NRC Research Press

ARTICLE

Understanding the genetic relationships between Indonesian bambara groundnut landraces and investigating their origins

E.S. Redjeki, W.K. Ho, N. Shah, O.O. Molosiwa, N.R. Ardiarini, Kuswanto, and Sean Mayes

Abstract: A total of 170 bambara groundnut (*Vigna subterranea*) accessions were evaluated using both simple sequence repeat (SSR) and single nucleotide polymorphism (SNP) markers generated using genotyping-by-sequencing (GbS), of which 56 accessions were collected from West and East Java. Principal coordinate analysis (PCoA), population structure, and cluster analysis suggest that the East Java accessions could be a result of the introduction of selected West Java accessions. In addition, the current Indonesian accessions were likely introduced from Southern Africa, which would have produced a very marked founding effect such that these accessions present only a fraction of the genetic variability that exists within this species.

Key words: bambara groundnut, microsatellite, marker, SNP, genetic relationship, population structure.

Résumé : Une collection de 170 accessions du pois bambara (*Vigna subterranea*) a été étudiée à l'aide de marqueurs SSR (séquences simples répétées) et SNP (polymorphismes mononucléotidiques) générés par une approche GbS (génotypage par séquençage). Au sein de cette collection, 56 accessions provenaient des parties occidentales et orientales de l'île de Java. Des analyses en coordonnées principales (PCoA), de structure de population et de groupement suggèrent que les accessions étudiées provenant de la partie orientale de l'île seraient le produit d'une introduction de certaines accessions issues de la partie occidentale. De plus, les accessions indonésiennes contemporaines ont vraisemblablement été introduites à partir de l'Afrique du Sud, ce qui aurait produit un effet fondateur très marqué faisant en sorte que ces accessions ne présentent qu'une fraction de la variabilité génétique qui existe au sein de cette espèce. [Traduit par la Rédaction]

Mots-clés : pois bambara, microsatellite, marqueur, SNP, relations génétiques, structure de population.

Introduction

Bambara groundnut (*Vigna subterranea* (L.) Verdc; $2n = 2x = 22$) belongs to the leguminous family Fabaceae, with Burkina Faso, Cameroon, Democratic Republic of the Congo, Mali, Niger, and Togo reported to be the main cultivation areas, producing approximately 180 000 t from 250 000 ha, annually (FAO 2017). An extensive survey conducted in Zimbabwe across seven districts has revealed that the cultivation of bambara groundnut is highly district dependent and largely driven by the end use purpose, either as cash crop or for own consumption (Mubaiwa et al. 2018), while surveys in Ghana and Nigeria suggest that the crop is valued for its drought tolerance and food value, although suffering from a lack of improved varieties and being very labour intensive (Adzawla et al. 2016*a*, 2016*b*; Olayide et al. 2018).

All of the bambara groundnut plant, including the leaf, stem, pod, seed, shell, and "offal", can be used for human consumption. In addition, it has been reported being used as herbal medicine, as animal feed, a green fertilizer, and a biopesticide (Mkandawire 2007; Daniel et al. 2016). The bambara groundnut seed is composed of 4.8% ash, 7.2% moisture, 47.0% carbohydrate, 19.0% protein, 7.0% oil, and 1.0% free fatty acid and compares well with other legumes, although systematic approaches to

Received 2 August 2019. Accepted 10 February 2020.

E.S. Redjeki. University of Muhammadiyah Gresik, Jl. Sumatera No. 101 GKB Gresik 61121, Jawa Timur, Indonesia.
W.K. Ho. Crops For the Future, Jalan Broga, 43500 Semenyih, Selangor, Malaysia; School of Biosciences, University of Nottingham Malaysia, Jalan Broga, 43500 Semenyih, Selangor, Malaysia.
N. Shah and O.O. Molosiwa. Plant and Crop Sciences, School of Biosciences, University of Nottingham, Sutton Bonington Campus, Loughborough, Leicestershire, LE12 5RD, United Kingdom.
N.R. Ardiarini and Kuswanto. University of Brawijaya, Veteran Street, Malang, 65145, Indonesia.
S. Mayes. Crops For the Future, Jalan Broga, 43500 Semenyih, Selangor, Malaysia; Plant and Crop Sciences, School of Biosciences, University of Nottingham, Sutton Bonington Campus, Loughborough, Leicestershire, LE12 5RD, United Kingdom.
Corresponding author: Sean Mayes (email: sean.mayes@nottingham.ac.uk).

comparing data on underutilised crops are limited to date (Okonkwo and Opara 2010; Halimi et al. 2019, 2020). Total calorific values for bambara groundnut are reported to be higher than other pulses, such as pigeon pea, lentil, and cowpea; 367, 343, 354, and 345 kcal, respectively (FAO 1982). Comparatively, bambara groundnut contains higher levels of lysine and methionine than other grain legumes, making bambara groundnut an important staple in the diet in combating food and nutrient security issues (Halimi et al. 2019). Nevertheless, the lack of improved varieties has limited its wider adoption to benefit subsistence farmers, particularly those with marginal lands.

Begemann (1988) carried out detailed analyses of the seed-pattern diversity within a large collection of bambara groundnut at the International Institute of Tropical Agriculture (IITA), Nigeria. The author's conclusion strengthened the hypothesis that the centre of origin of bambara groundnut is likely to be in the region of northeastern Nigeria and northern Cameroon. A recent study comprising of 33 landraces (total sample number = 128) from 14 countries suggested that the gene flow of bambara groundnut germplasm was not only influenced by geographic proximity but also the distribution was observed to contain a linguistic component (Santos 2018).

Java, having a clear dry season in the middle and east, is known as the main planting area for bambara groundnut in Indonesia. Although extensive cultivation data are still lacking, the production of bambara groundnut in 2007 from Sumedang district in West Java was recorded to be 138 t according to Widyasanti and colleagues (2019). Similarly, information on the origin of bambara groundnut in Indonesia is scarce, with this species being native to Africa. One of the hypotheses was that the crop was brought to Madagascar by the Arabians and subsequently spread to Brazil and Suriname in the early 17th century before being introduced to the Philippines and Indonesia (Adhi and Wahyudi 2018). Information on the origins of the original introduction(s) of germplasm into Indonesia are important for crop improvement and breeding programs in Indonesia, to widen the genetic base and to introduce new traits of value to farmers. This is one area where molecular genetic tools could help to reveal the likely source of the introduction of bambara groundnut to Indonesia. A previous study has shown that seeds derived from a single plant are essentially inbred, suggesting that selecting from a single plant is an effective method to develop near-homozygous pre-breeding lines in this strongly inbreeding species (Molosiwa et al. 2015). Understanding the ancestral origin of germplasm and the genetic base conserved in situ by the bambara groundnut farmers in Indonesia would facilitate the development of a structured breeding programme. This would also shed light on how this crop has adapted to local humid growing conditions.

Materials and methods

Plant materials and DNA extraction

The plant materials consisted of 12 accessions from East Java, 44 from West Java, 16 from East Africa, 30 from Central Africa, 24 from Southern Africa, and 44 from West Africa (Table S1[1]), with single genotypes derived and analysed from each accession. The plants were grown in a climate-controlled glasshouse located at the Sutton Bonington Campus of University of Nottingham, UK. DNA was extracted from young leaflets using the GenElute Plant Genomic DNA kit (Sigma-Aldrich) according to the manufacturer's instructions (Basu et al. 2007; Molosiwa et al. 2015). The DNA quality and quantity were evaluated under UV light on 1% Tris-borate-EDTA (TBE) agarose gel stained with ethidium bromide.

SSR genotyping

After quantification, the DNA samples were diluted to approximately 10 ng/μL. A total of 11 codominant markers developed by Molosiwa et al. (2015) were used to assess the variation of Indonesian materials (Table S2[1]). The allele sizes were scored after the fragments were separated using the CEQ™ 8000 Genetic Analysis System (Beckman Coulter) with a 400 bp internal standard. Visual investigation of the allele pattern combined with the automated scoring software were used to interpret the capillary electrophoresis results.

With the inclusion of data from samples reported by Molosiwa et al (2015), the allelic sizes of 11 SSR markers were scored from a total of 170 accessions.

SNP genotyping

SNP variation of samples (Table S1[1]) were supplied by Diversity Array Technologies Pty Ltd., Canberra, Australia (www.diversityarrays.com) using DArTseq™ genotype-by-sequencing method and a *Pst*I-*Taq*I genome complexity reduction method. Markers with minor allele frequencies >0.01 were considered as polymorphic. Population structure analysis was performed using fastSTRUCTURE (Raj et al. 2014) and the 'chooseK' function was used to suggest the optimal K value range.

Genetic diversity analysis

The genetic measures of both types of markers including number of alleles per locus (N_a), number of effective alleles (N_e), level of expected (H_e) and observed (H_o) heterozygosity, and fixation index (F, inbreeding coefficient) were computed using GenAlEx v6.5 (Peakall and Smouse 2012) while SSR marker information was generated using PowerMarker v3.25 (Liu and Muse 2005). GenAlEx v6.5 was also used for principal coordinate anal-

[1]Supplementary data are available with the article through the journal Web site at http://nrcresearchpress.com/doi/suppl/10.1139/gen-2019-0137.

Table 1. Summary statistics of genetic variation at 11 SSR loci and 3148 SNP loci across the entire germplasm collection with K = 3 and K = 5 for the genotyping-by-sequencing data.

Marker	Sample	N	Na	Ne	I	H_e	H_o	F	Polymorphism
SSR	Total	170	9.000±1.314	3.535±0.555	1.386±0.209	0.606±0.079	0.012±0.005	0.984±0.006	0.58*
SNP	Total	168	2.000±0.000	1.479±0.006	0.439±0.004	0.286±0.003	0.011±0.001	0.953±0.003	0.21**
	K = 3								
	Q1	76	1.923±0.005	1.228±0.004	0.280±0.003	0.163±0.002	0.010±0.001	0.940±0.004	—
	Q2	36	1.934±0.004	1.461±0.006	0.431±0.004	0.280±0.003	0.011±0.001	0.951±0.004	—
	Q3	56	1.931±0.005	1.507±0.006	0.446±0.004	0.295±0.003	0.012±0.001	0.949±0.004	—
	K = 5								
	Q1	20	1.152±0.006	1.069±0.004	0.063±0.003	0.041±0.009	0.009±0.001	0.600±0.010	—
	Q2	21	1.119±0.006	1.046±0.003	0.044±0.003	0.028±0.002	0.010±0.001	0.468±0.010	—
	Q3	25	1.845±0.006	1.305±0.005	0.330±0.004	0.204±0.003	0.009±0.001	0.947±0.004	—
	Q4	36	1.938±0.004	1.488±0.006	0.446±0.004	0.293±0.003	0.011±0.001	0.948±0.004	—
	Q5	56	1.921±0.005	1.511±0.006	0.449±0.004	0.298±0.003	0.011±0.001	0.951±0.004	—

Note: Mean values except N; *: polymorphic information content (PIC), **: minor allele frequency (MAF). N, sample size; Na, number of different alleles; Ne, effective number of alleles; H_e, expected heterozygosity; H_o, observed heterozygosity; F, fixation index (inbreeding coefficient).

ysis (PCoA) and analysis of molecular variance (AMOVA) with 999 permutations to assess the differentiation among subpopulations.

Hierarchical clustering analysis was carried out by using both neighbour-joining (NJ) with 10 000 bootstraps value and UPGMA methods calculated from 10 000 bootstraps of the 'simple matching' Dissimilarity Index in DARwin v6 (http://darwin.cirad.fr/darwin) (Perrier et al. 2003).

Results and discussion

To gain a better understanding of the genetic relatedness of Indonesian cultivars with those cultivated in African countries, bambara groundnut accessions collected from East Java, West Java, East Africa, West Africa, Central Africa, and Southern Africa (Table S1[1]) were evaluated by SSR (n = 170) and SNP markers (n = 168), with 85 common single seed descent derived accessions (Table S1[1]).

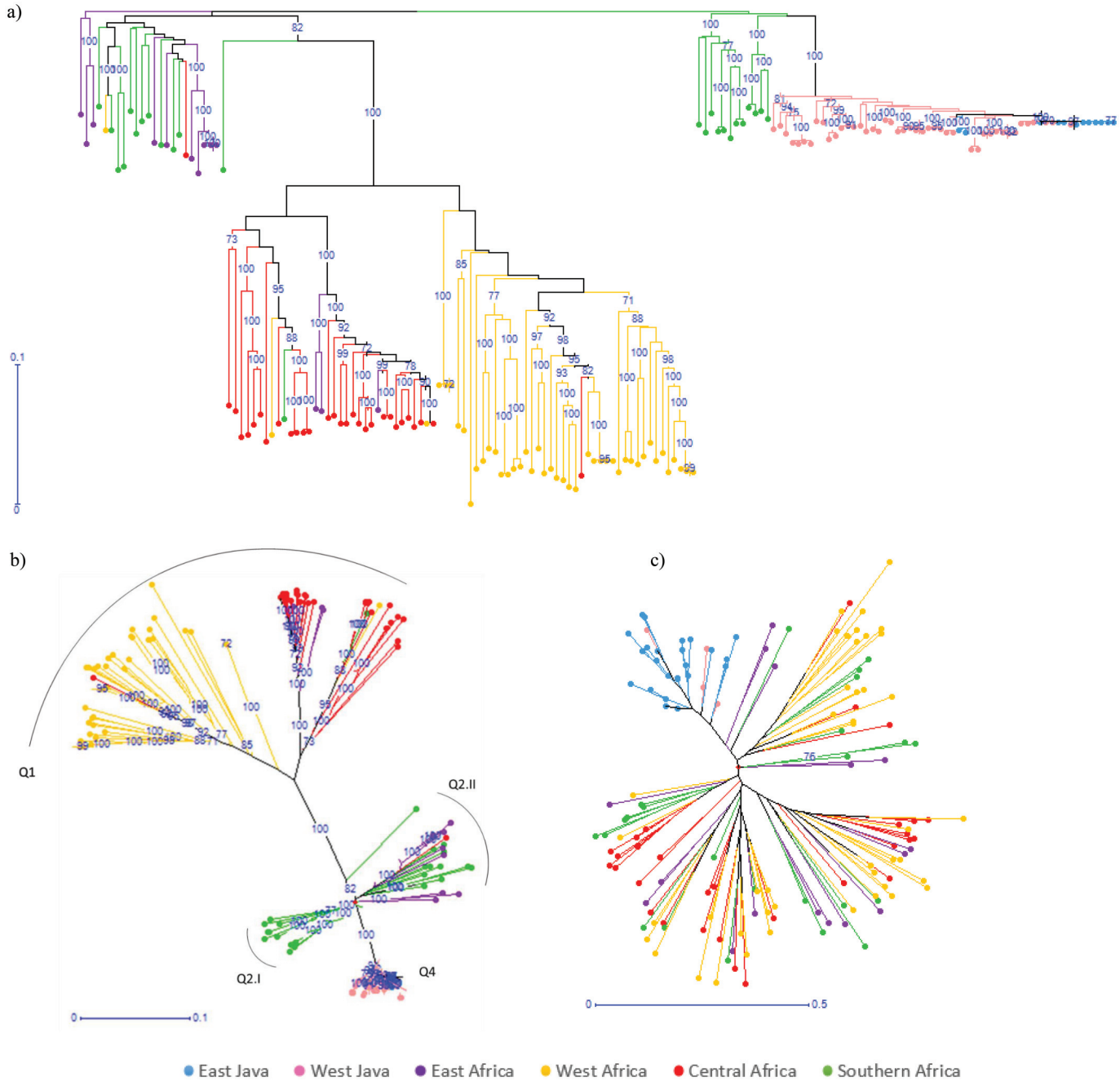
A total of 99 alleles, with an average of nine alleles per locus, were identified by 11 SSR markers (Table 1). The number of alleles (N_a) observed at each locus varied from 4 to 16 with the PIC values ranging from 0.11 to 0.83, and an average of 0.58 (Table S3[1]). Eight of these had a PIC value of more than 0.5 and are considered to be highly informative. Nevertheless, from the low bootstrap values for the nodes of the NJ tree (Fig. 1*c*), as well as the relatively low levels of molecular variation explained in the PCoA plot (25.6% of the total variance explained by first two components, Fig. 2*a*), the current set of SSR markers is not sufficiently informative to clearly distinguish the Central and West African accessions, although samples from East and West Java and also Southern and East Africa did cluster.

In terms of DArT Seq SNP markers, a total of 3148 SNPs were obtained after filtering with minor allele frequency (MAF) > 0.01 and with no missing data across samples. The majority of the SNP markers (34.8%) fall into the high H_e index category and are prevalently [CT] and [AG] types (Figs. 3*a* and 3*b*). Among these, there was a total of 649 SNPs that could be considered as "rare alleles" as their MAF values were less than 5%.

The genetic diversity within individuals revealed by the genotypes evaluated in this study by both types of markers was low (Table 1). It was consistent across both types of makers with the mean observed heterozygosity (H_o) far lower than the average expected heterozygosity (H_e), reflecting the cleistogamous nature of bambara groundnut. Low observed heterozygosity from these markers (0.012 ± 0.005 from SSR; 0.011 ± 0.001 from SNP) suggested that seed from a single plant are likely to represent an unselected cultivar (without trait selection) and that a single round of seed collection from a single plant would (on average) be sufficient to achieve homozygosity in pre-breeding materials, consistent with previous observation using SSR and dominant DArT markers (Molosiwa et al. 2015).

From Bayesian clustering analysis using SNP markers, three major clusters could be observed with a second peak at K = 5 (Fig. 4; Fig. S1[1]). When K = 3, the subpopulation clustering coincided largely with their geographical origins; Q1: 76 accessions (45.2%) mainly from Central and West Africa, Q2: 36 accessions largely consisted of accessions from Southern and East Africa, and Q3: solely 56 Indonesian accessions (Table 2). However, when compared with the PCoA plot (Fig. 2*b*), some of the accessions collected from Central African countries were at a distance from the West African group by the second principal component which explained 10.3% of the molecular variability. Interestingly, at K = 4, Q1 was not subdivided into two clusters as observed from the PCoA, instead Q2 was sub-divided into two; I: 23 accessions predominantly from East and Southern Africa whilst II: 12 accessions with 11 from Southern Africa. Accessions from Southern Africa were seen to be relatively clustered even in Cluster I and Cluster II. The phylogenetic NJ tree with most of the nodes having a bootstrap value of more than 70% supported this grouping (Figs. 1*a* and 1*b*). At K = 5, nine

Fig. 1. Neighbour-joining tree of bambara groundnut (*Vigna subterranea*) accessions in this study based on (*a* and *b*) SNP markers and (*c*) SSR markers. Colours reflect geographical origin and values in branches indicate bootstrap threshold ≥70. The clusters from SNP markers correlate with population structure when K = 4.



accessions (5.4%) were classified into an admixture (Q ≤ 70), three from Central Africa and the others from West Africa. Q1: 20 accessions (11.9%) with a majority from Central African, Q2: 22 accessions (13.1%) predominantly West African samples (*n* = 15), Q3: 25 accessions (14.9%), similar to Q2, 21 from West Africa and four from Central Africa, Q4: 36 accessions (21.4%) consisting of accessions primarily from Southern Africa (*n* = 23), and Q5: 12 East Java and 44 West Java accessions (33.3%). This is in good correspondence with the UPGMA tree (Fig. S2[1]). Figure 5 summarises the total variance explained by the two first coordinates when K = 3 and K = 5. There was no sample having a clear membership with any new cluster when K > 5. At K = 5, the distribution of accessions from Nigeria into Q2 and Q3 might suggest the existence of greater genetic diversity within the populations close to the centre origin. Furthermore, given that the fixation index was lowest in Q2 (when K = 5), higher genetic variability could be found in these accessions of which the majority are collected from Nigeria.

In brief, there are three major subpopulations that could be observed from the bambara groundnut acces-

Fig. 2. Principal coordinates analysis (PCoA) based on genetic distance derived from (*a*) 11 SSR markers and (*b*) 3148 SNP markers showing different clustering patterns.

sions evaluated in this study. Overall, the resulting subpopulations and genetic clusters were mainly correlated to the geographic origins of the collection sites for the samples, suggesting that region-specific selection and potentially a founder effect have had a major role in influencing the diversity of bambara groundnut germplasm, with partially limited gene flow being observed between locations. Nevertheless, the influences from dietary habit and a cultural role for bambara groundnut should not be underestimated. For example, a survey conducted in Zimbabwe has revealed that although peanut is a cash crop, in some districts the cultivation areas of bambara groundnut are comparable or have exceeded the amount of land allocated for growing peanut (Mubaiwa et al. 2018). Moreover, Santos (2018) detected a linguistic signal in the distribution of bambara groundnut.

Both NJ and UPGMA dendograms (Figs. 1*a*, 1*b*, 5*a*, and 5*b*) also suggested that the most likely origin of recent

Fig. 3. Distribution of (*a*) genetic diversity (expected heterozygosity; H_e) and (*b*) SNP type for 3148 SNP markers in the 168 bambara groundnut (*Vigna subterranea*) accessions.

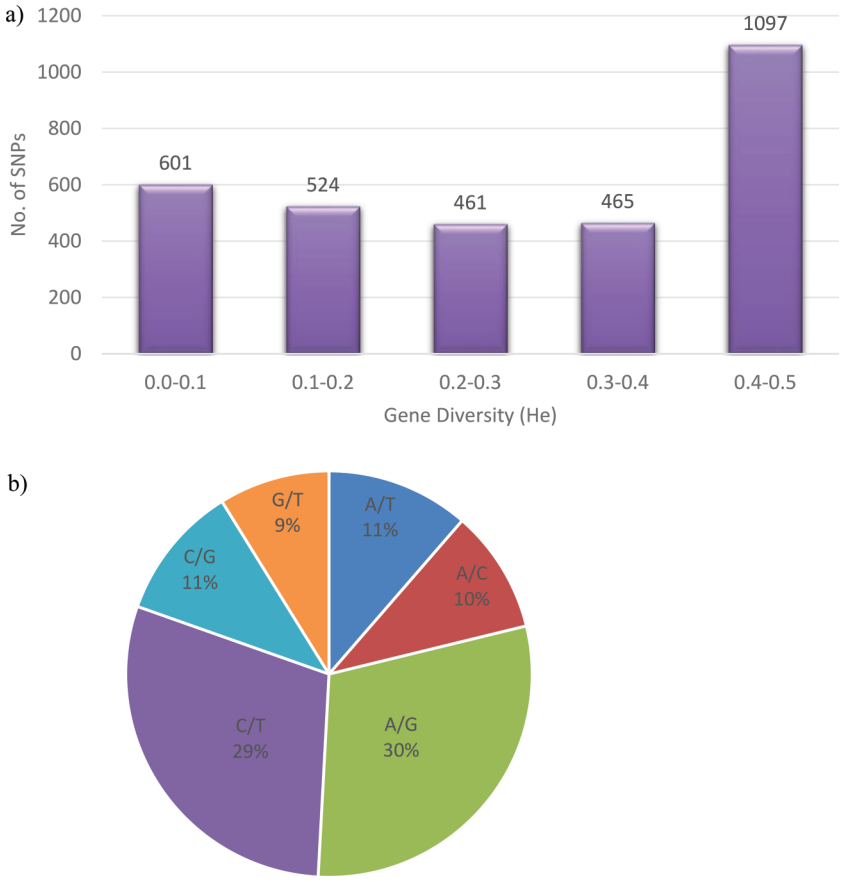
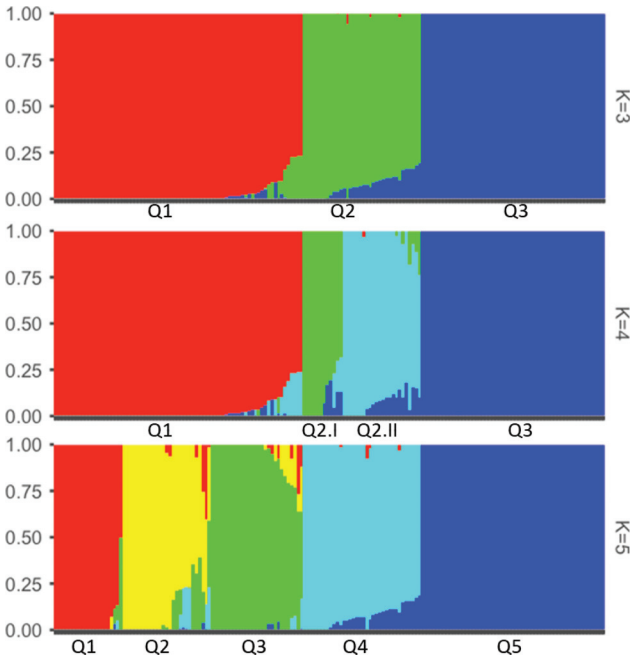


Fig. 4. Clustering analysis showing K values from 3 to 5, with strong geographic signals.



Indonesian materials is from Southern Africa. This is in good correspondence with the previous report of Molosiwa et al. (2015) even though those authors sampled a limited number of Indonesian lines (4 out of 123 accessions). The Dutch shipping routes between 1750–1800 could be speculated to be one of the plausible bambara groundnut introduction routes to Java (Burn-Murdoch 2012). In addition, the analysis provides evidence that the narrow genetic base of current East Java materials could result from the introduction of limited West Java materials to East Java. This preliminary observation could be further confirmed with the use of a wider germplasm set collected from the East Java cultivation regions. The genetic base of Indonesian accessions could potentially be widened through the introduction of genetic variation from another cluster; accessions grouped in Q2.I at K = 4. Four Southern African accessions sharing the highest similarity with the Indonesian groups were collected from Zambia, where the climate can be broadly classified into humid subtropical or semi-arid steppe in different ecoregions.

There is moderately strong differentiation between the subpopulations regardless of whether sub-clustered into three or five groups (F_{ST} = 0.251 and 0.259, respectively, Table 3), indicating that the groups are genetically distinct. The majority of the genetic variance occurred

Table 2. Number and percentage of bambara groundnut (*Vigna subterranea*) accessions assigned into clusters among six regions.

	K = 3			K = 4				K = 5					
Region	Q1	Q2	Q3	Q1	Q2	Q3	Q4	Q1	Q2	Q3	Q4	Q5	Admixture
Central Africa	29	1	0	29	0	1	0	16	6	4	1	0	3
West Africa	43	1	0	43	0	1	0	1	15	21	1	0	6
East Africa	3	11	0	3	1	10	0	3	0	0	11	0	0
Southern Africa	1	23	0	1	11	11	0	0	1	0	23	0	0
East Java	0	0	12	0	0	0	12	0	0	0	0	12	0
West Java	0	0	44	0	0	0	44	0	0	0	0	44	0
Percent (%)	45.2	21.4	33.3	45.2	7.1	13.7	33.3	11.9	13.1	14.9	21.4	33.3	5.4

Fig. 5. Principal coordinates analysis (PCoA) of the subpopulations when (*a*) K = 3 and (*b*) K = 5, along with the AMOVA analysis explaining the total variance found among and within groups.

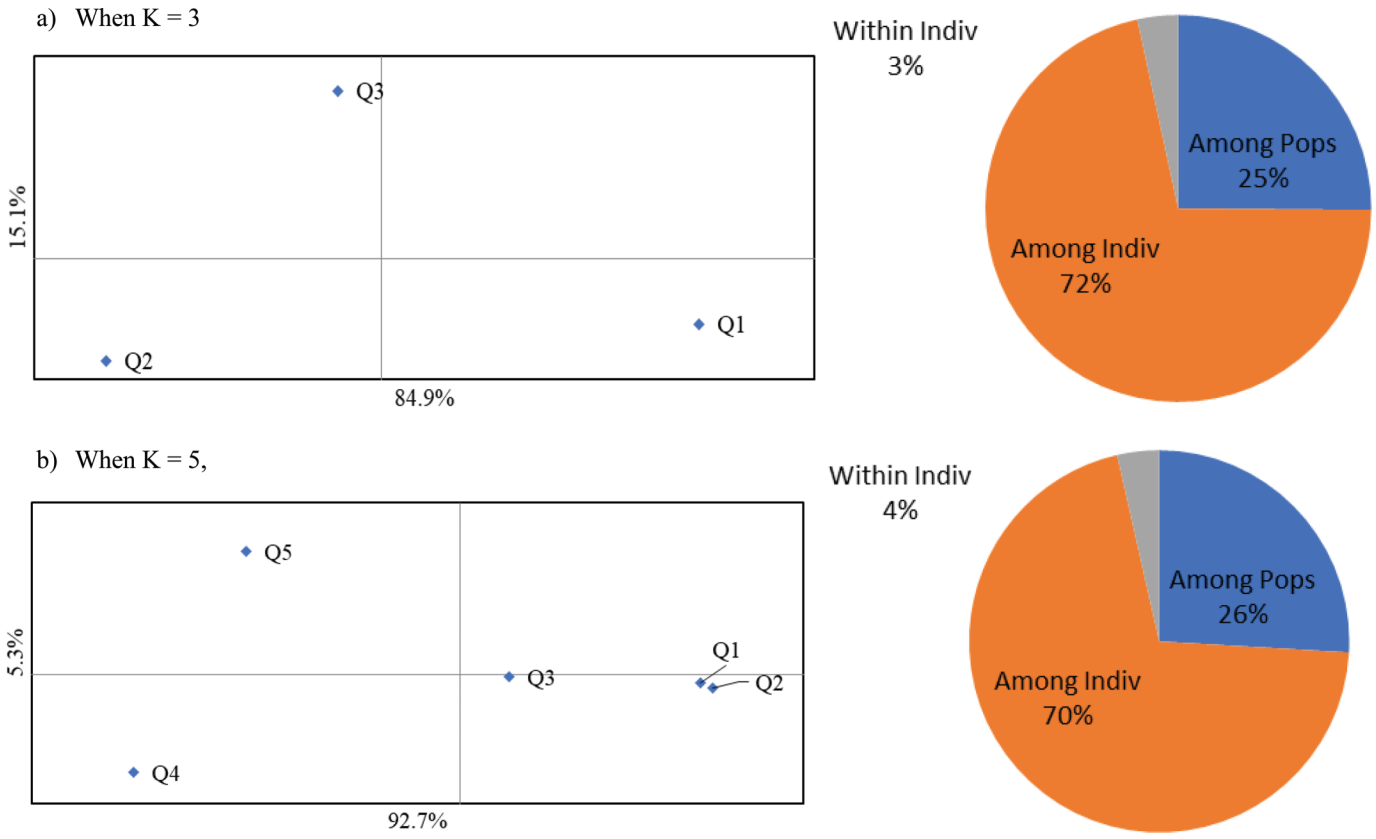


Table 3. Analysis of molecular variance among and within bambara groundnut (*Vigna subterranea*) populations for K = 3 and K = 5.

Variation source	df	SS	MS	Estimated variance	Percent (%)	F-statistics	*p*-value	Nm
K = 3								
Among groups	2	28238.63	14119.32	124.88	25	F_{ST} = 0.251	0.001	—
Among individuals	165	120067.98	727.69	355.38	71	F_{IS} = 0.955	0.001	—
Within individuals	168	2842.50	16.92	16.92	3	F_{IT} = 0.966	0.001	—
Total variation	335	151149.12	—	497.18	100	—	—	0.745
K = 5								
Among groups	4	31965.02	7991.25	121.24	26	F_{ST} = 0.259	0.001	—
Among individuals	153	103461.06	676.22	329.81	71	F_{IS} = 0.952	0.001	—
Within individuals	158	2623.50	16.60	16.60	4	F_{IT} = 0.964	0.001	—
Total variation	315	138049.58	—	—	100	—	—	0.714

within populations and accounted for 70%–72% of the total variation, whereas only 25%–26% was attributed to the difference between subpopulations (Fig. 5). This suggests that substantial genetic variability may be accessed from within the same clusters, perhaps minimising the disruption of adaptive complexes already in place. Cultivated at a small or subsistence scale for centuries without strong selection pressure from farmers, beyond matching the local agroecosystem, landraces may well contain many allelic variants that have not experienced strong selection. Both high subpopulation inbreeding coefficients, F_{IS} and F_{IT} values, indicate that the lines making up these groups are inbred lines, consistent with the self-pollination mechanism of bambara groundnut. Subpopulations Q2 and Q4 are the most diverged groups, which could be partially contributed to by geographical barriers limiting material exchange (Table S4[1]).

Integration with agronomic and phenotypic data following molecular characterisation would allow the informed development of crop improvement breeding programmes, particularly with the availability of the reference genome despite it being currently fragmented (Chang et al. 2019). Application in genome-wide association mapping (GWAS) would identify quantitative trait loci (QTL) or causal genes governing the traits of interest. Germplasm within the same subpopulation identified in this study, particularly those collected from humid subtropical regions, should be characterised in the field trials, if the goal is to improve bambara groundnut in Indonesia.

Conclusion

The genetic clusters postulated in this study have shed light on the potential origin of bambara groundnut cultivars in Indonesia from Southern African countries. Although the genetic base of bambara groundnut in Indonesian is generally narrow, an understanding of the diversity of bambara groundnut conserved in situ facilitates future breeding efforts towards development of new cultivars with a wider genetic base or to mine favourable alleles from traits of interest.

Acknowledgement

This research was conducted with financial support from the EU FP6 INCO-DEV “BAMLINK” project and an ITPGRFA R3 W3 Benefit Sharing Fund Project (P26). The authors would like to thank Rossuraya Abdullah and Nur Zaffan Ariffin in assisting in the graphical design.

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