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Full Length Research Paper

Random amplified polymorphic DNA (RAPD) and simple sequence repeat (SSR) marker efficacy for maize hybrid identification

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Knowledge as to genetic diversity and relationships among maize hybrids is important for breeding strategies. The main aims of this study were to (1) estimate molecular genetic diversity among 30 maize hybrids by random amplified polymorphic DNA (RAPD) and simple sequence repeat (SSR) markers; and (2) compare the genetic relatedness values obtained from these marker types. A set of 30 maize hybrids was assessed. To compare these two methods, genetic parameters were computed such as the number of polymorphic bands, average number of alleles per locus, effective number of alleles per locus, expected heterozygosity, effectiveness index of analysis and polymorphism information content (PIC). Better results were provided by SSR. The discrimination ability of individual markers was also determined. The SSR system provided an average PIC of 0.71 (ranging from 0.47 to 0.91) and RAPD provided an average value of 0.61 (ranging from 0.44 to 0.82). Genetic similarities (GS) were estimated using Nei and Li's coefficients for SSR and Jaccard's coefficient for RAPD. For SSR, GS ranged from 26.3 to 88.5% (with average of 58.8%); for RAPD, GS ranged from 6.7 to 86.7% (with average of 49.5%). Hybrids were clustered using unweighted pair group method with arithmetic mean (UPGMA). The correlation between similarity matrices for RAPD and SSR was 0.11.

Key words: Random amplified polymorphic DNA (RAPD), simple sequence repeat (SSR), genetic similarity, molecular marker, *Zea mays* L. var. indurata, *Zea mays* L. var. indentata.

INTRODUCTION

Information concerning the genetic diversity of maize hybrids is very important for germ plasm enhancement, hybrid breeding and preventing environmental damage that may occur due to the genetic uniformity of hybrids grown on large areas (Bauer et al., 2007). Unless there is sufficient genetic diversity in the germ plasm, it is practically not possible to increase the yield and other desirable characters of a crop, because the selection of improved genotypes depends on the availability of genetic variability within the breeding material. To improve genetic diversity of local germ plasm, it is important to know the extent of already existing genetic variability in the material. Various kinds of markers can be used to estimate genetic diversity in maize germ plasm. Morphological traits have been used as markers for assessing genetic diversity in the past, but these markers are very often influenced by the environment and are therefore unreliable. Later, biochemical and cytological markers were used to monitor germ plasm biodiversity. But these markers are limited in number and hence cannot be used to study the complete genome of a species (Islam and Shepherd, 1991). More recently, DNA-based markers

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Abbreviations: PIC, Polymorphism information content; **RAPD,** random amplified polymorphic DNA; **SSR**, simple sequence repeat; **GS**, genetic similarities; **UPGMA**, unweighted pair group method with arithmetic mean; **ASA,** allele-specific amplification; **CAPS,** cleavage amplification polymorphic sequences.

have enhanced the utilization of biotechnology in crop improvement (Miller et al., 1989). Molecular markers are also used to study the level of genetic diversity among different cultivars, closely related species and GenBank accessions (Erlich et al., 1991).

Among DNA marker methods, most commonly used is polymerase chain reaction (PCR). There are various kinds of PCR-based assays, for example, random amplified polymorphic DNA (RAPD), simple sequence repeats (SSRs), allele-specific amplification (ASA), cleavage amplification polymorphic sequences (CAPS), etc. RAPD consists in the random amplification of DNA fragments with a single primer having an arbitrary sequence, resulting in the final synthesis of several DNA fragments of different sizes. RAPD technique has been widely used in diversity studies because, in addition to its low cost, it allows polymorphism to be detected in a simple and rapid manner (Hahn et al., 1995; Liu et al., 1998; Lübberstedt et al., 2000; Wu, 2000).

SSRs (Jacob et al., 1991) are loci that are comprised of highly variable arrays of tandemly repeating DNA sequences 2 to 6 base pairs long (Senior et al., 1998). SSRs are useful molecular markers because they are (1) abundant; (2) uniformly distributed; (3) highly polymorphic; (4) co-dominant; (5) rapidly produced by PCR; (6) relatively simple to interpret; and (7) easily accessed by other laboratories via published primer sequences (Saghai-Maroof et al., 1994).

Molecular markers have been used to study the genetic diversity of maize inbred lines with the aim of predicting hybrid performance and heterosis of yield. Xu et al. (2004) reported that, based on genetic similarity, inbred lines have been divided into heterotic groups and used for hybrid development. Shieh and Thseng, (2002) evaluated genetic variability in 13 maize lines and assessed the relationship between genetic diversity and hybrids efficiency using RAPD analysis. The results showed that, this analysis based on RAPD technique cannot be used for accurate prediction of F1 hybrids efficiency and heterosis values because the determination coefficients are insignificant and the prediction value is limited. To determine the level of heterosis, they require detailed pedigree records, molecular markers and coefficients of parentage may also be used to estimate diversity (Liu et al., 1999; Sud et al., 2005). Identification of the hybrids obtained is essential for further breeding. Detailed knowledge regarding genetic diversity and relationships among breeding materials is indispensable for the development of new maize inbred lines (Xia et al., 2004). Molecular characterization and identification of materials is a very important part of the breeding process. Accurate identification of a maize hybrid's molecular spectra is a tool for determining seed authenticity and origin in potential business disputes. If a variety must be registered and legally protected according to the UPOV international convention (The International Union for Protection of New Varieties of Plants), it must fulfil the "DUS" test conditions (distinctness, uniformity and stability). There are morphological characters and physiological traits established for every cultivar and these should routinely be used for these purposes and subsequently be stated in the description of cultivars or hybrids. Therefore, molecular markers may be a suitable supplement to "classical" description methods and may be helpful for identifying and differentiating in some crop genera as molecular certificates. The objective of this study was to identify hybrids of maize (*Zea mays* L.) through RAPD and SSR marker systems for subsequent breeding strategies and to compare the effectiveness of these two methods based on genetic parameters.

MATERIALS AND METHODS

Plant materials

A set of 30 maize hybrids, all created by CEZEA – Plant Breeding Station, Inc. (Cejc, Czech Republic), was used for the analyses. A complete list of hybrids and their origins is presented in Table 1. Materials for DNA isolation were obtained from a set of 5 to 7 plants at the 3 to 4 leaf stage. The samples were ground into a very fine powder using liquid nitrogen and stored at -80°C.

DNA extraction

Total genomic DNA was isolated using the commercial GenEluteTM Plant Genomic DNA Miniprep Kit (SIGMA, Germany). To determine concentrations, the UV spectrophotometric absorption method was used. The quality of isolated DNA was verified by electrophoretic separation on 0.8% agarose gel.

RAPD assays

For the PCR reaction, 40 ng of DNA from each sample was used for amplification by RAPD markers. A total of 37 decanucleotide primers acquired from Metabion, GmbH (Germany) were used. The PCR reaction was performed using a final volume of 25 µl: 200 µM dNTPs, 1.5 mM $MgCl_2$, 8 µM primer, 1x reaction buffer (10 mM Tris-HCl, 50 mM KCl and 0.1% Triton X-100) and 2U DyNAzyme II polymerase (Finnzymes, Finland). The amplification cycle (Heun and Helentjaris, 1993) was carried out in the Touchgene gradient thermocycler (Techne, UK). The amplified products were separated on 1.5% agarose gel and subsequently stained with ethidium bromide. The fragment size was determined using the Bio Image advanced quantifier software v. 4.2.1 (Bio Image Systems, US).

SSR assays

Primers for amplification of SSR loci were synthesized on the basis of sequences from the MaizeGDB database (www.maizegdb.org). A total of 43 SSRs were used for polymorphism evaluation. The DNA content in 25 µl of the reaction mixture was 40 ng. The reaction contained 1x reaction buffer, 200 µM dNTPs, 1.5 mM of $MgCl_2$, 0.6 µM of each primer and 1U DyNAzyme II polymerase (Finnzymes, Finland). The thermal programme in the cycler was set for initial

Table 1. Materials used and information about their pedigrees.

Hybrid	Pedigree
CE-P 31 x CE-P 43	Flint EU, Lancaster
CE-P 31 x CE-P 44	Flint EU
CE-P 31 x CE-P 48	Flint EU, Iodent
CE-P 32 x CE-P 43	Lancaster
CE-P 32 x CE-P 44	Unknown
CE-P 33 x CE-P 44	Unknown
CE-P 33 x CE-P 48	Iodent
CE-P 34 x CE-P 43	Flint US, Lancaster
CE-P 34 x CE-P 44	Flint US
CE-P 34 x CE-P 48	Flint US, Iodent
CE-P 35 x CE-P 42	Flint US, Lancaster
CE-P 35 x CE-P 43	Flint US, Lancaster
CE-P 35 x CE-P 44	Flint US
CE-P 35 x CE-P 48	Flint US, Iodent
CE-P 36 x CE-P 44	Flint US
CE-P 36 x CE-P 46	Flint US, Flint EU
CE-P 36 x CE-P 47	Flint US, Flint EU
CE-P 36 x CE-P 48	Flint US, Iodent
CE-P 37 x CE-P 44	Iodent
CE-P 37 x CE-P 46	Iodent, Flint EU
CE-P 37 x CE-P 47	Iodent, Flint EU
CE-P 38 x CE-P 44	Iodent
CE-P 38 x CE-P 46	Iodent, Flint EU
CE-P 38 x CE-P 47	Iodent, Flint EU
CE-P 39 x CE-P 44	Unknown
CE-P 39 x CE-P 46	Flint EU
CE-P 39 x CE-P 47	Flint EU
CE-P 41 x CE-P 42	Lancaster
CE-P 40 x CE-P 42	Lancaster
CE-P 45 x CE-P 42	Lancaster

denaturation at 94°C for 1 min. Then followed 40 cycles of 15 s at 94°C, 15 s at 55°C, and 30 s at 72°C, with a final elongation at 72°C for 1 min. DNA samples were electrophoretically separated on 3% agarose in 1 x TBE buffer and then visualized using ethidium bromide.

Data analyses

Gels were evaluated with respect to the presence or absence of amplified products. To compare two different techniques, such genetic parameters were computed as the number of polymorphic bands, average number of alleles per locus, effective number of alleles per locus (Ne), expected heterozygosity (He) and effectiveness index of analysis (Ai) (Morgante et al., 1994). The polymorphism information content (PIC) was determined for each marker (Smith et al., 1997).

The genetic similarity among hybrids was calculated separately for each technique. The similarity matrix was created from binary data using Nei's (1972) coefficients for SSR and Jaccard's (1901) coefficient for RAPD. Based on similarity matrix cluster analysis, unweighted pair group method with arithmetic mean (UPGMA) was performed using FreeTree software v. 0.9.1.50 (Pavlíček et al., 1999). Matrices were compared using Pearson's correlation coefficient.

RESULTS AND DISCUSSION

For RAPD, a total of 37 primers were used. Six markers appeared to be polymorphic and provided sufficient and high polymorphic information content. The average PIC of 6 RAPD markers was 0.61, ranging from 0.44 to 0.82 (Table 2). The lowest and highest PIC values were recorded for primers OPG18 and UBC506, respectively.

Table 2. Polymorphic markers and their PICs in two marker systems.

RAPD	Sequence	PIC	SSR	Sequence (5´→3´)	PIC
0C16	CACACTCCAG	0.69	Phi053	CTGCCTCTCAGATTCAGAGATTGAC AACCCAACGTACTCCGG	0.79
OPG12	CAGCTCACGA	0.62	Umc1600	CGATCAGTGCGTGGAGAGTA TAGGCATGCATTGTCCATTG	0.83
OPG18	GGCTCATGTG	0.44	Bnlg1043	TTTGCTCTAAGGTCCCCATG CATACCCACATCCCGGATAA	0.57
OPX04	CCGGTACCGA	0.47	Umc1659	CAAGCTTGCTACTGTGATTTCTCG AACTTCTCGGTGATCTTGTCCATC	0.47
UBC401	TAGGACAGTC	0.64	Bnlg1867	CCACCACCATCGTAGGAGTT CAGTACACAGCAGGCAGCTC	0.72
UBC506	CCTTTCCCGA	0.82	Umc2258	AAGATTGTATAAATGGCAGCCACG GAATAAGACCAGACAGCACCGAAC	0.91
			Phi126	TCCTGCTTATTGCTTTCGTCAT GAGCTTGCATATTTCTTGTGGACA	0.67
			Bnlg1257	CGGACGATCTTAGCAAACA ACGGTCTGCGACAGGATATT	0.57
			Umc2071	ATACACGCAGTTACCCGAAGGTT ACTGATGGTGTTCTTGGGTGTTTT	0.76
			Bnlg1647	CGTCGTCTGTGGACGTACTG AGAAGCTCACAAGCCTGCTC	0.78
			Umc2101	CTAGCTAGTTTGGTGCGTGGTGAT CCCGGCTAGAGCTATAAAGCAAGT	0.57
			Umc2265	CTGGACGTGGACTCAGACACC AAGACGGTCCCGAAGAAAGC	0.82

PIC, Polymorphism information content; RAPD, random amplified polymorphic DNA; SSR, simple sequence repeat.

A total of 53 fragments were amplified using these RAPD markers, out of which 16 were polymorphic (30%). While working with maize hybrids, Heun and Helentjaris (1993) had observed that, 80% of the bands obtained were polymorphic by RAPD. From 43 SSR primers, 12 polymorphic SSRs were selected. A total of 49 polymorphic bands were amplified using these SSR markers. On the average, 4.08 alleles were obtained per locus. The markers exhibited a relatively high PIC (0.71), ranging from 0.47 to 0.91 (Table 2). The lowest and highest PIC values were recorded for primers Umc1659 and Umc2258, respectively. Sun et al. (2001) had used 17 primers with DNA obtained from 37 hybrids. A total of 79 bands were scored and an averages of 4.6 alleles were amplified per primer. Genetic diversity of the studied materials is the most important factor limiting average number of alleles identified per microsatellite locus during screening. However, such other factors as number of SSR loci, repeat types and the methodologies employed for detection of polymorphic markers have been reported to influence allelic differences. In this study, the average number of detected alleles per one locus (4.08) was

Table 3. Genetic parameters computed for comparison of two marker systems.

Parameter	RAPD	SSR
Number of polymorphic bands	16[a]	49
Average number of alleles per locus	2,67	4.08
Effective number of alleles per locus (N_e)	2.76	4.00
Expected heterozygosity (H_e)	0.63	0.74
Effectiveness index of analysis (Ai)	3.61	7.99

[a] Theoretically the maximum number of loci (Garcia et al., 2004).

lower than those reported previously in diversity studies of maize inbred. Lu and Bernardo (2001), using 40 U.S. inbred lines with 83 SSR markers, had reported 4.9 alleles per locus. Senior et al. (1998) had found 5.0 for 94 elite U.S. maize inbred with 70 SSR markers. Warburton et al. (2002) had found 4.9 alleles with 85 SSR loci and Vaz Patto et al. (2004) had determined 5.3 alleles using 80 SSR loci. On the other hand, Shah et al. (2009), using 17 Pakistani maize genotypes with 10 SSR primer sets, had reported 1.56 alleles per one genotype. The mean He value in this study (0.74) is comparable to those from previous studies of genetic diversity between U.S. maize inbred, for example, Pejic et al. (1998) had reported 0.72.

A comparative analysis is shown in Table 3. The average number of alleles per locus, as well as the number of effective alleles per locus was higher for the SSR system. This corresponds with the effectiveness index of analysis, which was higher in SSR than in RAPD. Matrices were developed using the suitable similarity coefficients and dendrograms of genetic similarities were generated using UPGMA.

Using the RAPD technique, the set of 30 maize hybrids was divided into 8 clusters (Figure 1). The first cluster included hybrids of lines CE-P 44 of unknown origin, excluding the CE-P 39 x CE-P 47 hybrid. The second cluster included hybrids of Iodent origin, excluding the CE-P 35 x CE-P 42 hybrid. The third cluster comprised hybrids from the Flint US x Flint EU cross. The third, fourth and fifth clusters corresponded to one of the clusters of the dendrogram of SSRs (fourth cluster) (Figure 2), excluding the CE-P 39 x CE-P 46 and CE-P 37 x CE-P 47 hybrids. In the sixth cluster there were hybrids of Lancaster origin, excluding the CE-P 35 x CE-P 48 hybrid. Hybrids of the Lancaster x Lancaster cross were separated from the others in the seventh cluster. The eighth cluster included hybrids of Iodent origin. The average genetic similarity was 49.5% and ranged from 6.7% of bands in common between the hybrids CE-P 36 x CE-P 46 and CE-P 33 x CE-P 48 and 86.7% of bands in common between the hybrids CE-P 38 x CE-P 44 and CE-P 38 x CE-P 46. Smith and Smith (1992) had studied the divergence of 45 American hybrids and found a mean value of 59% (GS = 41%). Sun et al. (2001), had shown that similarity values based on RAPD data among Ontario hybrids ranged from 31 to 86%.

In the SSR system, the set of 30 maize hybrids was divided into five main clusters (Figure 2). The first cluster comprised 2 hybrids from lines of unknown origin (CE-P 39). The second cluster included pure hybrids of Lancaster origin. The third cluster comprised hybrids of Flint EU, Iodent and unknown origin. The fourth cluster involved hybrids of Flint US origin, excluding the hybrids CE-P 37 x CE-P 46 and CE-P 37 x CE-P 44. There were also hybrids of lines CE-P 44 and 48, which were of similar but different origin (internal records of CEZEA – Plant Breeding Station, Inc.). A CE-P 32 x CE-P 44 hybrid of unknown origin was assessed separately. The similarity coefficient from the microsatellite markers showed a range of variation from 26.3% between hybrids CE-P 33 x CE-P 44 and CE-P 41 x CE-P 42 to the maximum similarity observed between hybrids CE-P 35 x CE-P 43 and CE-P 35 x CE-P 42, with a coefficient of 88.5%. The average genetic similarity was 58.8%. The similarity values from the microsatellite data generally showed similarity values a little above those of the RAPD-based analysis.

The matrices were compared using Pearson's correlation coefficient (r = 0.11), which was very low in the set of maize hybrids. A low correlation between RAPD and SSR markers had been obtained in the research of Garcia et al. (2004), who had observed a correlation of 0.33. On the other hand, there are studies showing high correlations between results obtained using these two molecular procedures, such as those of Pejic et al. (1998), Sun et al. (2001), Souza et al. (2008) and Leal et al. (2010), who had found correlations of 0.57, 0.43, 0.54 and 0.55, respectively, between RAPD and SSR markers. Among factors that might have contributed to the limited correlation between results obtained by the RAPD and microsatellites could be differences in those polymorphisms revealed by the two markers used. RAPD has a dominant character, while microsatellites have a co-dominant character. There could also be differences in the effectiveness and/or number of primers used (Liu and Furnier, 1993; Pejic et al., 1998; Sun et al., 2001).

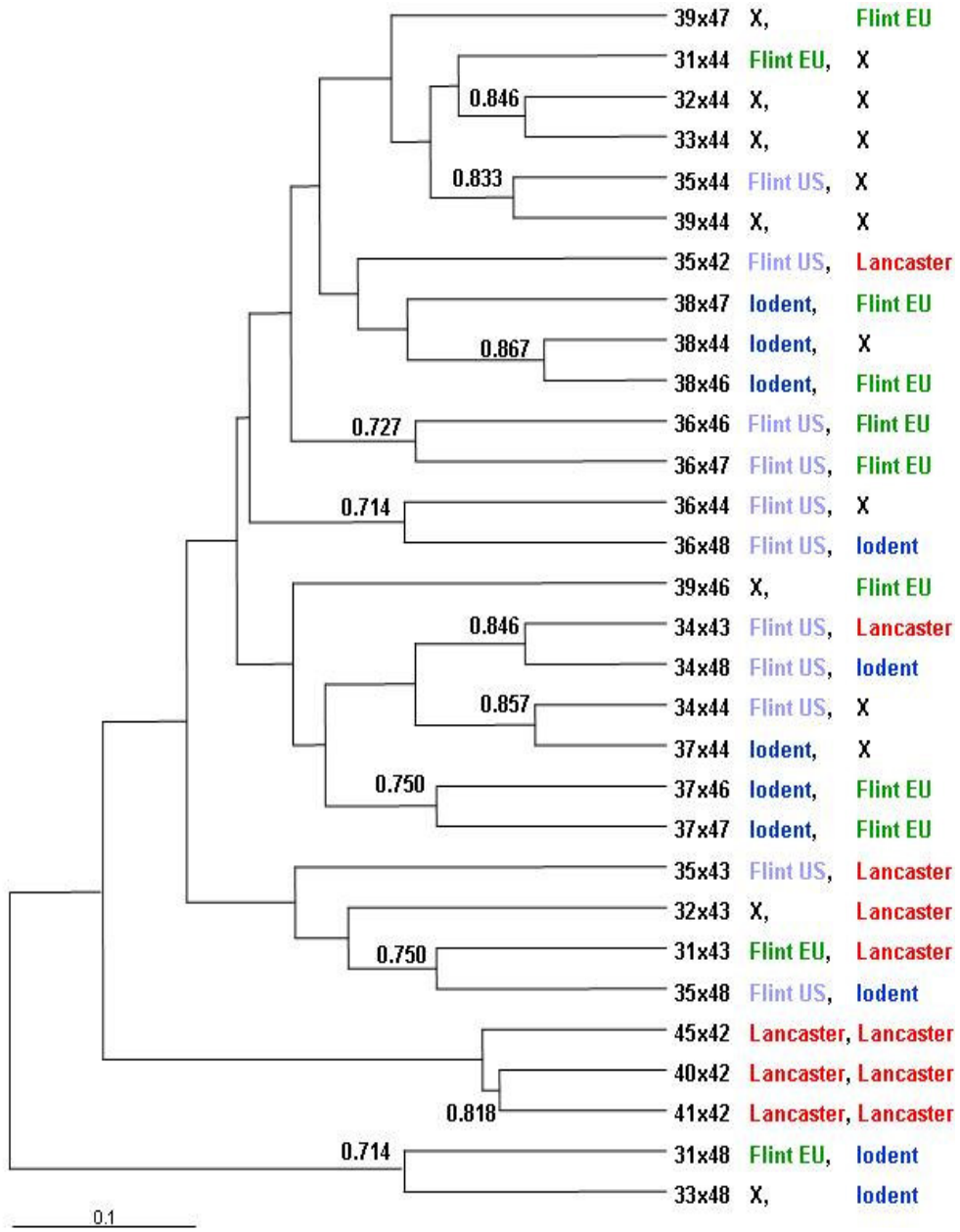

Figure 1. Dendrogram of 30 hybrids based on genetic similarity obtained by using 6 RAPD markers. X refers to lines of unknown origin.

DNA fingerprinting provides a detailed differentiation of plant material, including varieties, lines and even population accession. Such information may be utilized for verifying the authenticity of seeds and varieties, upholding copyright law, selecting the best lines for breeding, verifying pedigree originality, assessing a cultivars repertoire and its genetic diversity where heterosis breeding is used and for better estimating heterosis effect.

The selection of one or another marker in genetic diver-sity studies depends on the characteristics of the material to be used and on the objectives of the given project. The use of RAPD seems more appropriate when the objective is to cluster genotypes, because it showed similarities between the individuals. Microsatellites could be more appropriate for identifying genotypes, however, since they exhibited the genetic differences of each individual more extensively. These markers are important tools, in as much as they assess different DNA sequences in the genome of organisms and can indicate potential

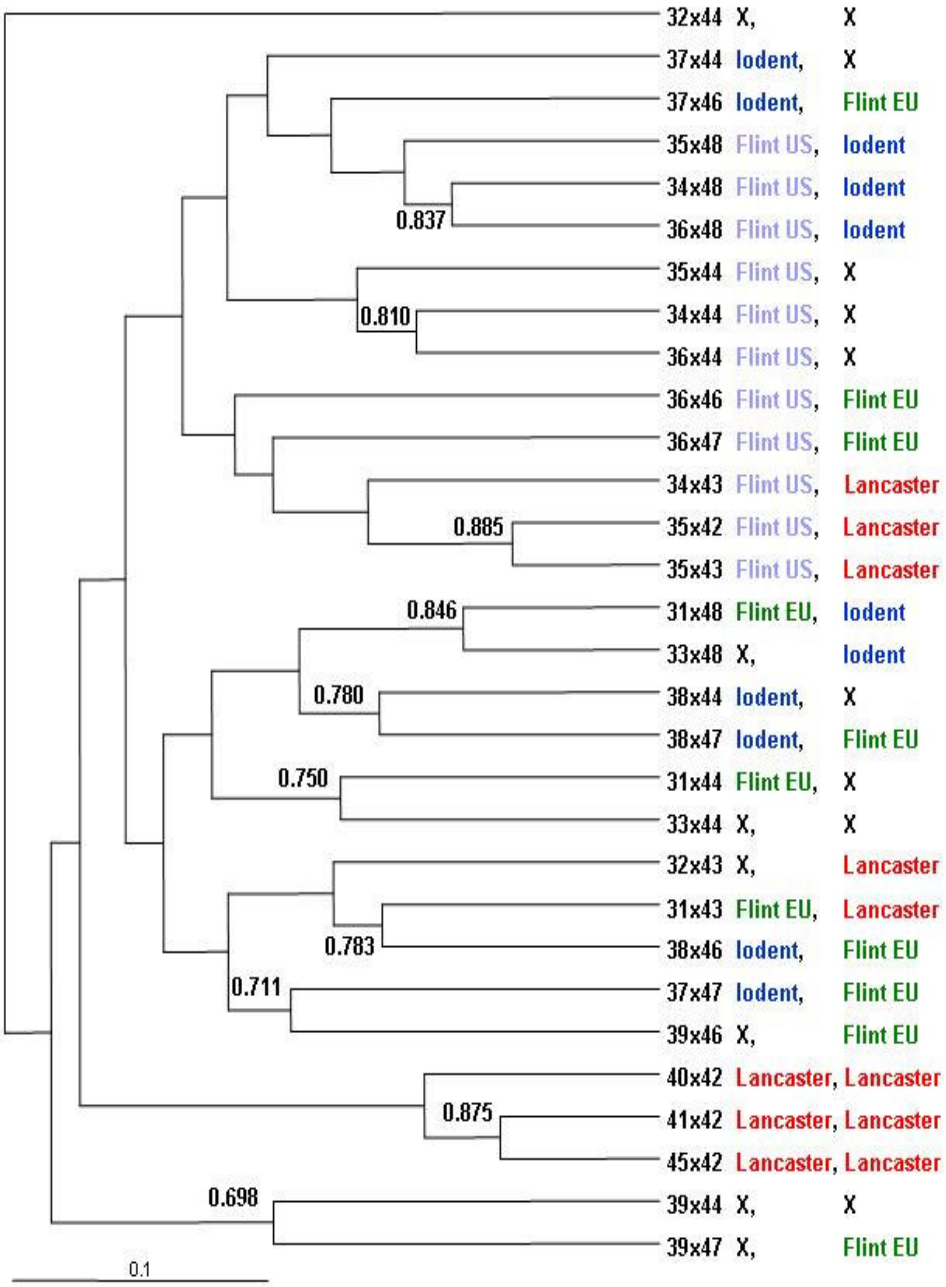

Figure 2. UPGMA dendrogram of 30 hybrids based on genetic similarity obtained using 12 SSR markers. X refers to lines of unknown origin.

results during the selection process within breeding programmes.

The set of 30 hybrids was divided into clusters based on genetic similarities using two different marker systems. The effectiveness of SSR markers is greater than that of RAPD. The genetic parameters computed demonstrated the reliability of the SSR marker system. Results obtained by SSR method are applicable for subsequent breeding strategies carried out by CEZEA– Plant Breeding Station, Inc. (Czech Republic).

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