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Identification of New Sources of Rust Resistance in Cowpea (*Vigna Unguiculata* L. Walp) Germplasm Using Simple Sequence Repeat Markers

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ABSTRACT

The demand for resistance cowpea to rust infection has currently increased due to considerable yield losses caused by the fungal pathogen. The study assessed available cowpea genotypes for rust resistance using simple sequence repeat (SSR) markers. Out of 100 cowpea genotypes screened, 97% showed the presence of the markers whilst 3% showed absence of the markers. Among the cowpea were 72% resistance, 16% moderate resistance and 9% low resistance to rust. The markers revealed a mean high allele frequency (0.86) and low gene diversity (0.24) and polymorphism information content (0.21) among the cowpea genotypes. The markers co-inherited with a mean regression value greater than 0.1. There was no clear pattern of clustering among the cowpea genotypes. The cowpea genotypes with rust resistance traits could serve as good sources of germplasm for cultivation or resilient genes with rust target in breeding programmes to improve the crop.

Key words: Cowpea, Genotype, Rust resistance, SSR marker.

INTRODUCTION

Cowpea [*Vigna unguiculata* (L.) Walp] is a universal legume and currently the most prominent leguminous crop cultivated in sub-Saharan Africa (Abate *et al.*, 2012). Development of the cowpea industry heavily depends on the improvement of existing cultivars and breeding for new varieties (Tan *et al.*, 2012). Grain yield (100–3000 kg ha^{-1}) and grain quality (size, colour, cookability and nutritional content) are the primary breeding goals of nearly all programmes (Simion, 2018; Timko and Singh, 2008). However, current programmes are concentrating on breeding for resistance to major cowpea pests due to tremendous economic losses (30–100%) from pests (Boa *et al.*, 2015). Unfortunately, development and screening procedures for resistance to cowpea rust disease still remain a challenge to plant breeders.

Cowpea rust caused by *Uromyces phaseoli* var. *vignae* is considered the most economically important and destructive disease of cowpea (Uma *et al.*, 2016). Rust disease adversely affects the yield and production of cowpea by reducing germination percentage, inert matter, trueness to variety, vigour and appearance of the grain (Emechebe and Florini, 1997; Wang, 2004). The use of resistant cultivars is the most effective and economical approach to control yield losses from cowpea rust disease (Li *et al.*, 2007). However, rust resistance genes are often effective only in a restricted region and resistance in cowpea may be overcome by the pathogen in a relatively short period upon release and production of the cultivar (Timko *et al.*, 2007). Besides, knowledge of cowpea germplasm resistant to rust disease is low, particularly in sub-Saharan Africa. There is an urgent need for systematic screening of available cowpea germplasm for adaptive genes to control cowpea rust and subsequently increase the yield and productivity of the crop (Boukar *et al.*, 2018).

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Conventional breeding methods such as pedigree, mass selection, pure line selection, backcross or bulk breeding do not provide ultimate solutions against many biotic stresses because they are easily influenced by the environment. Moreover, more time is normally required (12–15 years) to breed for a superior cultivar, which also depends on the source of the characteristics being introgressed (Ceballos *et al.*, 2015). Simple sequence repeat (SSR) markers, also called microsatellites, are currently the marker of choice for cowpea breeding programmes. The use of SSR markers that are linked to rust resistance genes can accelerate identification and selection of rust-resistant cowpea and enhance the overall efficiency, precision and effectiveness of cowpea improvement programmes (Semagn *et al.*, 2006). This study was therefore carried out to screen for rust resistance in some available cowpea germplasm using SSR markers and to detect genetic relatedness among cowpea genotypes.

MATERIALS AND METHODS

Sowing of cowpea genotypes for SSR marker screening was carried out in the Botanical Garden of the School of Biological Sciences, University of Cape Coast, Cape Coast, Ghana during the major cropping season of 2017. The area lies within the Coastal Savannah agro-ecological zone of Ghana and located within latitude 05°07' N and longitude 01°18' W.

One hundred (100) cowpea genotypes comprising 4 local landraces, 15 accessions from the International Institute of Tropical Agriculture (IITA) and 81 recombinant inbred lines (RILs) were obtained from the Department of Molecular Biology and Biotechnology, University of Cape Coast, Cape Coast, Ghana for the study. The cowpea genotypes are characterised by early to medium (55–75 days after planting) maturity periods, dual-purpose (grown for seeds and leaves) and high yielding with variations in flower colour (cream, white, violet, violet-pink) and seed coat colour (white, speckled white, cream, red, mottled red, brown, rough brown). Nursery plastic bags (5-inch diameter) were filled with sandy loam garden soil, watered and allowed to stand overnight. Two seeds each of the 100 cowpea genotypes were sown in each plastic bag. The experiment was laid out in a completely randomized design (CRD) with two replications.

Genomic DNA extraction

Young healthy leaves were harvested from 15 days old cowpea seedlings in the Botanical Garden, washed with 70% ethanol to remove dirt and mopped dry. Genomic DNA of each cowpea genotype was extracted using Whatman Flinders Technology Associates (FTA) card following the manufacturer's protocol (GE Healthcare Bio-Science Corp., USA). FTA discs (0.2 cm diameter) containing genomic DNA were made from the centres of the dried sample areas using Harris Uni-Core punch.

Genomic DNA amplification and agarose gel electrophoresis

Genomic DNA of the 100 cowpea genotypes were amplified with three SSR primers (Table 1) specific for rust resistance in cowpea (Uma *et al.*, 2016). The amplification reaction comprised 0.2 µM each of forward and reverse primer and 1 disc of FTA card in a 20 µL *AccuPower® Taq* PCR (polymerase chain reaction) PreMix tube (Bioneer Inc., USA). The amplification was performed in a Thermal Cycler (Bio-Rad Laboratories Inc., Singapore). The PCR condition consisted of initial denaturation at 94°C for 3 min followed by 35 cycles of denaturation at 94°C for 30 s, primer annealing at 55–58°C for 30 s and extension at 72°C for 30 s. The reaction ended with a final extension at 72°C for 5 min. The experiment was repeated two times after which the products were resolved in 2% agarose gel.

Genetic marker scoring and analysis

The SSR fragments were visually scored for presence (+) or absence (–) of bands against a 50 bp DNA ladder (Fermentas GeneRuler™). Cowpea rust gene diversity, allele frequency and polymorphism information content (PIC) of cowpea genotypes were determined in PowerMarker V3.25 software (Liu and Muse, 2005). Pairwise linkage disequilibrium (LD) test was done at a 5% significance level to evaluate the association between the SSR markers and rust resistance in the genotypes. Phylogenetic analysis was performed using Nei's genetic distance and the Unweighted Pair Group Average Method with Arithmetic mean (UPGMA) in MEGA 4.0.2 to illustrate clustering of rust-resistant and susceptible cowpea genotypes (Nei *et al.*, 1983; Sneath and Sokal, 1973; Tamura *et al.*, 2007).

Table 1: SSR primers used for screening cowpea genotypes for rust resistance.

Primer code	Primer sequences (5'–3')	Annealing temperature (°C)
VuUGM02F	GAAACTAGCACCAAATCCAACA	55.00
VuUGM02R	GAGCAAAAGCCTCCATCACT	
VuUGM08F	TCAAAAACACAGGTCCTCCA	58.00
VuUGM08R	CCTCGCCAATGATTCTGAG	
VuUGM19F	AGAACCCAGCAATACCTGCAT	57.00
VuUGM19R	CATCCCGTGAAATTCAACAA	

Table 2: Percentage of cowpea genotypes associated with rust resistance markers.

No. of SSR markers	Cowpea genotype (%)			Total (%)	Resistance status
	Local	RIL	IITA		
0	–	2.47	6.67	3.00	RS
1	–	9.88	6.67	9.00	LRR
2	–	16.05	20.00	16.00	MRR
3	100.00	71.60	66.67	72.00	RR

%: Percentage, –: Absence of cowpea genotype, RIL: Recombinant inbred line, RR: Rust resistance, MRR: Moderate rust resistance, LRR: Low rust resistance, RS: Rust susceptible.

RESULTS AND DISCUSSION

Screening of cowpea genotypes for rust resistance

The 100 cowpea genotypes showed differential expressions by either the presence or absence of a marker (Fig 1). All the markers gave the same monomorphic band size of 60 bp compared with an expected amplicon size of 200 bp (Uma *et al.*, 2016). The difference in amplicon size may be due to differences in the genetic composition of the cowpea genotypes and the differences in the inheritance of rust resistance in the genotypes (Uma *et al.*, 2011). Rust resistance in cowpea is generally controlled by recessive genes, dominance genes with additive effects, or polygenes located at different loci on the genome (Rangaiah, 1997; Uma and Salimath, 2004; Wu *et al.*, 2018). In line with the current work, the implication is that the cowpea genotypes may exhibit different resistance to cowpea rust isolate(s) as compared to rust isolate(s) infecting the genotypes from which VuUGM02, VuUGM08 and VuUGM19 SSR primers were developed (Uma *et al.*, 2016).

Presence of the SSR markers across the genome of 97% of the cowpea (Table 2) may suggest resistant genotypes of the crop. Except UCC-20 and UCC-226 (2.47%), all the RILs had the markers present which may be inherited. Cowpea genotypes (UCC-226, UCC-20 and IT04K-321-2) whose DNA were not amplified by the SSR primers may not have the rust resistance genes present. This indicates that the cowpea genotypes may have different levels of resistance to cowpea rust. The number and type of resistance genes in a genotype determine the degree of resistance by the genotype (Kourelis and van der Hoorn, 2018). The cowpea genotypes were characterised as resistant, moderately resistant and low resistant to rust based on presence of three, two and one SSR marker respectively. In contrast, genotypes that showed absence of the three SSR markers were characterised as rust susceptible. An inheritance study carried out by Rangaiah (1997) revealed two major genes associated with differences in rust resistance in cowpea genotypes.

Genetic relatedness in cowpea genotypes based on rust resistance

High allele frequency (average of 0.86) was observed among the cowpea genotypes (Table 3). However, the genetic variability and PIC were low with mean values of 0.24 and

Table 3: Allele frequency, gene diversity and PIC content of rust-resistant SSR markers across 100 cowpea genotypes.

Primer	Allele Frequency	Gene Diversity	PIC
VuUGM02	0.9300	0.1302	0.1217
VuUGM08	0.8200	0.2952	0.2516
VuUGM19	0.8200	0.2952	0.2516
Mean	0.8567	0.2402	0.2083

PIC: Polymorphism Information Content.

Table 4: Pairwise LD analysis for rust resistance.

Primer pair	MI	D'	r^2	Exact p-value
VuUGM02 and VuUGM08	0.0817	0.4774	0.0781	0.0037*
VuUGM02 and VuUGM19	0.1469	0.6516	0.1456	0.0001*
VuUGM08 and VuUGM19	0.1334	0.3902	0.1523	0.0005*
Mean	0.1207	0.5064	0.1253	

MI: Mutual information, D': Normalised LD coefficient, r^2: squared correlation coefficient. * Significant at $p \leq 0.05$.

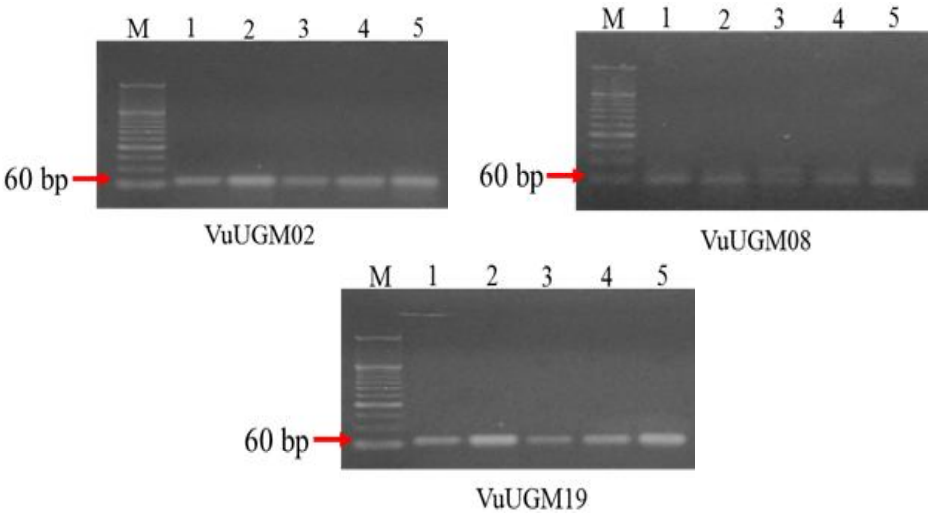

Fig 1: DNA bands resolved in 2% agarose involving VuUGM02, VuUGM08 and VuUGM19 SSR markers across cowpea genome. Lanes 1–5 denote DNA obtained from UCC-390 (1), UCC-285 (2), UCC-367 (3), Padi-Tuya (4) and IT10K-817-3 (5). Lane M represents 50 bp ladder.

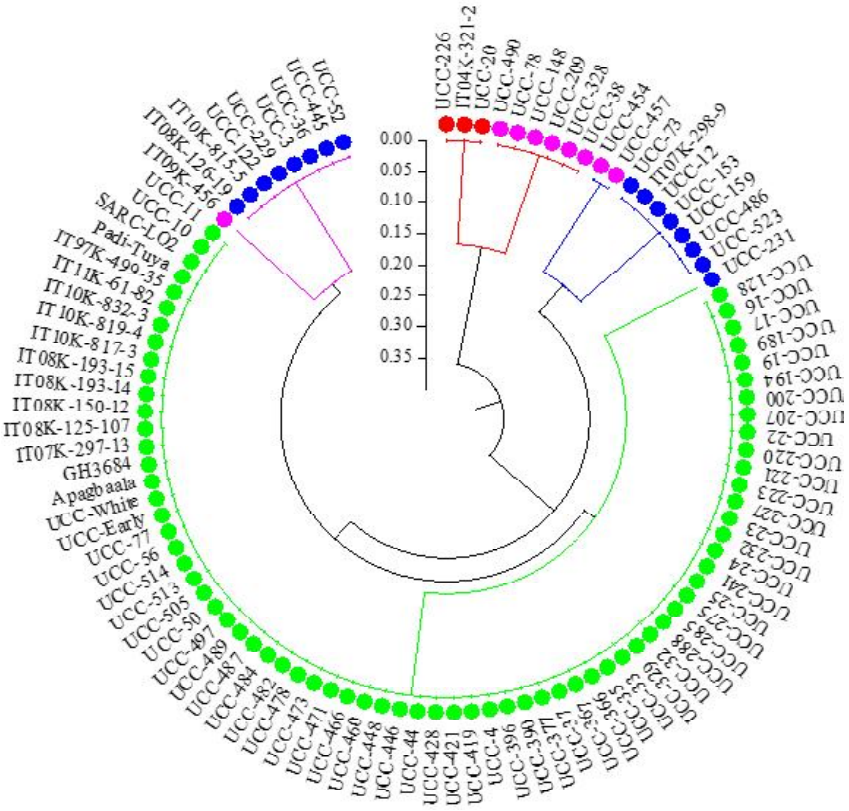

Fig 2: Phylogenetic relationships of 100 cowpea genotypes based on rust-resistant SSR primers. Red, blue, green and pink lines indicate clusters I, II, III and IV respectively. Red circles denote rust susceptible cowpea genotypes. Green, blue and pink circles denote rust resistant, moderate rust resistant and low rust resistant cowpea genotypes.

0.21 respectively. Similar findings have been reported by Deshpande *et al.* (2013) and Uma *et al.* (2011). This may suggest that SSR aided polymorphism for rust resistance in cowpea is low and may be attributed to the low genetic diversity as well as the inherent tendency for self-pollination in cowpea (Chen *et al.*, 2017). Besides, the diversity of cowpea germplasm and the number of genotypes and SSR primers used in the study may probably account for the low values of genetic variability and PIC obtained in the study.

Pairwise linkage disequilibrium (LD) for rust resistance in cowpea genotypes

The study revealed significant ($p < 0.05$) LD between the markers at theoretical maxima (D') of 47.74% (VuUGM02 and VuUGM08), 65.16% (VuUGM02 and VuUGM19) and 39.02% (VuUGM08 and VuUGM19) and regression values (r^2) > 0.078 (Table 4). These suggest that VuUGM02, VuUGM08 and VuUGM19 are tightly linked and closely associated with rust resistance in cowpea genotypes. These markers could be utilised in marker-assisted selection programmes on rust resistance in cowpea genotypes especially from diverse genetic backgrounds.

Phylogenetic analysis of cowpea genotypes based on rust resistance

In the current study, there was a lack of clear clustering pattern among the cowpea genotypes (Fig 2). Cowpea genotypes that showed rust resistance (presence of three markers) were found in Cluster III at dissimilarity coefficient of 18%. Clusters I, II and IV consisted of 9 cowpea genotypes each. Each cluster comprised genotypes that showed different resistances to rust. The low coefficient dissimilarity recorded in the cowpea genotypes further confirms that cowpea genotypes have low genetic diversity based on rust resistance. Similar observations have been made in the validation of SSR markers linked to Bean common mosaic virus (BCMV) resistance in cowpea genotypes (Manjunatha *et al.*, 2017). Absence of clear clustering pattern in rust-resistant and susceptible peanut (*Arachis hypogaea* L.) genotypes using SSR markers linked to rust have also been indicated (Gajjar *et al.*, 2014). UCC-52 and UCC-226 were most divergent and serve as invaluable genotypes for cowpea improvement.

CONCLUSION

The study showed the feasibility of SSR markers for rust resistance analysis in cowpea genotypes and recommends the identification of more SSR markers associated with other loci controlling rust resistance in cowpea to help pyramid the QTL for cowpea rust resistance. The genotypes displayed variations in their resistance to rust. Resistant cowpea

genotypes can be further assessed by phenotypic assaying and used in crossbreeding programmes to develop elite cowpea genotypes with durable resistance to cowpea rust fungus.

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