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Original article

Biochemical and molecular characterization of cowpea landraces using seed storage proteins and SRAP marker patterns

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ABSTRACT

Seven landraces of cowpea [*Vigna unguiculata* (L.) Walp.] were assessed for genetic variability in total proteins, protein fractions viz. albumins, globulins, prolamins, and glutelins by SDS-polyacrylamide gel electrophoresis and DNA polymorphism using sequence-related amplified polymorphisms (SRAP) markers. The solubility-based protein fractionation data indicated that the salt soluble fraction (globulin) and water-soluble fraction (albumin) proteins were the predominant fractions in cowpea seeds comprising 45–50.3% and 31.2–35.5% of total soluble proteins, respectively. The electrophoretic pattern revealed the molecular heterogeneity among total proteins as well as different protein fractions. The molecular weights of protein bands obtained by SDS-PAGE varied between 10 to 250, 15 to 110, 15 to 150, and 15 to 130 kDa for total proteins, albumins, globulins, and glutelins, respectively. A large number of bands were found common to the various landraces, indicative of their close relationship with one another. However, a few bands distinctive to some specific landraces were also detected, indicating varietal differences. A 34 SRAP primer pair combination generated a total of 1003 amplicons (loci) showed 100% polymorphism with an average of 0.93 polymorphism information content (PIC) value. Landraces displayed an average 0.50 similarity coefficient which clustered the landraces corresponding to their growth habit in main clusters and to their geographical origin in subcultures. Molecular and biochemical analysis were correlated with a medium level (Mantel test, $r = 0.56$, $P < 0.02$). These findings revealed that seed proteins and DNA polymorphism provide valuable information regarding the variability among landraces and this information could be utilized for breeding purposes in the enhancement of protein quality and quantity in grain legumes.

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1. Introduction

Cowpea [*Vigna unguiculata* (L.) Walp.] is one of the most important pulse crops which serves multiple purposes for human food

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and animal feed. The dry seeds can be used as a cheap source of protein and a vegetable for human consumption (Nielsen et al., 1997) and as supplement fodder to cereal for livestock (Singh et al., 2003). Cowpea can grow in a wide range of environments including Asia, Africa, Central and South America, the United States, and parts of southern Europe. Genetic variation and environmental adaption of the species are highly associated with the evolution of the species; in crops this is influenced by human activities included domestication, cultivation, and immigration (Xiong et al., 2018). Despite the significant difference in seed protein content among cowpea varieties, some studies have reported genetic control of protein accumulation by three to seven genes (Fernandes et al., 2012). In addition to genetics, location can also produce such variability in seed protein content in cowpea vari-

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eties (Fernandes et al., 2012). Genetic improvement of cowpea is mainly dependent on breeding and selection from existing landraces according to the existence of phenotypic variability, which is largely influenced by environmental conditions. In depth knowledge regarding the genetic mechanisms governing the variability of phenotypic traits, and the accurate assessment of genetic variability, is important for the preservation and utilization of germplasm resources. Subsequent improvement of cultivars would benefit the development of effective breeding strategies. However, selection according to genetic variability using biochemical and molecular markers proved advantageous compared with the use of other markers (phenotypic). Briefly, the low cost to generate large number of samples encompassed low genetic variability; high reproducibility and multi-locus nature make these markers particularly more attractive (Hayward et al., 2015; Teixeira-daSilva et al., 2017).

The proteins in seeds of different cowpea landraces ranged from 15.06% to 38.5% (Afiukwa et al., 2013), 20.57% to 24.95% (Itatut et al., 2013) and 25.80% to 28.95% (Oke et al., 2015), showing great variability among landraces. In addition to this, protein fractions i.e. albumins, globulins, glutelins, and prolamins of cowpea landraces also exhibited significant differences in molecular weights of protein bands using SDS-polyacrylamide gel electrophoresis as reported by Gupta et al. (2014). The different variants of seed protein types i.e. globulins (at least 16 protein variants), glutelins (21 variants), albumins (at least 20 variants), and prolamins (one variant) have been observed (Tchiagam et al., 2011). Variations in protein content, proteins fractions and other seed characteristics were also observed among other varieties of cowpea by Ajeigbé et al., (2008) and Vasconcelos et al., (2010). Typically, sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) is utilized for protein separation and molecular weight estimation as a qualitative tool for the analysis of seed proteins (Luo et al., 2004). DNA-based molecular markers have become the tool of choice; they have been widely used in genetic diversity research, variety identification, phylogenetic analysis, gene mapping and resource classification in various crops, including cowpea. Restriction fragment length polymorphism (RFLP) (Fatokun et al., 1993; Ouédraogo et al., 2002), amplified fragment length polymorphism (AFLP), (Kolade et al., 2016; Fang et al., 2007), simple sequence repeat (SSR), (Wamalwa et al., 2016; Asare et al., 2010; Desalegne et al., 2016; Chen et al., 2017), random amplified polymorphic DNA (RAPD) (Udensi et al., 2016; Prasanthi et al., 2012), single nucleotide polymorphisms (SNP) (Muchero et al., 2009; Carvalho et al., 2017), ISSR and start codon targeted (SCoT) (Igwé et al., 2017) have been used in cowpea genetic and breeding studies.

The sequence-related amplified polymorphism (SRAP) represents a simple and reliable PCR-based marker tool for genetic diversity analysis (Li and Quiros, 2001). SRAP marker characteristics include: a reasonable throughput rate, disclosure of numerous co-dominant markers, more reproducible than RAPDs and ease of performing assays compared to AFLPs and, most importantly, targeting of open reading frames (ORFs). In legumes SRAP was used to assess the genetic diversity in faba beans (Alghamdi et al., 2012; Ammar et al., 2015), lentils (Rana et al., 2009; Alghamdi et al., 2014), alfalfa (Vandemark et al., 2006; Ariss and Vandemark, 2007; Castonguay et al., 2010; Al-Faifi et al., 2013), peas (Esposito et al., 2007; Guindon et al., 2016) and chickpeas (Khan et al., 2016). However, SRAP markers have rarely been applied to assess genetic variation of cowpea, and to our knowledge, this is the first application of SRAP markers used for assessment of genetic variability in cowpeas. This study is aimed at evaluating the genetic differences in cowpea landraces through analysis of total seed protein and its four soluble fractions, by SDS-PAGE and DNA polymorphism using SRAP markers.

2. Materials and methods

2.1. Total protein extraction from cowpea seeds

Seven cowpea landraces, i.e. five landraces from Saudi Arabia and one each from Yemen and Egypt were used in this study. These landraces were deposited in KSU gene bank and designated as KSU-CO98 (Jizan, KSA), KSU-CO99 (Jizan, KSA), KSU-CO100 (Al Ahsa, KSA), KSU-CO101 (Jizan, KSA), KSU-CO102 (Jizan, KSA), KSU-CO103 (*cv.* Kafr El Sheikh, Egypt) and KSU-CO104 (YG 30119, Yemen). The seeds of these genotypes were obtained after growing these genotypes at the Dirab Agriculture Research Station, Riyadh by applying all cultural practices. The resulting seeds were ground to fine powder with a coffee grinder and flour so obtained was used for protein extraction.

The total proteins were extracted from cowpea flour (100 mg) using 1 ml of 0.1 M Tris-HCl buffer (pH 8.0) (Gupta et al., 2014). The crude homogenate was stirred intermittently for 2 h and then centrifuged at 10,000g for 20 min. The supernatant so obtained was designated as total protein of cowpea seeds and used for analysis using SDS-polyacrylamide gel electrophoresis.

2.2. Sequential extraction of proteins from cowpea seeds

Different fractions *viz.* albumin (water-soluble), globulin (salt-soluble), and glutelin (alkali-soluble) protein fractions were extracted sequentially according to the procedure of Agboola et al., (2005) with little modifications. Briefly, a sample (100 mg) of cowpea flour was mixed with distilled water (1 ml). The mixture was stirred intermittently for 2 h and then centrifuged at 10,000g for 20 min. The supernatant was designated as an albumin fraction. The residue was mixed with 5% NaCl and stirred for 2 h before being centrifuged at 10,000g for 20 min. The supernatant so obtained was designated as a globulin fraction. The residue from the globulin fraction was mixed with 1 ml of 70% ethanol and stirred intermittently for 2 h. The mixture was then centrifuged at 10,000 × g for 20 min and the supernatant was designated as a prolamin fraction. The residue from the prolamin fraction was mixed with 1 ml of 0.1 N NaOH and stirred intermittently for 2 h before the mixture was centrifuged at 10,000 × g for 20 min. The resulting supernatant was designated as a glutelin fraction. All these four fractions were used for SDS-PAGE analysis.

2.3. Protein estimation

The total protein content and proteins from different fractions were estimated by a modified Lowry assay (Bensadoun and Weinstein, 1976) using bovine serum albumin as standard.

2.4. SDS-PAGE analysis

The total protein and proteins from different fractions were analyzed using SDS-polyacrylamide gel electrophoresis (10% separating gel and 5% stacking gel). The 50 µL extract of each sample was mixed with an equal volume of 2X SDS sample loading buffer (125 mM M Tris-HCl buffer pH 6.8, containing 4% SDS, 10% glycerol, 0.5% bromophenol blue and 200 mM β-mercaptoethanol) and heated at 100 °C for 3–5 min in a water bath. After heating, each sample was loaded in the wells of the gel. Electrophoresis was performed at room temperature at a constant voltage of 80 V in the stacking gel and 120 V thereafter in the separating gel. The gel was run until the tracking dye reached 5 mm above the bottom of the gel. After electrophoresis, the gel was stained with Coomassie brilliant blue R-250 (SigmaAldrich) solution (0.2% R-250 in methanol (45%) water (45%) and acetic acid (10%)

and destained with frequent washings with destaining solution of methanol: acetic acid: water (50: 40: 10 v/v/v, respectively). Relative mobility (R_m) of the protein band was determined. For molecular weight determination, PageRuler broad range unstained protein ladder (containing a mixture of marker protein ranged from 5 to 250 KDa) was also run along with the samples during electrophoresis in the same gel.

2.5. DNA extraction

Total genomic DNA was extracted using the modified SDS based method (Al-faifi et al., 2013). Two-week-old cowpea leaves ground in liquid N_2 and 100 mg were mixed with 800 μ L of extraction buffer (100 mM Tris-HCl pH 8.0, 50 mM EDTA pH 8.0, 1.4 M NaCl, 2% SDS v/v, PVP 2% v/v, and 0.1% mercaptoethanol), and incubated at 65 °C for 30 min., then samples were treated by RNase1 (10 mg/mL). An equal volume (800 μ L) of chloroform-isoamyl alcohol 24:1 was added, mixed thoroughly and centrifuged at 13,680g for 20 min. A 1/3 vol (500 μ L) of 5 M potassium acetate was added to supernatant and vigorously mixed and centrifuged at 13,680g for 20 min. The supernatant was transferred to new 1.5 ml tubes, and 1/2 vol (500 μ L) of cold isopropanol was added, then centrifuged at 13,680g for 15 min at 4 °C. The pellets were re-suspended in 300 μ L of TE (10 mM Tris, 1 mM EDTA, pH 8.0), incubated at 65 °C for 30 min, centrifuged at 13,680g for 5 min at 4 °C and 1/10 vol (30 μ L) of 3 M Sodium acetate and 2/3 vol of ice-cold isopropanol were added. The samples were mixed thoroughly, incubated at 4 °C for 1 h, and centrifuged at 13,680g for 10 min at 4 °C to pellet the DNA. The pellets were washed with 80% EtOH for 10 min, centrifuged at 13,680g for 10 min at 4 °C, the solution discarded, and the tubes inverted to dry for 30 min. The DNA samples were dissolved in 100 μ L of TE. The integrity of the extracted DNA was detected using 1% agarose gel electrophoresis in TBE buffer and a Nano drop-2000 spectrophotometer was used for DNA quantification. Dilutions with TE were carried out and the concentration was fixed at 100 ng/ μ L.

2.6. SRAP-PCR

Out of forty-eight SRAP primer combinations (6 forward and 8 reverse) screened, 34 displayed consistently reproducible polymorphisms, were selected and used to analyze the cowpea accessions (Table 2). The forward primers were labeled with FAM dye at the 5' end. The PCRs were performed in 20 μ L reaction volumes containing 1 \times GoTaq Green Master Mix (Cat. No. M7123, Promega Corporation, Madison, WI, USA), 0.1 μ M of each forward and reverse primer, 50 ng DNA template, and nuclease-free water to 20 μ L. The thermal cycler profile for PCR amplification was set on a TC-5000 thermal cycler (Bibby Scientific, Staffordshire, UK) as follows: denaturation at 94 °C for 5 min, followed by five cycles of denaturing at 94 °C for 1 min, annealing at 35 °C for 1 min, and elongation at 72 °C for 1 min. In the remaining 30 cycles, the annealing temperature was increased to 50 °C for 1 min, followed by a final elongation step at 72 °C for 7 min. For electrophoresis, 1 μ L of the PCR amplified product was mixed with 0.5 μ L of the GeneScan 500 LIZ size standard (Applied Biosystems P/N 4322682), and 8.5 μ L of Hi-Di Formamide (Applied Biosystems P/N 4311320). The mixture was denatured and loaded on the 16-capillary system of the Applied Biosystems 3130xl Genetic Analyzer. A 36-cm capillary array (Applied Biosystems P/N 4315931) and 3130 POP-7 polymer (Applied Biosystems P/N 4352759) were used.

2.7. Statistical analysis

Finally, gels were visualized on a white light illuminator and only visibly clear bands, unambiguous, monomorphic, and poly-

morphic were scored. A score of 0 was given to the absence of a, while a score of 1 indicated its presence. Alike SRAP, the reproducible banding patterns of each primer were chosen for analysis, 0 and 1 binary data for absent and present bands were scored. Data analyses were conducted using PAST 3, version 3.18 (Hammer et al., 2001). Similarities between landraces were estimated using Jaccard coefficients (Jaccard, 1908). Lastly, the similarity coefficients were used to construct the dendrogram by using the unweight pair group method with the arithmetic average (UPGMA). The polymorphism information content (PIC) for each primer was calculated to estimate its allelic variation as follows:

$$PIC = 1 - \sum_{j=1}^n P_{ij}^2$$

where P_{ij} is the frequency of the i th allele for the marker j , and the summation extends over n alleles, being calculated for each SRAP marker (Anderson et al., 1993). Discrimination power was calculated by dividing the number of polymorphic markers amplified for each primer by the total number of polymorphic bands obtained (Brake et al., 2014). The possible correlation between SDS-PAGE and SRAP patterns was evaluated by a Mantel test (Mantel, 1967) based on Pearson's correlation (XLSTAT Pearson edition, version 2017).

3. Results and discussion

3.1. SDS-PAGE

Protein profiling of the seed storage proteins has been successfully used to detect differences within the populations of crop plants (Hameed et al., 2012). Legume seeds, in general, are largely comprised of albumins (20–35%), globulins (43–55%), prolamins (0.73–2.70%) and glutelins (11.84–32.21%) (Tchiagam et al., 2011). Of these, albumins are water soluble, globulins are salt soluble, prolamins are alcohol soluble and glutelins are alkali soluble. Albumin and globulin together account for 63–90% of the total seed proteins. Therefore, in the present study, the protein profile of the total proteins and various protein fractions viz. albumins, globulins, prolamins and glutelins of seven cowpea landraces was determined using SDS-PAGE.

Quantification results of different protein fractions are provided in Table 1. These results indicate that the salt-soluble fraction (globulins), which ranged from 45 to 50.3% of the total soluble proteins, with a mean value of 47.7%, was the major protein fraction. The second most abundant seed protein among the studied cowpea landraces was the water-soluble fraction (albumins), which ranged from 31.2 to 35.5% of the total soluble proteins with a mean value of 33.3%. The third most abundant seed protein was glutelins which varied from 15.1 to 20.5% of the total soluble protein with a mean value of 18.1%. Minor soluble fractions in cowpea landraces appeared to be prolamins, which ranged between 0.5 and 1.3% of

Table 1
The soluble protein contents in different fractions of seed storage protein.

| Genotype | Albumin (% SP) | Globulin (% SP) | Glutalin (% SP) | Prolamin (% SP) |
|-----------|----------------|-----------------|-----------------|-----------------|
| KSU-CO98 | 31.6 | 49.2 | 18.7 | 0.5 |
| KSU-CO99 | 32.6 | 46.3 | 20.5 | 0.6 |
| KSU-CO100 | 34.2 | 48.9 | 15.7 | 1.2 |
| KSU-CO101 | 33.9 | 45 | 20.3 | 0.8 |
| KSU-CO102 | 34.4 | 45.7 | 18.6 | 1.3 |
| KSU-CO103 | 35.5 | 48.3 | 15.1 | 1.1 |
| KSU-CO104 | 31.2 | 50.3 | 17.9 | 0.6 |
| Mean | 33.3 | 47.7 | 18.1 | 0.9 |
| Max | 35.5 | 50.3 | 20.5 | 1.3 |
| Min | 31.2 | 45 | 15.1 | 0.5 |

Table 2

Primers used for SRAP analyses, total number and polymorphic amplicons, total number of bands across landraces, polymorphism information content (PIC) and discrimination power (DP).

| Primer code | Forward sequence (5'-3') | Reverse sequence (5'-3') | Total # amplicons | Total # bands | PIC | DP |
|-------------|--------------------------|--------------------------|-------------------|---------------|------|------|
| SR1xSr6 | TGAGTCCAAACCGGATA | GACTGCGTACGAATTAGC | 42 | 63 | 0.97 | 4.19 |
| SR1xSr12 | TGAGTCCAAACCGGATA | GACTGCGTACGAATTCAT | 31 | 65 | 0.95 | 3.09 |
| SR1xSr16 | TGAGTCCAAACCGGATA | GACTGCGTACGAATTGAC | 31 | 46 | 0.96 | 3.09 |
| SR1xSr17 | TGAGTCCAAACCGGATA | GACTGCGTACGAATTGAT | 33 | 49 | 0.96 | 3.29 |
| SR1xSr20 | TGAGTCCAAACCGGATA | GACTGCGTACGAATTGGT | 31 | 47 | 0.96 | 3.09 |
| SR1xSr21 | TGAGTCCAAACCGGATA | GACTGCGTACGAATTGTA | 20 | 32 | 0.92 | 1.99 |
| SR1xSr29 | TGAGTCCAAACCGGATA | GACTGCGTACGAATTGTC | 26 | 40 | 0.94 | 2.59 |
| SR2xSr12 | TGAGTCCAAACCGGAGC | GACTGCGTACGAATTCAT | 51 | 80 | 0.97 | 5.08 |
| SR2xSr16 | TGAGTCCAAACCGGAGC | GACTGCGTACGAATTGAC | 44 | 70 | 0.97 | 4.39 |
| SR2xSr17 | TGAGTCCAAACCGGAGC | GACTGCGTACGAATTGAT | 25 | 35 | 0.94 | 2.49 |
| SR2xSr21 | TGAGTCCAAACCGGAGC | GACTGCGTACGAATTGTA | 57 | 86 | 0.98 | 5.68 |
| SR8xSr9 | TGAGTCCAAACCGGTAA | GACTGCGTACGAATTCAT | 31 | 83 | 0.95 | 3.09 |
| SR8xSr18 | TGAGTCCAAACCGGTAA | GACTGCGTACGAATTGCA | 28 | 57 | 0.95 | 2.79 |
| SR8xSr22 | TGAGTCCAAACCGGTAA | GACTGCGTACGAATTGTC | 49 | 75 | 0.95 | 4.89 |
| SR4xSr1 | TGAGTCCAAACCGGAGA | GACTGCGTACGAATTAAC | 19 | 36 | 0.92 | 1.89 |
| SR4xSr2 | TGAGTCCAAACCGGAGA | GACTGCGTACGAATTAAT | 8 | 14 | 0.84 | 0.80 |
| SR4xSr3 | TGAGTCCAAACCGGAGA | GACTGCGTACGAATTACA | 26 | 26 | 0.79 | 2.59 |
| SR4xSr5 | TGAGTCCAAACCGGAGA | GACTGCGTACGAATTACT | 21 | 47 | 0.93 | 2.09 |
| SR4xSr6 | TGAGTCCAAACCGGAGA | GACTGCGTACGAATTAGC | 17 | 27 | 0.91 | 1.69 |
| SR4xSr7 | TGAGTCCAAACCGGAGA | GACTGCGTACGAATTATG | 6 | 16 | 0.82 | 0.60 |
| SR4xSr8 | TGAGTCCAAACCGGAGA | GACTGCGTACGAATTATT | 11 | 11 | 0.77 | 1.10 |
| SR4xSr9 | TGAGTCCAAACCGGAGA | GACTGCGTACGAATTCAT | 9 | 19 | 0.84 | 0.90 |
| SR4xSr12 | TGAGTCCAAACCGGAGA | GACTGCGTACGAATTCAT | 19 | 36 | 0.92 | 1.89 |
| SR4xSr13 | TGAGTCCAAACCGGAGA | GACTGCGTACGAATTCCA | 38 | 62 | 0.96 | 3.79 |
| SR4xSr14 | TGAGTCCAAACCGGAGA | GACTGCGTACGAATTCTA | 36 | 56 | 0.96 | 3.59 |
| SR4xSr15 | TGAGTCCAAACCGGAGA | GACTGCGTACGAATTGAA | 13 | 28 | 0.88 | 1.30 |
| SR4xSr16 | TGAGTCCAAACCGGAGA | GACTGCGTACGAATTGAC | 35 | 63 | 0.96 | 3.49 |
| SR4xSr17 | TGAGTCCAAACCGGAGA | GACTGCGTACGAATTGAT | 29 | 49 | 0.95 | 2.89 |
| SR4xSr18 | TGAGTCCAAACCGGAGA | GACTGCGTACGAATTGCA | 22 | 34 | 0.94 | 2.19 |
| SR4xSr19 | TGAGTCCAAACCGGAGA | GACTGCGTACGAATTGGA | 19 | 25 | 0.92 | 1.89 |
| SR4xSr21 | TGAGTCCAAACCGGAGA | GACTGCGTACGAATTGTA | 24 | 45 | 0.94 | 2.39 |
| SR4xSr24 | TGAGTCCAAACCGGAGA | GACTGCGTACGAATTAC | 54 | 69 | 0.97 | 5.38 |
| SR4xSr28 | TGAGTCCAAACCGGAGA | GACTGCGTACGAATTGA | 67 | 153 | 0.97 | 6.68 |
| SR4xSr30 | TGAGTCCAAACCGGAGA | GACTGCGTACGAATTTTA | 31 | 53 | 0.94 | 3.09 |
| Total | | | 1003 | 1697 | – | – |
| min | | | 6.00 | 11.00 | 0.77 | 0.60 |
| max | | | 67.00 | 153.00 | 0.98 | 6.68 |
| Average | | | 29.50 | 49.91 | 0.93 | 2.94 |

the total soluble protein with a mean value of 0.9%. These results are more or less agreed with those described previously for cowpea genotypes (Tchiagam et al., 2011; Gupta et al., 2010). The variations among the contents of different protein fractions within cowpea genotypes also depend on different factors especially extracting method used, the cultivars and also on environmental and genetic variability (Vasconcelos et al., 2010).

The electrophoretic pattern of the Coomassie blue stained SDS-polyacrylamide gel for the total seed storage protein extracts of seven landraces of cowpea is shown in Fig. 1. The total proteins extracted covered a wide range of molecular weights on SDS-PAGE, within the region 10–250 kDa. The protein band profiles of all seven landraces showed considerable similarities with respect to the number of protein bands and their band intensities. No variability among the landraces was observed in the protein bands with respect to the presence or absence of bands, except one. However, there were slight differences in intensities of protein bands among the landraces. The number of clearly visible protein bands exhibited, ranged from 24 to 25 bands in different landraces, with a maximum of 25 in KSU-CO98, KSU-CO99, and KSU-CO104, followed by 24 each in KSU-CO100, KSU-CO101, KSU-CO102, and KSU-CO103. Only one polymorphic polypeptide with a molecular weight of approximately 22 kDa was absent in KSU-CO100, KSU-CO101, KSU-CO102, and KSU-CO103, compared to the other three landraces. The overall band intensities in landraces KSU-CO101 and KSU-CO102 were more intense than other landraces, followed by KSU-CO103, KSU-CO104, KSU-CO100, KSU-CO99 and KSU-CO98, respectively. The similarities between protein bands among

landraces highlighted the close genetic relationships among them. Gupta et al. (2014) and Kalloo et al. (2001) were able to distinguish cowpea landraces individually by SDS-PAGE and suggested that the electrophoretic technique was the most appropriate technique to differentiate among closely related varieties, and to unveil the molecular heterogeneity of proteins. Similarly, protein polymor-

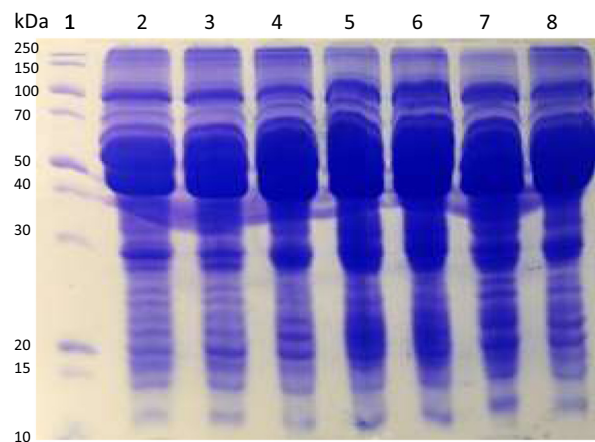


Fig. 1. SDS-PAGE showing the profiles of total seed storage protein of cowpea genotypes. Lane 1: Protein ladder, lane 2: KSU-CO98, lane 3: KSU-CO99, lane 4: KSU-CO100, lane 5: KSU-CO101, lane 6: KSU-CO102, lane 7: KSU-CO103, lane 8: KSU-CO104.

phism indicated that the existence of genetic variability among landraces of cowpea and the results are in accordance with earlier findings of Gupta et al. (2014) in cowpea landraces, Goyal and Sharma (2003) in cluster bean and Dhillon and Nainawatee (1989) in mung bean.

Characterization of different protein fractions of seed storage proteins is the prerequisite for both basic and applied studies. Therefore, individual protein fractions viz. albumin, globulin, prolamin and glutelin were characterized for their electrophoretic pattern by SDS-PAGE as shown in Figs. 2–5. Fig. 2 depicts the electrophoretic pattern of the albumin protein fraction of seven cowpea landraces. The Albumin fraction was resolved into maximum 21 and minimum 19 major polypeptides and their molecular weights varied between 15 and 110 kDa. The intensity of proteins bands is almost equal except KSU-CO104 which has a low intensity. This protein fraction distribution is similar to that found in others such as blackgram, where Chavan and Djurtoft (1982) reported 8 albumin subunits having Mr 27 to 140 kDa. Sathe and Salunkhe (1981) also separated albumins in *Phaseolus vulgaris* into 14 bands which ranged from 14.7 to 55.4 kDa. The only three polypeptides of molecular weights 52, 37 and 25 kDa showed polymorphism among seven landraces, according to their presence and absence in some landraces. The 52 kDa polypeptide was present only in KSU-CO102 and KSU-CO103 landraces, while 37 kDa

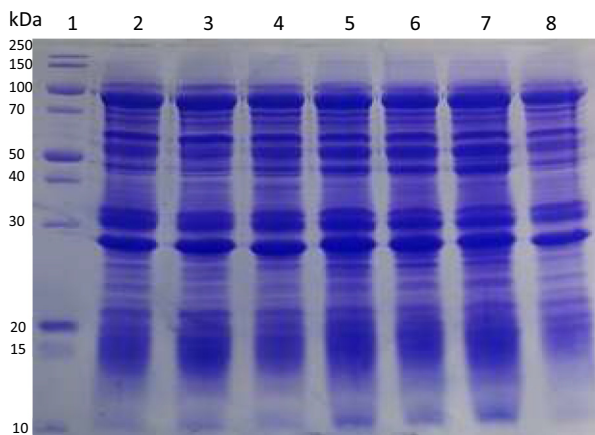


Fig. 2. SDS-PAGE showing the profiles albumin fraction of seed storage protein of cowpea genotypes. Lane 1: Protein ladder, lane 2: KSU-CO98, lane 3: KSU-CO99, lane 4: KSU-CO100, lane 5: KSU-CO101, lane 6: KSU-CO102, lane 7: KSU-CO103, lane 8: KSU-CO104.

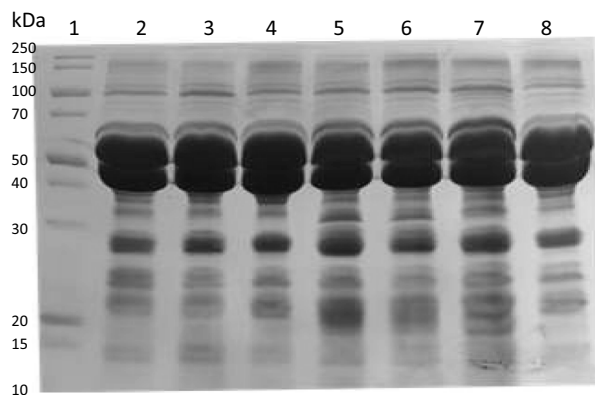


Fig. 3. SDS-PAGE showing the profiles globulin fraction of seed storage protein of cowpea genotypes. Lane 1: Protein ladder, lane 2: KSU-CO98, lane 3: KSU-CO99, lane 4: KSU-CO100, lane 5: KSU-CO101, lane 6: KSU-CO102, lane 7: KSU-CO103, lane 8: KSU-CO104.

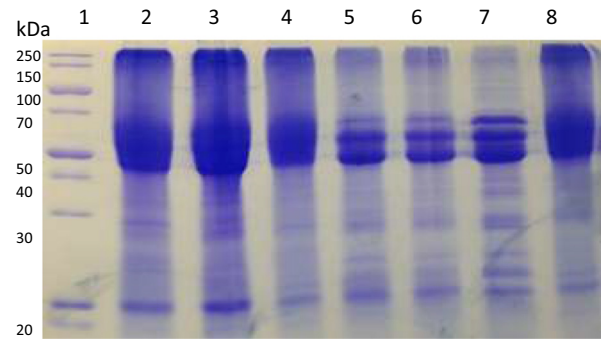


Fig. 4. SDS-PAGE showing the profiles glutelin fraction of seed storage protein of cowpea genotypes. Lane 1: Protein ladder, lane 2: KSU-CO98, lane 3: KSU-CO99, lane 4: KSU-CO100, lane 5: KSU-CO101, lane 6: KSU-CO102, lane 7: KSU-CO103, lane 8: KSU-CO104.

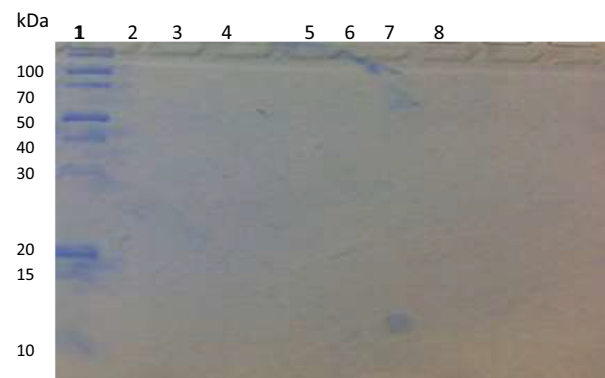


Fig. 5. SDS-PAGE showing the profiles prolamin fraction of seed storage protein of cowpea genotypes. Lane 1: Protein ladder, lane 2: KSU-CO98, lane 3: KSU-CO99, lane 4: KSU-CO100, lane 5: KSU-CO101, lane 6: KSU-CO102, lane 7: KSU-CO103, lane 8: KSU-CO104.

polypeptide was absent only in KSU-CO102 and 25 kDa was absent only in KSU-CO100. These polypeptide results are closely matched with molecular weight of the pea proteins separated by SDS-PAGE where they reported the polymorphism of only 3 albumin proteins of 26, 47 and 78 kDa (Grant et al., 1976). The electrophoretic pattern of the globulins protein fraction is shown in Fig. 3. The globulin fraction was separated into maximum 19 (KSU-CO101, KSU-CO102 and KSU-CO103) and minimum 17 (KSU-CO98, KSU-CO99, KSU-CO100 and KSU-CO104) polypeptide bands with molecular weights ranging from 15 to 150 kDa. Two highly intense bands of molecular weights 47 and 52 kDa were present in all landraces. However, they were slightly less intense in KSU-CO101, followed by KSU-CO102 and KSU-CO103. Three polypeptides of molecular weights approximately 70, 30 and 17 kDa were polymorphic among seven landraces. The 70 kDa polypeptide was present only in KSU-CO103 landrace, while 30- kDa polypeptide was present only in KSU-CO101 and KSU-CO102, and 17 kDa was present only in KSU-CO101, KSU-CO102 and KSU-CO103. Similar heterogeneity in globulin protein fractions has also been reported by Gupta et al. (2014) in cowpea, Rao et al. (1992) in Vigna species and Mendoza et al. (2001) in mung bean with SDS-PAGE.

The glutelin fraction was separated into maximum 11 (KSU-CO103) and minimum 7 (KSU-CO98, and KSU-CO100, KSU-CO104) polypeptide bands with molecular weights ranging between 15 and 130 kDa (Fig. 4). The other landraces KSU-CO99, KSU-CO101, and KSU-CO102 have maximum 8, 10 and 9 polypeptide bands respectively. The polypeptide of molecular weight 48 and 55 kDa was more intense in KSU-CO98, KSU-CO99, KSU-

CO100 and KSU-CO104, as compared to other landraces. The four polypeptides of molecular weights 38, 29, 28 and 25 kDa showed heterogeneity among seven landraces. Heterogeneity in glutelins was reported in glutelins of rice (Orth and Bushuk, 1973) and maize (Paulis and Wall, 1975). The electrophoretic pattern of prolamin protein fraction is shown in Fig. 5; no visible bands appeared on the gel. This may be due to the low abundance of prolamin in legume seeds, as formerly reported by Tchiagam et al., (2011).

3.2. Clustering analysis

A dendrogram was constructed using Jaccard's similarity coefficients obtained for electrophoretic binary data, from the total seed storage proteins and various protein fractions of the seven landraces of cowpea employing past3 statistical software (Fig. 6). The dendrogram clustered the seven landraces into two distinct clusters according to their growth patterns. Cluster one landraces consists of indeterminate landraces (KSU-CO98, KSU-CO99, KSU-CO100 and KSU-CO104) while cluster two consists of determinate landraces (KSU-CO101, KSU-CO102 and KSU-CO103). Cluster one is further separated into two sub clusters, sub cluster A1 and A2. Sub cluster A1 consists of three landraces (KSU-CO104, KSU-CO98 and KSU-CO99) while sub cluster A2 separated into one individual landrace, KSU-CO100. Cluster A1 followed the geographical origin as KSU-CO104 and KSU-CO98 are from Yemen, while KSU-CO99 is from Jizan Saudi Arabia, yet Jizan is near to the Yemen border. The landrace, KSU-CO100 in sub cluster A2 belongs to Al Ahsa region. Similarly, the second main cluster is further divided into two sub clusters, sub cluster B1 and sub cluster B2. Sub cluster B1 is separated into two landraces (KSU-CO101 and KSU-CO102) which are from the Jizan region, and sub cluster B2 separated into an individual landrace, KSU-CO103, which is from Egypt. Clustering showed the pattern of geographic origin as almost all landraces are separated into different clusters based on their geographical origin.

3.3. Molecular characterization using SRAP markers

Vigna unguiculata is a diploid species ($2n = 2x = 22$ chromosomes), nuclear DNA content of 1.27 pg/2C and a genome size of 613 Mbp (Arumuganathan and Earle, 1991). It is a highly self-

pollinated plant (Ehlers and Hall, 1997). Depending on the subspecies, outcrossing occurs at different rates (Pasquet, 1996) varying between 1 and 9.5%, as well as possible gene flow between domesticated to wild cowpea var. *spontanea* (Kouam et al., 2012).

The genetic diversity among seven Saudi cowpea landraces was analyzed using SRAP markers. Among 48 (6*8) SRAP primer pair combinations tested, 34 primers showed consistent polymorphisms and were used for the analysis of genetic diversity in cowpea landraces. A total of 1003 amplicons (loci) with an average of 29.5 amplicons per primer pair combinations were obtained, ranging in size from 100 to 500 bp. All loci showed 100% polymorphism (Table 2). A maximum of 67 amplicons was generated for primer pair combinations SR4*SR28, while SR4*SR7 primer pair combinations resulted in the generation of 6 amplicons. The SRAP profiling revealed an average of 29.5 amplicons per primer pair combinations, while 16 out of 34 primers generated more than 30 amplicons. Across landraces, primer pair combinations SR4*SR28 generated a total of 153 bands, while only 11 bands were produced by SR4*SR8 primer pair combinations. All primers produced 1697 bands with an average of 49.9 bands per primer pair combination. Fourteen primer pair combinations produced more than 50 bands across landraces. Polymorphism information content (PIC) ranged between 0.77 for primer pair combinations (SR4*SR8) to 0.98 for SR2*SR21 primer pair combinations; all primers showed an average 0.93 value and 22 primer pair combinations over exceeded the PIC average. The highest discrimination power, 6.68, was recorded for primer pair combinations SR4*SR28, while the lowest value was recorded for SR4*SR7 primer pair combinations. The primers showed an average DP value of 2.94. Based on Jaccard's similarity coefficients, the variation in genetic diversity among these landraces ranged from 0.70 among KSUCO99 and KSUCO103 to 0.28 among KSUCO100 and KSUCO104. The landraces showed an average similarity index of 0.5. At this value, cluster analysis based on UPGMA with high bootstrap values revealed two distinct clusters comprised of three and four landraces according to their growth patterns (Fig. 7). The first cluster encompassed the determinate landraces (KSU-CO101, KSU-CO102 and KSU-CO103), while, the second cluster encompassed the four indeterminate growth habit landraces (KSU-CO98, KSU-CO99, KSU-CO100 and KSU-CO104).

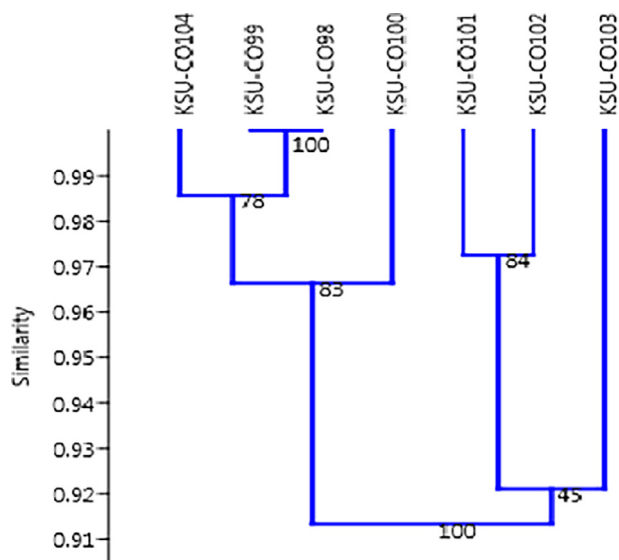


Fig. 6. Dendrogram of 7 cowpea landraces generated by Jaccard's coefficient and the unweighted pair group method with arithmetic average (UPGMA) clustering methods based on SDS-PAGE analysis.

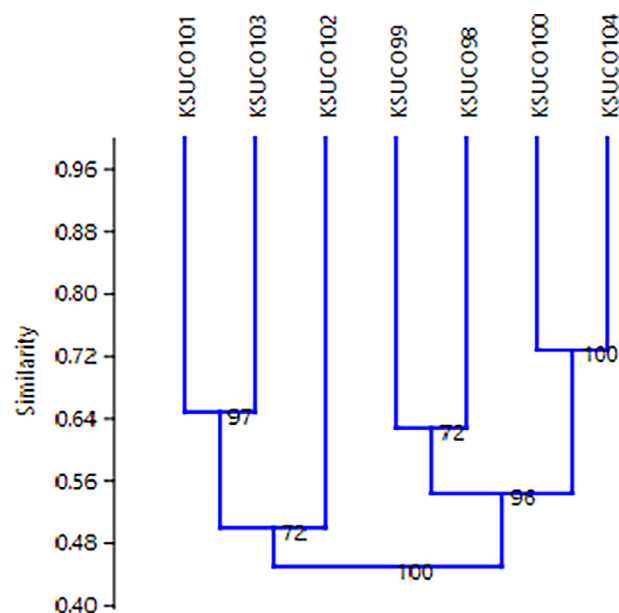


Fig. 7. Dendrogram generated using Unweighted Pair Group Method with Arithmetic average (UPGMA) analysis, showing relationships between seven cowpea genotypes using SRAP data based on Jaccard genetic similarity coefficient.

Genetic differentiation assessed by the biochemical markers among cowpea landraces correlated with the differentiation assessed by the molecular markers in medium level (Mantel test, $r = 0.56$, $P < 0.02$).

Genetic diversity in cowpea has been estimated using phenotypic, qualitative and quantitative agronomic traits that do not necessarily reflect real genetic relationships (Patil et al., 2013). In addition, environmental conditions strongly influence the expression of these traits; hence, it limits the knowledge of the germplasm structure for specific ecological adaptations (Kameswara, 2004). It is vital to understand and manage the natural variability present in the landraces, which is a prerequisite for their genetic improvement programs.

DNA based markers have been used for characterization and assessing genetic diversity in legume crops including cowpea. This generated a vast amount of information to be used in crop breeding programs. However, it is crucial to choose suitable markers for genome analysis. The SRAP marker system has been found to be very useful in several crops and has additional advantages compared with other markers. SRAP exhibits good levels of polymorphism in many plant species, reliability, a reasonable throughput rate, targets open reading frames (ORFs) in genomes, numerous co-dominant and clear high-intensity bands rarely overlap, not crop-specific, wide flexibility in the design of SRAP primers, easy isolation of bands for sequencing and cost-effective (Li and Quiros, 2001).

The high polymorphism value obtained in this study, compared with other studies using alternative molecular markers could be attributed to the type of markers and primers used, the fragment analysis method (our study utilizing the fluorescent dye-labeling and detection system) and germplasm used. High PIC values indicated a higher polymorphism existed between landraces at the test locus (Li and Nelson, 2001).

The extent of the correlation reported here was to a certain degree consistent with those reported from similar studies using other molecular markers. RAPD markers were used to assess genetic variability among 10 Indian cowpea cultivars and generated 81.7% polymorphism with an average of 15 bands per primer, and the genetic diversity ranged from 0.17 to 0.41 (Malviya et al., 2012). Moreover, Algerian cowpeas showed a range of 0.03 to 0.34 for genetic diversity using RAPD markers (Ghalmi et al., 2009). A 64.5% polymorphism has been reported for six cowpea landraces using RAPD markers (Sharawy and Fiky, 2003), 55% recorded in Bangladeshi landraces using RAPD markers and genetic distance between landraces was correlated with their source of origin (Khan et al., 2015). AFLP markers were used to study genetic relationships among a collection of advanced breeding lines and landrace accessions worldwide. Polymorphic bands (54.2%) sharing a minimum 86% genetic similarity were obtained, indicating narrow genetic diversity. Asian and US accessions have common origins and were distinct from West African accessions, which suggest incorporating the germplasm from West Africa with US and Asian breeding programs, while introgression of Asian germplasm in West Africa programs to ensure long-term gains from selection (Fang et al., 2007). Moreover, Fatokun et al., (1997) whom used AFLP and Li et al., (2001) whom used microsatellite markers, revealed a relatively low genetic diversity among the cowpea germplasms investigated. The low level of genetic diversity in cultivated cowpea compared with landraces or wild accessions has been attributed to the severe genetic bottleneck that occurred during the process of cowpea domestication (Kouam et al., 2012; Huynh et al., 2013). Genetic diversity was also attributed to the size of the germplasm tested; a high genetic diversity was related to the higher number of accessions tested (Egbadzor et al., 2014). However, Chen et al., (2017) whom evaluated 33 cowpea accessions from Niger and 27 accessions from China reported a low

genetic diversity in Niger (genetic diversity = 0.23) than accessions from china (genetic diversity = 0.31), suggesting that the number of accessions may not influence genetic diversity among populations, which was consistent with that of Chen et al. (2015), who reported that high levels of genetic variation were indirectly deduced among the tested landraces. Moreover, it was reported that although human activities played a vital role in increasing species genetic diversity, this may increase the rate of species evolution towards a single direction, especially in the process of breeding, which leads to a decline in genetic diversity (Zhou et al., 2012; Bauchet et al., 2017).

In spite of the limited number of landraces used in this study (7 landraces), a large number of polymorphic markers were produced, and a high level of genetic diversity has been expressed using the SRAP system indicating that this marker system is a reliable and powerful tool to evaluate genetic polymorphisms and relationships among cowpea landraces. In our opinion, each landrace should be preserved on the respective region from which it came. Clustering of landraces is not always related to geographical area, landrace KSUCO103 from Egypt was grouped with landraces KSUCO101 and KSUCO102 from Jizan region (Saudi Arabia) and landrace KSUCO100 from Alhsa (Saudi Arabia) grouped with landraces originating from Yemen (KSUCO98, KSUCO99 and KSUCO104). This may be due to seed movement and/or high rates of gene flow between the populations, where Yemen and Egypt are border countries of Saudi Arabia and the movement of people between these counties is unrestricted. This movement of genetic material may contribute to the higher genetic diversity among landraces tested.

4. Conclusion

This study explored the protein profile and DNA polymorphism of seven cowpea landraces using the SDS-PAGE and SRAP marker approach. Through use of SDS-PAGE on total proteins and its various fractions (albumins, globulins, and glutelins) and SRAP profiling in cowpea, it is possible to detect a useful band polymorphism to explore the diversity of the landraces. It is probable to cluster landraces which have a similar band pattern. Clustering of the landraces indicates the close genetic relationships among them. Some of the landraces may be identified by their specific banding pattern and information could be helpful to choose a landrace for improvement of cowpea productivity through breeding and other techniques.

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