


Association analysis of salt tolerance in cowpea (*Vigna unguiculata* (L.) Walp) at germination and seedling stages

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Abstract

Key message This is the first report on association analysis of salt tolerance and identification of SNP markers associated with salt tolerance in cowpea.

Abstract Cowpea (*Vigna unguiculata* (L.) Walp) is one of the most important cultivated legumes in Africa. The worldwide annual production in cowpea dry seed is 5.4 million metric tons. However, cowpea is unfavorably affected by salinity stress at germination and seedling stages, which is exacerbated by the effects of climate change. The lack of knowledge on the genetic underlying salt tolerance in cowpea limits the establishment of a breeding strategy for developing salt-tolerant cowpea cultivars. The objectives of this study were to conduct association mapping

for salt tolerance at germination and seedling stages and to identify SNP markers associated with salt tolerance in cowpea. We analyzed the salt tolerance index of 116 and 155 cowpea accessions at germination and seedling stages, respectively. A total of 1049 SNPs postulated from genotyping-by-sequencing were used for association analysis. Population structure was inferred using Structure 2.3.4; *K* optimal was determined using Structure Harvester. TASSEL 5, GAPIT, and FarmCPU involving three models such as single marker regression, general linear model, and mixed linear model were used for the association study. Substantial variation in salt tolerance index for germination rate, plant height reduction, fresh and dry shoot biomass reduction, foliar leaf injury, and inhibition of the first trifoliate leaf was observed. The cowpea accessions were structured into two subpopulations. Three SNPs, Scaffold87490_622, Scaffold87490_630, and C35017374_128 were highly associated with salt tolerance at germination stage. Seven SNPs, Scaffold93827_270, Scaffold68489_600, Scaffold87490_633, Scaffold87490_640, Scaffold82042_3387, C35069468_1916, and Scaffold93942_1089 were found to be associated with salt tolerance at seedling stage. The SNP markers were consistent across the three models and could be used as a tool to select salt-tolerant lines for breeding improved cowpea tolerance to salinity.

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Introduction

Cowpea (*Vigna unguiculata* (L.) Walp.) ($2n = 2x = 22$) is one of the most consumed legumes worldwide, especially in Africa. It is a mandated crop for the International Institute of Tropical Agriculture (IITA) (<http://www.iita.org/crop/cowpea.htm>). Cowpea is a protein-rich crop, which constitutes an affordable source of protein in developing countries.

Worldwide cowpea production is 5.4 million metric tons (Olufajo 2012). However, salinity is a growing threat to cowpea production, and most other crops, in semi-arid regions (Zhang et al. 2012). In the USA, 19.6 million hectares of cultivated areas are under salinity threat (Shannon 1997).

Strategies for addressing the negative impacts of salinity need to be developed. Providing salt-tolerant cowpea cultivars through breeding could be the most cost effective way to lessen the impacts of salinity. Salt-tolerant variations in cowpea genotypes have been reported. Ashebir et al. (2013) evaluated 19 cowpea genotypes for salt tolerance at germination stage; Win and Oo (2015) tested 21 cowpea genotypes for tolerance to salt stress at seedling stage; and both identified the genetic variations for salt tolerance in cowpea genotypes. Conventional cowpea breeding has been extensively undertaken by different national and international research programs to strengthen the crop from biotic and abiotic stresses. Nevertheless, such a process is time consuming, labor intensive, and expensive. Molecular plant breeding is a successfully demonstrated approach to pyramiding desired traits in crops (Moose and Mumm 2008; Collard and Mackill 2008; Xu and Crouch 2008). DNA markers have been proven to be an effective screening method used by plant breeders to screen for salt-tolerant genotypes (Foolad 2007). Despite the fact that these molecular markers will speed up the screening process for salt tolerance, genetic research related to salt tolerance on cowpea is very limited.

The single nucleotide polymorphism (SNP) marker is a potential and cost-efficient breakthrough for molecular plant breeding. SNP is defined as the individual nucleotide base difference between two DNA sequences (Xu 2010). SNP discovery is essential for research related to genetic variation, genome mapping, association analysis, and gene isolation (Ganal et al. 2009; Varshney et al. 2009). Regarding cowpea, SNP markers have been used for different purposes. Muchero et al. (2009) were able to design a consensus map for cowpea using EST-derived SNPs. In addition, these SNPs and the genetic map were used to conduct a study of synteny between cowpea, soybean (*Glycine max* L.), and Arabidopsis (*Arabidopsis thaliana* L.). Another study conducted by Egbadzor et al. (2013) reported SNP markers associated with seed size in cowpea. Eighteen SNPs were found after conducting an association analysis involving 78 cowpea genotypes. These SNPs were distributed across the cowpea chromosomes. Such results are of interest because they provide substantial information on SNP markers, which can be used for marker-assisted selection (MAS) for seed size-related traits in cowpea. Egbadzor et al. (2014) analyzed the diversity of 113 cowpea accessions using SNPs. They reported 477 SNPs, and 458 of them showed polymorphisms. Their results suggested that these markers were efficient to discriminate

the accessions in their study. Shi et al. (2016) conducted an association analysis to study the bacterial blight resistance in cowpea using 1031 SNP markers. They worked on a panel of 400 cowpea accessions and identified four SNP markers (C35046071_1260, C35084634_455, scaffold96328_3387, and scaffold96765_4430) to be highly associated with bacterial blight resistance in cowpea.

Genotyping-by-sequencing (GBS) is a next generation DNA sequencing approach and can be used to identify SNP markers associated with important traits in plants (Elshire et al. 2011; Sonah et al. 2013; Bastien et al. 2014). GBS is a cost effective and fast way for conducting an association analysis. Such a method deals with a reduced library representation of the genome; GBS provides high throughput genotyping of populations using a large number of SNP markers (Bradbury et al. 2007; Elshire et al. 2011).

Association mapping and QTL analysis-based studies have been important components in molecular plant breeding. They have been extensively used to genetically unravel traits related to salt and drought tolerance in different crops (Li and Xu 2007; Li et al. 2007; Ben-Hayyim and Moore 2007). In tomato (*Solanum lycopersicum* L.), QTL analysis pertaining to salt tolerance was performed during seed germination. Foolad and Jones (1993) identified five QTLs located on chromosomes 1, 3, 7, 8 and 12 linked to salt tolerance in tomato. They worked on an F₂ population resulting from a cross between salt-sensitive and salt-tolerant parents. Subsequent investigations have been conducted to validate these aforementioned QTLs (Foolad et al. 1997, 1998). At the tomato seedling stage, Foolad (1999) identified five QTLs associated with salt tolerance, which were located on chromosomes 1, 3, 5, and 9. In soybean, Kan et al. (2015) reported eight SNPs highly correlated to the ratio between the germination index under salinity stress and the germination index under no-salt conditions, and the ratio of the germination rate under salinity conditions to the germination rate under a non-saline environment. They identified eight candidate genes associated with these SNPs markers, and five of them (Glyma08g12400.1, Glyma08g09730.1, Glyma18g47140.1, Glyma09g00460.1, and Glyma09g00490.3) controlled tolerance to salinity at germination stage in soybean.

Despite the fact that SNP markers are useful in plant breeding, no molecular markers associated with salt tolerance have been reported in cowpea. Discovering SNP markers for salt tolerance will help plant breeders to select cowpea lines which are tolerant to salinity. The objectives of this study were to conduct an association analysis for cowpea salt tolerance at germination and seedling stages, and to identify salt-tolerant-related SNP markers in cowpea.

Materials and methods

Plant materials

A total of 116 cowpea genotypes were used for salt tolerance evaluations at germination stage and 155 cowpea genotypes at seedling stage. The 116 cowpea genotypes consisted of 30 University of Arkansas cowpea advanced lines and 86 USDA cowpea germplasm accessions, which were from 22 countries (Table S1). The 155 genotypes for assessing cowpea salt tolerance at seedling stage consisted of 37 cowpea advanced lines from the University of Arkansas, and 118 USDA cowpea germplasm accessions from 25 different countries (Table S2). Among the cowpea genotypes involved in this study, 111 genotypes overlapped between seedling and germination stages; among which 29 genotypes were from the University of Arkansas and 82 from USDA germplasm. All original seeds of the USDA Germplasm Resources Information Network (GRIN) germplasm were obtained from USDA Plant Genetic Resources Conservation Unit at Griffin, GA and were increased at the Research and Extension Station of University of Arkansas at Fayetteville, AR in the summers 2014 and 2015.

Phenotyping of salt tolerance

Forty seeds from each cowpea genotype were germinated on a 9-cm diameter Petri dish in an incubator New Brunswick Scientific Innova 4230[®] (Manasquan, NJ) set at 28 °C (Souza et al. 2004). Seed germination was performed over 48 h. Controls consisted of adding 14 ml of distilled water to each dish. 150 mM NaCl was added to each salt-treated dish (Lobato et al. 2009). The experiment design was a randomized complete block design (RCBD) with three replicates. The experiments were conducted in multiple runs. Data on seed germination rate for genotype without salt treatment and under salt stress were collected.

Phenotyping pertaining to seedling stage was carried out in the greenhouse of Harry R. Rosen Alternative Pest Control of the University of Arkansas, Fayetteville, AR,

USA. The average temperature in the greenhouse was maintained at 26 and 21 °C during day and night, respectively. Daylight length was set to 14 h. A total of 24 germinated seeds from each cowpea genotype were planted in six plastic pots, each containing four germinated seeds. Plastic pots were filled with 85 g Sunshine[®] Natural & Organic (Agawam, MA). Holes were designed at the bottom of each pot to avoid water logging, and paper towel was placed at the bottom to prevent the soil medium from leaking.

For experiments in seedling, three of the six pots were irrigated with deionized water and the other three irrigated with 200 mM NaCl solution (Abeer et al. 2015). The experiments were carried out in five runs due to space limitations for phenotyping. Each run involved approximately 40 genotypes. Growing conditions including soil medium type and weight, and experimental procedures were kept uniform across the different runs. In addition, the commercial variety “Early Scarlet”, a sensitive to salt tolerance from our preliminary experiment was used as control. For every run, measurements were adjusted to “Early Scarlet” data. The idea of adjusting values when space limitations constraint was found in other abiotic stress-related studies. For instance, Stratonovitch and Semenov (2015) adopted a similar approach due to space limitations to phenotype transgenic wheat for drought tolerance in greenhouse.

When the plants reached VI stage (exhibition of the first trifoliolate leaf) (Fehr et al. 1971), the salt treatment was initiated. To each pot, a 100 ml solution of deionized water or NaCl was added every 2 days over 2 weeks. Deionized water represented the control, and NaCl the treatment. Measurements were done 14 days after the first treatment application. Some plants were completely dead after 14 days. Foliar leaf injury associated with the salt treatment was scored visually (1 = no apparent leaf injury, 2 = first symptoms of leaf injury, 3 = moderate leaf injury, 4 = severe leaf injury, and 5 = dead leaves) (Fig. 1), inhibition of first trifoliolate leaf development (1 = untrifoliolate, 9 = trifoliolate), plant height reduction, and fresh and dry biomass reduction due to salt stress. Data were collected on a per plant basis.

Fig. 1 Foliar leaf injury due to salinity in cowpea: 1 = no apparent leaf injury, 2 = first symptoms of leaf injury, 3 = moderate leaf injury, 4 = severe leaf injury, and 5 = dead leaves



Salt tolerance index (STI) was calculated for germination rate, plant height, and fresh and dry shoot biomass. The STI was obtained using the following formula (Fernandez 1992; Saad et al. 2014).

$$STI = (Y_{\text{non salt stress}} \times Y_{\text{salt stress}}) / (\bar{Y}_{\text{non salt stress}})^2,$$

where $Y_{\text{non salt stress}}$ is the seed germination rate without salt stress/plant height, fresh and dry shoot biomass without salt stress, $Y_{\text{salt stress}}$ is the seed germination rate under salt stress/plant height, fresh and dry shoot biomass under salt stress, and $\bar{Y}_{\text{non salt stress}}$ is the average of seed germination rate without salt stress/plant height, fresh and dry shoot biomass without salt stress.

Descriptive statistics were generated using “Tabulate” function of JMP Genomics 7. Data distribution was drawn using “Distribution” function in JMP Genomics 7.

Genotyping for association analysis

DNA extraction, library preparation, and genotyping-by-sequencing (GBS)

Genomic DNA was extracted from young fresh leaves when the cowpea plants reached V1 stage (first trifoliate). These leaves were stored at $-80\text{ }^{\circ}\text{C}$ overnight and then dried in a Lypophilizer[®] (Salt Lake City, UT). A CTAB (hexadecyltrimethyl ammonium bromide) protocol described by Kisha et al. (1997) was used for DNA extraction. Cowpea leaves were ground using Mixer Mill MM 400[®] (Haan, Germany). The DNA extraction buffer was added to each sample, which was centrifuged at 13,000 rpm for 10 min. Then, the aqueous solution was transferred to 2 ml tubes. Then 1 ml of chloroform–isoamyl alcohol (24:1) was added to each sample to subtract proteins. The samples were centrifuged at 10,000 rpm for 5 min, and the supernatant liquid was transferred to additional 2 ml tubes. The use of 1 ml of isopropanol added to each tube helped the DNA precipitate from the solution. The samples were then stored overnight at $-20\text{ }^{\circ}\text{C}$. DNA pellets were subsequently washed with 70 and 90% ethanol and dried for 30 min. A total of 200 μl of $0.1\times\text{ TE}$ per tube were used to solubilize the DNA. Afterwards, 3 μl of RNase was added to each tube.

DNA was quantified using a NanoDrop 200c spectrophotometer (Thermo SCIENTIFIC, Wilmington, DE). 1% agarose gel with ethidium bromide gel stain permitted to check on DNA qualities. Genotyping-by-sequencing libraries were constructed according to the *ApeKI* protocol described by Elshire et al. (2011). DNA sequencing was performed using GBS (Elshire et al. 2011; Bastien et al. 2014) by HiSeq series in Beijing Genome Institute (BGI). The GBS protocol of BGI consisted of several steps. DNA was digested using the restriction enzyme *ApeKI*, which resulted in sticky end fragments. Two adaptors, P1 and P2, were ligated to

the restriction fragments. P1 adaptor or barcode consisted of a forward Illumina Sequencing primer and a 4–8-bp barcode. P2 adaptor or common adaptor had a reverse Illumina Sequencing primer. The 3' end of each fragment was repaired and adenylated. In situ PCR was conducted using the two specific primers (forward and reverse Illumina Sequencing primers). However, DNA fragments could be ligated to either barcode/common adaptors, barcode/barcode adaptors, or common/common adaptors. Those which were same ended could not be sequenced on Illumina platform, hence would not provide any reads. Only fragments which had both barcode and common adaptors on their ends were sequenced. Prior to sequencing, quality check on GBS libraries was performed. The data were filtered afterwards (GBS protocol of BGI).

SNP assembly, mapping, discovery, and filtering

SNP assembly, mapping, and discovery were conducted by BGI using SOAP family software (<http://soap.genomics.org.cn/>). The short reads were aligned to a cowpea genome reference (cowpea_Genome_0.03.fa) by SOAPalinger/soap2 (<http://soap.genomics.org.cn/>). Initial SNP calls were performed using SAOPsnp v 1.05 (Li et al. 2009). Dr. Timothy J. Close from the University of California Riverside, CA, provided the cowpea_Genome_0.03.fa (644,126 scaffolds or contigs).

Cowpea genotypes having more than 35% missing SNP data were discarded from the analysis. For each SNP, if its heterozygous calls were greater than 35%, the SNP was considered as missing data; the minor allele frequency threshold was set at 0.05 for SNP callings. SNPs containing more than 35% missing data were also removed from the analysis. Finally, 1049 SNPs postulated from GBS were used for association analysis after SNP filtering.

Population structure analysis

STRUCTURE 2.3.4 was used to infer the population structure of the cowpea accession panel (Pritchard et al. 2000). An admixture model along with a correlated allele frequency model, independent for each run, were involved in the analysis to assess the population structure (K). The use of such models has been shown to provide conclusive results for cowpea association mapping-related studies (Shi et al. 2016).

For each estimated K value, ten runs were performed. Markov chain Monte Carlo (MCMC) length of the burn-in period was 30,000. After the burn-in period, the number of MCMC iterations was adjusted to 50,000. To screen appropriate K values, values of delta K and optimal K were computed using STRUCTURE Harvester (Earl and VonHoldt

2011; <http://taylor0.biology.ucla.edu/structureHarvester/>) based on the formula developed by Evanno et al. (2005).

After defining K optimal, Q matrix with the K vectors were generated and used for association analysis using TASSEL 5. Each cowpea genotype was allocated to each cluster (Q). The cut-off probability for assigning an accession to a Q cluster was 0.5. To visualize the structure among the cowpea panel, bar plots using STRUCTURE PLOT with the option “sort by Q ” and based on K optimal were designed (Ramasamy et al. 2014).

Genetic diversity

The analysis of genetic diversity and the drawing of phylogenetic trees were performed using MEGA 7 (Kumar et al. 2016). The maximum likelihood tree was adopted as statistical method and the parameters were set as described by Shi et al. (2016).

The population structure along with the outputs containing the Q clusters was imported to MEGA 7 for combined analysis of genetic diversity while drawing the phylogenetic trees. For the sub-trees for each cluster (Q), the shape of “node/subtree marker” and the “branch line” was colored similarly as the bar plots displayed by STRUCTURE PLOTS.

Association analysis

Association analysis was conducted using TASSEL 5 (Bradbury et al. 2007) and R package (Liu et al. 2016). Four types of models were used. These models consisted of a single marker regression (SMR) for all SNPs without structure and kinship, a general linear model (GLM) using structure outputs, a mixed linear model (MLM) involving structure and kinship from TASSEL 5, and a fixed and random model Circulating Probability Unification (FarmCPU) using R. The LOD threshold for significant SNPs was between 2 and 3 (Lander and Botsteins’b 1989).

Results

Phenotyping of salt tolerance

Evaluation of cowpea salt tolerance was performed on 116 genotypes at germination stage and 155 genotypes at seedling stage. Data on germination rate under normal condition, salt tolerance index for seed germination, foliar leaf injury, inhibition of the development of first trifoliolate leaf, salt tolerance index for plant height, fresh and dry shoot biomass were collected in 14 days after the first salt treatment (Tables S1, S2, and S3). At this time, some plants were completely dead.

Salt tolerance index for seed germination, plant height, fresh and dry shoot were calculated (Tables S1, S2, and S3). Data were normally distributed (Fig. 2) except for visual leaf injury, which showed a right-skewed distribution (Fig. 3). Germination rate under non-saline condition varied from 60.8 to 99.2%, with a mean of 81.1% and a standard deviation of 11.1%. Envoy (99.2%), PI583194 (99.2%), PI487518 (98.3%), PI218123 (97.5%), and PI255765 (97.5%) had the highest germination rate, whereas PI293570 (62.5%), PI582809 (61.7%), PI582852 (61.7%), PI225922 (60.8%), and PI339610 (60.8%) showed the lowest germination rate (Table S1). Salt tolerance index for seed germination ranged between 0.00 and 0.92, with a mean of 0.47 and a standard deviation of 0.23. Highest salt tolerance index for germination was recorded for PI582422 (0.92), 09-529 (0.88), PI293584 (0.86), PI201498 (0.84), and PI582420 (0.83), indicating that these genotypes were salt-tolerant. 09-745 (0.07), PI582665 (0.02), 09-393 (0.01), PI582522 (0.01), and PI582813 (0.00) had the lowest salt tolerance index for germination rate, suggesting that they were salt-sensitive at germination stage.

The development of first trifoliolate leaf was inhibited for 54% of the genotypes tested at seedling stage. Foliar leaf injury scores ranged between 1.0 and 4.9, with a mean of 2.2 and a standard deviation of 0.8. Leaf damage was substantial for PI582665 (4.9), PI255815 (4.8), PI255774 (4.7), PI583201 (4.7), and PI527561 (4.6), indicating that these genotypes were salt-susceptible. Leaf injury was almost absent for PI582420 (1.2), PI582428 (1.2), PI582422 (1.1), PI582812 (1.1), and PI293570 (1.0) (Table S2), indicating that these accessions were salt-tolerant based on leaf injury at seedling stage.

Salt tolerance index for plant height varied from 0.26 to 0.76, with a mean of 0.52 and a standard deviation of 0.09. PI257463 (0.76), 09-175 (0.74), 09-529 (0.73), 09-295 (0.71), and 09-655 (0.67) had the highest salt tolerance index for plant height, indicating that these genotypes were salt-tolerant based on plant height reduction. Lowest salt tolerance index for plant height was found for PI339610 (0.36), PI582697 (0.36), PI582812 (0.35), PI582852 (0.26), and PI582866 (0.26), indicating that these genotypes were highly susceptible to salt stress based on plant height reduction (Table S3).

Fresh shoot biomass salt tolerance index ranged between 0.17 and 0.82, with a mean of 0.54 and a standard deviation of 0.12. PI582340 (0.82), ENCORE (0.81), PI152195 (0.79), PI666252 (0.78), and 09-105 (0.77) had the highest salt tolerance index for fresh shoot biomass, indicating that they were salt-tolerant based on fresh shoot biomass; whereas PI293476 (0.30), PI223023 (0.28), PI582852 (0.28), PI582468 (0.17), and PI255774 (0.17) showed the lowest fresh biomass salt tolerance index, suggesting that these genotypes were highly susceptible to salt stress (Table S3).

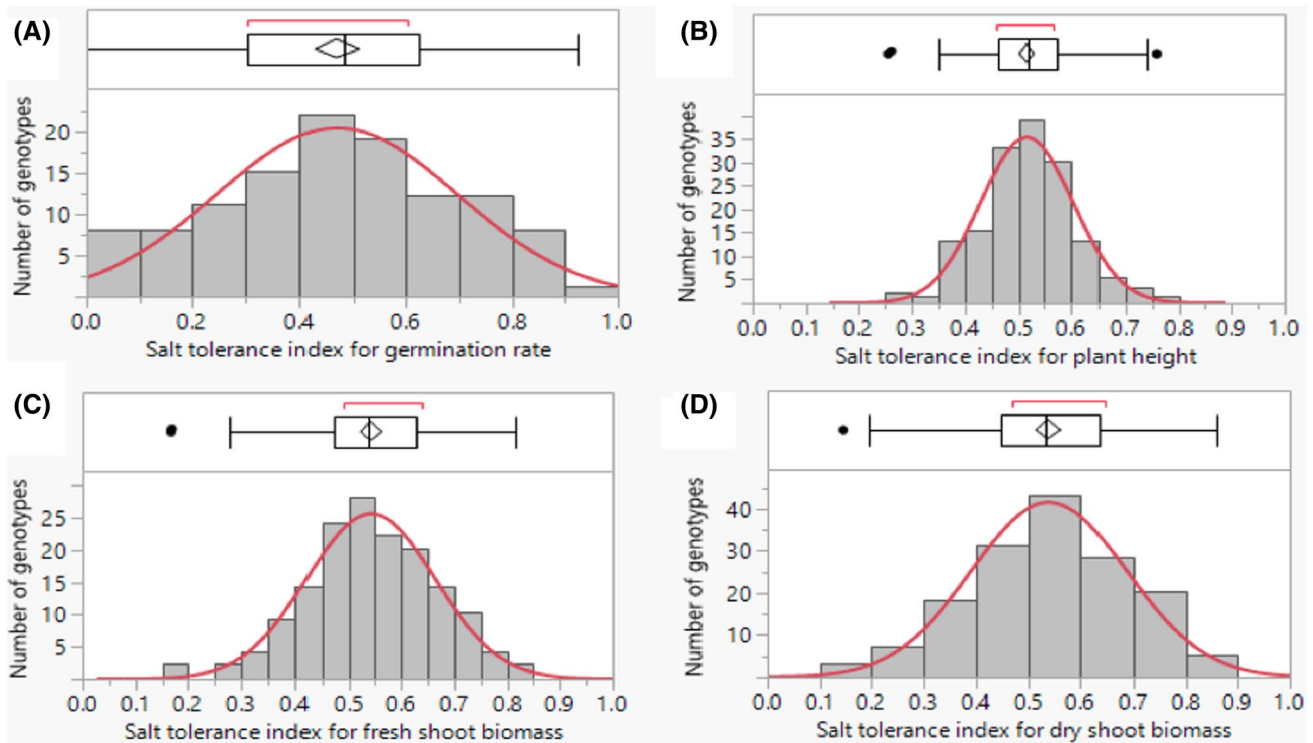


Fig. 2 Distributions of salt tolerance index: **a** seed germination rate, **b** plant height, **c** fresh shoot biomass, and **d** dry shoot biomass in cowpea

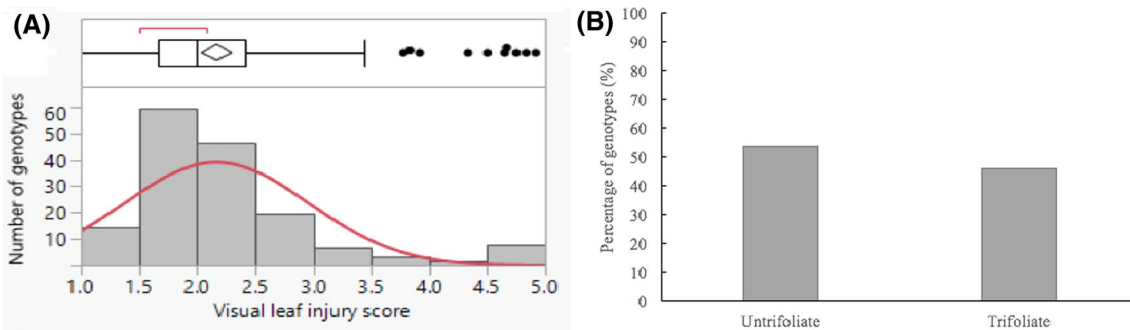


Fig. 3 Distribution of the visual leaf injury scores: **a** inhibition of first trifoliolate leaf development, and **b** at seedling stage

Salt tolerance index for dry shoot biomass varied from 0.15 to 0.86, with a mean of 0.54 and a standard deviation of 0.15. Highest dry shoot biomass salt tolerance index was recorded for PI666260 (0.86), PI293469 (0.84), PI293582 (0.82), PI582340 (0.81), and PI292891 (0.81), indicating that these genotypes were salt-tolerant based on dry shoot biomass; whereas PI487518 (0.25), PI582468 (0.22), PI582852 (0.20), PI225922 (0.19), and PI255774 (0.15) had the lowest salt tolerance index for dry shoot biomass, indicating that these lines were salt-sensitive (Table S3).

Genetic diversity and population structure

The population structure was inferred using STRUC-TURE 2.3.4 in 116 cowpea accessions at germination stage and 155 cowpea genotypes at seedling stage. For both stages, Structure Harvester indicated an optimal delta K for K equal to 2, suggesting that two main populations were found in the cowpea accession panel (Figs. 4, 5). Each population cluster was termed as Q_1 or Q_2 . In regard to the germination stage, Q_1 made up 58% (67 cowpea accessions) of the total cowpea accessions; the remaining 42% fell under Q_2 (49

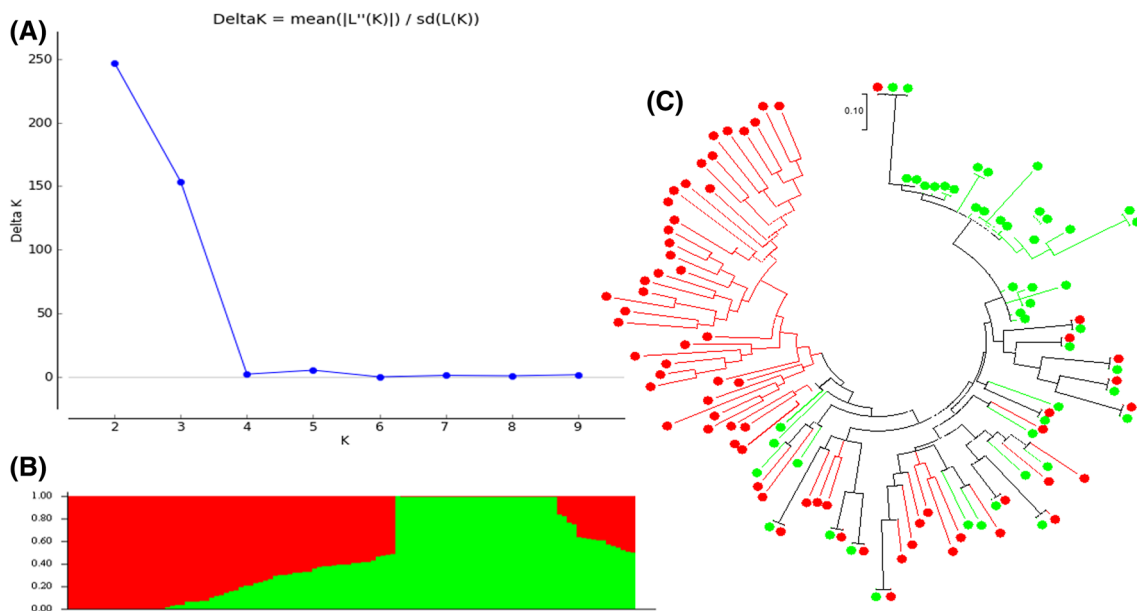


Fig. 4 Model-based populations of 116 cowpea genotypes at germination stage: **a** Delta *K* values for different numbers of populations assumed (*K*) in STRUCTURE analysis drawn by STRUCTURE HARVESTER, **b** classification of the 116 cowpea accessions into two subpopulations using STRUCTURE 2.3.4, where the subgroup mem-

bership is presented on the y-axis, and each accession on the x-axis. The color code shows the distribution of the different cowpea accessions (*Q*1 or cluster 1: green, and *Q*2 or cluster 2: red), and **c** maximum likelihood (ML) tree of the 116 cowpea accessions drawn using MEGA 7. Color coding in **b**, **c** are consistent (color figure online)

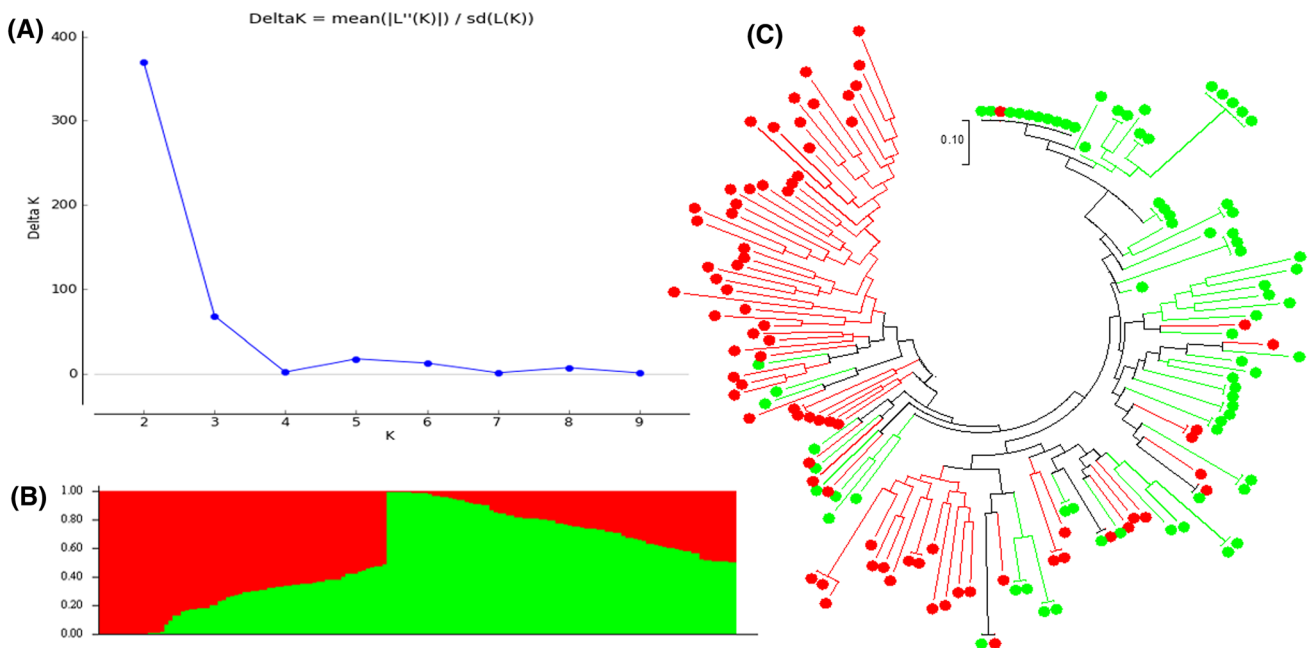


Fig. 5 Model-based populations of 155 cowpea genotypes at seedling stage: **a** Delta *K* values for different numbers of populations assumed (*K*) in STRUCTURE analysis drawn by STRUCTURE HARVESTER, **b** classification of the 155 cowpea accessions into two subpopulations using STRUCTURE 2.3.4, where the subgroup mem-

bership is presented on the y-axis, and each accession on the x-axis. The color code shows the distribution of the different cowpea accessions (*Q*1 or cluster 1: red, and *Q*2 or cluster 2: green), and **c** maximum likelihood (ML) tree of the 155 cowpea accessions drawn using MEGA 7. The color codes are consistent in **b**, **c** (color figure online)

cowpea accessions). With respect to the seedling stage, *Q1* accounted for 45% (70 cowpea accessions) of total cowpea accessions, and *Q2* represented 55% (85 cowpea accessions).

The phylogenetic trees were drawn using the maximum likelihood (ML) method in MEGA 7 (Figs. 4, 5). Two main populations were identified for the cowpea accessions at germination and seedling stages. The first cluster *Q1* was represented in red, and the second cluster *Q2* in green (Figs. 4, 5). The same colors were used in the phylogenetic trees where the red circles show the accessions belonging to *Q1* and the green ones for those representing *Q2*. The option radiation was used to draw both genetic trees. The accessions under each cluster tended to be clustered together in the phylogenetic tree (Figs. 4, 5), which indicated that the results from the structure analysis were consistent with that of the genetic diversity. Therefore, two distinct populations were found within the cowpea accession panel.

Association analysis

Association analysis was conducted using TASSEL 5 and R package. Four different models, single marker regression (SMR) without structure and kinship, general linear model (GLM) with structure, mixed linear model (MLM) using structure and kinship, and fixed and random model circulating probability unification (FarmCPU) were used in this study. SNPs having an LOD (= $-\log(p \text{ value})$) higher than 2 were selected. SNPs related to cowpea germination rate under normal condition, salt tolerance index for germination, inhibition of the development of first trifoliolate leaf, visual leaf injury, salt tolerance index pertaining to plant height, and fresh and dry biomass were identified.

C35042053_245, Scaffold27032_5665, and Scaffold94454_419 were found to be highly associated with cowpea germination. A range of 8.43–10.97% of the variation in cowpea germination were attributed to these SNPs. C35042053_245 had an LOD greater than 2.5 across the four different models (Table 1). Five SNPs, C35017374_128, Scaffold36825_365, Scaffold51130_55, Scaffold87490_622, and Scaffold87490_630 were associated with germination salt tolerance index in cowpea. The highest LOD was recorded for Scaffold87490_630, having an R^2 value up to 15% (Table 1).

At seedling stage, Scaffold68489_600 and Scaffold93827_270 were associated with the inhibition of first trifoliolate leaf development after salt stress in cowpea. R^2 values pertaining to Scaffold68489_600 were 13.35, 13.09, and 7.74% in SMR, GLM, and MLM, respectively. R^2 values were 6.45, 10.06, and 8.30% for Scaffold93827_270 in SMR, GLM, and MLM, respectively. Five SNPs, C35051519_114, C35070194_1643, Scaffold87490_633, Scaffold87490_640, and Scaffold87665_2770 were highly associated with visual leaf injury due to salinity. LOD values

related to these SNPs decreased from SMR to FarmCPU except for C35051519_114 whose LOD was 3.21 in MLM. Scaffold87490_640 had the highest LOD value (2.02) using FarmCPU. Values for R^2 varied from 9.50 to 11.05% for foliar leaf injury-related SNPs (Table 1).

Eight SNPs, C35043181_374, Scaffold22931_1172, Scaffold6972_9093, Scaffold70430_30, Scaffold70430_31, Scaffold82042_3387, Scaffold87665_2770, and Scaffold9649_28 were identified to be associated with plant height salt tolerance index in cowpea. C35043181_374 had the highest LOD value, ranging from 2.15 to 4.10 across the four models. The second highest LODs were recorded for Scaffold82042_3387, varying between 2.44 and 3.03 for four models. Values for R^2 ranged from 4.64 to 11.84% (Table 1), indicating that SNPs could have effects on plant height salt tolerance index in cowpea. Scaffold87665_2770 was associated to both visual leaf injury and plant height salt tolerance index in cowpea.

Regarding salt tolerance index for fresh shoot biomass, six SNPs, C35069468_1916, Scaffold2771_4351, Scaffold3150_538, Scaffold32980_10968, Scaffold77319_293, and Scaffold93942_1089 were identified. LOD values varied from 1.71 to 2.88 under four different models (Table 1). Higher LOD values were observed using SMR. R^2 values ranged between 4.17 and 12.64%. Highest R^2 values were recorded for C35069468_1916.

C35069468_1916, Scaffold2771_4351, Scaffold32980_10968, Scaffold61254_2828, and Scaffold93942_1089 were associated with dry shoot biomass salt tolerance index (Table 1). LOD values ranged between 1.60 and 3.00, and R^2 values varied from 7.58 to 13.15%. C35069468_1916, Scaffold2771_4351, Scaffold32980_10968, and Scaffold93942_1089 overlapped between salt tolerance index for fresh shoot biomass and that of dry shoot biomass, indicating that these SNPs can be used to screen for cowpea salt tolerance at seedling stage using fresh and dry shoot biomass as phenotypic markers.

Discussion

Large variability in salt tolerance-related traits among cowpea genotypes was observed in this study. Traits included germination under normal condition, salt tolerance at germination stage, visual leaf injury due to salinity, inhibition of first trifoliolate leaf development, salt tolerance index related to plant height, and fresh and dry shoot biomass. Trait distribution was approximately normal except for visual leaf injury. These phenotypic results indicated that cowpea salt tolerance could be controlled by QTLs. Zhang et al. (2014) reported a normal distribution of salt tolerance index related to main root length, fresh and dry root biomass, and hypocotyl length in soybean.

Table 1 SNP markers associated with cowpea germination rate, foliar leaf injury, inhibition of the development of the first trifoliolate leaf, salt tolerance index using germination, plant height, and fresh and dry shoot biomass as phenotypic markers

Trait	SNP marker ^a	SNP type	Contig/scaffold	SNP position	LOD (−log(p)) value			R ² (%) value			
					SMR	GLM (Q)	MLM (Q + K)	FarmCPU	SMR	GML (Q)	MLM (Q + K)
Germination rate under normal condition	C35042053_245	C/T	C35042053	245	2.51	2.80	2.58	2.88	9.61	10.97	10.31
	Scaffold27032_5665	A/G	Scaffold27032	5665	2.18	2.52	2.17	2.28	8.43	10.13	9.54
	Scaffold94454_419	A/T	Scaffold94454	419	2.15	2.34	2.03	1.77	8.84	9.82	8.88
Germination	C35017374_128	G/T	C35017374	128	2.80	2.58	1.86	1.90	15.24	14.14	12.24
	Scaffold36825_365	A/G	Scaffold36825	365	2.37	2.57	2.63	1.96	7.58	8.39	9.22
	Scaffold51130_55	A/G	Scaffold51130	55	2.26	2.39	2.25	2.21	8.83	9.52	9.31
First trifoliolate leaf development	Scaffold87490_622	C/T	Scaffold87490	622	2.48	2.47	2.36	1.91	13.46	13.54	14.12
	Scaffold87490_630	G/T	Scaffold87490	630	2.56	2.78	1.94	3.17	13.84	14.99	13.31
	Scaffold68489_600	G/T	Scaffold68489	600	3.36	3.27	1.63	2.72	13.35	13.09	7.74
Foliar injury	Scaffold93827_270	A/G	Scaffold93827	270	2.18	3.23	2.46	1.66	6.45	10.06	8.30
	C35051519_114	A/T	C35051519	114	2.94	2.92	3.21	1.94	10.07	10.11	11.05
	C35070194_1643	A/C	C35070194	1643	2.64	2.58	2.34	1.58	9.94	9.81	9.84
Plant height	Scaffold87490_633	C/T	Scaffold87490	633	2.53	2.58	2.36	1.92	10.06	10.28	9.50
	Scaffold87490_640	A/T	Scaffold87490	640	2.55	2.45	2.35	2.02	9.94	9.65	9.52
	Scaffold87665_2770	A/C	Scaffold87665	2770	2.57	2.56	2.54	1.76	10.28	10.33	9.53
Fresh shoot biomass	C35043181_374	C/T	C35043181	374	3.10	2.91	2.15	4.10	9.94	9.26	7.72
	Scaffold22931_1172	C/T	Scaffold22931	1172	2.20	2.23	2.06	2.93	7.03	7.17	7.09
	Scaffold6972_9093	C/T	Scaffold6972	9093	2.63	3.01	2.39	1.78	10.34	11.84	11.48
Fresh shoot biomass	Scaffold70430_30	C/G	Scaffold70430	30	2.37	2.19	1.88	2.48	5.81	5.29	4.67
	Scaffold70430_31	G/T	Scaffold70430	31	2.42	2.24	1.87	2.52	5.90	5.38	4.64
	Scaffold82042_3387	C/T	Scaffold82042	3387	3.03	2.87	2.44	2.46	11.16	10.56	10.31
Fresh shoot biomass	Scaffold87665_2770	A/C	Scaffold87665	2770	2.37	2.52	1.86	1.91	9.53	10.09	9.44
	Scaffold9649_28	C/T	Scaffold9649	28	3.22	2.99	2.59	1.65	9.69	8.93	8.44
	C35069468_1916	A/T	C35069468	1916	2.88	2.85	2.28	1.82	12.64	12.49	12.25
Fresh shoot biomass	Scaffold2771_4351	A/C	Scaffold2771	4351	2.22	2.19	1.83	1.72	9.10	9.04	8.45
	Scaffold3150_538	A/T	Scaffold3150	538	2.74	2.81	2.53	1.97	11.12	11.36	11.76
	Scaffold32980_10968	C/T	Scaffold32980	10,968	2.33	2.21	1.75	2.27	9.70	9.26	8.05
Fresh shoot biomass	Scaffold77319_293	A/T	Scaffold77319	293	2.03	2.21	1.71	2.25	4.76	5.31	4.17
	Scaffold93942_1089	C/T	Scaffold93942	1089	2.31	2.22	1.78	2.63	9.44	9.16	8.27

Table 1 (continued)

Trait	SNP marker ^a	SNP type	Contig/scaffold	SNP position	LOD (–log(p)) value				R ² (%) value			
					SMR	GLM (Q)	MLM (Q + K)	FarmCPU	SMR	GML (Q)	MLM (Q + K)	
Dry shoot biomass	C35069468_1916	A/T	C35069468	1916	3.00	2.96	2.16	1.73	13.15	13.09	12.08	
	Scaffold2771_4351	A/C	Scaffold2771	4351	2.26	2.24	1.77	1.60	9.27	9.27	8.66	
	Scaffold32980_10968	C/T	Scaffold32980	10968	2.09	2.05	1.56	1.68	8.78	8.69	7.58	
	Scaffold61254_2828	C/G	Scaffold61254	2828	2.39	2.41	1.71	1.94	9.61	9.77	7.89	
	Scaffold93942_1089	C/T	Scaffold93942	1089	3.00	2.96	2.38	2.71	12.12	12.05	11.86	

Salt tolerance index is obtained by multiplying the value of the phenotypic marker (germination, plant height, and fresh and dry shoot biomass) by under non-stress and salt-stress conditions for each replication, which is divided by the average value of the phenotypic marker at non-stress condition

^aSNP marker refers to the contig/scaffold name along with the SNP position. LOD is obtained by computing $-\log(p)$ value, and p value corresponds to the p value associated with the SNP marker. p value and R^2 were from TASSEL 5 (Bradbury et al. 2007) using three models. SMR (single model regression) = phenotype + genotype, GLM (general linear model) = phenotype + genotype + Q _matrix, Q _matrix is obtained from STRUCTURE (Pritchard et al. 2000), MLM (mixed linear model) = phenotype + genotype + Q matrix + Kinship (K), K is obtained from TASSEL 5. FarmCPU (Fixed and random model circulating probability unification) is a method using GAPIT (Liu et al. 2016)

Overall, a decreasing value in LOD was identified from SMR to FarmCPU. R^2 values decreased from SMR to MLM. Such results were expected since the four models involved different factors aiming at reducing false positives as described by Yu et al. (2006). The first model was “single marker regression” (SMR), which only consisted of the phenotypes and genotypes (SNPs). The second model was “general linear model” (GLM) including the phenotypic data, genotypic data (SNPs), and Q matrix from structure analysis using STRUCTURE 2.3.4. The third one was “mixed linear model” (MLM) including the phenotype, genotype, Q matrix, and Kinship K from TASSEL 5, where K had random effect. The fourth model was FarmCPU (Liu et al. 2016). In spite of the discrepancy in factors used in each model, consistency of some significantly reported SNPs were found in this report, thus providing more robustness to the SNP markers. Values of R^2 associated with SNPs varied from 4 to 15% for germination, plant height, and fresh and dry shoot biomass salt tolerance indexes. These findings were similar to those of Xu et al. (2013) who reported QTLs explaining 3–13% of salt tolerance in rice. Overall, cowpea salt tolerance at germination and seedling stages appeared to be quantitatively inherited. In this study, several SNP markers were found to be associated with each of the salt stress-related traits, indicating that cowpea salt tolerance could be controlled by QTLs, which were consistent with the fact that traits were approximately normally distributed. In addition, these results were in agreement with that of found in sorghum (*Sorghum bicolor* L.) salt tolerance. Wang et al. (2014) reported multiple QTLs controlling sorghum salt tolerance at germination and seedling stages. In soybean, Kan et al. (2016) reported 11 QTLs associated with salt tolerance. Elakhdar et al. (2016) identified 46 QTLs associated with salt tolerance in a barley population consisting of 60 progenies, suggesting that salt tolerance is a complex trait controlled by multiple loci.

In this study, we found that Scaffold87665_2770 was associated with both visual leaf injury and plant height salt tolerance index in cowpea. Foliar injury results from the accumulation of Na^+ and Cl^- (Yeo and Flowers 1983), whereas plant height reduction is due the osmotic inhibition of growth and the accumulation of salt ion in plants (Munns et al. 1995; Neumann 1997). Therefore, we could suggest that Scaffold87665_2770 is likely associated with salt ion regulation in cowpea since salt ions are common features in foliar leaf injury and plant growth inhibition.

Scaffold87490_622 and Scaffold87490_630 were associated with salt tolerance index for germination and Scaffold87490_633 and Scaffold87490_640 were associated with foliar leaf injury due to salinity. Since these SNPs belong to the same contig/scaffold and only had 19 bp range, we suggest that there could be a QTL on

Scaffold87490 which controls salt tolerance at both germination and seedling stages in cowpea.

Significant overlapping SNPs were found between salt tolerance index for fresh shoot biomass and dry shoot biomass, which supports our results since fresh shoot biomass and dry shoot biomass are two highly correlated traits. In barley, overlapping SNPs for fresh and dry shoot biomass were identified by Long et al. (2013). Kordrostami et al. (2016) also reported overlapping SNPs for salt tolerance index related to shoot dry biomass and shoot fresh biomass in rice (*Oryza sativa* L.).

To date, few studies have dealt with association mapping analysis for important traits in cowpea (Agbicodo et al. 2010; Qin et al. 2016; Shi et al. 2016; Xiong et al. 2016). Cowpea salt tolerance research at the genetic level is very limited. So far, this present report could be one of the first investigations to do so. The salt-tolerant SNP markers identified in this study have practical applications. Cowpea breeders can use these SNPs as a tool to select cowpea salt-tolerant plants. Breeding programs could benefit from these molecular markers to rapidly identify salt-tolerant plants. In addition, the results could significantly advance knowledge on the genetic architecture governing salt tolerance in cowpea. However, more studies are required to unravel the genetic mechanisms underlying salt tolerance in cowpea.

Conclusion

The phenotypic evaluation of salt tolerance indicated large variation among the cowpea accessions in this study. Association analysis was conducted to identify SNPs associated with salt tolerance at both germination and seedling stages in cowpea. Scaffold87490_622, Scaffold87490_630, and C35017374_128 were highly associated with salt tolerance at germination stage. Scaffold93827_270, Scaffold68489_600, Scaffold87490_633, Scaffold87490_640, Scaffold82042_3387, C35069468_1916, and Scaffold93942_1089 were associated with salt tolerance at seedling stage. To our knowledge, this is one of the first investigations reporting SNP markers for salt tolerance in cowpea. These SNPs could be used as a tool in marker-assisted selection for improving salt tolerance of cowpea genotypes.

Author contribution statement WR, YW, DM, JQ, LD, WY, GB, YS carried out phenotyping and genotyping. AS and BM managed the project, WR and AS analyzed the data. WR, AS, BM, JC, PC, VS wrote, reviewed, and edited the paper.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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