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Genetic diversity among turf grasses by ISSR markers

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ABSTRACT

In this study, leaves sample of eight turf grass genera were used to standardized the ISSR protocol and quantities of template DNA, dNTPs, $MgCl_2$, Taq DNA polymerase, primer concentration and annealing temperature for each primer. Analysis of molecular variance (AMOVA), Genetic diversity, Nei's gene diversity, Shannon's index, and Nei's unbiased genetic distance, partition, within- and among-group, of all parameters was analyzed. Levels of genetic divergence between samples were calculated with the fixation index PhiPT. Statistics with AMOVA revealed 84 and 16 % variance among and within various mutants, respectively. Cluster analysis based on the Unweighted-pair group method arithmetic average (UPGMA), Principal coordinate analysis (PCA) and Spatial correlation is a measured that looks at the relationship (genetic distance) amongst mutants. PCOA analysis of ISSR data showed that the first three factors comprised about 94.5% of total variance when the first, second and third axis comprised about 49.85, 29.29 and 15.36% of total variance, respectively. Statistically non-significant genetic similarity exists among population which varies from -1.0000 to 0.2419. Maximum similarity was recorded between the two samples of *Lolium perenne* and *Agrostis stolonifera* (0.0682) followed by *Stenotaphrum secundatum, Poa pratensis* (0.0419). Cluster analysis was conducted to generate a dendogram elucidating for relationships among turf grass. The first cluster divided into three sub-clusters comprising: (i) *Poa pratensis* and *Agrostis stolonifera* (ii) DFR-NS-1 and *Lolium perenne* and (iii) the DFR-NS-1 *Festuca arundinacea*.The second cluster consisted of *Stenotaphrum secundatum, Zyosia junsia* and *Paspalum vaginatum*. Within the second cluster *Paspalum vaginatum* was separated from the zoysiagrass. As revealed by ISSR analysis, *Stenotaphrum secundatum*, and Paspalm were quite distinct from bermudagrass (DFR-NS-1) and rest of the turf grasses.

Keywords: Genetic diversity, Geographical populations, Inter simple sequence repeat (ISSR), Turf grasses

Turf grasses are used worldwide for lawns of home and office buildings, athletic fields, other recreational facilities and roadsides (Tiwari *et al*. 2015a). Each turf grass species has a number ofcultivars, varieties or hybrids, each of which is genetically different (Humaid *et al*. 2011). *Cynodon dactylon* Pers. is highly valued warm season turf grass having global adaptability, robustness and resistance to trampling. In India, it is widely used as turf grass, and is also effective in soil conservation (Tiwari *et al.* 2014, 2015b).The aims of the present study were: (1) to form an optimal protocol of ISSR-PCR reaction condition (2) to screen 12 primers and select those with high clarity and repetition for further downstream applications and (3) to specify genetic diversity for investigation of differences or resemblances among different 8 species from different geographical regions and clarify their phylogenic relationship in order to establish breeding programs.

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MATERIALS AND METHODS

Leaves sample of eight turf grass genera (viz.*Cynodon dactylon, Poa pratensis, Lolium perenne, Zyosia junsia, Agrostis stolonifera, Paspalum vaginatum, Stenotaphrum secundatum* and *Festuca arundinacea)* were collected from Research Field of Directorate of Floriculture Research, ICAR-IARI and experiment was laid out during 2013-14 in the Molecular Laboratory of Plant Pathology division of ICAR-IARI, New Delhi. Fresh young leaves of 1.5 g were harvested for DNA isolation.

Fresh young leaves weighing 1.5 gm were harvested and wrapped in foil paper for DNA isolation. These were immediately taken to the laboratory and kept in refrigerator at -20°C to retain freshness of the material. The leaves were vigorously rinsed in distilled water to remove particles on leaf surfaces. About 200 mg of each sample was gently ground into paste in a mortar with 2 ml cTAB extraction buffer (pre-heated at 65°C for 10 min). To facilitate and speed up grinding, leaves were chopped into smaller bits with scissors. As a precaution, scissors were dipped in

absolute ethanol before reuse. After grinding, equal volume (approximately 1 ml each) of the resultant paste was distributed into two separate 2 ml microcentrifuge tubes. This stage took 3 to 5 min/sample.

A volume of 2 µl β-mercaptoethanol was added to each tube, which was then mixed thoroughly by gently rocking the rack. Samples were incubated in a water bath at 65°C for 30 min and allowed to return to room temperature for 5 to 10 min. An equal volume of chloroform:isoamyl alcohol (24:1) (i.e.1 ml into each tube) was added for extraction. This was mixed gently by continuously shaking and inverting the tubes for up to 5 min. Samples were centrifuged at 11 000 rpm for 10 min at 25°C to separate the phases. The top light green-colored aqueous phase was transferred to new 1ml microcentrifuge tubes, along with 0.75 volume chilled isopropanol (e.g., for 1 ml aqueous phase, 750 µl chilled isopropanol added) to precipitate the DNA. Samples were mixed gently by continuous inversion, kept at -20°C overnight followed by centrifugation to recover the DNA pellets.

The samples were centrifuged at 12 000 rpm for 10 min at 4°C. Carefully, the supernatant was discarded being mindful of the DNA pellet, the pellets were washed in 70% ethanol and air-dried until ethanol evaporated completely from the samples. This was facilitated by inverting tubes on tissue paper or paper towel or using a vacuum blower. The DNA pellets were rehydrated/dissolved in 400 µl T10E1 buffer and treated with 3 µlRNase (10 mg/ml). Samples were incubated for 50 min at 37°C. For purification, 400 µl phenol:chloroform:isoamyl alcohol (25:24:1) was added followed by centrifugation for 5 min at 10 000 rpm. The top layer was carefully pipetted into new 1.5-ml microcentrifuge tubes, with the addition of an equal volume of chloroform:isoamyl alcohol (24:1) and centrifugation at 5 000 rpm for 5 min at 22°C. The supernatant was transferred to new 1.5-ml tubes with the addition of 0.6 volumes (0.6 × 300 =180 µl) of chilled isopropanol followed by several but slow inversions of the tubes. Sodium acetate (3 M) 1/10 volume (30 µl), was added to facilitate DNA precipitation. DNA was further precipitated at -20°C for overnight. A volume of 200 µl 70% ethanol was added onto the pellet with centrifugation at 12 000 rpm for 10 min at 4°C. The supernatant was discarded, and the pellet dried and dissolved in TE (volume of TE depended on the visible quantity of pellet in the tube).

DNA yield and purity were determined by two methods: agarose gel electrophoresis and spectrophotometric analyses. Aliquots (1 µl) of DNA samples were run on a 0.8% agarose gel and compared with band intensities from known concentration of lambda DNA standards. The yield was further measured by checking the optical density (OD) in a UV spectrophotometer at 260 nm. DNA purity was determined by calculating the absorbance ratio at A260/280.

ISSR amplified fragments, with the same mobility according to molecular weight (bp),were scored manually for band presence (1) or absence (0). Data recording followed the three principles: 1) Only the easily recognizable bands can be recorded, and the obscure bands are excluded, 2) the bands that cannot be precisely identified should be excluded, and 3) the bands with the same mobility but different intensity should not be treated as the same bands (Weising *et al*. 2005). Analysis of molecular variance (AMOVA), Genetic diversity within and among populations was measured as the percentage of polymorphic bands, Nei's gene diversity (Nei 1973), Shannon's index, and Nei's unbiased genetic distance, partition, within- and among-group, of all parameters was analyzed using the version 6.5 of software in GenALEx (Peakall and Smouse, 2006). Phi-statistics (PhiPT) is a modified version of Wright's F that refers to the relative contributions of between-population separation to the overall genetic variation in the whole sample. Levels of genetic divergence between samples were calculated with the fixation index PhiPT (Excoffier *et al.* 1992) and principal coordinate analysis (PCA) (calculated with GenAlex, ver. 6.5). Spatial autocorrelation analysis were performed to quantify the spatial scale of clonalspread and its statistical significance in the population. Pairwisecor relation and Cluster analysis based on the Unweighted-Pair Group Method arithmetic Average (UPGMA) were analysed by JMP.9 software.

RESULTS AND DISCUSSION

ISSR Amplification

The 12 selected ISSR primers produced 109 bands with an average of 9.08 bands per primer, of which 97 (88.99%) were polymorphic ranging from 250 to 2 000 bp in size (Table 1). Each primer produced 6 to 10 polymorphic bands with an average of 8.08. The most polymorphism was shown by nine primers (P1, P2, P3, P4, P5, P6 P7, P9 and P10), which showed 100% polymorphism. Statistics with AMOVA revealed 16 and 84% variance within and among various germplasm, respectively, (Table 2). Variance differentiation was significant ($P < 0.01$) for all turf grasses (Table 3). This result suggests that genetic variance was high among turf grasses and low within population. Phi-statistics values ranges from 0.841 to 0.915 which refers to the relative contributions of between-population to the overall genetic variation in the whole sample of population. The greater the Fst values are, the greater the differences between Population. The *Festuca arundinacea*, *Lolium perenne* and *Paspalum vaginatum* showed highest diversity judged from the means of *Ne*, *He*, *I* and Unbiased Diversity, while *Cynodon dactylon*, *Poa pratensis*, *Agrostis stolonifera* showed the lowest diversity (Table 4). The band patterns across turf grasses reveals low degree of similarity indicated high divergence amongst population (Fig 1). Results indicated that there was variation between studying population considering to Shannon's information index (I) and Nei's gene diversity (He) which, revealed that turf grass population had highest variation amongst population. Genetic variation between populations of *Festuca campestris* were reported by Fasih *et al.* 2013 based on Shannon's information index and molecular variance analysis using ISSR markers. Cai

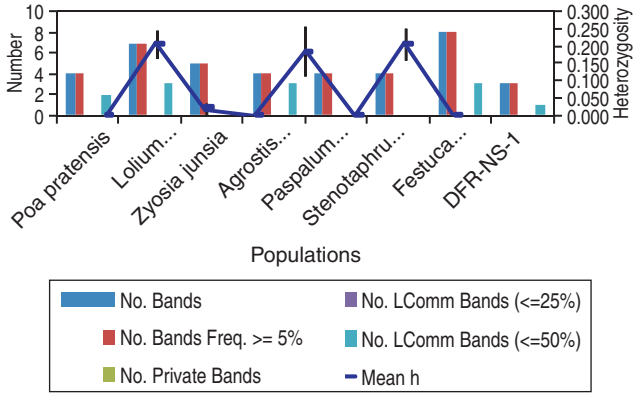

Fig 1 Band patterns across turfgrasses

Table 1 Characteristics of the 12 ISSR primers used for polymorphism in various turf grasses

Primer	Sequence (5′⟶3′)	Annealing temperature (°C) *TA* (°C)	No. of polymorphic loci scored/ No. of loci scored	% of polymorphism bands	Size range of fragments (bp)
P 1	(GA)8CTA	55	9/9	100	450-1500
P 2	(GA)8AGC	55	10/10	100	600-2000
P 3	(GGGGT)3	55	8/8	100	250-1900
P 4	(AC)8GCT	55	6/6	100	500-1000
P 5	(AC)8TG	55	9/9	100	270-1800
P 6	(TCC)5TG	55	8/8	100	400-2000
P 7	(AC)8GT	55	10/10	100	270-1300
P 8	(AG)8TC	55	8/10	80	400-1600
P 9	(GA)8GCC	55	9/9	100	300-1600
P 10	ACT ACG ACT (TG)7	55	7/10	100	500-2000
P 11	ACTC-GTACT (AG)7	55	6/10	60	400-1700
P 12	CGT AGT CGT (CA)7	55	7/10	80	500-1000
			97/109		

et al. (2005) developed ISSR markers for zoysia grass identification. Also, Kamps *et al*. (2007) found that PCR amplification resulted in SSR marker profiles having up to 17 unique bands to discern tested bermudagrass genotypes. Therefore, ISSR represents an excellent technique for bermudagrass cultivar identification, varietal protection, and for the identification of mistakes inplantings, mislabeled plant materials, and contamination or substitutions of sod fields (Humaid *et al.* 2011).

Genetic variation and similarity among genotypes

The average values of observed number of alleles (Na), effective number of alleles (Ne), Nei'sgene diversity (He), Shannon's information index (I) unbiased diversity and percentage of polymorphic loci for all population were 0.891, 1.112, 0.078, 0.125, 0.085 and 28.125%, respectively (Table 3). Correlogram of the population was calculated at various distance classes with 99% confidence intervals. The r was used to evaluate the genetic relationship within, and among individual families. Average kinship coefficients among the individuals (represented by r) were 0.2000 in the population. At the population level, a total of 66 discernible bands were analyzed by GeneAlExGenetic Analysis Software. The spatial autocorrelation revealed no autocorrelation at short distances of various grasses. These observations are in agreement with many studies showing the higher reproducibility and efficiency of ISSR markers Galvan *et al.* (2003) and Kameli *et al*. (2013).

Cluster analysis

PCOA analysis of ISSR data showed that the first three axes comprised about 94.5% of total variance when the first, second and third axis comprised about 49.85, 29.29 and 15.36% of total variance respectively. The pair-wise genetic distance estimates of the turf grass cultivars in this study were analysed and are given in Table 4. Statistically non-significant genetic similarity exists among population which varies from -1.0000 to 0.2419. Maximum similarity was observed between the two samples of *Lolium perenne* and *Agrostis stolonifera* (0.0682) followed by *Stenotaphrum secundatum, Poa pratensis* (0.0419). Cluster analysis was conducted to generatea dendogram elucidating for relationships among turf grass. The dendogram constructed with UPGMA analysis revealed two main clusters (Fig 2). The first cluster divided into three sub-clusters comprising: (i) *Poa pratensis* and *Agrostis stolonifera* (ii) DFR-NS-1 and *Lolium perenne* and (iii) the DFR-NS-1*Festuca arundinacea*. The second cluster consisted of *Stenotaphrum secundatum, Zyosia junsia* and *Paspalum vaginatum*. Within the second cluster *Paspalum vaginatum*was separated from

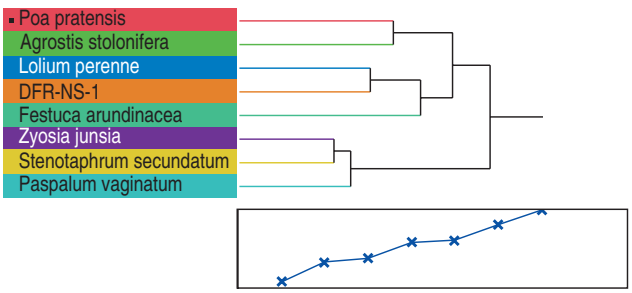

Fig 2 Dendrogram of cluster analysis for populations by UPGMA method

Table 2 Analysis of molecular variance (AMOVA) of profiles developed from inter-simple sequence repeats markers in various turf grasses

Source of variation	df	Sum of squares	Variance of component(MS)	Est. Var.	Percentage of variation	P-valuea
Among pops	7	153.906	21.987	1.804	84%	>0.01
Within pops	88	29.917	0.340	0.340	16%	>0.01
Total	95	183.823		2.144	100%	

Phi-statistics	*Value*	*P(r and >= data)*
PhiPT	0.841	0.010
PhiPT max	0.915	
Phi'PT(Min)	0.920	

PhiPT = AP / (WP + AP) = AP / TOT

Where, AP = Est. Var. Among Pops, WP = Est. Var. Within Pops

Variation within population	*Poa*	*Lolium*	*Zyosia*	*Agrostis*	*Paspalum*	*Stenotaphrum*	*Festuca*	*DFR-NS-1*	*Total SS within population*
SSWP	0.00	10.00	0.91	0.00	9.00	0.00	10.00	0.00	29.917

[a]Levels of significance were obtained through nonparametric procedures using 99 permutations. Probability, P(r and >= data), for PhiPT is based on standard permutation across the full data set.

Table 3 Variation of genetic parameters developed from inter-simple sequence repeats markers for different turf grasses

Population	Poa	Lolium	Zyosia	Agrostis	Paspalum	Stenotaphrum	Festuca	DFR-NS-1	Total Mean
No. of alleles Na = *No. of Different Alleles*									
Mean	0.500	1.750	0.750	0.500	1.000	0.500	1.750	0.375	0.891
Standard Error	0.189	0.250	0.250	0.189	0.378	0.189	0.164	0.183	0.102
Effective number of alleles, *Ne = No. of Effective Alleles = 1 / (p^2 + q^2)*									
Mean	1.000	1.287	1.023	1.000	1.300	1.000	1.288	1.000	1.112
Standard Error	0.000	0.066	0.023	0.000	0.113	0.000	0.063	0.000	0.025
Nei's gene diversity, *He = Diversity = 1 - (p^2 + q^2)*									
Mean	0.000	0.208	0.019	0.000	0.188	0.000	0.208	0.000	0.078
Standard Error	0.000	0.041	0.019	0.000	0.071	0.000	0.045	0.000	0.017
Shannon's information index, *I = Shannon's Information Index = -1* (p * Ln (p) + q * Ln(q))*									
Mean	0.000	0.347	0.036	0.000	0.281	0.000	0.338	0.000	0.125
Standard Error	0.000	0.061	0.036	0.000	0.106	0.000	0.074	0.000	0.026
uh = Unbiased Diversity = (N / (N-1)) * h									
Mean	0.000	0.227	0.021	0.000	0.205	0.000	0.227	0.000	0.085
Standard Error	0.000	0.045	0.021	0.000	0.077	0.000	0.050	0.000	0.018
% of Polymorphic Loci	0.00%	87.50%	12.50%	0.00%	50.00%	0.00%	75.00%	0.00%	28.125%

the zoysiagrass. As revealed by ISSR analysis, *Stenotaphrum secundatum*, and paspalum were quite distinct from bermudagrass (DFR-NS-1) and rest of the turf grasses. Similar results were reported by Humaid *et al.* (2011) using ISSR markers. Extremely high variability and high mapping density as compared with RFLP and RAPD data make these ISSR new dominant, microsatellite based molecular markers ideal for producing genetic maps of individual species (Karaca *et al.* 2002). These features, combined with greater robustness in repeatability of experiments and less prone to changing band patterns with changes in constituent or DNA template concentrations, make them superior to other readily available marker systems in investigations of genetic variation among very closelyrelated individuals and in crop cultivar classification (Karaca *et al.* 2002). The results clearly demonstrate that a methodology based on ISSR markers can be used to identify and fingerprint turf grass cultivars.

In summary, results indicated that the level of polymorphism among turf grassesis high. It also suggests

Table 4 Pairwise correlations amongst various turf grasses

Variable	by Variable	Correlation	Lower 95%	Upper 95%	SignifProb
Lolium perenne	*Poa pratensis*	-0.9025	-0.9163	-0.8864	<.0001*
Zyosia junsia	*Poa pratensis*	0.0153	-0.0651	0.0954	0.7098
Zyosia junsia	*Lolium perenne*	-0.0394	-0.1192	0.0410	0.3370
Agrostis stolonifera	*Poa pratensis*	-0.0419	-0.1218	0.0385	0.3065
Agrostis stolonifera	*Lolium perenne*	0.0682	-0.0121	0.1477	0.0957
Agrostis stolonifera	*Zyosia junsia*	-0.9735	-0.9774	-0.9690	<.0001*
Paspalum vaginatum	*Poa pratensis*	0.0316	-0.0488	0.1116	0.4406
Paspalum vaginatum	*Lolium perenne*	-0.0467	-0.1265	0.0337	0.2544
Paspalum vaginatum	*Zyosia junsia*	0.7375	0.6986	0.7720	<.0001*
Paspalum vaginatum	*Agrostis stolonifera*	-0.7575	-0.7898	-0.7211	<.0001*
Stenotaphrum secundatum	*Poa pratensis*	0.0419	-0.0385	0.1218	0.3065
Stenotaphrum secundatum	*Lolium perenne*	-0.0682	-0.1477	0.0121	0.0957
Stenotaphrum secundatum	*Zyosia junsia*	0.9735	0.9690	0.9774	<.0001*
Stenotaphrum secundatum	*Agrostis stolonifera*	-1.0000	-1.0000	-1.0000	<.0001*
Stenotaphrum secundatum	*Paspalum vaginatum*	0.7575	0.7211	0.7898	<.0001*
Festuca arundinacea	*Poa pratensis*	-0.3400	-0.4091	-0.2671	<.0001*
Festuca arundinacea	*Lolium perenne*	0.3321	0.2588	0.4017	<.0001*
Festuca arundinacea	*Zyosia junsia*	-0.2412	-0.3153	-0.1641	<.0001*
Festuca arundinacea	*Agrostis stolonifera*	0.2530	0.1763	0.3266	<.0001*
Festuca arundinacea	*Paspalum vaginatum*	-0.1916	-0.2677	-0.1130	<.0001*
Festuca arundinacea	*Stenotaphrum secundatum*	-0.2530	-0.3266	-0.1763	<.0001*
DFR-NS-1	Poa pratensis	-0.7877	-0.8163	-0.7552	<.0001*
DFR-NS-1	*Lolium perenne*	0.6950	0.6510	0.7343	<.0001*
DFR-NS-1	*Zyosia junsia*	-0.2483	-0.3221	-0.1715	<.0001*
DFR-NS-1	*Agrostis stolonifera*	0.2820	0.2064	0.3542	<.0001*
DFR-NS-1	*Paspalum vaginatum*	-0.2195	-0.2945	-0.1417	<.0001*
DFR-NS-1	*Stenotaphrum secundatum*	-0.2820	-0.3542	-0.2064	<.0001*
DFR-NS-1	*Festuca arundinacea*	0.2419	0.1649	0.3160	<.0001*

that the ISSR marker offers apowerful means to analyze the genetic diversity among accessions. Cluster analysis based on the Unweight-Pair Group Method arithmetic Average (UPGMA), principal coordinate analysis (PCA) and Spatial correlation are measures that look at the relationship (genetic distance) amongst mutants.

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REFERENCES

Cai H W, Inoue M, Yuyama N, Takahashi W, Hirata M and Sasaki T. 2005. Isolation, characterization and mapping of simple sequence repeat markers in zoysiagrass (*Zoysia* spp.). *Theoretical and Applied Genetics* **112**(1): 158–66.

Excoffier L, Smouse P E and Quattro J M.1992. Analysis of molecular variance Inferred from metric distances among DNA haplotypes - Application to Human Mitochondrial-DNA Restriction Data. *Genetics* **131:** 479–91.

Fasih Z farshadfar M and Safari H. 2013. Genetic diversity evaluation of within and betweenpopulations for *Festuca arundinacea* by ISSR markers. *International Journal of Agriculture and Crop Sciences* **5**(13): 1 468–72.

Humaid A Al, Ibrahim G H and Motawei M I.2011. Molecular identification of some turf grass cultivars and their resistance to *Fusarium graminearum. Australian Journal of Crop Science* **5**(13): 1 754–9.

Kameli M, Hesamzadeh S M and Hejazi Ebadi M. 2013. Assessment of genetic diversity on populations of three *satureja* species in Iran using ISSR markers. *Annals of Biological Research* **4**(3): 64–72.

Kamps T L, Ortega V M, Williams N R, Chamusco K C, Scully B T and Chase C D. 2007. Fingerprinting cultivars: DNA polymorphisms of bermudagrass (*Cynodon* spp.) microsatellite loci. *Plant and Animal Genomes XV Conference,* January 13-17, San Diego, USA.

Karaca M, Saha S, Zipf A, Jenkins J N and Lang D J .2002.Genetic diversity among forage bermudagrass (*Cynodon* spp.).*Crop Science* **42**: 2 118–27.

Nei M. 1973. Analysis of gene diversity in subdivided populations, *Proceedings of the National Academy of Sciences USA* 70: 3

321–3.

Peakall R and Smouse P. 2006. GENALEX 6: Genetic analysis in Excel.Population genetic software for teaching and research. *Molecular Ecology Notes* **6:** 288–95.

Tiwari A K, Kumar R, Kumar G, Kadam G B, Saha T N, Girish K S and Tiwari B. 2014a. Mutagenesis and digital image analysis of mutants for quality attributes of native *Cynodon dactylon*. *Indian Journal of Agricultural Sciences* **84**: 733–6.

Tiwari A K, Kumar R, Kumar G, Kadam G B, Saha T N and Tiwari A K, Kumar R, Kumar G, Kadam G B and Saha T N. 2015a. Comparing digital image analysis and visual rating of gamma ray induced Bentgrass (*Agrostis stolonifera*) mutants. *Indian Journal of Agricultural Sciences* **85**(1): 93–106.

Weising K, Nybom H, Wolff K and Kahl G. 2005. *DNA Fingerprinting in Plants: Principles, Methods, and Applications*, 2nd Edition, pp 207–33. CRC Press, Florida