Intra-specific variation in West African and Asian germplasm of okra (*Abelmoschus* spp L.)

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ABSTRACT

Ten quantitative agromorpho-economic traits, six inter-simple sequence repeat (ISSR) primers, and three sequenced regions were employed to study intra-specific genetic diversity among twenty-eight accessions of West African and Asian okra (*Abelmoschus* spp L.) collected from eight geographical regions of Ghana. Pod yield per plant was analysed as dependent variable in relation to other agromorpho-economic traits, showing the correlation and contribution of each trait to crop yield. 50% germination and flowering were the most significant traits followed by plant height and average seeds per plant. Principal coordinate analysis defined three sets of traits, while Agglomerative Hierarchical Clustering (AHC) defined three clusters of the germplasms. ISSR detected very low level of polymorphism among the accessions. Testing the correlation between molecular data and morphological traits using Mantel test showed a significant positive correlation (r-value = 0.71, 0.90) with 50% flowering, fruiting and number of leaves per plant. Eclectic variation between Indiana and the rest of the accessions for both agromorpho-economic traits and molecular markers affirms its potential usefulness as a source of diverse genes for future breeding programmes. Sequencing of regions from all accessions, suggests that they are identical with a common ancestry. Outcomes of this study is timely for an ongoing okra hybridisation programme in Ghana.

Introduction

Okra (*Abelmoschus* spp L.) also known as okro, is a semi-fibrous herbaceous annual cultivated throughout the tropics and sub-tropics as a vegetable for its edible immature fruits (Tindall, 1983; Norman, 1992; Siemonsma and Kouame, 2004; Yıldız et al., 2015a,b). Every part of the crop seems to offer some useful purpose or the other. Hence, it is put to diverse uses by different end-users. Varieties may be used as sources of protein, fibre, bio-mass, oil, mucilage, colourants, medicine, pharmaceuticals and ornamentals (National Academis Press, 2006). Okra is ostensibly traceable to the Abyssinian centre of origin of cultivated plants,

an area that includes present-day Ethiopia, the mountainous or plateau portion of Eritrea, and the eastern part of the Anglo-Egyptian Sudan. Considering the little contact between that region and the rest of the world within historic times, it is not unanticipated that little is known about the early history and dispersal of okra (Siemonsma and Kouame, 2004; Kumar et al., 2010; Ahiakpa et al., 2013; Siemonsma and Hamon, 2013). The Asian species (*Abelmoschus esculentus* L.) also referred to as common okra is an amphidiploid (2n = 130–140) while the West African okra (*Abelmoschus caillei* Stev.) is amphipolyploid (2n = 196–200). The Asian okra accounts for 95% of cultivated area while the West African okra accounts for 5% cultivated area globally (Kumar et al., 2010; Ahiakpa et al., 2013). Okra can be regarded as a polytypic complex (Singh and Bhatnagar, 1975) that exhibits both high polyploidy and hybridity of which the parental wild species is yet to be determined (Ahiakpa et al., 2014).

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Broad genetic diversity has been reported among the cultivated species of the genus *Abelmoschus* (Bisht et al., 1995; Omonhinmin and Osawaru, 2005), with West Africa alone holding 1769 out of the 2283 accessions collected globally (Oppong-Sekyere et al., 2012; Ahiakpa et al., 2013). Variability is more pronounced particularly in plant height, days to flowering and fruiting, tolerance to the yellow vein mosaic virus, yielding potential and most fruit characteristics (Ariyo and Odulaja, 1991; Gulsen et al., 2007).

Traditional farmers often engage in selection for different purposes facilitating its diversification into a vast number of landraces adapted to various agro-ecological systems (De Lannoy, 2001; Ahiakpa et al., 2014). In Ghana, perennial varieties which are early-maturing and simultaneously combine high yields with a long harvest duration as well as resistance to pests and diseases are considered ideal (Oppong-Sekyere et al., 2012; Amoatey et al., 2015a,b). However, with the emerging status of Okra as an export crop, there is a need for standardisation of traits to meet specific end-uses and markets.

Molecular characterisation provides the ultimate tool for accurate identification of the genetic variability within this crop from different geographic areas. This precedes any efforts aimed at enhancing the value of the crop, through improvements in yield, conditions for cultivation, nutritional value, and nutraceuticals (National Academies Press, 2006; Kumar et al., 2010).

The Inter-Simple Sequence Repeat (ISSR) marker, developed by Zietkiewicz et al. (1994) is based on PCR amplification with a single primer containing sequence complementary to a microsatellite 'core' one. The sequence is often anchored at the 5′ or 3′ end by a set of 2–4 purine or pyrimidine residues; offering a high degree of reproducibility with the detection of a rich level of polymorphism in a relatively simple procedure. It has been extensively utilised in assessment of crop genetic diversity (Jonah et al., 2011), cultivar identification (Bhattacharya et al., 2010) and recently in okra genetic diversity studies (Yuan et al., 2015, 2014).

One of the most commonly used polymorphic regions is the Internal Transcribed Spacer (ITS), a segment of non-coding RNA situated between structural ribosomal RNAs on a common precursor transcript (Wheeler and Honeycutt, 1988). Since its first application by Porter and Collins (1991), it has been widely employed for phylogeny reconstruction, due to its bi-parental inheritance, easy PCR amplification, multi-copy structure, and moderate size. ITS is also suitable for evolutionary studies at the species or generic level (Maggini et al., 1998; Viard et al., 2002).

Most higher-plant chloroplast genomes have conserved quadripartite structure-two copies of the inverted repeat and the large and small single-copy regions (Jansen et al., 2005). Borsch et al. (2003, 2005) and Löhne and Borsch (2005) showed that rapidly evolving introns and spacers of the chloroplast genome single-copy regions possess high performance as phylogenetic markers. Therefore, they have been widely used in the phylogenetic analysis field (Borsch et al., 2007; Moore et al., 2010).

The aim of the study was to assess the extent of polymorphism and intra-specific genetic variation within and among the 28 accessions of okra collected from eight geographic regions of Ghana using ISSR markers, 3 sequenced polymorphic regions and 10 selected agromorpho-economical traits. This will provide information for subsequent breeding purposes. In the current study, the chloroplast *trn*L (UAA) intron, *ITS* and *rpl*16 intron were chosen.

Materials and methods

Field layout and experimental design

Field cultivation of the 28 accessions of okra obtained from eight geographical regions (Fig. 1 and Table 1) was done at the research farm of the Biotechnology and Nuclear Agriculture Research Institute (Kwabenya, Ghana).

A total land area of 60 m × 32 m was cleared, ploughed and harrowed to a fine tilth for planting. The Randomised Complete Block Design (RCBD) was used with four replications; each replicate measuring 30 m × 12.5 m, separated by a distance of 2 m with 30 subplots (within blocks). Each subplot had a dimension of 3.5 m × 2.5 m, spaced by a distance of 1 m. Seeds were sown at a depth of 2 cm and a spacing of 0.70 m × 0.50 m within and between rows with 3–4 seeds per hill, later thinned to 2 after germination. No fertiliser was applied, but weeds were controlled fortnightly and water was supplied during the dry season using watering can (Amoatey et al., 2015a,b).

Agromorpho-economical traits record

Data were collected on five tagged plants (data plant) within the central rows, using the '*International Plant Genetic Resources Institute Descriptor List*' for okra (IPGRI, 1991). Data were collected on 10 agromorpho-economic traits, 50% Germination (G50), Number of Ridges Per Pods (NRpP), Number of Leaves Per Plant (NLpP), Stem Diameter (SDi) in cm, Average Seeds Per Pod (ASpP), Average Pods Per Plant (APpP), 50% Flowering (Fl50), 50% Fruiting (Fr50), 1000 Seed Weight (SWK) in g and Plant Height (PHi).

DNA extraction

DNA extraction was done using SIGMA® Plant High Molecular DNA extraction KIT® (SIGMA Inc., USA). Okra tissues were disrupted by grinding in liquid nitrogen. Total DNA was extracted according to the manufacturer's instructions. DNA quality was detected using 1% Agarose Gel Electrophoresis (Macrogen Europe, Netherlands), visualised by pre-added RedSafe® (5 ul/100 ml) under UV light and quantified using Eppendorf® Spectrophotometer X100 device (BioRad, Germany). About 50 μg of DNA were obtained from 2 g of grounded leaf powder.

ISSR-PCR amplification

The PCR reactions were undertaken in a total volume of 25 μl consisting of 1X Flexi buffer (Macrogen Europe, Netherlands), 50 ng DNA template, 25 mM MgCl$_2$, 10 uM dNTPs, 0.4 uM of each primer (common ISSR primers: C4 ((GA)7GYT), O4 ((GA)7YA), O9 ((ATG)5), O11 ((AG)6 G), O12 ((GTG)5), UBC888 (BDB(CA)6)), and 1 U Promega© Green Go Taq™ enzyme (Promega, U.S.A) (Table 2). Reactions were undertaken using Techne™ 96 Thermocycler (BioRad, Germany) with the following PCR program: denaturing cycle at 94 °C for 5 min, followed by 35 cycles each consisting of 94 °C, 1 min, 40 °C, 1 min, and 72 °C, 1 min for denaturing, annealing and extension, respectively, and final extension of 5 min at 72 °C. PCR products were then kept at 4 °C.

The amplicons were separated by 3% (w/v) Agarose (25 cm × 25 cm) gel electrophoresis (Macrogen Europe, Netherlands) on a constant power (I = 120) for 1 h. Samples were loaded with Fermentas™ 100 bp DNA ladder and revealed using a pre-added RedSafe® staining solution (5 ul/100 ml) (Macrogen Europe, Netherlands) under UV trans-illuminator. The visualised bands were documented by the Gel documentation system VILBER E-BOX VX2 (Rohlf, 2000).

Multi-locus genotyping

PCRs were performed in 50 ul reaction mixture (1X Flexi buffer, 50 ng DNA template, 2.5 mM MgCl2, 10 uM dNTPs, 0.4 uM of each primer, and 1 U Promega© Green Go Taq™ enzyme). Standard PCR profile with 55 °C annealing temperature was used to amplify all
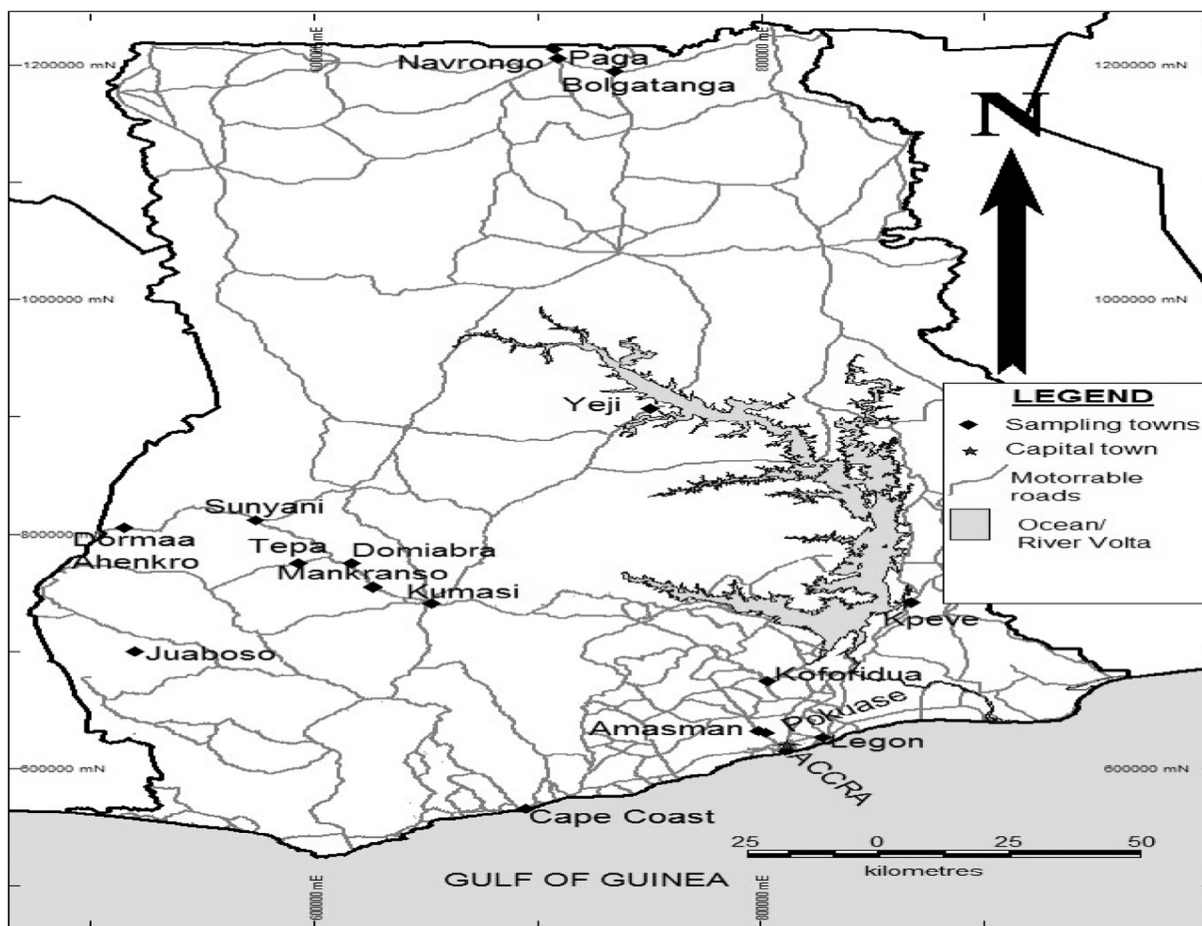
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Fig. 1. Map of Ghana showing various Localities where Accessions were collected.

Table 1
Characteristics and origins of accessions of okra used in the study.

Region	Accession
Ashanti	Agric short fruit, Agric type I, Asante type II, Asontem-ASR, Debo, Kortebortor-ASR
Brong Ahafo	Asontem-BAR, Asontem-NV., Kortebortor-BAR, Nkran Nkuruma, Yeji-Local
Central	Cape
Eastern	Amanfrom, Asontem-ER
Greater Accra	Asontem-GAR, Atomic, Clemson spineless, Cs-Legon, Labadi, Legon fingers, Volta, Indiana
Upper East	Mamolega, Mapelega, Wune mana
Western	Juaboso
Volta	Akrave, Kpeve

Table 2
Sequences of six ISSR primers applied to the current study.

Primer	Sequence
C4	$(GA)_7GYT$
O4	$(GA)_7YA$
O9	$(ATG)_5$
O11	$(AG)_6G$
O12	$(GTG)_5$
UBC888	$BDB(CA)_6$

These were available markers during the period of study.

regions (ITS, *trn*L and *rpl*16) using specific primers: ITS4 and ITS5 for ITS region (White et al., 1990); Tab-C and Tab-D for *trn*L intron (Taberlet et al., 1991); and F71 and R1516 for *rpl*16 intron (Jordan et al., 1996).

Results were tested on 1.5% agarose gel electrophoresis and visualised by pre-added 1x RedSafe® (Macrogen Europe, Netherlands) using a UV light. When successful, amplified fragments were purified and concentrated using Fermentas (GeneJET PCR Purification Kit #K0702, BioRad, Germany). Purified fragments were sequenced by sequencing service (Macrogen Europe, Netherlands).

Data analysis

Using XLSTAT statistical software, principal coordinate analysis (PCoA) for all agromorpho-economical traits, agglomerative hierarchical clustering (AHC) analysis of all accessions based on recorded traits, correlation test among the recorded traits and regression model of all traits contributing to the average pod per plant were performed. The model was tested using normalised residuals distribution.

To analyse ISSR gels, based on the band mobility, clear bands were scored using Totallab120® (Nonlinear, Durham, NC) gel analysis program as (1) for presence; while (0) for absence in a binary data form. The unclear unidentified bands were excluded. Genetic dissimilarity matrix estimation and AHC analyses among accessions were performed using XLSTAT statistical package. The Mantel test was used to estimate correlation between the genetic distances and the Euclidean distance (based on clustered traits determined for PCoA analysis) among all 28 accessions under study, the p-value was calculated using the distribution of r (AB) estimated from 10,000 permutations.

Sequence chromatograms were compiled using Bioedit V3 (Hall, 1999) to assemble the sequences. All sequences were manually

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aligned, while gaps were inserted to preserve nucleotide homology. Haplotype sequences were submitted into the NCBI GenBank database.

Results

Morphological characterisation

Correlation and regression analyses

Based on the selected agromorpho-economical trait prioritised by breeders (number of pods per plants); accessions Asontem-NV and Kortebortor-BAR recorded the highest number of pods (58.25 and 57.5) per plants, respectively, while Wune mana recorded the lowest number of pods (7) per plant.

Correlation among traits showed interesting positive and negative significant relationships, mainly the most significant and recurrent traits were number of leaves and stem diameter (cm) among the key agromorpho-economic traits studied (Fig. 2 and Table 3).

Linear regression was found significant, when average pods per plants were used as dependent variable. Thus, linear prediction model was estimated and a statistically supported model equation was defined. Plant germination and flowering were the most significant traits followed by plant height and average seeds per plant. Normalised residuals were shown as a function of the explanatory traits; the residuals were found to be distributed randomly around the X-axis (Eq. (1) and Fig. 3).

Intercept (a) and coefficient by each trait contributing to the dependent trait Average pod per plant.

$$APpP = -7.496 + 0.437G50 + 0.404Fi50 - 0.034Fr50$$
$$+ 0.279Phi + 0.062NLpP + 0.109SWK - 0.027SDi$$
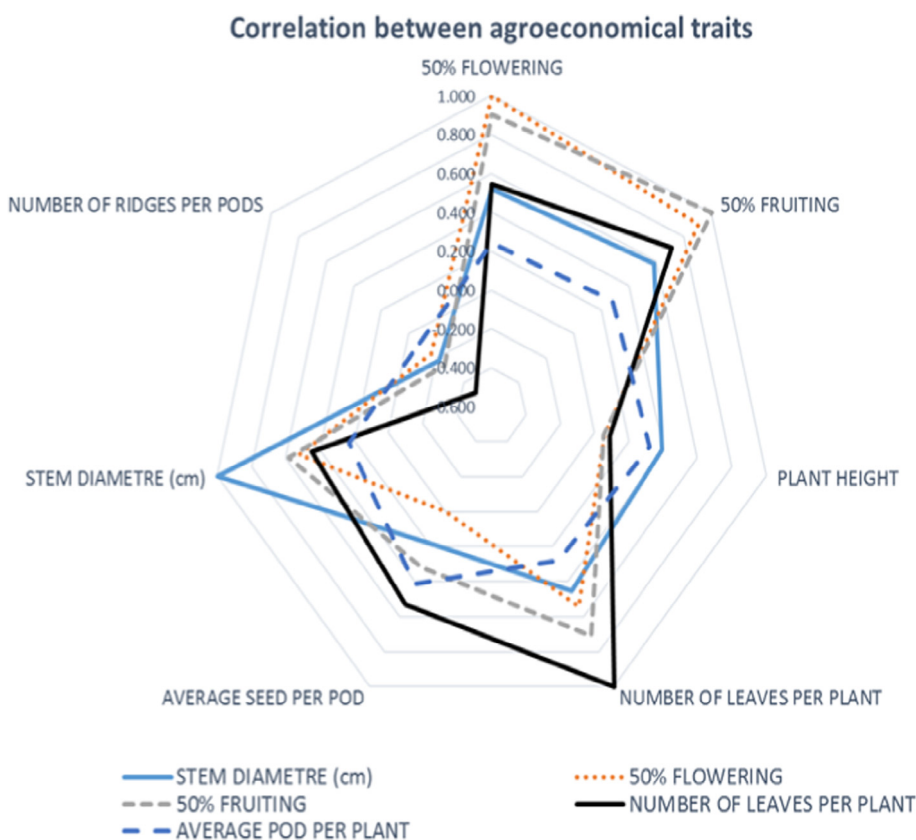$$+ 0.018NRpP \tag{1}$$

Fig. 2. Significant Correlation (r-values) among selected traits studied.

Table 3
Correlation coefficients among agromorpho-economic traits studied.

Traits	G50	NLpP	SDi	NRpP	ASpP	APpP	Fl50	Fr50	SWK	PHi
G50										
NLpP	−0.14									
SDi	−0.18	0.45								
NRpP	0.37	−0.48	−0.22							
ASpP	0.29	0.53[*]	0.18	−0.34						
APpP	0.21	0.28	0.23	−0.08	0.41					
Fl50	−0.18	0.54[*]	0.52[*]	−0.16	0.00	0.24				
Fr50	−0.10	0.71[**]	0.58[*]	−0.26	0.32	0.28	0.90[***]			
SWK	−0.18	0.33	0.20	−0.25	0.18	0.11	−0.03	0.03		
PHi	−0.32	0.09	0.39	−0.18	0.08	0.32	0.06	0.05	0.37	

[*] = significant (P ≤ 0.05); [**] = very significant (P ≤ 0.001); [***] = highly significant (P ≤ 0.0001) computed using standard linear Pearson correlation. G50 = 50% Germination; NRpP = Number of Ridges per Pods; NLpP = Number of Leaves per Plant; SDi = Stem Diameter in cm; ASpP = Average Seeds per Pod; APpP = Average Pods Per Plant; Fl50 = 50% Flowering; Fr50 = 50% Fruiting; SWK = 1000 Seed Weight in g and PHi = Plant Height.
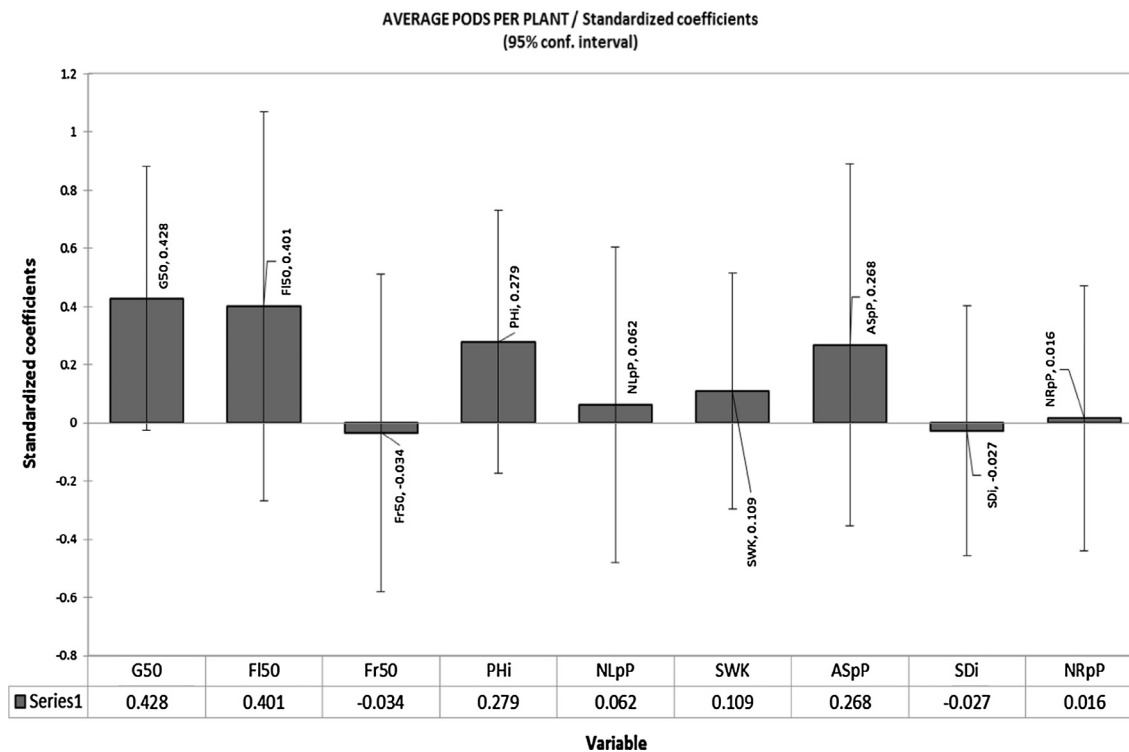
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Fig. 3. Standardised coefficient for each trait to predict the "average pod per plant" according to the estimated linear model. Above corner shows the normalised residuals distributed randomly around x-axis.

Recorded data from 10 agromorpho-economic traits were standardised using z-score transformation method. Principal coordinate analysis was performed to describe traits clustering based on statistical variation among accessions. Three sets of traits were defined, set 1 includes 50% germination and number of ridges per pods; set 2 includes plant height and 1000 seed weight in grams; while set 3 includes 50% flowering, 50% fruiting, average pods per plants, average seed per pod, number of leaves per plant and stem diameter in cm.

Agglomerative cluster analysis (AHC)

AHC analysis performed based on all traits regardless of the PCoA results, clustered the 28 accessions into three main clusters, two which were subdivided into two more sub clusters (Fig. 4). Cluster (I)-Sub 1, includes CS-Legon, Kortebortor-ASR, Mapelega and Kpeve, while sub 2, includes Yeji-Local, Amanfrom, Cape, Asontem-ASR and Mamolega. Cluster (II), includes Asontem-ER, Juaboso, Debo and Volta. Cluster (III)-sub 1, includes Agric short fruit, Asontem-NV, Labadi, Indiana, Asante type II, Legon fingers

Fig. 4. Clustering analysis based on all agromorpho-economic traits, where three clusters can be defined.

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and Akrave, while sub cluster 2, includes Atomic, Clemson spineless, Wune mana, Agric type I, Asontem-BAR, Asontem-GAR, Nkran Nkuruma and Kortebortor-BAR.

ISSR polymorphism
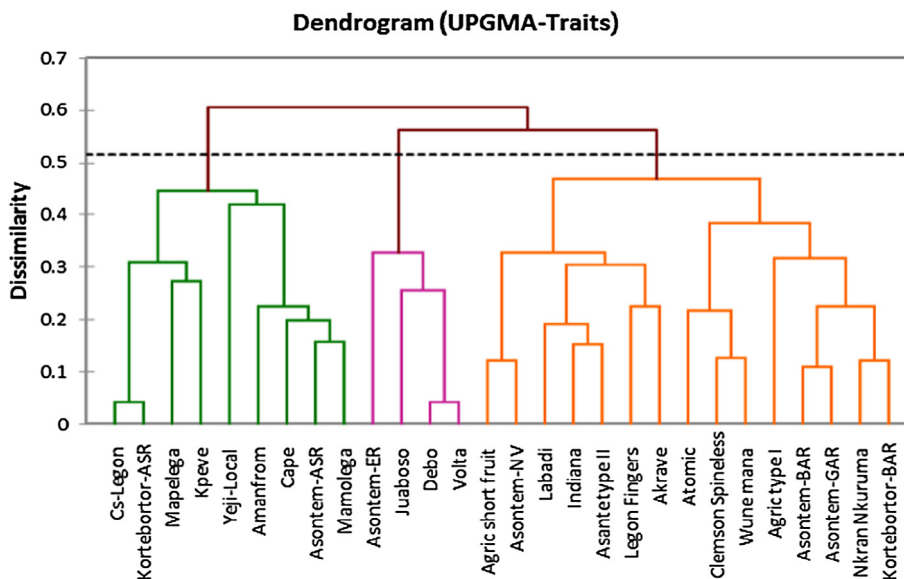
All the six primers used showed low percentage of polymorphic bands (15.38%) for determination of variability/polymorphism among the accessions. Primers, C4 and UBC888, each amplified three bands where only a unique polymorphic band was detected. Both bands showed low amplification rate among accessions (14.28% for CA unique polymorphic band and 10.71% for UBC888 unique polymorphic band). Testing the correlation of the genetic distance matrix and Euclidean distance matrix (based on each PCoA set) showed no significant correlation (p-value > 0.05) between the genetic distance and set 2 and set 3, while significant negative correlation was only found with set 1 (r-value = −0.109, p-value = 0.035).

Cluster analysis based on both polymorphic ISSR loci and agromorpho-economic traits echoes a clear divergence among the studied accessions. Indiana was clearly separated from other accessions. Yeji-Local, Asante type II and Atomic accessions formed a separate cluster, while the rest were contained in the other cluster (Fig. 5).

Multi-locus genotyping

ITS, *trn*L and *rpl*16 sequenced regions generated a unique haplotype for all accessions (GenBank accession numbers: KF514667, KF514666 and KF514665, respectively). Comparing the three sequences with the other accessions in NCBI database confirms the sample species. However, the sequences of each region from all samples showed single haplotype per sequenced region among accessions studied.

Discussion

Agromorpho-economical traits

Number of leaves per plant and Stem diameter reveal their potential as direct indicators for selection-based breeding program. Interestingly, the most economically desirable trait (number of pods per plant) was not significantly correlated with any trait except with average seeds per pod. However, no correlation or linkage was found between any of the other studied traits and the number of pods per plant. Thus, all traits may contribute equally to the number of pods per plants as presented by the regression model. Based on each trait, a standard coefficient was estimated to determine the exact contribution of the trait measurement on the prediction of the average pod per plant, which is considered the preferred agromorpho-economic trait among okra consumers. Singh et al. (2012) detected high genetic divergence for fruit yield/plant of 50 different genotypes of Indian okra germplasms, using multivariate analysis. Fifteen clusters were defined, where three showed higher inter-cluster distance and proposed as a potential breeding stock. Such information would provide insight to breeding programmes based on trait selection of direct and indirect associated traits.

The very low polymorphism detected by the ISSR is not a common case among originally diverse collections. For example, 85% polymorphism was detected in Cashew germplasms (Thimmappaiah et al., 2009) from different sources, and 73% polymorphism in Lentil germplasm (Seyedimoradi and Talebi, 2014). However, a more revealing AFLP technique on international and Greek okra genotypes showed a very low level of polymorphism (12%) and distinct geographical grouping (Kyriakopoulou et al., 2014) as confirmed with the current ISSR results.

The clustering of the agromorpho-economical traits in three clusters reflect a unique relation among those traits. Only one set correlated with the ISSR polymorphism, suggesting the necessity to establish a breeding programme based on each set, rather than including variant sets together, which would provide more precise and biologically-reflecting results. The broad genetic similarity indices recorded and clustering patterns displayed suggest useful variability within the collection for future genetic improvement of the crop. Direct selection of accessions with the desired characteristics can be hybridised using genetically divergent ones as parents (Yao et al., 2008; Pasqualone et al., 2012) as against results from the molecular studies. The pattern of clustering did not show distinct association between agro-morphological characters and geographic origin of the collections. This is in consonance with earlier studies by Jonah et al. (2011); Kalivas et al. (2011) and Pasqualone et al. (2012).
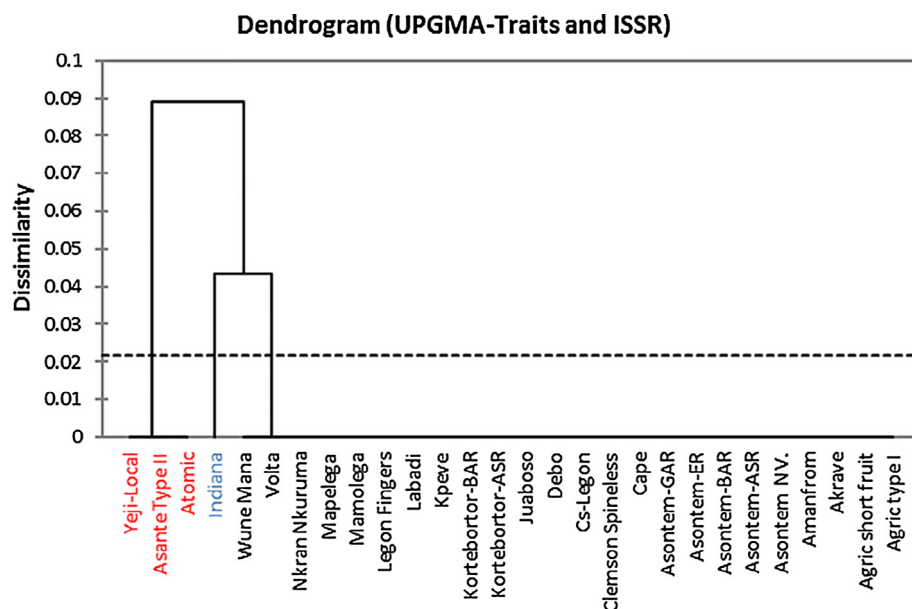

Dendrogram (UPGMA-Traits and ISSR)

Fig. 5. Clustering analysis based on all agromorpho-economic traits and polymorphic ISSR locus, where two clusters and a singleton can be defined.

Assessment of the extent of relatedness among potential parents forms the basis for selection of genetically distant and diverse parents in any breeding programme. Based on both morphological and genetic variation, the accessions Asante type II, Yeji-Local, Atomic were contained in a single cluster (from different geographic area of collection), Indiana was clustered as a single group and the remaining 24 accessions were grouped together in a cluster. The wide variation between Indiana and the rest of the accessions indicates its potential usefulness as a source of diverse genes for future breeding work (Ahiakpa et al., 2013, 2014; Amoatey et al., 2015a,b).

Indiana is early-maturing and possesses fruit qualities (slim and uniform in shape and size, smooth, yellowish green in colour) desired for export. On the other hand, Asante type II, Yeji-Local, and Atomic are all indigenous cultivars, well adapted to local conditions and very robust to both biotic and abiotic stresses yet non-uniform in shape and size. The desirable traits in Indiana could be introgressed into any of these three accessions (Asante type II, Yeji-Local and Atomic) to improve them for both local consumption and the export market.

Few of the ISSR primers however, were able to amplify more than one band per genotype. Hence, less residual heterogeneity could be suspected within the lines (Ogunbayo et al., 2005). This low level of polymorphism detected with the six ISSR primers among the accessions could be attributed to the dominant nature and low level of locus specificity associated with ISSR primers, and/or accessions may be possible duplicates (Andersson et al., 2007) or with a recent common ancestry (Kalivas et al., 2011). This is however inconsistent with reports by Yuan et al. (2015, 2014) where ISSR primers detected high level of polymorphism in 48 and 24 accessions of okra in China.

The *trn*L and *rpl*16 introns are expected to be more conserved than the ITS. However, all sequences were identical which confirms the possibility that the accessions may be duplicates, or originated from a relatively proximate source of collection. Even though, results from the sequenced regions contradict the presence of two groups revealed by ISSR technique, this is perhaps ascribable to the nature of the sequenced regions being more conservative than the regions targeted by ISSR markers, in addition to the very low polymorphism existing between the two groups. However, two ISSR markers were significantly correlated with two morphological traits.

West African okra was reported as *Abelmoschus caillei* (A. Chev.) Stevels (Siemonsma and Kouame, 2004; Siemonsma and Hamon, 2013). Results from both the morphological and molecular studies confirm identities of the accessions (Akrave, Nkran Nkuruma, Debo, Kortebortor-BAR, Kortebortor-ASR, Amanfrom, Juaboso, Kpeve, Wune mana, Yeji-local, Mamolega and Mapelega) as West African okra. Interestingly, the Indiana variety expected to be the common okra (*Abelmoschus esculentus* L.) showed similar sequence on both maternal and nuclear levels. This raises an important issue of speciation of *Abelmoschus* spp (Werner et al., 2016). More studies will be needed including more accessions from a wider scale, while a more robust technique can be proposed (such as Next generation sequencing, "NGS") to efficiently assess the genetic diversity among the studied populations of the *Abelmoschus* spp (Hohenlohe et al., 2012; Yıldız et al., 2015b).

Conclusion

Indiana was the most distantly distinct accession revealed by both morphological and ISSR primers and stands as a unique source of genes in Ghana for improvement programmes. Yeji-Local, Asante type II and Atomic were also detected as divergent genotypes. The use of the six ISSR primers detected low level of polymorphism among the 28 accessions of okra. Sequencing of ITS, trnL and rpl16 of all the accessions generated a unique haplotype, suggesting that most of the accessions are identical with a common ancestry. Intra-specific variation among accessions was more pronounced in the West African okra than the Asian okra. Findings of this study could be useful for ameliorating the local landraces and broadening the genetic base of commercially grown West African and Asian okra varieties in Ghana. Ongoing okra hybridisation programme involving introgression of the desirable traits of Indiana, Yeji-Local, Asante type II and Atomic into landraces that are well adapted to local agro-ecological conditions is underway.

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