphism or incomplete lineage sorting and hybridization do exist in plants, and information from all genomes is required (Naciri and Linder 2015). This is especially the case in cowpea. Chloroplast captures and lineage sorting were detected between cowpea infraspecific groups (Pasquet et al. 2021). Within cowpea, chloroplast DNA restriction fragment length polymorphisms (cpDNA-RFLPs) are interesting markers that can characterize the different subspecies (Pasquet et al. 2021), but this technique is obsolete and tedious, limiting its use. While the interesting restriction site mutations can be converted into much more convenient single nucleotide polymorphisms (SNPs), as Feleke et al. (2007) did for the *BamHI* s13.3 mutation, more cpDNA mutations should be found after full sequencing of the chloroplast genome of the various subspecies.

However, regarding the nuclear genome, there are no available markers for the molecular characterization of cowpea subspecies. Recent molecular research did not explore the cowpea gene pool beyond domesticated cowpea and its wild progenitor, i.e., subsp. *unguiculata* (Gupta et al. 2012; Huynh et al. 2013; Chen et al. 2017a; Otwe et al. 2017; Fatokun et al. 2018; Ketema et al. 2020; Gbedevi et al. 2021; Sarr et al. 2021; Munoz-Amatriain et al. 2021; Sodedji et al. 2021; Zuluaga et al. 2021; Dagnon et al. 2022; Gumede et al. 2022; Guimaraes et al. 2023). The only work focusing on the wild gene pool is that of Ogunkamni et al. (2008) based on simple sequence repeats (SSRs), but they did not try to characterize the different subspecies. However, SSRs were successfully used for the identification of closely related species in some complex taxonomic groups, e.g., *Psidium* (Tuler et al. 2015), *Rhododendron* (Wang et al. 2019), and Mediterranean *Tamarix* (Terrones et al. 2022).

Since several hundred cowpea SSRs are available, especially from functional regions (Timko et al. 2008; Andargie et al. 2014), the objective of the present work is to prove that SSRs can be used for molecular characterization of the nuclear genomes of cowpea subspecies. SSRs could improve the cowpea phylogeny, help cowpea gene bank managers characterize their wild cowpea accessions, and, ultimately, help breeders use wild cowpea genetic resources.

Materials and methods

Plant materials

The plant materials consisted of 30 wild cowpea accessions provided by Meise Botanical Garden, Belgium (http://db.plantentuinmeise.be/RESEARCH/COLLECTIONS/LIVING/PHASEOLUS/index.html), 3 wild accessions from Senegal and the breeding line Melakh provided by ISRA (Institut Sénégalais de Recherches Agricoles). All subspecies and taxonomic groups were represented, except subsp. *dekindtiana* (Harms) Verdc. sensu stricto from the South Angola Mountains, which is still absent from living collections (Table 1). Most of these accessions were already included in previous works (Pasquet 1999; Feleke et al. 2006; Pasquet et al. 2021), and the MT and SP numbers used previously were kept instead of their equivalent four-digit NI numbers from Meise Botanical Garden. *Vigna vexillata* (L.) A.Rich. NI 1014 was added as an outgroup. Plants were grown in pots filled with noninoculated sandy soil and watered with tap water twice a week.

DNA isolation and genotyping

The DNA extraction, PCR, and electrophoresis methods followed those of the Sarr et al. (2021) protocol. Considering the goal of our study, highly polymorphic SSRs were discarded, especially those showing polymorphism within subsp. *unguiculata* alone (Li et al. 2001; Diouf and Hilu 2005; Asare et al. 2010; Badiane et al. 2012; Ogunkamni et al. 2014; Ali et al. 2015;

Table 1 List of wild cowpea accessions

Taxonomic group	Accessions
subsp. *pawekiae* Pasquet	MT 53, SP 200, SP 575
subsp. *baoulensis* (A.Chev.) Pasquet	NI 933, SP 36, SP 170
subsp. *letouzeyi* Pasquet	NI 456, SP 95, SP 403
subsp. *protracta* (E.Mey.) Pienaar var. *protracta*	SP 302, SP 313, SP 322
subsp. *protracta* var. *kgalagadiensis* Mithen	MT 546, MT 593
subsp. *stenophylla* (Harv.) Maréchal et al	SP 196, SP 309, SP 363
subsp. *alba* (G.Don) Pasquet	SP 74, SP 144
subsp. *tenuis* (E.Mey.) Maréchal et al. upland	MT 340, SP 73
subsp. *tenuis* lowland	SP 167, SP 304
subsp. *pubescens* (R.Wilczek) Maréchal et al	NI 979, NI 1029
subsp. *unguiculata* var. *spontanea* BWA group	NI 817, SP 143
subsp. *unguiculata* var. *spontanea* IOCP group	SP 141, SP 219, SP 582
subsp. *unguiculata* var. *spontanea*	SN 19, SN 51, SP 595
subsp. *unguiculata* var. *unguiculata*	Melakh
Vigna vexillata	NI 1014

Var. *spontanea* BWA group (BWA for Botswana since most of these accessions come from Botswana) is thought to be introgressed with subsp. *dekindtiana* sensu stricto (Pasquet 1999). The var. *spontanea* IOCP group consists of perennial accessions from the Indian Ocean Coastal Plain, from mid-Mozambique to Kenya (Pasquet et al. 2021)

Desalegne et al. 2016; Xiong et al. 2016; Xu et al. 2010; Chen et al. 2017b; Desalegne et al. 2017; Sarr et al. 2021). A total of 61 SSR primers were selected and tested. The SSR primers can be downloaded from the Cowpea Genomics Knowledge Base (CGKB) (http://cowpeagenomics.med.virginia.edu/CGKB) (Timko et al. 2008).

Data analysis

Parsimony analysis was performed with Paup* 4.0a169 (Swofford 2017). The two most variable markers, i.e., SSRs 6193 and 6220, were removed from the dataset for this parsimony analysis.

Chromosomal location of the SSR markers and map construction

Each polymorphic SSR marker used in this study was blasted against the cowpea genome available in Phytozome (https://phytozome-next.jgi.doe.gov/). The markers were mapped to the chromosomes presented by Munoz-Amatriain et al. (2017) based on their physical position using MapChart 2.3 (Voorrips 2002).

Results

SSR polymorphism

Out of the 61 SSR primers tested, 27 yielded amplification products across all cowpea subspecies. Some primers, such as SSR 6326, amplified subsp. *unguiculata* and accessions from close subspecies but not the accessions of subspecies far from subsp. *unguiculata*, which suggests mutations in the anchoring region. The results from these primers were not included in the analysis.

Vigna vexillata was initially included as an outgroup, but the primers did not amplify the DNA for half of the accessions. For the other half, the *V. vexillata* allele was different from all the *V. unguiculata* alleles. The only exception was SSR 6209, which yielded an allele for NI 1014 that was similar to the allele of subsp. *baoulensis*. Therefore, NI 1014 was not included in the parsimony analysis, and the tree was not rooted.

Finally, 18 SSR markers were polymorphic (average 3.83 alleles per locus). With the exception of the very variable SSR 6193 (8 alleles) and SSR

Table 2 Genetic diversity information provided by the polymorphic markers used in this study

SSR	Number of alleles	Subspecies/variety characterized
SSR 6189	3	subsp. *pubescens*
SSR 6191	2	
SSR 6193	8	
SSR 6209	4	subsp. *baoulensis*, var. *protracta* subsp. *unguiculata*
SSR 6212	2	var. *protracta*, subsp. *letouzeyi*
SSR 6220	1	
SSR 6222	2	
SSR 6225	2	
SSR 6229	3	
SSR 6246	3	subsp. *stenophylla*, subsp. *unguiculata*
SSR 6274	4	subsp. *stenophylla*, var. *kgalagadiensis* var. *protracta*
SSR 6276	2	
SSR 6619	4	subsp. *letouzeyi*, subsp. *pubescens*
SSR 6674	3	
SSR 6860	2	subsp. *letouzeyi*
SSR 6920	3	subsp. *pawekiae*, subsp. *stenophylla* var. *protracta*
SSR 6924	5	subsp. *baoulensis*
SSR 7067	5	subsp. *pawekiae*

6620 (12 alleles), the number of alleles varied from 2 to 5 for the polymorphic loci (Table 2). The 18 polymorphic SSRs were distributed among 10 chromosomes (Fig. 1). Some markers were located in close vicinity (SSR 6193 and 6222, SSR 6225 and 6246, SSR 6274 and 6674), but within these marker pairs, both markers behaved very differently.

Regarding SSRs that could be used for molecular characterization, i.e., that showed no variability within a subspecies or a group, 11 SSRs characterized 6 subspecies or varieties (Table 2). A combination of SSRs 6246, 6274, and 6920 characterized not only subsp. *stenophylla* but also SP 304. A combination of SSRs 6209, 6212, 6274, and 6920 characterized var. *protracta*. Var. *protracta* was the taxonomic group most difficult to characterize.

A unique combination of three alleles from SSRs 6246, 6274, and 7067 characterized most accessions from subsp. *alba*, subsp. *tenuis*, subsp. *pubescens*, and the BWA group of var. *spontanea*, as well as accession SP 141 from the IOCP group of var. *spontanea*.

Parsimony analysis

The parsimony analysis (Fig. 2) yielded numerous trees with a length of 52 single characters. They differed in the position of MT 340 (with subsp. *pawekiae* or with var. *kgalagadiensis*), SP 167 and SP 304, and SP 219 and SP 582 (with subsp. *unguiculata*, with the subsp. *alba*—subsp. *pubescens* polytomy, or in a fourth clade). The tree presented here has a consistency index of 0.6346 and a homoplasy index of 0.3654.

Although this tree is not rooted, we can consider a basal polytomy with 3 clades. The first clade includes subsp. *baoulensis*, subsp. *letouzeyi*, subsp. *pawekiae*, subsp. *stenophylla*, var. *kgalagadiensis*, and var. *protracta*, i.e., the main subspecies (Pasquet et al. 2021).

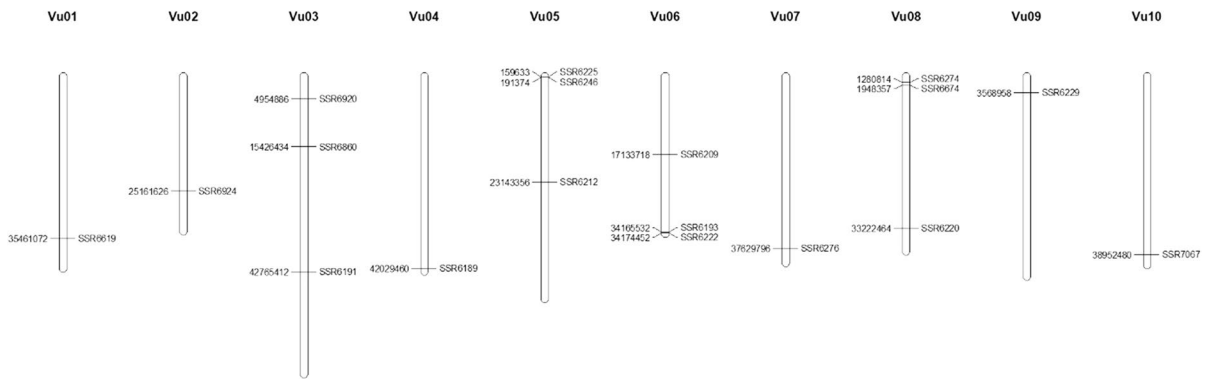

Fig. 1 Distribution of the 18 polymorphic simple sequence repeat (SSR) loci on 10 cowpea chromosomes

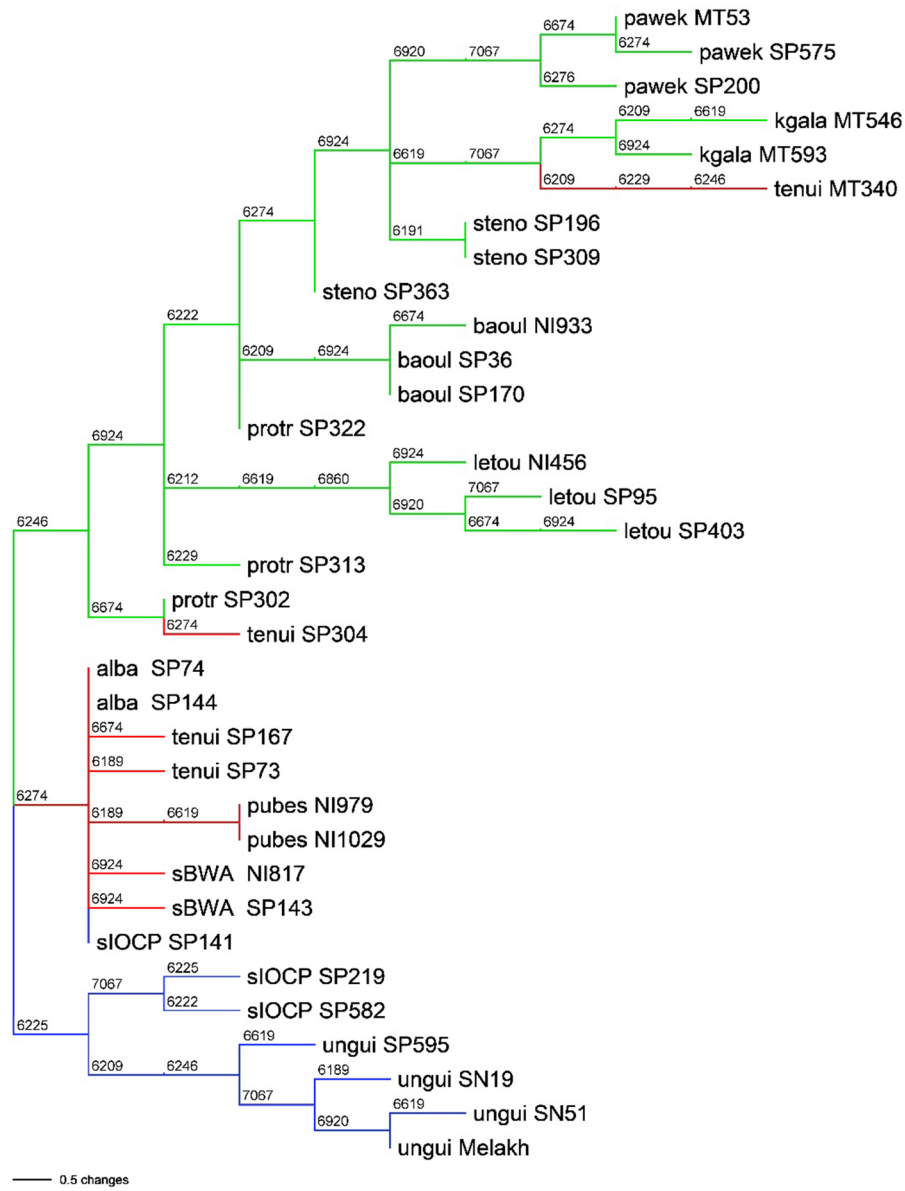

Fig. 2 Parsimony analysis. SSR 6193 and SSR 6220 were not included in this parsimony analysis

The second clade includes subsp. *pubescens*, subsp. *alba*, the BWA group, and subsp. *tenuis*, i.e., the subspecies of hybrid origin (Pasquet et al. 2021). The third clade comprises subsp. *unguiculata*, including two accessions from the IOCP group.

Discussion

The SSRs tested are spread throughout the genome. They are not concentrated on a few chromosomes and are representative of the whole genome. The SSRs tested can characterize all the main subspecies (Pasquet et al. 2021), i.e., subsp. *pawekiae*, subsp. *letouzeyi*, subsp. *baoulensis*, var. *protracta*, var. *kgalagadiensis*, and subsp. *stenophylla*, as well as the annual subsp. *unguiculata*, but they failed to characterize most of the subspecies and groups of hybrid origin (Pasquet et al. 2021), i.e., subsp. *alba*, subsp. *tenuis*, and the BWA group and the IOCP group of var. *spontanea*. There is still no set of SSRs for characterizing subsp. *tenuis* or subsp. *alba*.

Although Pasquet et al. (Pasquet et al. 2021) performed parsimony analysis of cowpea chloroplasts, this is the first cowpea gene pool parsimony analysis based on nuclear DNA. The chloroplast DNA led to a seven-clade polytomy, while we observed a three-clade polytomy. Even though subsp. *unguiculata* formed a single clade in both analyses, there are major differences between the two analyses.

Chloroplast DNA clades A, B, D, and E and the accessions not belonging to any clade are here pooled into the main clade, with the exception of subsp. *alba* accessions, which here are included in the hybrid origin clade. Regarding the organization of the cowpea gene pool, this work confirms the opposition between the main subspecies and the subspecies of hybrid origin. With the exception of the paraphyletic subsp. *stenophylla* and var. *protracta*, all the main subspecies as well as the annual subsp. *unguiculata* are monophyletic. According to this nuclear phylogeny, var. *kgalagadiensis* could deserve subspecies status.

The split between the forest subspecies from the Mensensis group and the savanna subspecies from the Dekindtiana group does not appear in this analysis. The forest subspecies do not form a monophyletic group, nor do the savannah subspecies. Instead of the forest versus savannah opposition, there seems to be opposition between the main subspecies with a keel twisted toward the left (with the exception of subsp. *letouzeyi*) and the subspecies that show a keel twisted toward the right, i.e., subsp. *unguiculata* and the subspecies with a hybrid origin.

The subspecies of hybrid origin appear in a clade between the main subspecies and subsp. *unguiculata*, along with the BWA and IOCP groups. There are alleles (from SSR 6246, 6274 and 7067) which group all these accessions in this clade. Such a grouping did not appear in Pasquet (1999) or in Ogunkanmi et al. (2008). Although grouped by these SSR markers, these accessions belong to three different chloroplast clades (Pasquet et al. 2021). Chloroplast clades C and F are consistent with the present hybrid origin clade. Subsp. *alba* having a var. *kgalagadiensis* chloroplast but being located far from var. *kgalagadiensis* seems to be a clear example of old chloroplast capture. This confirms the hybrid origin of subsp. *alba* and suggests that the male ancestor capturing the var. *kgalagadiensis* chloroplast was subsp. *tenuis* (or a taxon close to subsp. *tenuis*) instead of subsp. *unguiculata*.

As observed with cpDNA (Pasquet et al. 2021), few accessions from the subspecies of hybrid origin were not in their expected clade. Subsp. *tenuis* MT 340 is associated with var. *kgalagadiensis*. It has 3 alleles in common with var. *kgalagadiensis* and 3 alleles in common with the other subsp. *tenuis* accessions. Subsp. *tenuis* SP 304 is also misplaced due to its allele at SSR 6246, which is mainly observed in var. *protracta* (SP 304 was collected in Port Saint Johns in South Africa, a few kilometers away from a var. *protracta* area). Similarly, SP 141 is close to subsp. *alba*, subsp. *tenuis* and subsp. *pubescens* due to its allele at SSR 6246. These accessions are from a geographic area where different subspecies are known to overlap and where numerous intermediate plants are encountered. These discrepancies are likely due to recent hybridizations or to incomplete lineage sorting (Naciri and Linder 2015).

This work also confirms the special status of the annual subsp. *unguiculata*. In all the analyses, subsp. *unguiculata* was separated from the different perennial subspecies. This can be explained by its annual status. More generations should lead to the accumulation of more mutations, as observed previously with cpDNA (Pasquet et al. 2021). This should contribute to the isolation of this subspecies in the different analyses.

Var. *protracta*, located at the bottom of the clade including all the main subspecies in the parsimony analysis and not as well grouped as the other main subspecies, appears to be the oldest subspecies. Since the parsimony analysis tree is not rooted, we could also consider var. *protracta* as a hinge between the main subspecies and the group composed of subsp. *unguiculata* and the subspecies of hybrid origin. This should be in agreement with the hypothesis that the species *Vigna unguiculata* originated in southern Africa (Padulosi 1993).

Conclusion

Unfortunately, subsp. *dekindtiana* sensu stricto from southern Angola is still unavailable, and the outgroup accession was too distantly related, which hampered the reconstruction of the complex evolutionary history of *V. unguiculata*. However, this work can be considered the first attempt to perform parsimony analysis of the *V. unguiculata* nuclear genome.

Of course, a larger set of primers would need to be tested on a larger set of accessions, but the SSRs tested allowed us to characterize subsp. *pubescens* and all the main subspecies (Pasquet et al. 2021).

We can conclude that SSR markers from functional regions are an ideal tool for cowpea subspecies molecular characterization, especially since SSR analyses can be multiplexed (e.g., Mitchell et al. 1997). SSRs are robust and very reliable molecular markers that are widely used in cowpea, and they are most cost effective than sequencing. They do not require costly equipment or bioinformatic skills. In addition, since subsp. *dekindtiana* sensu stricto accessions are still unavailable, it is too early to develop several thousand SNPs based on next-generation sequencing. In the meantime, as long as subsp. *dekindtiana* sensu stricto accessions continue to be unavailable, SSRs could be used on a large scale for characterizing wild cowpea accessions in different gene banks.

Associated with SNPs derived from chloroplast restriction site mutations, such a tool should help understand the complex evolutionary history of the cowpea gene pool as well as improve its taxonomy. Perhaps more importantly, it should help breeders access the greatest part of the cowpea gene pool diversity.

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Authors' contributions RSP, NC, and AB conceived and designed the work; RD, DF, and AM helped AJCQ with the laboratory work; AJCQ, RSP and DD analyzed the results and wrote the first draft; and all authors contributed to the final manuscript.

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Data availability The datasets analyzed in the current study are available from the corresponding author upon reasonable request.

Declarations

Conflict of interest The authors have no relevant financial or nonfinancial interests to disclose.

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