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Genetic diversity and association mapping of Ethiopian and exotic finger millet accessions

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Abstract. Combining morphological and molecular data to identify genetic variation and marker–trait association is one of the most important prerequisites for genomics-assisted selection in crop improvement. To this end, a total of 138 finger millet (*Eleusine coracana* subsp. *coracana*) accessions including five improved varieties were evaluated to assess the genetic variation and population structure and undertake association mapping. These accessions were basically collected from Ethiopia (96), Eritrea (8), Kenya (7), Zambia (9) and Zimbabwe (13). Finger millet accessions were evaluated in the field for 10 important agronomic traits and also characterised using a set of 20 microsatellite markers. Mean polymorphism information content of 0.61 was observed from a total of 222 alleles with an average of 11.1 alleles per microsatellite locus. About 61% of alleles detected were rare (<5%) and specific allele amplification was observed in 34 accessions. Both weighted neighbour-joining based clustering using molecular data and hierarchical clustering using phenotypic trait data grouped the 138 accessions into four major clusters that were not entirely based on their geographical origins. Genome-wide association studies depicted 16 significant ($P < 0.01$) associations between 13 microsatellite markers and six agronomic traits. Our results reveal a unique abundance of rare alleles in finger millet and highlight the need for more careful selection of genome-wide association studies in the future in order to capture the contribution of rare alleles to important agronomic traits.

Additional keywords: *Eleusine*, genome-wide association studies, microsatellites, PIC.

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Introduction

Finger millet (*Eleusine coracana* subsp. *coracana*, 2n = 4x = 36), of the family Poaceae and subfamily Chloridoideae, is a cereal crop cultivated in the tropical and sub-tropical regions of Africa and India. It ranks third in cereal production in semiarid regions of the world after sorghum and pearl millet (Barbeau and Hilu 1993). It occupies 12% of the global millet area and is cultivated in more than 25 countries in Africa and Asia (Vetriventhan *et al*. 2015). Finger millet is adapted to adverse agro-ecological conditions, requires minimal inputs, is tolerant to moisture stress (Hittalmani *et al*. 2017) and acidic soils, and generally thrives on marginal land where other crops cannot perform (Barbeau and Hilu 1993). Due to its high nutritional value and good storage quality (Dida *et al*. 2007), finger millet also plays an important role in food security, especially in marginal areas. However, finger millet remains one of the few under-researched cereal crops in the world.

Archaeological records revealed that the primary centre of origin for finger millet is East Africa, particularly Ethiopia (Purseglove 1985) and Uganda (de Wet 1995). Understanding the extent of genetic diversity of finger millet genotypes cultivated in Ethiopia, therefore, represents an important resource for the study of finger millet genetics and genome evolution. Genetic diversity assessment aids in understanding intra-species crop performance that can be exploited in crop improvement (Aremu 2011) and provides information on the extent of genetic divergence. Diversity assessment also serves as a platform for specific breeding objectives (Thompson *et al*. 1998) and identifies parental combinations exploitable to create segregating progenies with maximum genetic potential for further selection (Djè *et al*. 2000). Successful genetic conservation and utilisation of finger millet will depend on understanding the genetic diversity and its distribution in a given region, as has been done in other crops (Bekele 1983; Varshney *et al*. 2007).

Being an important centre of diversity, Ethiopia also harbours several wild cross-compatible *Eleusine* species, which grow alongside cultivated finger millet. The expected gene flow between cultivated and wild accessions is likely to result in unique genetic combinations but would also create complex genome structures if not fully monitored and characterised. However, the true extent of genetic diversity and genome evolution in finger millet, especially from Ethiopia, is not known. Morphological (Tsehaye and Kebebew 2002; Bezawuletaw *et al*. 2006; Daba and Debelo 2008; Wolie and Dessalegn 2011) and molecular (Bezawuletaw 2011) diversity studies focusing on Ethiopia or eastern Africa (Upadhyaya *et al*. 2007; Reddy *et al*. 2009; Manyasa *et al*. 2015) have been limited and therefore not conclusive. The lack of knowledge on genetic variability in finger millet coupled with limited breeding efforts has consequently resulted in the production of low-yielding finger millet cultivars (Dida *et al*. 2007; Neves 2011). Given the recent interest in the potential of finger millet to improve nutrition security in the region, a refined understanding of the extent of genetic variation and population structure among finger millet germplasm is urgently required to guide future crop improvement programs.

Some of the most commonly used molecular markers in finger millet diversity study and also linkage mapping to date include random amplification of polymorphic DNA (RAPD) (Salimath *et al*. 1995; Babu *et al*. 2007; Das and Misra 2010; Gupta *et al*. 2010; Panwar *et al*. 2010; Bezawuletaw 2011), restriction fragment length polymorphism (Parani *et al*. 2001), inter simple sequence repeats (ISSRs) (Gupta *et al*. 2010) and simple sequence repeats (SSRs) (Dida *et al*. 2007, 2008; Panwar *et al*. 2010; Sinha and Pande 2010) as well as some efforts to investigate blast-resistant R-genes (Panwar *et al*. 2011; Bheema Lingeswara Reddy *et al*. 2011). Although still very limited in finger millet, microsatellite markers, also known as SSRs, are the most reliable molecular markers in finger millet so far available. Due to their high polymorphism (Kubik *et al*. 2009), microsatellite markers can be used to determine genetic distance between genotypes and identify parental lines for new variety development with maximum hybrid vigour. Microsatellites therefore remain markers of choice for practical breeding applications, particularly in developing countries (Sharma *et al*. 2010) and for a crop such as finger millet where single nucleotide polymorphism (SNP) markers have not been developed. Microsatellite markers can also be used to identify association with important agronomic traits, which is essential for cultivar development. Association mapping is increasingly being applied to plants (Nordborg and Weigel 2008) and has been advocated as the method of choice for identifying loci involved in the inheritance of complex traits (Risch and Merikangas 1996; Pritchard *et al*. 2000; Ramakrishnan *et al*. 2016*a*; Hittalmani *et al*. 2017).

Therefore, this study was initiated to assess the genetic variation and association mapping in 96 finger millet accessions collected from various regions of Ethiopia using morphological and microsatellite markers, along with five improved varieties and 37 additional accessions introduced from Eritrea (eight), Kenya (seven), Zambia (nine) and Zimbabwe (13). We also established likely associations of the molecular markers to traits of interest.

Materials and methods

Experimental accessions and testing locations

Ninety-six finger millet accessions collected from major finger millet producing regional states of Ethiopia, together with five improved varieties and 37 introduced accessions (Table 1) were grown in the 2011 main cropping season under field conditions at two sites in Ethiopia where finger millet is widely grown: Arsi Negele Research Sub-site (altitude 1947 m a.s.l., 07°19′N, 38°39′E), 250 km south-east of Addis Ababa and Gute Research Sub-site (1906 m a.s.l., 09°00′N, 36°38′E), 321 km west of Addis Ababa. Each entry was planted in a single 2-m row using a randomised complete block design. Field data were recorded for 10 major agronomic traits following the finger millet descriptors (IBPGR 1985): days to 50% heading, days to maturity, productive tiller number, plant height (cm), finger length (cm), finger number per ear, ear weight (g), number of grains per spike, thousand-grain weight (g) and grain yield per plant (g). Additionally, 20 published microsatellite markers (Dida *et al*. 2007) were used to determine the extent of molecular diversity among the germplasm set.

DNA extraction and PCR

The DNA was extracted from three-week-old young leaf samples according to the modified CTAB protocol of Mace *et al*. (2003). Extracted DNA was visualised on a 0.8% (w/v) agarose gel and quantified using a Nanodrop® 1000 spectrophotometer (Thermo Scientific, St Louis, MO, USA). The DNA samples were subjected to genotyping using 20 published SSR markers for finger millet (Dida *et al*. 2007) (Table 2). All forward primers contained an M13-tag (5′-CACGACGTTGTAAAACGAC-3′) on the 5′-end that was fluorescently labelled to allow detection of amplification products (Schuelke 2000). The PCR amplification was performed in 10-μL reaction volume comprising 1 × PCR buffer (20 mM Tris-HCl, pH 7.6; 100 mM KCl; 0.1 mM EDTA; 1 mM DTT; 0.5% (w/v) Triton X-100; and 50% (v/v) glycerol), 2 mM $MgCl_2$, 0.16 mM dNTPs, 0.16 μM fluorescent-labelled M13-forward primer, 0.04 μM forward primer, 0.2 μM reverse primer, 0.2 units of Taq DNA polymerase (SibEnzyme Ltd, Russia) and 30 ng of template DNA. The PCR reactions were performed in 384-well microtitre plates on a GeneAmp 9700 thermocycler (Applied Biosystems) with initial denaturation of 94°C for 5 min, followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 59°C for 1 min, extension at 72°C for 2 min and final elongation at 72°C for 20 min.

SSR fragment detection and data analysis

Amplification was confirmed by running the PCR products on a 2% (w/v) agarose gel stained with GelRed® (Biotium, USA) and visualised under UV light. Amplification products (1.5–3.5 μL of each) were co-loaded in sets of 3–4 markers together with the size standard GeneScan™ –500 LIZ® (Applied Biosystems) and Hi-Di™ Formamide (Applied Biosystems), and separated by capillary electrophoresis using an ABI Prism® 3730 Genetic analyser (Applied Biosystems). Allele calling was performed with GeneMapper 4.0 (Applied Biosystems). PowerMarker ver. 3.25 software (Liu and Muse 2005) was used to calculate polymorphism information content (PIC), analysis of molecular variance (AMOVA) and allelic frequency. The weighted

Table 1. Finger millet accessions evaluated for phenotypic and molecular diversity with their passport information

Acc. name	Code[A]	Altitude (m)	Latitude (°-′-″)N	Longitude (°-′-″)E	Acc. name	Code	Altitude (m)	Latitude (°-′-″)	Longitude (°-′-″)E
242133	AM-1	1825	12-01-00	37-43-00	230105	ER-38	1600	14-32-00N	37-47-00
215989	AM-2	2000	11-55-00	37-35-00	230102	ER-39	1850	14-33-00N	37-46-00
215985	AM-3	1940	12-17-00	37-4-00	230109	ER-40	1800	14-32-00N	37-46-00
235835	AM-4	1930	12-39-00	37-33-00	230107	ER-41	1800	14-33-00N	37-46-00
229731	AM-5	1950	11-31-00	37-18-00	230101	ER-42	1740	14-32-00N	37-46-00
235782	AM-6	1860	12-02-00	45-11-00	230104	ER-43	1800	14-33-00N	37-46-00
242117	AM-7	1915	11-36-00	37-25-00	230103	ER-44	1700	14-33-00N	37-46-00
215802	AM m-8	1950	08-32-00	36-46-00	203545	KN-45	1590	00-14-00S	34-32-00
215887	AM-9	1880	11-06-00	37-44-00	203542	KN-46	1540	00-54-00S	34-30-00
229730	AM-10	1850	11-34-00	37-21-00	203546	KN-47	1620	00-18-00S	34-46-00
215982	AM-11	1850	12-17-00	37-7-00	203543	KN-48	1514	00-54-00S	34-30-00
215976	AM-12	1860	12-25-00	37-20-00	203547	KN-49	1510	NR	NR
242111	AM-13	2100	10-39-00	36-56-00	203544	KN-50	1485	NR	NR
243639	AM-14	2070	NR	NR	203351	KN-51	1490	18-54-00S	31-02-00
225892	AM-15	1710	12-31-00	37-19-00	BKFM0034	OR-52	1454	NR	NR
215990	AM-16	1910	11-50-00	37-32-00	BKFM0042	OR-53	1867	NR	NR
237443	AM-17	2100	12-09-00	39-39-00	BKFM0028	OR-54	1608	NR	NR
238344	AM-18	2000	10-09-00	36-09-00	BKFM0032	OR-55	1390	NR	NR
238341	AM-19	1780	10-09-00	36-09-00	BKFM0006	OR-56	1479	NR	NR
242135	AM-20	1910	11-50-00	39-35-00	BKFM0022	OR-57	1926	NR	NR
242105	AM-21	1860	11-14-00	37-38-00	216039	OR-58	1950	09-26-00-N	35-36-00
238346	AM-22	1940	10-02-00	37-09-00	BKFM0047	OR-59	1334	NR	NR
235783	AM-23	2000	12-01-0	45-12-00	BKFM0029	OR-60	1251	NR	NR
235140	AM-24	1950	36-30-00	37-05-00	BKFM0039	OR-61	2144	NR	NR
242112	AM-25	2125	10-39-00	36-57-00	BKFM0010	OR-62	1484	NR	NR
242109	AM-26	2060	11-39-00	37-09-00	BKFM0062	OR-63	1923	NR	NR
242132	AM-27	1910	12-03-00	38-00-00	BKFM0052	OR-64	2200	NR	NR
215981	AM-28	1850	12-18-00	37-8-00	BKFM0048	OR-65	1337	NR	NR
229725	AM-29	1650	NR	NR	BKFM0024	OR-66	1913	NR	NR
242120	AM-30	1850	11-49-00	37-36-00	BKFM0005	OR-67	1449	NR	NR
229723	BG-31	1300	11-04-00	36-33-00	BKFM0018	OR-68	1667	NR	NR
229728	BG-32	1440	NR	NR	208726	OR-69	1880	NR	NR
216033	BG-33	1930	09-15-00	35-44-00	BKFM0055	OR-70	1723	NR	NR
229722	BG-34	1750	11-11-00	36-39-00	BKFM0011	OR-71	1428	NR	NR
229724	BG-35	1520	NR	NR	BKFM0057	OR-72	1707	NR	NR
229738	BG-36	1830	11-06-00	37-44-00	BKFM0060	OR-73	1852	NR	NR
230106	ER-37	1800	14-33-00	37-46-00	245091	OR-74	1991	NR	NR
216056	OR-75	1600	08-57-00	35-15-00	242624	TG-107	1400	13-77-07N	38-20-07
216057	OR-76	1800	08-34-00	34-55-00	216036	TG-108	1900	09-19-00N	35-44-00
BKFM0001	OR-77	1580	NR	NR	237472	TG-109	1800	14-21-00N	38-11-00
236446	OR-78	1930	NR	NR	238327	TG-110	1900	14-05-00N	38-04-00
BKFM0051	OR-79	2227	NR	NR	AAUFM-23	TG-111	2100	NR	NR
BKFM0004	OR-80	1445	NR	NR	AAUFM-20	TG-112	2142	NR	NR
BKFM0008	OR-81	1459	NR	NR	AAUFM-14	TG-113	1568	NR	NR
216040	OR-82	1940	09-28-00	35-33-00	AAUFM-21	TG-114	1722	14-07-12.7N	38-37-20
BKFM0058	OR-83	1725	NR	NR	AAUFM-12	TG-115	1502	NR	NR
Tadesse	IMV-84	N/A	N/A	N/A	AAUFM-11	TG-116	1502	14-20-06.2N	38-48-26
Wama	IMV-85	N/A	N/A	N/A	214995	ZA-117	1130	11-08-00N	24-08-00
Bereda	IMV-86	N/A	N/A	N/A	214991	ZA-118	1330	11-35-00N	24-28-00
Gute	IMV-87	N/A	N/A	N/A	214994	ZA-119	1160	11-10-00N	24-10-00
Boneya	IMV-88	N/A	N/A	N/A	214996	ZA-120	1130	11-15-00N	24-15-00
241768	SN-89	1500	05-24-90	37-14-06	214988	ZA-121	1300	11-58-00N	24-05-00
237584	SN-90	1990	NR	NR	214987	ZA-122	1310	11-44-00N	24-18-00
241769	SN-91	1500	05-20-67	37-14-99	214993	ZA-123	1340	11-25-00N	24-18-00
235700	SN-92	1530	05-41-00	36-38-00	214989	ZA-124	1210	11-58-00N	24-18-00
235699	SN-93	1440	05-16-00	37-30-00	214997	ZA-125	1100	13-32-00N	25-00-00
AAUFM-42	TG-94	2058	NR	NR	203350	ZI-126	1400	19-32-00S	30-42-00
AAUFM-22	TG-95	2142	NR	NR	203353	ZI-127	1420	18-53-00S	31-24-00
AAUFM-4	TG-96	1896	13-41-25.6	39-01-09.7E	203354	ZI-128	1420	18-53-00S	31-24-00

(*continued next page*)

Table 1. (*continued*)

Acc. name	Code[A]	Altitude (m)	Latitude (°-′-″)N	Longitude (°-′-″)E	Acc. name	Code	Altitude (m)	Latitude (°-′-″)	Longitude (°-′-″)E
AAUFM-34	TG-97	1568	NR	NR	203356	ZI-129	1420	18-53-00S	31-24-00
AAUFM-32	TG-98	1630	NR	NR	203358	ZI-130	1420	18-53-00S	31-24-00
AAUFM-33	TG-99	1620	NR	NR	203360	ZI-131	1420	18-53-00S	31-24-00
AAUFM-8	TG-100	1812	13-59-6.7	38-58-39.5E	203355	ZI-132	1420	18-53-00S	31-24-00
AAUFM-19	TG-101	1811	NR	NR	203362	ZI-133	1420	18-53-00S	31-24-00
AAUFM-2	TG-102	1896	13-41-25.6	39-01-09.7E	203352	ZI-134	1490	18-54-00S	31-24-00
237475	TG-103	1750	14-00-00	38-00-00	203361	ZI-135	1420	18-53-00S	31-24-00
245087	TG-104	1923	NR	NR	203363	ZI-136	1420	18-53-00S	31-24-00
238300	TG-105	1980	14-04-00	38-04-00	203357	ZI-137	1420	18-53-00S	31-24-00
242616	TG-106	1400	14-37-96	38-79-81	203359	ZI-138	1420	18-53-00S	31-24-00

[A]The codes represent the accessions listed under column 1. The first two letters in the codes represent the country or region of origin as follows: AM, Amhara; BG, Benishangul Gumuz; ER, Eritrea; KN, Kenya; OR, Oromia; IMV, improved varieties; SN,- Southern Nations; TG, Tigray; ZA, Zambia; ZI, Zimbabwe. 'NR' denotes 'not recorded' data and 'N/A' denotes 'not applicable'.

Table 2. List of SSR primers used and allelic diversity information

N_A, Number of alleles; PIC, polymorphism information content

Marker	Repeat motif	Primers	Linkage group	N_A	PIC
UGEP24	(GA)26	F: gccttttgattgttcaactct R: cgtgatcctctcctctctg	3B	27	0.94
UGEP53	(AG)26	F: tgccacaactgtcaacaaaag R: cctcgatggccattatcaag	2A	13	0.79
UGEP84	$(CT)_{24}$	F: ggaacttccgtcagtcctt R: tggggaaggtgttgaatc	ND	2	0.37
UGEP27	$(GA)_{19}$	F: ttgctctgaggttgtgtgttgc R: tcaagcatagtgccctcctc	ND	15	0.82
UGEP95	$(TC)_{14}$	F: aggggacgcttggagtttg R: gcctctacctgtctccgttg	ND	7	0.77
UGEP64	$(CT)_{23}$	F: gtcacgtcgattggagtgtg R: tctcacgtgcatttagtcat	ND	16	0.88
UGEP33	$(TC)_{18}$	F: tagccgtttgcttgttgttttg R: aaggccctagaacgtcaagc	ND	11	0.62
UGEP67	$(TC)_{22}TT(GT)_5$	F: ctcctgatgcaagcaaggac R: aggtgccgtagtttgtgctc	ND	20	0.9
UGEP106	(AC)12	F: aattccattctctcgcatcg R: tgctgtgctcctctgttgac	9B	7	0.52
UGEP110_1[A]	(CT)12	F: aaattcgcatccttgctgac	7AB	7	0.31
UGEP110_2[A]		R: tgacaagagcacaccgactc		7	0.36
UGEP57	$(AG)_{16}$	F: ccatgggttcatcaaacacc R: acatgagctcgcgtattgc	ND	8	0.69
UGEP96	$(CT)_{10}$	F: taatgggcctaatggcaatg R: caaaatccgagccaagattc	ND	5	0.1
UGEP66	$(AG)_{29}$	F: cagatctgggtagggctgtc R: gatggtggttcatgccaac	ND	19	0.87
UGEP46	$(GA)_{14}$	F: caagtcaaaacattcagatgg R: ccactccattgtagcgaaac	ND	10	0.71
UGEP20	$(GA)_{20}$	F: ggggaaggcaatgatatgtg R: ttggggagtgccaacaatac	ND	16	0.84
UGEP79	$(CT)_{12}$	F: ccactttgccgcttgattag R: tgacatgagaagtgccttgc	ND	5	0.45
UGEP12	(CT)22	F: atccccacctacgagatgc R: tcaaagtgatgcgtcaggtc	8B	11	0.74
UGEP73	$(CT)_4CC(CT)_{10}$	F: ggtcaaagagctggctatcg R: accagaaccgaatcatgagg	ND	6	0.15
UGEP05	(TC)12AC(TC)4	F: tgtacacaacaccacactgat R: ttgtttggacgttggatgtg	9B	10	0.34
Mean				11.1	0.61

[A]UGEP110 amplified sets of fragments in two clearly different size ranges and hence was split and scored as two separate markers (UGEP110_1 and UGEP110_2).

neighbour-joining based clustering was computed using DARwin v.5 (Perrier and Jacquemoud 2006).

Structure 2.3.3 (Pritchard *et al*. 2000) was used to establish the number of hypothetical distinct populations. The optimum number of subpopulations was inferred by running an admixture ancestry model with correlated allele frequencies starting from $K=2$ to $K=8$, with 10 independent runs at each K. For each run, 5000 burn-ins followed by 50 000 Markov chain Monte Carlo simulations were performed. The ideal number of k according to Pritchard *et al*. (2000) was used as the criterion for defining the number of groups (k). The most trustworthy value was estimated based on the lowest negative number of Ln (the log-likelihood of the data) and the lowest standard deviation found during statistical analysis.

Phenotypic data for 10 agronomic traits were analysed at each individual and across locations. Best Linear Unbiased Predictors were estimated for all accessions and all traits. A dendrogram was built by the average linkage method of squared Euclidian distance using the MINITAB 14 statistical package (MINITAB 2003). The means of phenotypic traits across locations were standardised to the mean 0 with a variance of 1 for cluster analysis to avoid any bias due to differences in measurement scales. Individual accessions were assigned to the different clusters using 80% cluster assignment probability as a cut-off (Garris *et al*. 2005; Keneni *et al*. 2012).

Marker–trait association analyses were performed with the compressed mixed linear model (Zhang *et al*. 2010) implemented in the GAPITR package (Lipka *et al*. 2012) to test 20 polymorphic pre-screened SSR loci (Dida *et al*. 2007) across 10 major agronomic traits to detect marker–trait association. Association was considered significant if $P<0.01$.

Results

Allele diversity

All SSR markers tested detected polymorphism except UGEP98, which was subsequently omitted from the analysis. Marker UGEP110 amplified sets of fragments in two clearly different size ranges and hence was split and scored as two separate markers: UGEP110_1 and UGEP110_2 (Table 2). A total of 222 alleles were observed with an average of 11.1 alleles per locus (varying from 2 for UGEP84 to 27 for UGEP24) and PIC values ranged from 0.10 (UGEP96) to 0.94 (UGEP24) with an average of 0.61 (Table 2). Markers UGEP24, UGEP67, UGEP64 and UGEP66 revealed the highest polymorphism of 0.94, 0.90, 0.88 and 0.87 respectively.

Rare alleles (<5% occurrence) were the most abundant (up to 18 per locus for UGEP24) with a total of 136 (Table 3) out of the 222 alleles reported. Abundant alleles (>50%) were only observed in seven cases and common alleles (5–50%) in 79 cases. There were 48 specific allele amplifications observed in 34 accessions with AM-1 (Acc. 242133) having the highest number (4) (Table 3). The Benishangul Gumuz region of Ethiopia had the highest proportion (50%) of its accessions showing specific allele amplification although only six accessions were included in the study (Fig. 1).

Regional genetic diversity

Weighted neighbour-joining based clustering revealed a complex accession distribution pattern and grouped the 138 accessions into four major clusters from the main node (Fig. 2). The first cluster comprised mainly accessions from Northern Ethiopia (Tigray and northern Amhara). A second cluster comprised an admixture of collections from different

Table 3. Distribution of alleles of the 20 SSR loci across 138 finger millet accessions

R_A, Rare alleles present in <5% of accessions; C_A, common alleles present in 5–50% of accessions; A_A, alleles present in >50% of accessions

Marker	R_A	C_A	A_A	Specific alleles[A]
UGEP24	18	9	0	IMV-88 (194), OR-74 (192), AM-1 (258), OR-56 (232), TG-96 (206)
UGEP53	7	6	0	ZI-135 (225), SN-89 (235)
UGEP84	0	1	1	–
UGEP27	10	5	0	ZI-131 (249)
UGEP95	2	5	0	TG-96 (222)
UGEP64	9	7	0	IMV-88 (253)
UGEP33	7	4	0	OR-60 (211), TG-101 (207), OR-72 (225), ZI-133 (221), TG-98 (227), OR-57 (229)
UGEP67	14	6	0	OR-65 (231), ZI-131 (233), BG-32 (241), OR-79 (253)
UGEP106	4	2	1	AM-1 (177), OR-54 (185)
UGEP110_1	5	1	1	OR-72 (187), KN-50 (215)
UGEP110_2	5	1	1	AM-1 (205), TG-94 (209), ER-38 (213)
UGEP57	4	4	0	ZI-127 (476)
UGEP96	4	0	1	AM-1 (233)
UGEP66	14	5	0	OR-74 (206), KN-47 (236), BG-35 (246), TG-105 (252), OR-56 (256)
UGEP46	6	4	0	AM-12 (197), ZI-133 (181)
UGEP79	2	2	1	KN-48 (185)
UGEP20	9	7	0	BG-35 (149), SN-92 (165), TG-100 (181), AM-23 (185)
UGEP12	4	7	0	OR-74 (237), BG-32 (255)
UGEP73	5	0	1	ER-37 (248)
UGEP05	7	3	0	IMV-85 (226), OR-68 (238), AM-12 (242), BG-33 (252)
Total	136	79	7	

[A]These alleles were observed only in one genotype for that specific locus and therefore referred to as specific alleles. The genotypes are shown and allele included in brackets.

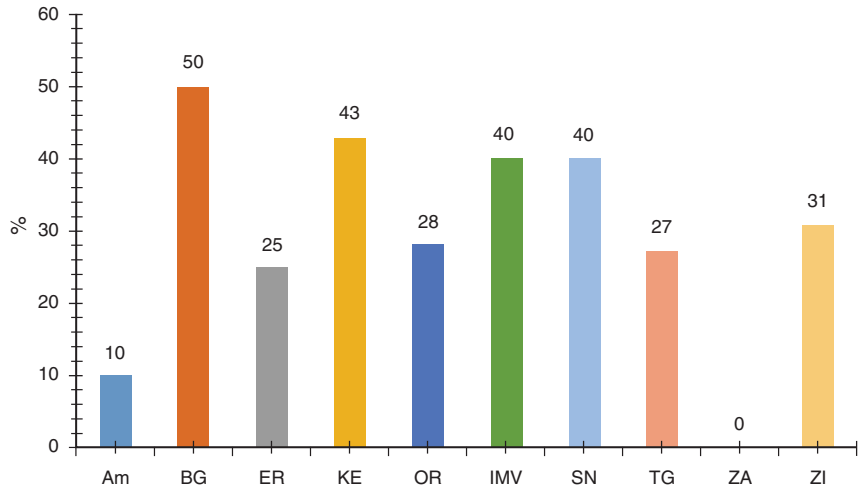

Fig. 1. Proportion of accessions from each of the regions or countries showing specific allele amplification. The codes on the *x*-axis denote the region as explained in Fig. 2 legend while the figures on the *y*-axis denote the percentage.

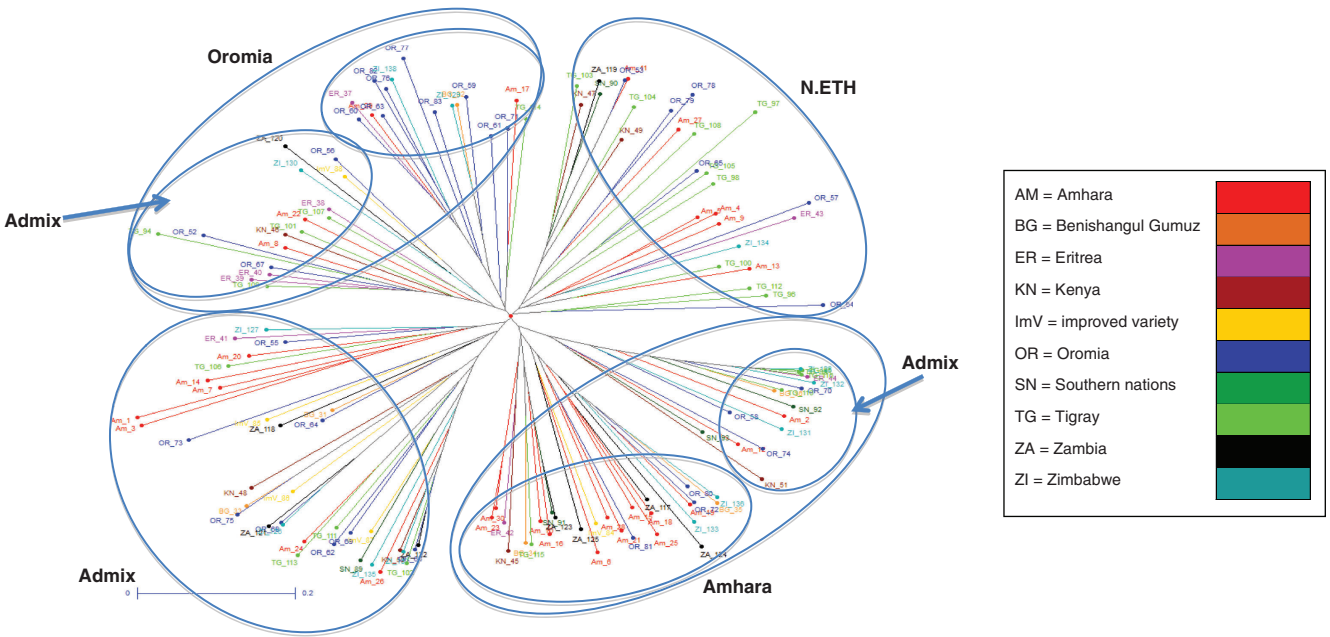

Fig. 2. Weighted neighbour-joining based clustering of 138 finger millet accessions for 19 polymorphic SSR markers. The key is shown with respective colour codes. Four clusters are shown with accessions predominantly from Tigray and Northern Amhara clustering within the Northern Ethiopia group; the majority of samples collected from Oromia clustered within the 'Oromia' cluster and those from Amhara clustered within the 'Amhara' cluster. In each of the 'Oromia' and 'Amhara' clusters, there was a distinct cluster made up of admixtures. A bigger cluster of admixture was also observed.

regions or countries and three improved varieties: Wama, Gute and Bereda. The other two major clusters were further grouped into subclusters that distinguished accessions from the south (Southern Region and southern Oromia) and the western region (western Oromia, Benishangul Gumuz and south-western Amhara).

Similar to the neighbour-joining results using molecular markers, accessions collected from the northern region (Tigray and northern Amhara) and from Eritrea shared strong phenotypic similarity and grouped together in the first cluster (Fig. 3) and this cluster recorded high scores for finger length and grain yield per plant. The second cluster comprised accessions from the western region (western Oromia, Benishangul Gumuz and south-western Amhara), which were characterised by late heading (107 days after planting) and consequent late maturity (163 days after planting). A third cluster comprised collections from western and south-western Oromia (14), Zimbabwe (13) and Zambia (7) (Fig. 3).

The Oromia region, which had the highest number of accessions (32), contributed the highest proportion of

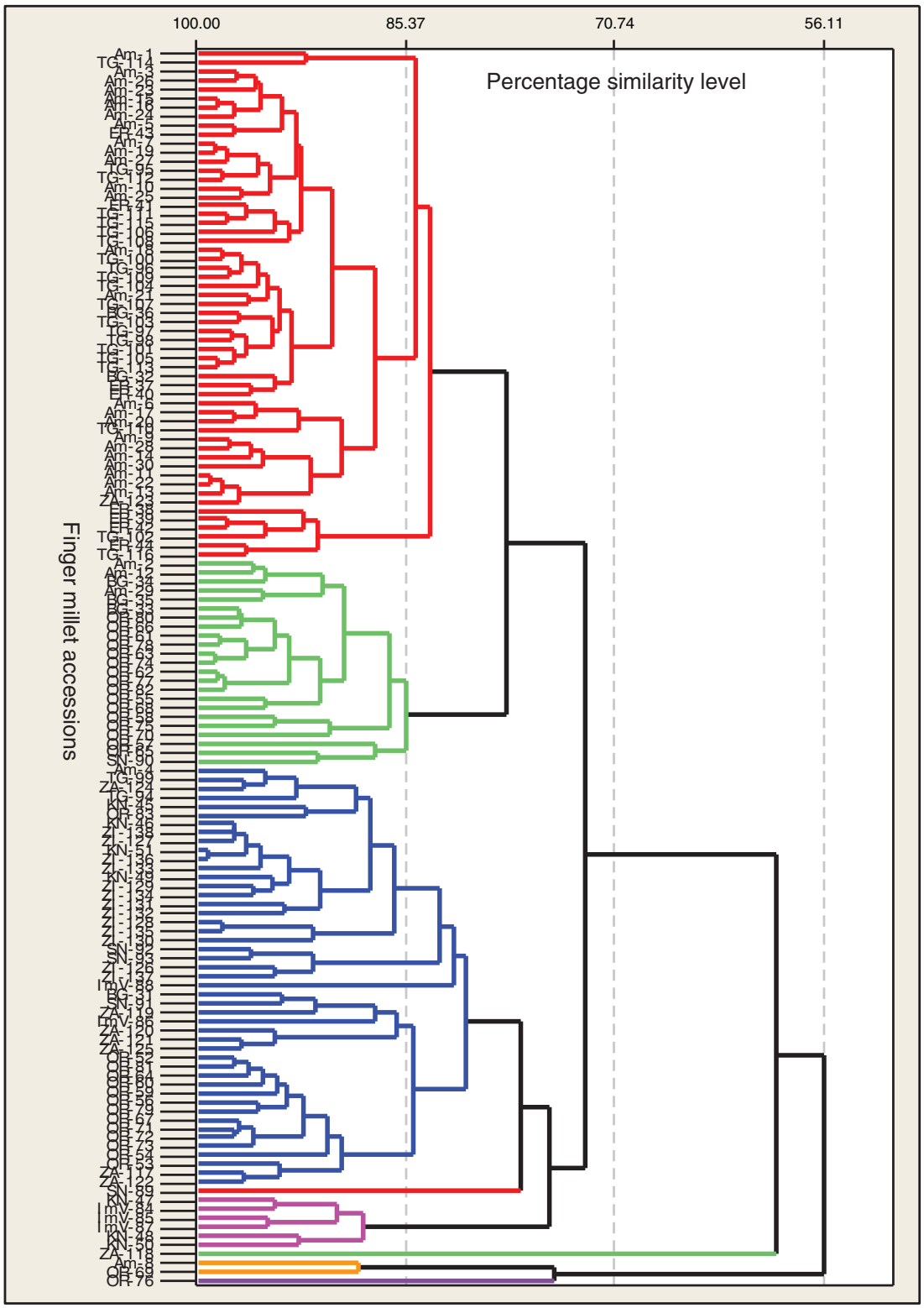

Fig. 3. Dendrogram showing the clustering of finger millet accessions based on 10 major phenotypic traits at 80% similarity for standardised data. The respective accession numbers correspond to the codes indicated in Table 1.

Table 4. Analysis of molecular variance showing contribution for the variation observed within accessions of the same region and among accessions of different regions or countries

d.f., Degrees of freedom; MS, mean square; St. dev., standard deviation; f-calc, f-calculated; f-tab, f-tabulated; Eth., Ethiopia; $^{**}P < 0.01$

Source	d.f.	Sum of squares	MS	Percentage contribution for variation	Variance	St. dev.	f-calc	F-tab.
Amhara (Eth.)	29	97.521	3.251	16.93	3.32	1.822	9.93	1.459**
Benishangul Gumuz (Eth.)	5	7.333	1.222	1.27	5.38	2.318	1.39	2.802
Eritrea	7	13.819	1.974	2.4	2.96	1.719	3.25	2.639**
Kenya	6	13.548	2.258	2.35	3.53	1.878	3.18	2.802**
Oromia (Eth.)	31	121.55	4.052	21.1	6.68	2.584	8.73	1.459**
Improved varieties	4	5.033	1.258	0.87	9.24	3.040	0.93	2.379
SNNP region (Eth.)	4	6.65	1.330	1.15	6.73	2.593	1.26	2.214
Tigray (Eth.)	22	77.334	3.362	13.43	1.83	1.351	12.19	1.758**
Zambia	8	19.2	2.400	3.33	1.64	1.280	5.63	2.511**
Zimbabwe	12	38.384	2.953	6.66	0.01	0.080	13.78	1.783**
Within population total	137	400.37	2.922	69.5	28.56	5.344	6.42	1.120**
Among populations	9	175.573	19.508	30.48	61.52	7.844	7.86	2.407**

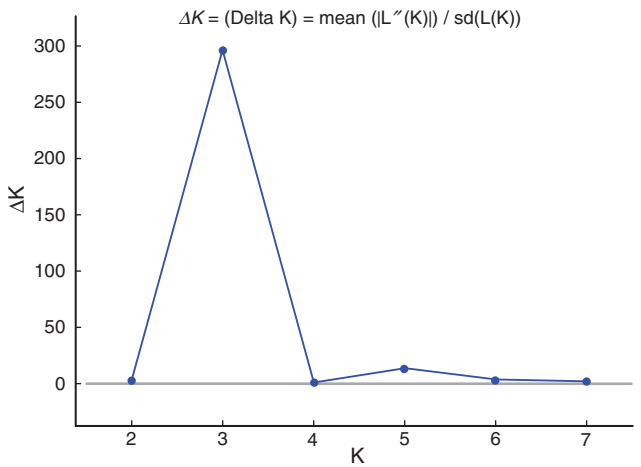

Fig. 4. The number of groups (Δk) from the STRUCTURE runs. The peak of the graph at $k = 3$ indicates the likeliest number of groups (subpopulations) present in the panel.

observed genetic variation (21.10%) followed by Amhara (16.93%), Tigray (13.43%) and Zimbabwe (6.66%) (Table 4). There was also a significant level of variation ($P < 0.01$) among accessions from the same region except for Benishangul Gumuz (1.27%) and Southern Nation Nationalities and Peoples Region (SNNPR) (1.15%), both of which had only six and five accessions, respectively. The AMOVA allocated 69.5% of the total allelic variation within accessions and 30.48% among accessions (Table 4).

Population structure

Analysis of population structure distinguished three groups among the 138 finger millet accessions with a ΔK of around 300 (Fig. 4). Population I consisted of 28 accessions from all regions or countries except Zambia; populations II and III consisted of 54 and 56 accessions respectively from all regions or countries (Fig. 5). There was a fair distribution of collections from all regions of Ethiopia in the three populations except for those from Amhara, which were predominantly in population III (Fig. 6). Most of the improved varieties seemed to be derived from all three populations, with the greater proportion (>60%) of ancestry from population II (Fig. 6). The fixation index (F_{ST}) values of the subpopulations ranged from 0.0723 (population II) to 0.3322 (population I) indicating that population II was less distinct compared with populations I and III (data not shown). Nearly half of the accessions (15 out of 34) that showed specific allele amplification also clustered in population II.

Association analysis between agronomic traits and molecular markers

Genome-wide association analysis for 10 agronomic traits and 19 polymorphic pre-screened SSR markers depicted significant ($P < 0.01$) association of 16 alleles of 13 markers (UGEP05, UGEP20, UGEP24, UGEP27, UGEP53, UGEP64, UGEP66, UGEP67, UGEP73, UGEP79, UGEP95, UGEP106 and UGEP110) with six agronomic traits, i.e. days to maturity, finger number, grain yield per plant, number of grain per spikelet, productive tiller number and thousand-grain weight using a mixed linear model (Table 5). In two cases, different alleles of the same marker were found to be associated with the same or a similar trait; such as for grain yield per plant and thousand-grain weight for UGEP66 and productive tiller number for UGEP24 (Table 5). One accession from Amhara (AM-1) showed specific allele amplifications for two markers that showed significant associations with productive tiller number. These unique associations provided further confirmation that the marker–trait associations detected were reliable.

Discussion

The microsatellite markers used in this study were informative and successfully distinguished the examined finger millet accessions. Our results are comparable to those of Manyasa *et al*. (2015), who used the same markers to analyse 340 accessions from Kenya, Uganda and Tanzania. The present study showed higher SSR allelic diversity in comparison to Arya *et al*. (2013), who used 17 SSRs to assess diversity across 67 finger millet accessions originating from India (48) and Africa (19). These authors also reported lower PIC values for four of the SSRs (UGEP24, UGEP53, UGEP67 and

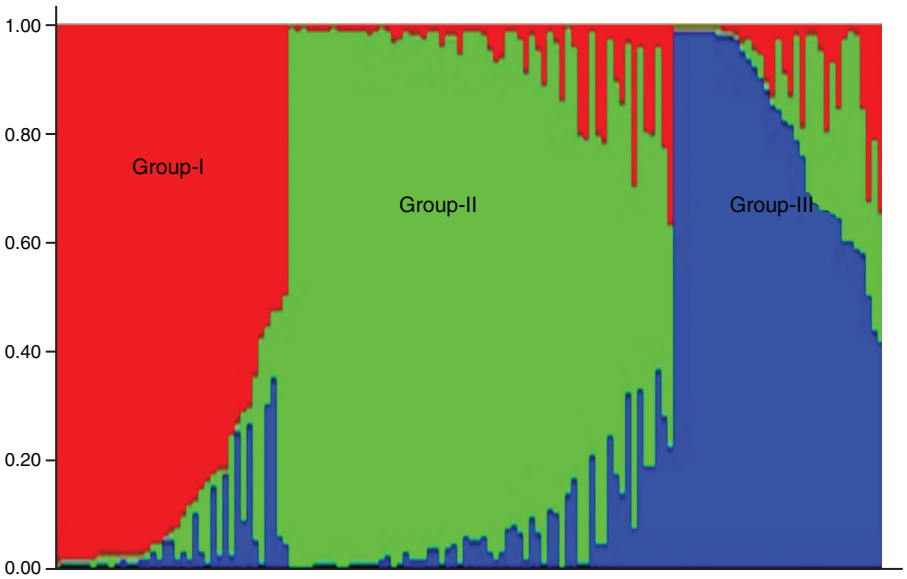

Fig. 5. Estimated population structure of the 138 finger millet genotypes sorted by ancestor coefficient Q for $k=3$. The *y*-axis is the group (subpopulation) membership, and the *x*-axis is the individual finger millet genotypes.

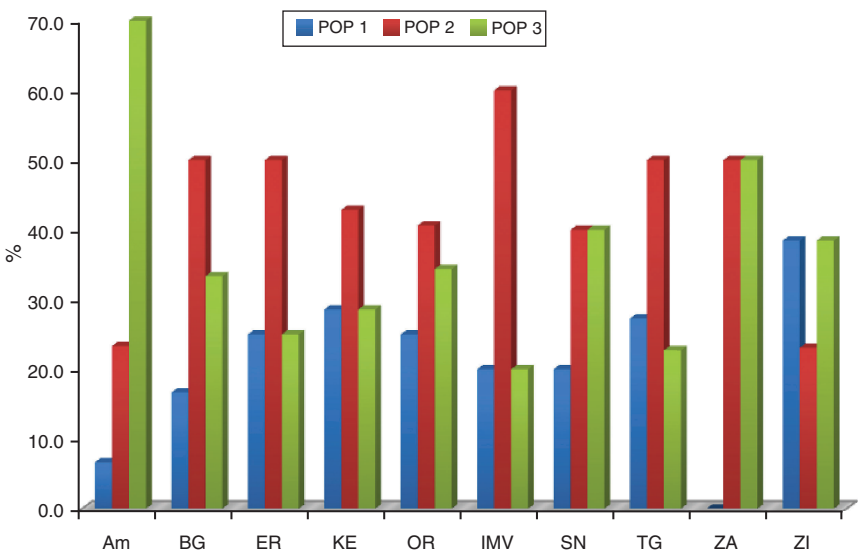

Fig. 6. The proportion of accessions from different regions or countries that clustered under different STRUCTURE-generated populations. The codes for countries or regions are described in Table 1. The codes on the *x*-axis denote the region and the figures on the *y*-axis denote percentage contributions of the respective regions or countries to the populations observed in Fig. 5.

UGEP12) when tested across the Indian collections compared with the African collections even though fewer (<50%) African accessions were used in their study. Ramakrishnan *et al*. (2016*b*) evaluated 128 finger millet accessions using 87 genomic SSR primers and reported the means of polymorphic alleles were 2.13 per primer and 1.45 per genotype. The origin (Africa) of accessions used in our study and other similar studies (Dida *et al*. 2008; Manyasa *et al*. 2015) could explain these differences. The eastern Africa region, particularly around Ethiopia and Uganda, is believed to be the centre of origin for finger millet and therefore would have a higher genetic variation compared to Indian accessions (Purseglove 1985; de Wet 1995).

The different PIC values observed across studies could also be attributed to the numbers and types of accessions used. Previous studies reported that the degree of polymorphism depended on the sample size (Sharma *et al*. 2010), sampling strategy (Kong *et al*. 2011) and types of test material (He *et al*. 2011). It would therefore be expected that our study and that of Manyasa *et al*. (2015) reported higher PIC given that larger numbers of 138 and 340 African accessions were used respectively. However, Babu

Table 5. Association of marker alleles with phenotypic traits using mixed linear models

Traits	Locus with allele position	Specific allele amplification	Linkage[A] group	*P*-value	Minor allele frequency	R^2
Days to maturity	UGEP110_215	KN-50	7AB	0.008	0.362	0.065
	UGEP27_279	–	ND	0.007	0.093	0.068
	UGEP53_235	SN-89	2A	0.001	0.005	0.099
Finger number	UGEP67_249	–	ND	0.003	0.110	0.069
	UGEP73_246	–	ND	0.008	0.449	0.054
Grain yield per plant	UGEP66_224	–	ND	0.003	0.043	0.109
Number of grain per spikelet	UGEP79_187	–	ND	0.008	0.076	0.050
Productive tiller number	UGEP106_177	AM-1	9B	0.005	0.005	0.068
	UGEP24_212	–	3B	0.007	0.044	0.064
	UGEP24_258	AM-1	3B	0.006	0.005	0.065
	UGEP67_267	–	ND	0.007	0.059	0.064
	UGEP95_230	–	ND	0.004	0.018	0.073
Thousand-grain weight	UGEP05_226	IMV-85	9B	0.006	0.004	0.101
	UGEP20_163	–	ND	0.009	0.007	0.096
	UGEP64_245	–	ND	0.007	0.009	0.101
	UGEP66_236	KN-47	ND	0.006	0.004	0.103

[A]ND, Linkage group not determined.

et al. (2014) used more accessions (190) than the current study but reported less polymorphism.

Consistent with studies in other crops, SSR markers used in the current and previous studies (Dida *et al*. 2008; Arya *et al*. 2013) gave higher PIC values than other genetic diversity studies in finger millet with other markers such as ISSRs (Gupta *et al*. 2010) and RAPDs (Das and Misra 2010; Bezawuletaw 2011; Ramakrishnan *et al*. 2015). Although SNP markers have also been shown to be informative in other polyploid cereal crops such as wheat (Wang *et al*. 2014), there are currently no known SNP assays developed for finger millet. Our results therefore indicate that SSRs are sufficiently informative and powerful to assess the genetic variability of both natural populations and improved finger millet cultivars. Additionally, SSR markers are very efficient in identifying genetic diversity among finger millet accessions due to their higher polymorphism compared with other markers (Kubik *et al.* 2009) and hence are the marker of choice for practical breeding applications, particularly in developing countries (Sharma *et al.* 2010).

The abundance of rare alleles confirms the recent report of Manyasa *et al*. (2015), who reported 57.7% rare alleles (<5%) while analysing genetic diversity among African finger millet collections. Our results indicate the significant contribution of rare alleles to the overall genetic diversity of cultivated finger millet. More studies are needed to determine whether the Asian finger millet populations are equally abundant in rare alleles, and the extent of contribution of these alleles to traits of agronomic importance. More importantly, a more informed conservation decision will need to be made in eastern Africa in order to preserve and retain the unique genotypes harbouring the rare alleles.

The weighted neighbour-joining tree constructed from pairwise genetic distances in the current study revealed that the clustering of African accessions was not entirely based on their geographical origin, confirming earlier reports for cultivated finger millet (Babu *et al*. 2007; Dida *et al*. 2008; Bezawuletaw 2011; Nethra *et al*. 2014; Manyasa *et al*. 2015). Previous studies that included both Asian and African finger millet collections revealed a distinct clustering pattern between Asian and African collections but not within each collection (Dida *et al*. 2008; Arya *et al*. 2013; Ramakrishnan *et al*. 2015). The lower levels of geographical clustering within African collections could be attributed to cross-boundary farmer-to-farmer seed exchange, inter-population seed dispersal, migration and gene flow. Several authors have reported similar findings in other crops such as chickpea (Keneni *et al*. 2012) and sesame (Park *et al*. 2014).

In some cases, close genetic similarities were observed between accessions collected from countries or regions geographically far apart. For example, accessions ZA-121 and ZA-122 collected from Zambia shared strong similarities with OR-68 collected from Oromia (Ethiopia) and TG-95 collected from Tigray (Ethiopia) respectively. A possible explanation is that the materials from Zambia were originally selected from Oromia and Tigray respectively, probably by an international research and genetic resource centre. Arya *et al*. (2013) also observed tight clustering between a South Indian accession and an accession from Tanzania, and Dida *et al*. (2008) reported tight clustering between an accession from Pakistan and a Ugandan one. This unpredictable clustering reveals a complex history of finger millet genotypes grown throughout eastern and southern Africa and also calls for a more extensive joint analysis of germplasm from Asia and Africa in order to derive more reliable conclusions.

The AMOVA showed higher genetic variation within accessions of the same region (69.52%) than among accessions of different regions (30.48%) (Table 4). This implied that most genetic differentiation was observed when all 138 accessions were analysed together than when collections from different regions or countries were analysed independently.

Although these results would be expected due to the self-pollinating nature of finger millet, they remain inconclusive given the low numbers of accessions examined from each region. In regions where relatively higher numbers of accessions were used – such as Amhara, Oromia and Tigray – significant levels of variation were observed among accessions, suggesting that analysing higher numbers from other regions would provide more conclusive results. It was also encouraging to observe significant levels of genetic variation among the improved lines from Eritrea, Kenya, Zimbabwe and Zambia despite analysing fewer than 10 cultivars from each country. This suggests that the breeding programs in the respective countries have released improved genotypes that are genetically distinct and fairly representative of the genetic diversity within the finger millet gene pool.

Our analysis of the population structure showed that the 138 accessions could be separated into three major genetic groups (Fig. 5), which were not based on geographical origin. Previous studies in finger millet that included African cultivated, Asian cultivated and wild (*E. coracana* subsp. *africana*) accessions (Dida *et al*. 2008) also reported the grouping of accessions into three populations: an African cultivated subpopulation, an Asian cultivated subpopulation and a wild *africana* subpopulation. Ramakrishnan *et al*. (2016*b*) also reported that 128 finger millet genotypes were divided into three subpopulations – all three had an admixture of alleles and no pure line was observed. The clustering of African accessions into three similar subpopulations may reveal a complex history of cross-pollination between cultivated and wild accessions, as well as between Asian and African cultivated germplasm. The abundance of rare alleles also suggests high gene flow from cross-compatible wild accessions. The complex structure within finger millet will only be resolved if an extensive study of Asian cultivated, African cultivated and cross-compatible wild accessions is undertaken as has been done in other cereals (Mace *et al*. 2003; Thurber *et al*. 2013; Qiu *et al*. 2014).

Genome-wide association analysis showed highly significant ($P < 0.01$) marker–trait associations between 13 markers and six agronomic traits using a mixed linear model. A similar study undertaken with 190 finger millet accessions using 21 polymorphic SSR loci (Babu *et al*. 2014) also reported association of four markers with basal tiller number, 50% flowering, flag leaf width and plant height. Although the results from our study and that of Babu *et al*. (2014) report associations with different traits and different markers, they provide a basis for future association studies in finger millet. Our study also reported significant associations of rare alleles with agronomic traits, suggesting that more studies are needed with higher sample numbers in order to determine the overall contribution of these rare alleles to traits of agronomic importance. Given that several genome-wide association studies tend to exclude most markers below a certain level of minor allele frequency (Lipka *et al*. 2012), more integrative methods such as recommended by Zhu *et al*. (2011) would be most appropriate in finger millet. Candidate quantitative trait loci identified through genome-wide association studies will also need to be further validated using multiple bi-parental mapping populations such as Nested Association Mapping and Multi-parent Advanced Generation Inter-Cross populations in order to capture the rare alleles.

Conclusion and recommendation

Finger millet is widely produced in the tropical and sub-tropical regions of Africa and India and serves as a main staple food for rural populations in those countries. Despite Ethiopia being the centre of origin and domestication of finger millet, little research attention has been given to improve finger millet and molecular marker-based investment has been neglected compared with other cereals. Analysis of genetic polymorphism for 138 finger millet accessions genotyped using a set of 20 SSR markers confirmed the presence of genetic variability among accessions studied, within and between regions of origin. Furthermore, neighbour-joining based clustering discriminated the potential variability existing within accessions from the same region. Similarly, analysis of the population structure separated the 138 accessions into three major genetic groups which were not based on geographical origin. Genome-wide association analysis depicted highly significant ($P < 0.01$) marker–trait associations between 13 markers and six agronomic traits using a mixed linear model, providing a basis for future association studies in finger millet. The present study also reported significant associations of rare alleles with agronomic traits, suggesting that more studies are needed with higher sample numbers in order to determine the overall contribution of these rare alleles to traits of agronomic importance. In general, the present study contributed to the application of molecular tools in discovering the extent of genetic variation among finger millet accessions and eco-geographical regions and is thus crucial input for further breeding and variety development.

Conflicts of interest

The authors declare no conflicts of interest.

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