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Full Length Research Paper

Use of biplot approach for genetic analysis of yield and related traits in cotton (*Gossypiumbarbadense*)

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Combining ability is an important genetic attributes to cotton breeders in anticipated improvement via hybridization and selection. Seven parents were involved in a half diallel mating design which was analyzed by genotype × environment interaction (GGE) biplot graphical method. General combining ability (GCA) and specific combining ability (SCA) effects were significant for all traits. None of the parents were found to be a good combiner for all traits. The combinations of Giza 75 x Sea, 10229 x Giza 86 and Giza 86 had the best SCA for seed cotton yield/plant, lint percentage and boll weight, respectively. The graphical demonstration proposed by the biplot analysis provided an effective overview of GCA and SCA effects and mean performances in crosses. In addition, the biplot also provided an opportunity for assessing the interrelationship among the genotypes.

Key words: Cotton, diallel, biplot, correlation, general combining ability, specific combining ability.

INTRODUCTION

In terms of production and value, cotton is still a very important crop in Egypt. The whole plant has commercial use directly or indirectly and also has capability to meet the demand for natural fiber and oil. However, fluctuation in price and high cost of production affect negatively on cotton in dedicated area from year to year. The crop has been gradually forced out of the Delta region and cultivated under marginal conditions. Therefore, varieties suitable for new conditions need to be developed through appropriate hybridization and selection techniques. Combining ability estimates provide information useful for the selection of parents and also provides information regarding the nature and magnitude of gene action involved. The knowledge of genetic structure and made of inheritance of different characters helps breeders to select appropriate breeding methodology (Kiani et al., 2007). Diallel crossing was usually done by using Griffing's methods (1956). These methods are less interpretative, difficult to understand without the aid of some graphical display (Dehghani et al., 2012), Yan and Hunt (2002) have developed a quick evaluation method called GGE biplot model for analyzing the diallel data, this technique enhances the capability of interpreting the phenotypic variation to obtain combining ability and interrelationships of parents based on graphical presentation using PC1 and PC2 which are derived through PC analysis of environment-centered yield data. GGE biplot is recent method and has been widely used to determine combining ability and heterotic responses (Shang et al., 2006; Darvishzadeh et al., 2009). The GGE biplot methodology was developed for multi-environments trial (MET) data, in which, genotypes are entries and

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Table 1. Origin and abbreviation for each genotype.

Entry code	Tester code	Origin	Categories	Current position
G86	g86	Giza 86	Long staple	Commercial variety
G93	g93	Giza 93	Extra long staple	Commercial variety
G92	g92	Giza 92	Extra long staple	Commercial variety
G75xS	g75xS	Giza 75 x Sea	Long staple	New promising hybrid
10xG86	10xg86	10229 x Giza 86	Long staple	New promising hybrid
G85	g85	Giza 85	Long staple	Commercial variety
G88	g88	Giza 88	Long staple	Commercial variety

environments are testers. Yan's GGE biplot is also preferred to the conventional diallel approach because it gives jointly GCA and SCA effects of the population and the preferences of the crosses as well as grouping pattern of similar genotypes (Bertoia et al., 2006). The present study was undertaken to analyze diallel data using GGE biplot model to gather information about genetic interrelationships among parents, general and specific combining ability and to identificate heterotic combination for three important traits, that is, seed cotton yield (SCY/P), lint percentage (L%) and boll weight (BW).

MATERIALS AND METHODS

Five varieties and two promising crosses from different categories in Egyptian cotton (Table 1) were used in this study. These genotypes were crossed following in a half diallel fashion to produce 28 F_1 crosses during season 2011. All the F_1 crosses were evaluated along with parents in the following season with the planting date of April 24, 2012 at Sakha Agriculture Research Station under two system of irrigation. The first system was normal irrigation system that irrigated at about 15 day's intervals; the other one was under drought regime which irrigated at 30 day's intervals both irrigation systems, a randomize complete block design was used with three replication for each genotypes and each replication consisting of one row (4 m long, 70 cm wide, 40 cm between hells and one plant per hell after thinning). Standard cultural practices were applied uniformly at all experimental units. Six plants from each plot were separately harvested to estimate the three traits, seed cotton yield/plant (SCY/P), lint percentage (L %) and boll weight (BW).

An analysis of variances (ANOVA) was done using average environments values. Griffing's (1956) method 2 model 1 (fixed effect of parents) was applied to estimate GCA and SCA. The significance of genotypes, GCA and SCA mean squares were estimated using F test.

In GGE biplot, mean and stability of genotypes referred to GCA and SCA parents, respectively. The mean values for hybrids and parental populations across environments are used to form a symmetrical diallel data matrix from which the first two principle component (PC1 and PC2) were extracted. Each population corresponded to one row and one column of data, after obtaining the first two principle component of the adjusted data. The model for data analysis is:

$$Yij - \beta j = \lambda 1 \varepsilon i1 \, \eta j1 + \lambda 2 \varepsilon_{i2} \, \eta_{j2} + \varepsilon ij$$

Where Yij is genotypic values of the combination between entry i and tester j for a given trait; βj average value of all combinations with tester j, λ1 and λ2 are singular values for PC1 and PC2. εi1 and εi2 are PC1 and PC2 eigenvectors for entry i, respectively; ηj1 and η j2 are PC1 and PC2 eigenvectors for tester j, respectively; εij is the residual of model for entry i and tester j. In diallel crosses, a parent is both an entry and a tester. This statistical method has been described by Yan and Hunt (2002) and Yan and Kang (2003). This analysis is done using GGE biplot software (Yan, 2001).

The analysis of interrelationship between parents entries/testers can be approximated by cosine of the angle between parents:

$$\cos (aij) = rij$$

Where, aij is the angle between parent i and parent j and rij is the correlation coefficient between both parents. Two parent are positively correlated if the angle between their vectors is < 90°, negatively correlated if the angle is > 90°, and independent if the angle between them is 90°. 0° means correlation (r) is 1 and 180 means correlation is -1. Entry with longer vector are more discriminating of the entries, those with short vectors are less discriminating and those located at the biplot origin are not discriminating.

The GCA and SCA effects of entries were examined by drawing an average tester coordinate (ATC) abscissa view for entries. The GCA effect of the entries was approximated by the projection of their markers to the ATC abscissa (the single arrowed lines) with the direction indicating the positive end. While the SCA of the entries was approximated by the projection of their markers to the ATC ordinate (double arrowed line) (Yan and Hunt, 2002). The polygon view of the biplot is drawn by connecting the entries. The perpendicular line to each side drawn from the origin of the plot divides the biplot into several sectors, and each tester falls into one sector. Tester falling in a sector shares the best mating partner with another entry present at the vertex of the polygon in that sector. Entries located near the biplot origin are less responsive to change of the testers.

RESULTS AND DISCUSSION

The analysis of variance (Table 2) showed widespread significant differences among genotypes suggesting a great level of genetic variability to among the parents for all traits. GCA and SCA based on conventional method (Griffing's model) showed high significance suggesting the role of both additive and non-additive gene action. For seed cotton yield/plant and boll weight, the ratio of (δ^2 of GCA)/(δ^2 of SCA) was less than one indicating non-additive gene action in the inheritance of these traits. Meanwhile, for the lint percentage the ratio of (δ^2 of

Table 2. Analysis of variance and variances and estimates of combining ability for seed cotton yield, lint percentage and boll weight.

Source of variation	df	SCY/P	L%	BW
Replication	2	119.5	0.147	0.071
Genotypes	27	1348.6**	4.235**	0.150**
Error	54	315.62	0.750	0.018
Combining ability variances				
GCA	6	772.2**	3.463**	0.086**
SCA	21	357.3**	0.825**	0.040**
Error	4	105.21	0.250	0.006
Combining ability estimates				
δ^2 of GCA		66.699	0.3213	0.008
δ^2 of GCA		126.045	0.2875	0.017
(δ^2 of GCA/δ^2 of SCA)		0.727	1.057	0.685
S.Eg		3.42	0.154	0.023
S E sij		9.21	0.448	0.061

**Significant at the 0.01 probability level.

GCA)/(δ^2 of SCA) was more than one indicating additive gene action was predominant for the trait.

Seed cotton yield/plant (SCY/P)

In GGE biplot, the graphical representation of concentric ring with vectors showed that parental Giza75x Sea had the longest vector (the largest variation), while parents Giza 85 and 10229 x Giza 86 had the shortest vectors (the lowest variation) as seen in Figure A1. The vectors of the parent Giza 85 with the all parents except Giza75x Sea and Giza 92 had acute angle (less than 90°), which suggests positive correlations among them. The parent Giza75 x Sea had obtuse angle with 10229 x Giza 86, Giza 85, Giza 86 and Giza 88 which suggests lowest correlations among them and should produce heterosis crosses. Meanwhile, Giza 75 x Sea had acute angle with Giza 92. The angle between Giza 75 x Sea and Giza 93 was 90°, which means independent relation between them.

The biplot for seed cotton yield explained 73% (50 and 23%, by PC1 and PC2) of the total variation in Figure A1, Hamoud et al. (2012) reported 86.6% of variation for sum of PC1 and PC2 for the same trait. The remaining proportion of the total variation was not accounted by biplot analysis due to much complexity in genetics involved in this trait among the seven parents.

As shown in Figure A2 entry Giza75 x Sea and Giza 92 had the largest projection onto ATC (Average tester coordination) abscissa exhibiting the highest and positive GCA effect for seed cotton yield / plant. Whereas, the entries 10229 x Giza 86, Giza85, Giza G88, Giza 93 and Giza 86 were located on the left side of the ATC ordinate (in the opposite direction of ATC abscissa) indicating the lowest and negative GCA effects. Entry Giza 92 followed by Giza75 x Sea and Giza 93 had highest SCA based on the largest projections onto the ATC ordinate. Whereas entry Giza 86, Giza 88 and Giza 85 showed the smallest SCA effects (small projection on ATC ordinate). Based on heterosis, two different groups were suggested in in Figure A2. First group contained Giza 75 x Sea and Giza 93 and the other contained Giza 92. Meanwhile, the others entries located in intermediate positions. Therefore, two crosses, that is, (Giza 75 x Sea) x Giza 92 and Giza 93 x Giza 92 are expected to be heterotic, better than their parents.

Figure A3 provided the best way to demonstrate the interaction patterns between entries and testers as mentioned by Yan et al. (2000) and Yan and Hunt (2002). Four entries are on the vertex on which they have the largest distances from the origin. The polygon view provides a way to group testers based on their best mating partners. Testers falling in the same sector share the same best mating partner and those falling in different sectors have different best mating partners. Subsequently the entry Giza 75 X Sea is the best mating partner for Giza 92, 10229 x Giza 86, Giza 85 and Giza 88. Giza 75 x Sea had the highest GCA, because four of the other seven testers were located in this sector. Moreover, the parent Giza 75 x Sea, as a tester was not found in this sector, so heterosis was suggested in hybrids Giza 75 x Sea with the testers (Giza 92, Giza 85, Giza 88 and 10229 x Giza 86). In the same manner, Giza 92 was in the second arrange for GCA, because 3 testers (Giza 93, Giza 86 and Giza 75 x Sea) were located in this sector. The parent Giza 92 as a tester was not found in sector Giza 92 as entry, so heterosis was suggested in

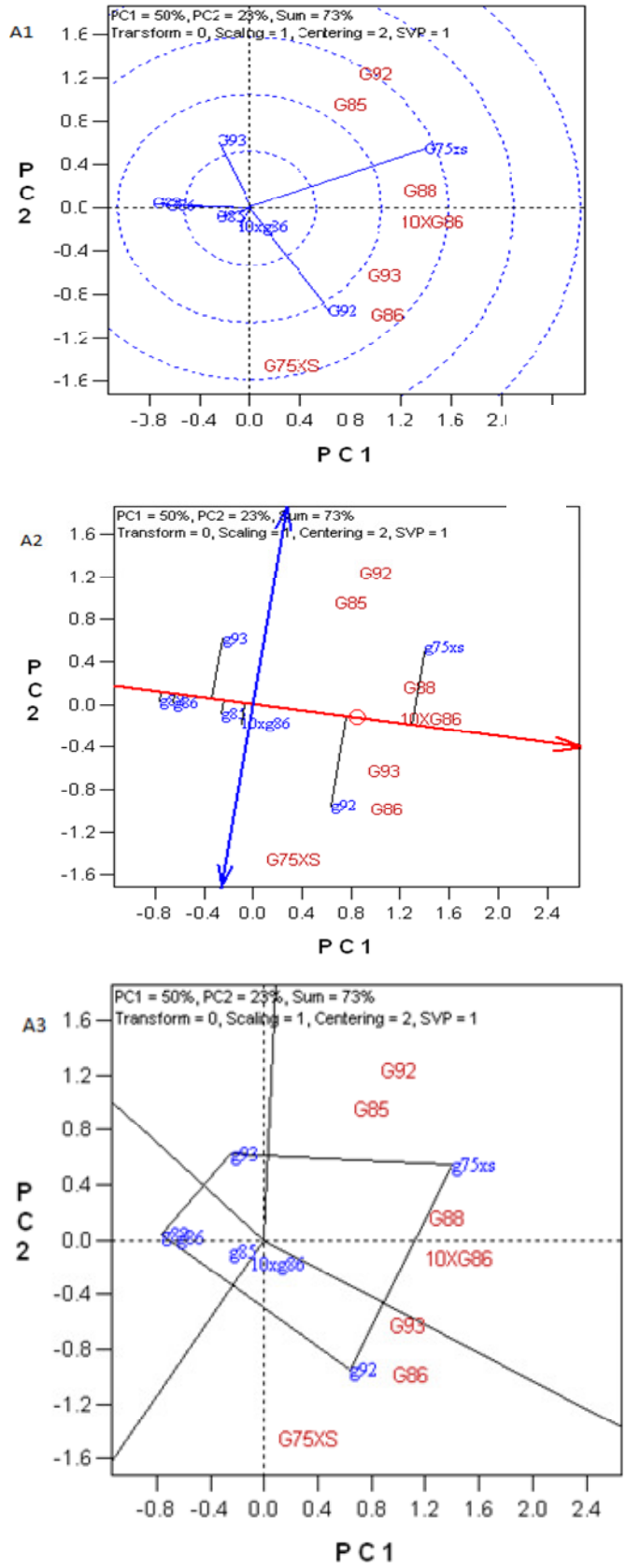

Figure 1. Biplot based on diallled data of seven cotton genotypes of seed cotton yield (scy/p). (A1) Relationship among entries; (A2) Average tester co-ordination; (A3). Polygon view.

the hybrids of Giza 92 with the testers (Giza 93, Giza 86 and Giza 75 x Sea). Rastogi et al. (2011) reported that all the heterotic crosses obtained through biplot analysis showed similar heterotic effects for same crosses analyzed manually by following standard formula. Meanwhile, no tester fell in sectors of Giza 93 and Giza 88, indicating that entries were not the best partner with any of the other testers. In addition, in sector of Giza 75 x Sea, Giza 75 x Sea was predicted to be the best mating partner for Giza 92 and in sector of Giza 92, Giza 92 was also predicted to be the best mating partner for Giza 75 x Sea. Giza 75 x Sea and Giza 92 were therefore identified to be the best partner for each other, and the cross (Giza 75 x Sea) x Giza 92 must be the best of all possible combination (Yan and Kang, 2003).

Lint percentage (L%)

Graphical representation of centric ring system in Figure B1 revealed that the parent 10229 x Giza 86 had the largest vector and so had the largest variation. Meanwhile the parents Giza 88, Giza 93 and Giza 75 x Sea had the lowest variation which were located at the same circle around origin. The parents 10229 x Giza 86 with Giza 88, and Giza 92 with each of Giza 86 and Giza 75 x Sea had the acute angles, which suggests positive correlations among them. Meanwhile, 10229 x Giza 86 with Giza 93, Giza 86, Giza 92 and Giza 75 x Sea had obtuse angles, which suggests negative correlations. Independent relation was found between 10229 x Giza 86 and Giza 85 because the angle between them equal to 90°.

Figure B2 showed that GCA and SCA biplot explained 83% of variation (PC1= 65.3%, PC2 = 17.7%). Hamoud et al. (2012) reported that PC1 and PC2 explained 95.9% of total variation. GCA for entries increased in the direction of arrow on ATC (average tester coordination) abscissa. The parents on the right of the ATC ordinate had positive GCA, while the other parents had negative GCA. The ranking of the entries for GCA was: 10229 x Giza 86> Giza 88 >Giza 85 > Giza 93 > Giza 75 x Sea > Giza 86 > Giza 92.

For SCA, unlike the conventional methods of diallel analysis, which gave an insight only into SCA of crosses (Bocanski et al., 2011) biplot analysis enables the SCA of the parent to estimate. Based on projections on the ATC ordinate, that the highest SCA related to Giza 85 and the lowest was found for Giza 92 and Giza 88.

The biplot in Figure B3 provided the best way to demonstrate the interaction patterns between entries and testers as mentioned by Yan et al. (2000) and (Yan and Hunt (2002). A polygon view is shown in the biplot such that six entries are on the vertices while one is inside the polygon. Since the vertex entries have the largest distances from the origin, they are most responsive to the change of testers relative to other entries within respective groups. The biplot was divided into six sectors. The testers

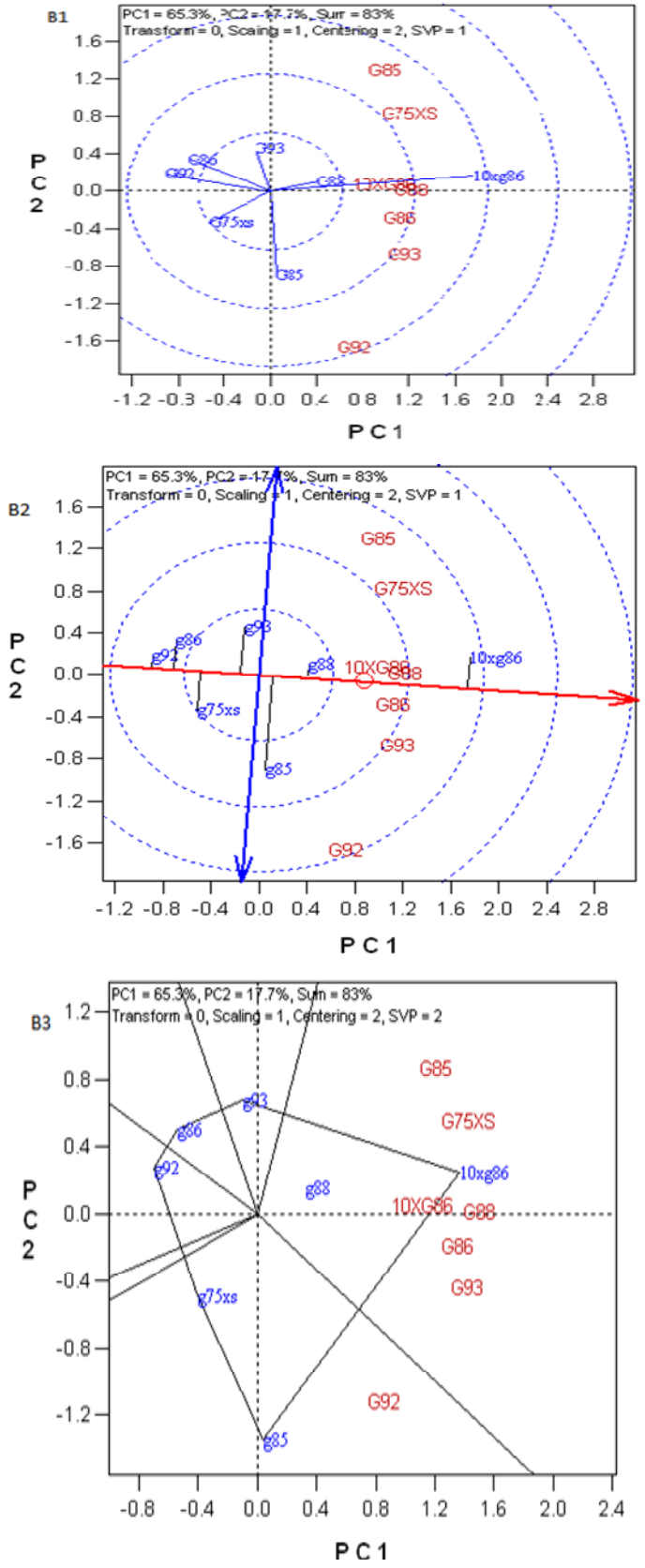

Figure 2. Biplot based on diallled data of seven cotton genotypes for lint percentage (L%). (B1) Relationship among entries; (B2) Average tester co-ordination; (B3). Polygon view.

Giza 85, Giza 75 x Sea, Giza 86, Giza 93 and Giza 88 fell in the sectors that have the vertex 10229 x Giza 86. The biplot clearly shows why 10229 x Giza 86 had the highest GCA, since it was the vertex entry in a sector in which four of the other seven testers, are Giza 85, Giza 75 x Sea, Giza 86, and Giza 93. Since tester 10229 x Giza 86 fell in sector 10229 x Giza 86, the combination (10229 x Giza 86) x (10229 x Giza 86) would be the best among all crosses involving 10229 x Giza 86 and therefore heterosis between 10229 x Giza 86 and any of the other parents is not possible. Meanwhile, the only tester Giza 93 is located in the sector that has the vertex Giza 85 which represents the best mating partner. For the other sectors including Giza 93, Giza 86, Giza 92 and Giza 75 x Sea, there is no testers fell in, indicating that these parents were not the best partner with any of the other parents

Boll weight (BW)

Biplot explained 75% of variation in BW (50 and 25%, by PC1 and PC2, respectively) (Figure C1). The interrelationships among genotypes are visualized in Figure C1. The entry Giza 93 is very close to Giza 85 and Giza 88 is very close to the entries 10229 x Giza 86 and Giza 86, which have angles < 90°, and predict positive relationships among them. However, the negative relationship was observed between Giza 86 and each of Giza 85 and Giza 93, and negative relationship between Giza 75 x Sea and each of 10229 x Giza 86 and Giza 88, which have angle > 90° indicating that these genotypes were apparently different. The entry Giza 92 is located very close to origin, which implies the lowest discriminate entry.

GCA and SCA can be detected from in Figure C2. Based on the projections onto abscissa, the entries ranking for GCA were: Giza 86 > Giza 88 > 10229 x Giza 86 > Giza 75 x Sea ≈ Giza 92> Giza 93 ≈ Giza 85. Abdel-Bary et al. (2008) reported that Giza 86 was the best combiner for boll weight. To display SCA effects of the entries, the vector length helps in ranking the entries as shown in Figure C2. Since the entry Giza 75 x Sea has the longest projection on the ATC ordinate (located on the same perpendicular line which had the grand mean for GCA for all traits) exhibiting that it has the highest SCA effects for boll weight. Similarly, Giza 86 and 10229 x Giza 86 followed by Giza 88 showed positive SCA effects. Whereas, the entries Giza 92 and Giza 85 showed smallest the SCA effects (small projection on to ATC ordinate).

Polygon view in biplot as shown in Figure C3. Five entries are on the vertices while two are inside the polygon. Since the vertex entries have the largest distance from the origin; they are the most responsive to the change of testers relative to other entries within respective groups. The sector that has the vertex Giza 86 contains several testers, that is, 10229 x Giza 86, Giza

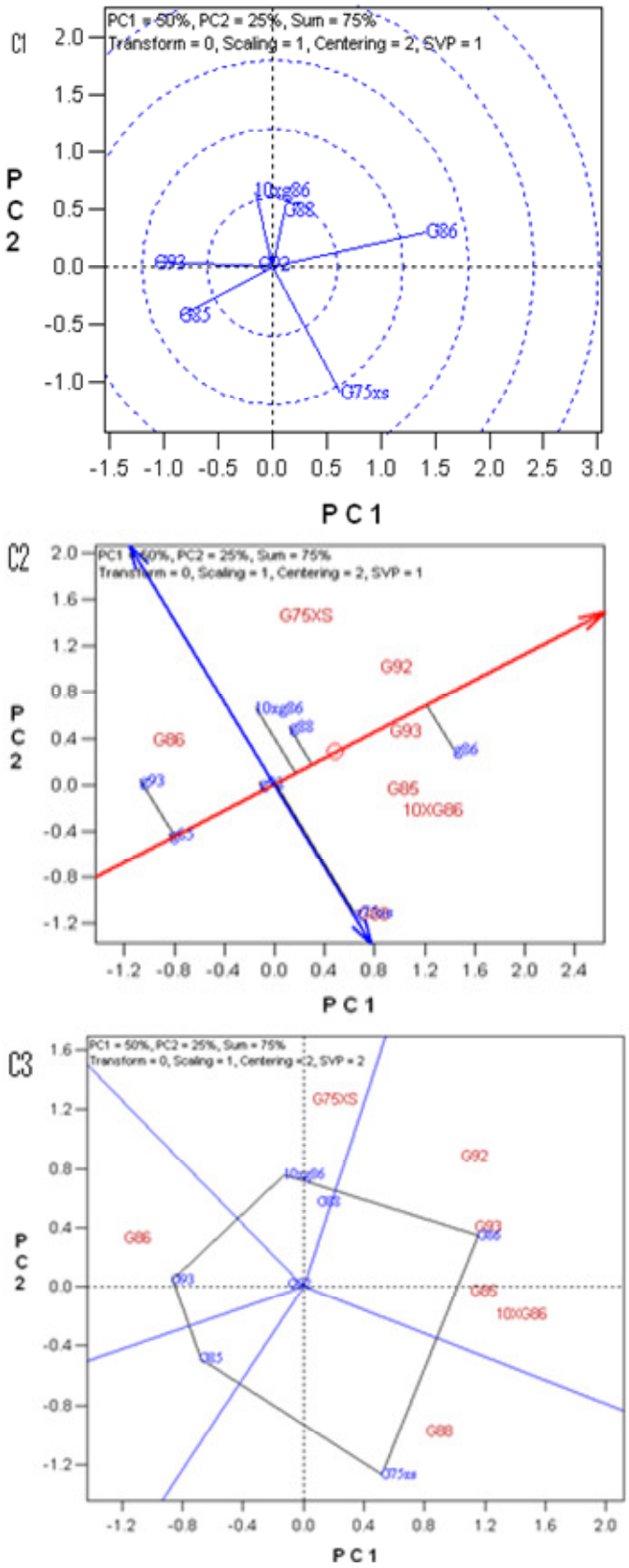

Figure 3. Biplot based on diallled data of seven cotton genotypes for boll weight (b. w). (C1) Relationship among entries; (C2) Average tester co-ordination; (C3). Polygon view.

85, Giza 93 and Giza 92. The biplot clearly shows why entry Giza 86 had the highest GCA effect because it was the vertex entry in a sector in which four testers from seven fell. Also, in each of the next three sectors 10229 x Giza 86, Giza 93 and Giza 75 x Sea only a single tester, that is, Giza 75 x Sea, Giza 86 and Giza 88 can be seen. These represent the three best mating partners including (10229 x Giza 86) x (Giza 75 x Sea), Giza 93 x Giza 86 and (Giza 75 x sea) x Giza88.

Conclusion

The significance of GCA and SCA effects discovered in this study suggested the importance of both additive and non-additive gene action for all traits under investigation in cotton. The study evidently proved the authenticity of Yan's model is useful for analysis of diallel data. The first advantage of the biplot is its graphical presentation of the diallel data, which greatly enhances our ability to understand the patterns of data. The second is that it is more interpretative. Third is its display of a complete picture of the interrelationship among parents. Several researchers have used this method to analyze and interpret diallel data (Khalil et al., 2010; Borghi et al., 2012).

Conflict of Interests

The author(s) have not declared any conflict of interests.

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Full Length Research Paper

Reliability and comparison of the polymorphism revealed in amaranth by amplified fragment length polymorphisms (AFLPs) and inters simple sequence repeats (ISSRs)

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The present study reported the effectiveness of two PCR-based molecular techniques, inters simple sequence repeats (ISSRs) and amplified fragment length polymorphisms (AFLPs), for genetic assessment of amaranth. The polymorphic loci ranged from 110 among *A. caudatus* to 228 among *A. cruentus* and 16 among *A. tricolor* to 56 among *A. hypochondriacus* for AFLP primer combinations and ISSR primers, respectively. Among the two marker systems used, ISSR fingerprinting detected the highest number of alleles per locus (1.83) compared to AFLPs (1.63). However, the assay efficiency index for AFLP was 14.49, more than five-fold higher than ISSR (1.75). The study also revealed that ISSR primers with di-nucleotide repeats gave a good fingerprint, indicating that di-nucleotide repeats are more frequent in amaranth genome. The reproducibility of the two marker systems was confirmed by the narrow gene diversity (0.03 ± 0.11 to 0.07 ± 0.17) observed between the controls. Bayesian consensus and neighbor-joining trees were constructed to describe the cluster arrangement among the *Amaranthus* spp. The cluster pattern was similar for both markers, though the cluster order in the trees was slightly different. The results of this study confirm the usefulness of AFLPs and ISSRs for the genetic assessment of amaranth.

Key words: Amplified fragment length polymorphisms (AFLPs), *Amaranthus* spp., gene, inter simple sequence repeats (ISSRs), markers, nucleotide, polymorphism.

INTRODUCTION

The species within the *Amaranthus* are very closely related and literature shows that misclassifications among the grains, vegetable as well as their weedy and wild relatives occur frequently. Comprehensive genetic diversity studies have been conducted in major crops, using passport, agro-morphological (Ben-Har et al.,

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Table 1. List of amaranth accessions used in the study.

S/N	Accessions	Genetic resource unit
1	EXZIM (*A. cruentus*)	AVRDC, Tanzania
2	AHNL (*A. hypochondriacus*)	AVRDC, Tanzania
3	AM25 (*A. cruentus*)	AVRDC, Tanzania
4	AM44 (*A. cruentus*)	AVRDC, Tanzania
5	Z017 (*A. caudatus*)	RICP, Czech Republic
6	Z018 (*A. caudatus*)	RICP, Czech Republic
7	Z014 (*A. tricolor*)	RICP, Czech Republic
8	Z024 (*A. tricolor*)	RICP, Czech Republic
9	Z076 (*A. tricolor*)	RICP, Czech Republic
10	Z009 (*A. hypochondriacus*)	RICP, Czech Republic
11	Z123 (*A. hypochondriacus*)	RICP, Czech Republic
12	Z150 (*A. hypochondriacus*)	RICP, Czech Republic
13	Z150 (Laboratory duplicate)	RICP, Czech Republic
14	Z006 (*A. hypochondriacus*)	RICP, Czech Republic
15	Z081 (*A. cruentus*)	RICP, Czech Republic
16	Z010 (*A. cruentus*)	RICP, Czech Republic
17	Z152 (*A. cruentus*)	RICP, Czech Republic
18	Z151 (A. *cruentus*)	RICP, Czech Republic
19	Z151 (Blind check)	RICP, Czech Republic

AVRDC, Asian Vegetable Research and Development Centre, Tanzania; NACGRAB, National Center for Genetic Resources and Biotechnology, Nigeria.

1995), and biochemical data obtained by analyses of isozymes (Hamrick and Godt, 1997) or storage proteins (Smith et al., 1987). However, their usefulness for obtaining reliable estimates of genetic similarity is limited because of the small number of marker loci available and the low degree of polymorphism generally found in improved local breeding materials (Messmer et al., 1991). The advantage in the use of molecular markers technique present or absent. Fingerprinting techniques have the their ability to detect genetic variation at levels of resolution that exceed those achievable with other, previously applied methods (Karp, 2002). Owing to the great number of polymorphic marker loci and nature, DNA-assays are more robust and independent of environmental conditions. PCR-based DNA markers are less labour- and time-consuming, and provide an estimate of genetic similarity by direct sampling from the entire genome with unprecedented precision (Peleman and van der Voort, 2003). However, the nature of the marker system, genome coverage and the crop determines the extent of their utility.

Most widely applied DNA marker techniques differ not only in principle, but also in the type and amount of polymorphism detected. Techniques such as non-PCR based restriction fragment length polymorphisms (RFLPs) (Botstein et al., 1980) and PCR-based microsatellites or simple sequence repeat polymorphisms (SSRs) (Tautz, 1989) possess the ability to distinguish multiple bands (alleles) per locus, thus giving more information on a single locus. By contrast, individual bands detected with PCR-based fingerprinting techniques, such as randomly amplified polymorphic DNA (RAPDs) (Williams et al., 1990) and amplified fragment length polymorphisms (AFLPs) (Vos et al., 1995), are scored on a biallelic basis, as marker band ability to generate multiple marker bands in a single assay.

Molecular markers are also employed for the genetic characterization of amaranth germplasm. They have been used to differentiate genotypes under environmental conditions that confounded their phenotypes (Costea et al., 2006). Simple sequence repeats (SSR's) are one of the frequently used molecular markers for genotyping crops (Tautz, 1989). A number of research studies have demonstrated the use of SSRs and ISSRs to detect polymorphism and diversity in amaranth (Mallory et al., 2008; Xu and Sun, 2001; Ray and Roy, 2007), and quinoa (Mason et al., 2005; Jarvis et al., 2008; Fuentes et al., 2009). However, inter-simple sequence repeat (ISSR) markers are simpler to use than SSR technique (Ray and Roy, 2007; Nolan et al., 2010). The use of ISSRs does not require prior knowledge of the target sequences flanking the repeat regions, is not expensive and is relatively easy to score manually compared to SSR. This aim of this study was to evaluate the reliability and compare the application of ISSRs and AFLPs to reveal genetic polymorphism in amaranths.

MATERIALS AND METHODS

Plant materials and DNA extraction

Seeds of seventeen genotypes of *Amaranthus* spp., representing *A. caudatus, A. cruentus, A. hypochondriacus* and *A. tricolor* were obtained from the genetic resource unit of Asian Vegetable Research and Development Centre (AVRDC), Tanzania, and Research Institute and Crop Production (RICP), Czech Republic (Table 1). Two control genotypes were included, a genotype sown twice under coded numbers (blind check) and a randomly chosen genotype duplicated after DNA extraction and re-duplicated in consecutive steps of the analyses (laboratory duplicate). Total genomic DNA was extracted from fresh young leaves of each genotype following the manufacturer protocol of the DNeasy Plant Mini Kit (QIAGEN, Hilden, Germany).

DNA amplification

AFLP protocol

AFLP analysis was performed as described in Vos et al. (1995) with modification (Baránek et al., 2009). Genomic DNA (0.30 µg) was digested with restriction enzymes *Eco*RI and *Mse*I (10 units each) at 37°C for 6 h in 1 × NEB buffer, and *EcoR*I and *Mse*I adapters were ligated to both ends of the restriction fragments with 1 unit of T4 DNA ligase at 16°C overnight. The adapter and primer sequences used in this study are given in Table 2. PCR was carried out with specific, commercially produced primers exactly complementary to the adaptors, but whose 3´ ends are extended for selective nucleotides into the fragments.

Table 2. Oligonucleotide adapters and primer combinations used for AFLP analysis.

Name	Sequence
*EcoR*I adapter	5' -CTCGTAGACTGCGTACC- 3' 3' -CTGACGCATGGTTAA- 5'
*Mse*I adapter	5' -GACGATGAGTCCTGAG- 3' 3' -TACTCAGGACTCAT- 5'
Primer pair used in pre-amplification	
E-A	5' –GACTGCGTACCAATTC-A- 3'
M-0	5' -GATGAGTCCTGAGTAA- 3'
Primer pair used in selective amplification	
**EcoR*I + 3-AGG (FAM – blue)	5' -GACTGCGTACCAATTC + AGG- 3'
*Mse*I + 2-CT	5' -GATGAGTCCTGAGTAA + CT- 3'
**EcoR*I + 3-ACT (JOE – green)	5' -GACTGCGTACCAATTC + ACT-3'
*Mse*I + 2-CT	5' -GATGAGTCCTGAGTAA + CT-3'
**EcoR*I + 3-AGC (NED – yellow)	5' -GACTGCGTACCAATTC + AGC-3'
*Mse*I + 2-CT	5' -GATGAGTCCTGAGTAA + CT-3'
**EcoR*I + 3-AGG (FAM – blue)	5' -GACTGCGTACCAATTC + AGG- 3'
*Mse*I + 2-GC	5' -GATGAGTCCTGAGTAA + GC- 3'
**EcoR*I + 3-ACT (JOE – green)	5' -GACTGCGTACCAATTC + ACT-3'
*Mse*I + 2-GC	5' -GATGAGTCCTGAGTAA + GC-3'
**EcoR*I + 3-AGC (NED – yellow)	5' -GACTGCGTACCAATTC + AGC-3'
*Mse*I + 2-GC	5' -GATGAGTCCTGAGTAA + GC-3'
**EcoR*I + 3-AGG (FAM – blue)	5' -GACTGCGTACCAATTC + AGG- 3'
*Mse*I + 2-AG	5' -GATGAGTCCTGAGTAA + AG- 3'
**EcoR*I + 3-ACT (JOE – green)	5' -GACTGCGTACCAATTC + ACT-3'
*Mse*I + 2- AG	5' -GATGAGTCCTGAGTAA + AG-3'
**EcoR*I + 3-AGC (NED – yellow)	5' -GACTGCGTACCAATTC + AGC-3'
*Mse*I + 2- AG	5' -GATGAGTCCTGAGTAA + AG-3'

*Fluorescent labeled primer

Five microliters of template DNA from a 1:10 diluted ligation mixture was used for pre-amplification in a total volume of 50 µl with primers *EcoR*I-A (5´-GACTGCGTACCAATTCA-3´) and M-0 (5´-GATGAGTCCTGAGTAA-3´); 26 PCR cycles were carried out at 94°C for 60 s, 52°C for 60 s and 72°C at 60 s. Statistics showed that with one nucleotide extension in the primer, 1 fragment will be selectively amplified in every 16 fragments (Xu and Sun, 2001).

The pre-amplification products were diluted five times and used as templates for subsequent selective amplifications. Nine primer pair combinations were used for selective amplifications (Table 3). Three *EcoR*I primers with three selective nucleotides (*E*-AGG, *E*-ACT, *E*-AGC) were each combined with one of three *Mse*I primers with two selective nucleotides (*M*-CT, *M*-GC, *M*-AG). *EcoR*I primers were end-labeled with fluorescence T4 polynucleotide kinase (Invitrogen Life Technologies GmbH, Karlsruhe, Germany). Selective PCR amplification was performed following the protocol as suggested in the AFLP Analysis System I AFLP starter Primer Kit (GibcoBRL) with modification: first cycle at 94°C for 30 s, 65°C for 30 s, and 72°C for 1 min; the annealing temperature was then lowered 0.7°C each cycle during the following 9 cycles, and the optimal annealing temperature of 56°C was reached after a touch-down phase of 10 cycles; the amplification was then continued for an additional 24 cycles (94°C for 30 s, 56°C for 30 s, and 72°C for 1 min).

All amplifications were performed in T-Gradient thermocycler (Biometra).

The AFLP products were analyzed using an automated ABI PRISM 310 genetic analyzer (Applied Biosystems). GeneScan software (Applied Biosystems) was used to overlap the signals from all samples making it possible to evaluate the peak and intensity of each sample, and to translate the product into a descriptive computer files. Detailed manual evaluation was undertaken by two ISSCR-3$(CA)_8$GG, ISSCR-5$(CA)_8$AC, ISLA-$(AGC)_4$G, ISLA-$(CA)_6$GT, ISLA-$(CT)_8$AC and ISLA-$(CT)_8$TG, produced polymorphic

Table 3. Twenty-one ISSR primers screened using five randomly selected amaranth species

S/N	Primers	Nucleotide sequence (5' → 3')	Annealing temperature (°C)
1	UBC-825 $(AC)_8T$	AC AC AC AC AC AC AC AC T	50
2	UBC-842 $(GA)_8YG$	GA GA GA GA GA GA GA GA YG	54
3	UBC-846 $(CA)_8RT$	CA CA CA CA CA CA CA CA RT	52
4	UBC-847 $(CA)_8RC$	CA CA CA CA CA CA CA CA RC	54
5	UBC-857 $(AC)_8YG$	AC AC AC AC AC AC AC AC YG	54
6	UBC-866 $(CTC)_6$	CTC CTC CTC CTC CTC CTC	55
7	ISSCR-2 $(CA)_8AG$	CA CA CA CA CA CA CA CA AG	52
8	ISSCR-3$(CA)_8GG$	CA CA CA CA CA CA CA CA GG	54
9	ISSCR-4 $(CT)_8TG$	CA CA CA CA CA CA CA CA TG	52
10	ISSCR-5 $(CA)_8AC$	CA CA CA CA CA CA CA CA AC	52
11	ISLA-$(AGC)_4G$	AGC AGC AGC AGC G	50
12	ISLA-$(CA)_6AG$	CA CA CA CA CA CA AG	50
13	ISLA-$(CA)_6GG$	CA CA CA CA CA CA GG	50
14	ISLA-$(CA)_6GT$	CA CA CA CA CA CA GT	50
15	ISLA-$(CT)_8$	CA CA CA CA CA CA CA CA	50
16	ISLA-$(CT)_8AC$	CT CT CT CT CT CT CT CT AC	50
17	ISLA-$(CT)_8TG$	CT CT CT CT CT CT CT CT TG	50
18	ISLA-$(GA)_6CC$	GA GA GA GA GA GA CC	50
19	ISLA-$(GAG)_3GC$	GAG GAG GAG GC	50
20	ISLA-$(GTG)_3GC$	GTG GTG GTG GC	50
21	ISLA-$(GT)_6CC$	GT GT GT GT GT GT CC	50

R = A, G; Y = C, T.

bands and were subsequently used in the study. PCR reactions for ISSR analysis were done using the method of Ray et al. (2006). PCR mixtures were carried out in 25 µl volumes containing of 18.75 µl dH_2O, 0.5 µl primer, 2.5 µl Finnzymes Buffer (1x), 0.5 µl $MgCl_2$, 0.25 µl dNTP, 0.5 µl Taq DNA polymerase and 2 µl of template DNA (10 ng/µl). The amplification was performed in a T-Gradient thermocycler (Biometra). The amplification reaction involved an initial denaturation at 94°C for 2 min followed by 45 cycles of denaturation at 94°C for 60 s, hybridization of primers at 50°C for 60 s and polymerization by Taq at 72°C for 2 min. Final extension at 72°C for 7 min followed by 4°C hold. The annealing temperature was adjusted according to specification.

The amplification products were mixed with DNA gel loading buffer and fragments were separated by horizontal electrophoresis on a 1.4% resolute agarose gel, using 1 × TAE buffer (pH 8.0) at 110V for 1 h. The bands were detected under UV light and digitized with trans-illuminator ECX-20.M (VILBER LOURMAT).

Data scoring and genetic analysis

AFLP and ISSR fragments were used for each individual and primer combination to score the presence (1) or absence (0) of bands. Fragment sizes were estimated based on GS 500 ROX size standard and 1-kb DNA ladder size (according to the BioMax ID software algorithm) for AFLP and ISSR, respectively. This information generated the binary matrix that was used for analysis. Only bands that could be scored consistently among the genotypes were used. It was interpreted as dominant markers and was scored as diallelic regardless of band intensity. The binary matrix was used to determine the allele frequency, number of polymorphic loci and percentage of polymorphism using POPGEN Version 1.32 software program (Yeh et al., 1997).

The efficiency of AFLP and ISSR marker systems was compared by computing the assay efficiency index (Ai). The index combines the effective number of alleles identified per locus (calculated as $^{1}/_{\sum fi^2}$, where *fi* is the frequency of *i*th marker allele) and the number of polymorphic bands detected in each assay. Ai = $^{\sum n_e}/_{p}$, where $\sum n_e$ is the total number of effective number of alleles detected over all loci and *P* is the total number of assay performed for their detection (Pejic et al., 1998). FREETREE program (Pavlicek et al., 1999) was used to calculate the degree of similarity using the Nei and Li/dice similarity index (Nei and Li, 1979). Similarity matrices obtained were used to calculate the average intra- and interspecific similarity between the amaranth genotypes, and also used to construct a Bayesian tree using the unweighted pair group mathematical average (UPGMA), and a dendrogram using neighbor-joining. The bootstrap re-sampling method was used to evaluate the reliability of phylogenetic groupings, with bootstrap support values obtained over 500 replications.

RESULTS

The number of polymorphic loci generated with the nine AFLP primer combinations varied among intraspecfic genotype and was higher compared to the number of polymorphic loci detected by ISSR primers (Table 4). The polymorphic loci ranged from 110 among *A. caudatus* to 228 among *A. cruentus* and 16 among *A. tricolor* to 56 among *A. hypochondriacus* for AFLP primer combinations and ISSR primers, respectively. However, the percentage of polymorphic loci detected relative to the total number

Table 4. Gene diversity and polymorphism at the intraspecific level detected with 14 ISSR primers and 9 AFLP primer combinations.

Species	Number of accessions	AFLP primers combination			ISSR primers		
		Polymorphic loci	Polymorphic percentage	Gene diversity	Polymorphic loci	Polymorphic percentage	Gene diversity
A. tricolor	3	167.00	25.46	0.11 ± 0.19	16	17.4	0.18 ± 0.22
A. caudatus	2	110.00	16.77	0.08 ± 0.20	39	42.4	0.09 ± 0.19
A. hypochondriacus	6	218.00	33.23	0.13 ± 0.18	56	60.9	0.23 ± 0.19
A. cruentus	8	228.00	34.76	0.14 ± 0.20	46	50.0	0.21 ± 0.22
Laboratory duplicate	2	22.00	3.35	0.03 ± 0.09	10	10.9	0.05 ± 0.16
Blind check	2	36.00	5.49	0.03 ± 0.11	12	13.0	0.07 ± 0.17
Average number of alleles		1.63 ± 0.48			1.83 + 0.38		
Effective number of alleles per locus		1.29 ± 0.33			1.45 ± 0.34		
Assay efficiency index		14.49			1.75		

± Standard deviation.

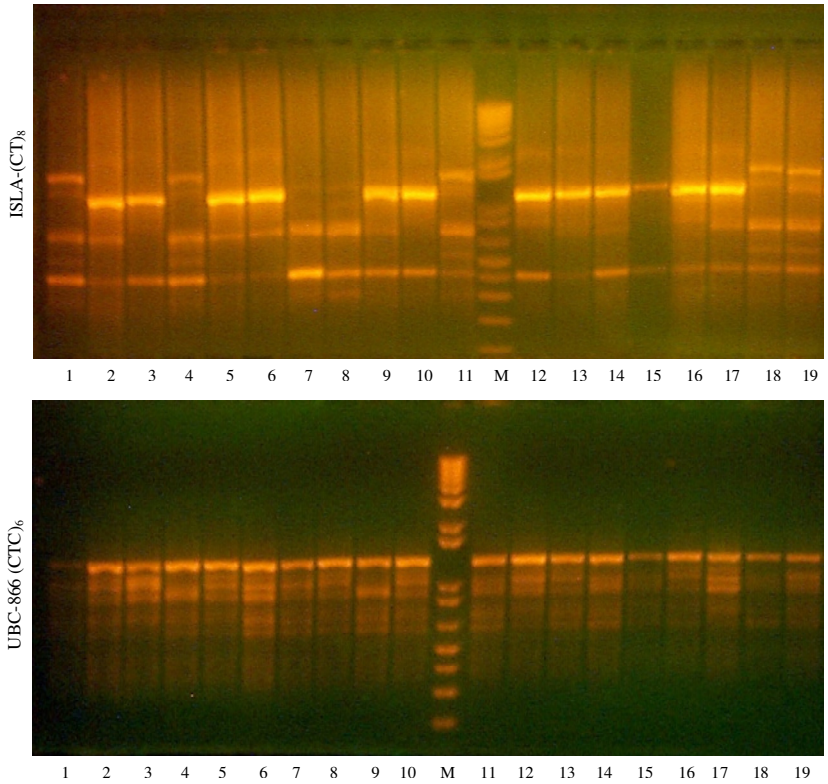

Figure 1. ISSRs gel electrophoresis profile of 19 amaranths generated by ISLA-$(CT)_8$ and UBC-866 $(CTC)_6$ in 1.4% resolute agarose gel. Lane M = 1-kb DNA ladder.

of fragments generated varied from 17 to 35% for AFLP primer combinations and 17 to 61% for ISSR primers. At intraspecific level, higher gene diversity was detected by ISSR primers compared to AFLP primer combinations. Furthermore, low gene diversity estimates was observed within laboratory duplication, 0.03 ± 0.09 for AFLP and 0.05 ± 0.16 for ISSR, and blind check, 0.03 ± 0.11 for AFLP and 0.07 ± 0.17 for ISSR. The average number of alleles per locus was 1.63 ± 0.48 for AFLP and 1.83 ± 0.38 for ISSR while the assay efficiency index of these alleles was 14.49 and 1.75 for AFLP and ISSR, respectively.

ISSRs gel electrophoresis profile generated by ISLA-$(CT)_8$ (di-nuclotide repeats) and UBC-866 $(CTC)_6$ (tri-nucleotide repeats) is presented in Figure 1. Generally, ISSR primers composed of di-nucleotide repeats motif

Table 5. Average genetic similarity matrix between four *Amaranthus* spp. with AFLP primer combinations (above diagonal) and ISSR primers (below diagonal) based on Nei and Li/Dice coefficients.

Species	*A. cruentus*	*A. hypochondriacus*	*A. caudatus*	*A. tricolor*
A. cruentus	-	0.84	0.77	0.86
A. hypochondriacus	0.66	-	0.78	0.85
A. caudatus	0.64	0.64	-	0.78
A. tricolor	0.69	0.68	0.66	-

gave a clear and distinct finger-printing pattern in the study.

Nei and Li/Dice's similarity coefficients ranged from 0.77 to 0.86 and 0.64 to 0.69 with AFLP primer combinations and ISSR primers, respectively. Both primers indicated the highest similarity coefficient between *A. cruentus* and *A. tricolor* and the lowest between *A. cruentus* and *A. caudatus* (Table 5).

The Bayesian consensus trees (BCT) of the *Amaranthus* spp. based on AFLP primer combinations and ISSR primers using Nei and Li/Dice's genetic similarity matrix are presented in Figure 2. In both molecular technique, the *Amaranthus* spp., Z151 and Z150, were closely associated with the blind check (Z151) and laboratory duplicate (Z150), respectively. The same result was also observed with the neighbor-joining tree (NJT) obtained from the same primer set (Figure 3). NJT produced a better resolution of species relationship compared to the BCT. Although minor differences exist among the NJ and BC trees, similar clustering pattern was generated by AFLP and ISSR primer sets. Generally, however, the trees from AFLP data set had stronger bootstrap support values than ISSR-based trees.

DISCUSSION

Molecular marker approaches are considered efficient in fingerprinting plant genome. This study investigated the usefulness and effectiveness of two PCR-based molecular techniques, ISSRs and AFLPs in detecting polymorphism in amaranth. The number of alleles per locus detectable by ISSRs was higher compared to AFLPs. Such high level of polymorphism is to be expected with molecular techniques that are based on replication slippage (Tautz et al., 1986). Similarly, studies have shown that when ISSRs are compared to other marker systems they revealed the highest level of polymorphism (Ray and Roy, 2007; Xu and Sun, 2001).

The study also revealed that the assay efficiency index was more than five-fold higher for AFLPs than ISSRs. Therefore, ISSRs have the ability of revealing the highest level of information per single marker while AFLPs can detect the highest number of polymorphisms in a single assay. The high assay efficiency index is a reflection of the efficiency of AFLPs to simultaneously analyze a large number of bands rather than the levels of polymorphism detected at each locus (Pejic et al., 1998). This principle account for the high similarity coefficients observed among the amaranth species for AFLP relative to ISSR. The assay efficiency index for ISSRs, however, can be considerably higher if multiplex PCR and gel-running procedures are adopted, where several microsatellites are simultaneously amplified using multicolour fluorescent technologies (Lindqvist et al., 1996; Heyen et al., 1997; Fuentes et al., 2009).

Di-nucleotide repeat ISSR primers produced the highest average number of bands and generally gave a clear fingerprint pattern compare to tri-nucleotide repeats. This suggested that di-nucleotide repeat ISSR primers are more frequent in amaranth genome compared to tri-nucleotide repeats. However, using di-nucleotide repeats alone may not be efficient and sufficient to differentiate between amaranth genotypes. Similarly, di-nucleotide repeats ISSR primers yielded the highest amount of polymorphic bands in rice (Blair et al., 1998) and *Diplotaxis* (Brassicaceae) (Martin and Sanchez-Yelamo, 2000).

Both molecular markers used in the study revealed narrow gene diversity between the laboratory duplicate and the blind check, thus, indicating the reproducibility of the two marker systems in genetic assessment of amaranth. The slightly high polymorphism observed in the blind check relative to laboratory duplicate may be due to heterogeneity present in the amaranth genotype. The distribution pattern of the *Amaranthus* spp. into different clusters was similar for AFLPs and ISSRs, although the cluster order in the trees was slightly different. Furthermore, the tree robustness was lower nearly for all relationships in the ISSR-based trees than the AFLP-based trees. This could result from a smaller ISSR data set compared to the AFLP data set.

In conclusion, this study may not be sufficient to justify that both markers used in the study strongly support the phylogenetic assessment of amaranth species because fewer number of species were used. However, the phylogenetic trees obtained from these marker systems were related, even though AFLPs and ISSRs differ in nature and principles of mechanisms. The current study has shown that AFLPs and ISSRs are highly reproducible and can generate informative characters useful for

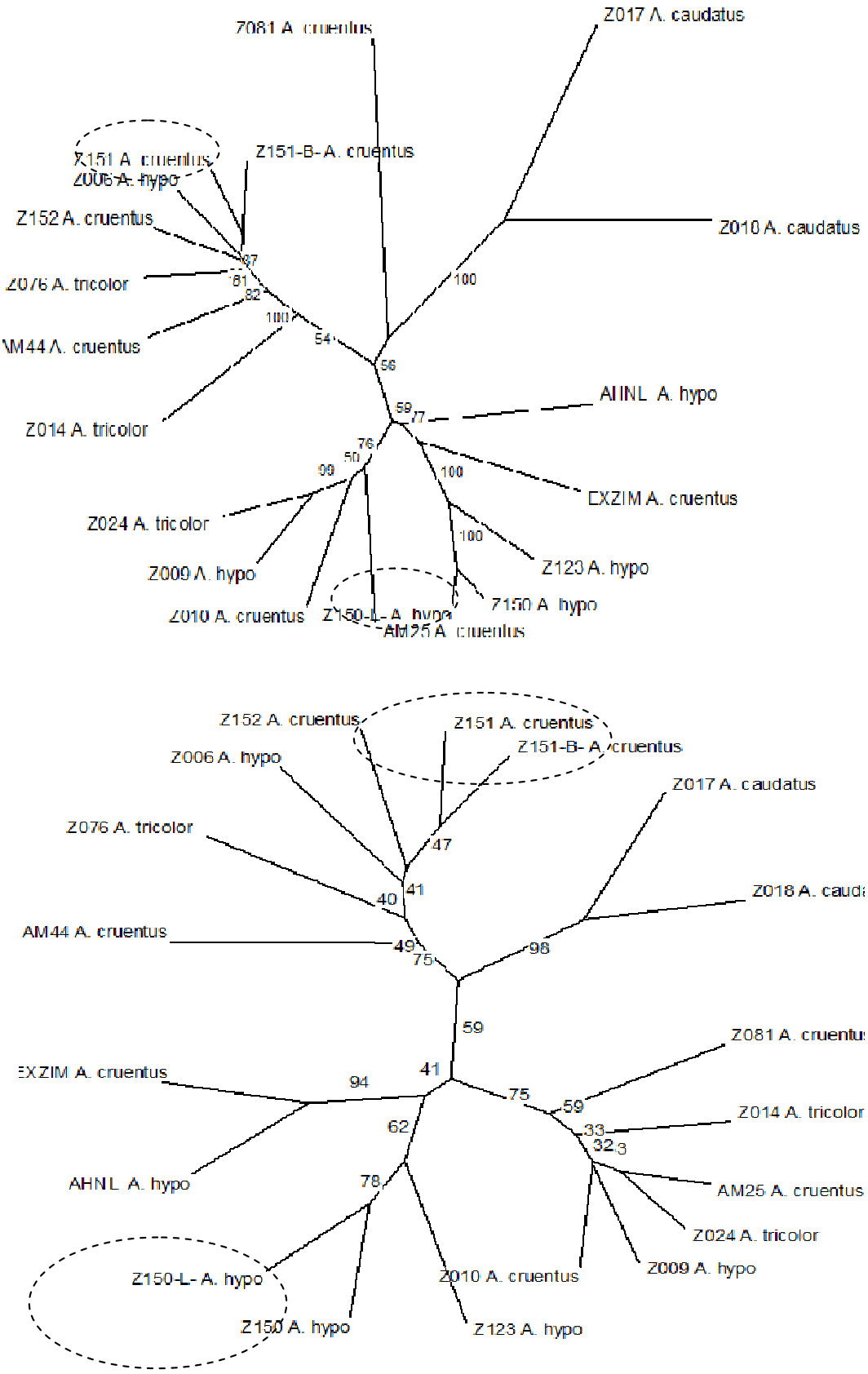

Figure 2. Bayesian consensus tree of the *Amaranthus* spp. based on AFLP primer combinations and ISSR primers; bootstrap support values are given above branches. *A. hypo.* – *A. hypochondriacus.*

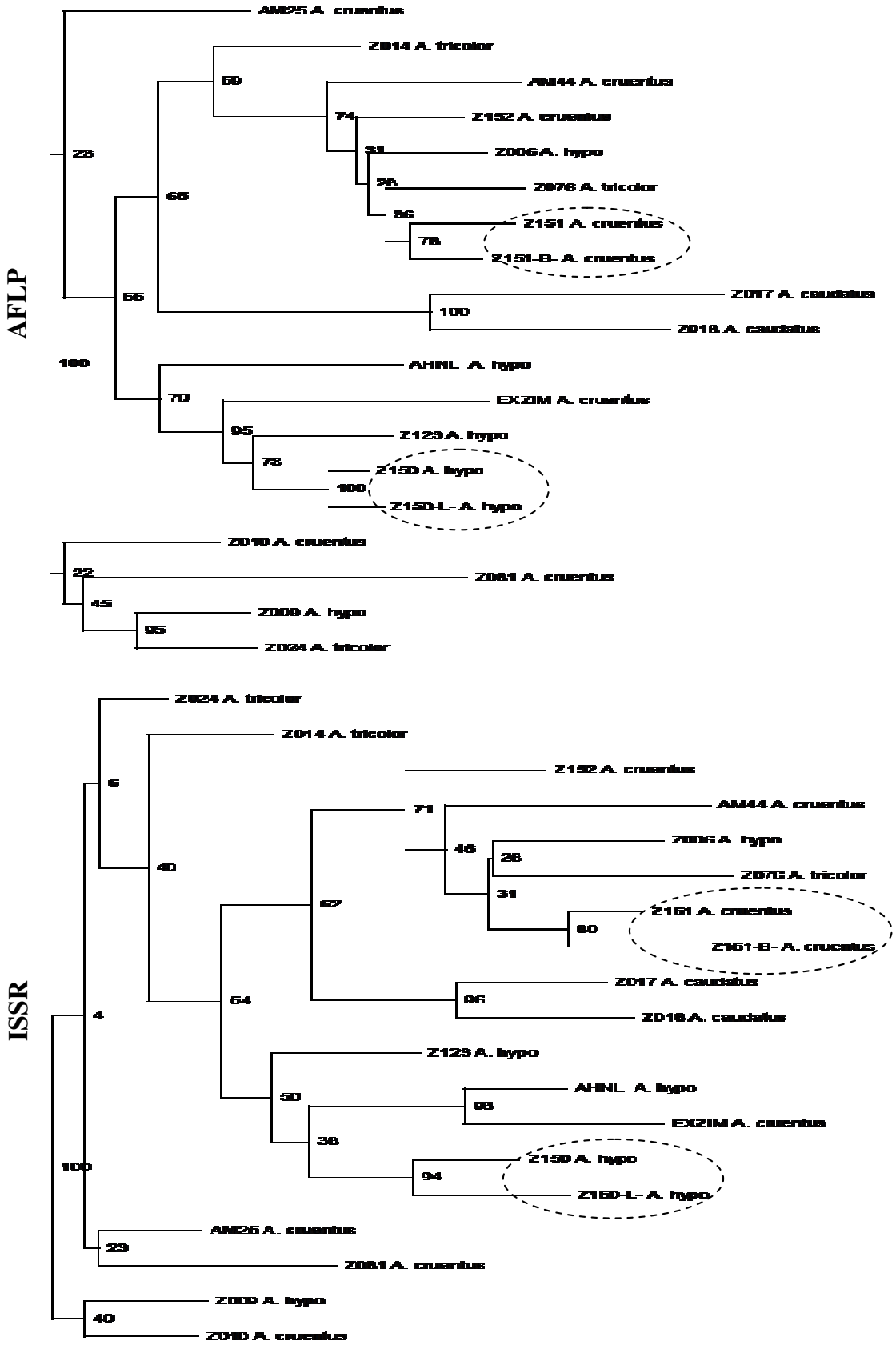

Figure 3. Neighbor-joining tree obtained from the AFLP primer combinations and ISSR primers; bootstrap support values are given below branches. *A. hypo. – A. hypochondriacus.*

phylogenetic assessment of amaranth. The use of these molecular markers can be valuable for efficient germplasm management and breeding programmes of amaranth.

Conflict of Interests

The author(s) have not declared any conflict of interests.

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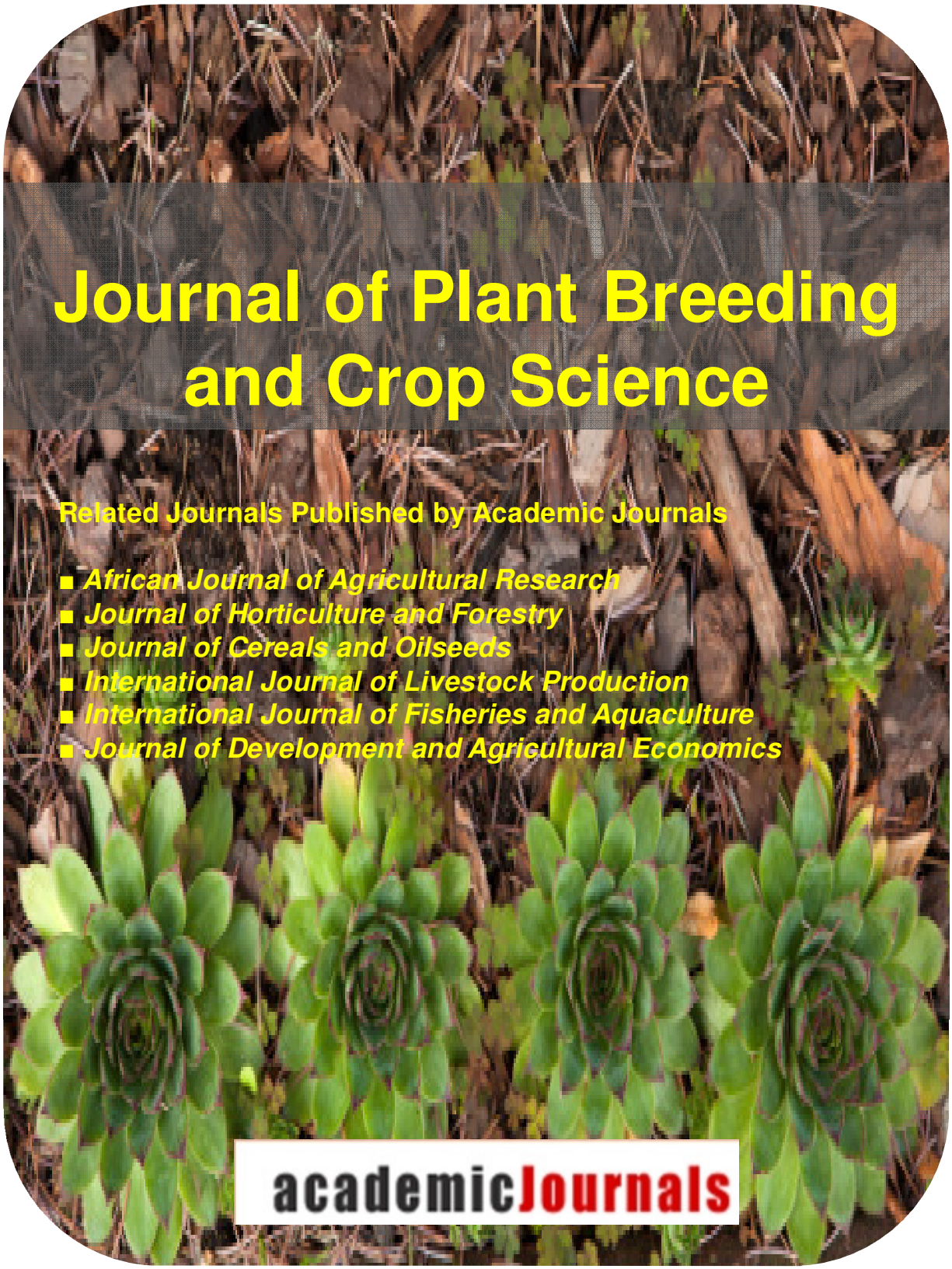

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