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AFLP and SSR analysis of genetic diversity among landraces of bread wheat (*Triticum aestivum* L.em.Thell) from different geographic regions.

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Abstract

A set of 44 bread wheat landraces was used to determine the efficacy of 16-amplifed fragment length polymorphism (AFLP) primers and 63 wheat simple sequence repeat (SSR) markers in identifying polymorphisms between accessions. The SSR markers detected approximately 10 alleles per locus with a mean gene diversity (*Hz*) of 0.63, whereas AFLP primers identified approximately 147 fragments per primer with mean gene diversity of 0.25. A set of 54 SSR markers and 11 AFLP primers were identified as highly polymorphic (PIC $\geq$ 0.5 and 0.3 for SSR and AFLP, respectively), and suitable for molecular characterisation of germplasm. Principle co-ordinate analysis suggested that the AFLP and SSR loci could be used to discriminate between accessions collected from North Africa and Southern Europe from those collected from the Middle East. Both marker types indicate that accessions from North Africa and Southern Europe, the Middle East and Southern and Eastern Asia are genetically diverse. The results indicate the usefulness of the molecular markers to assess genetic diversity present within germplasm collections.

Keywords: Wheat, landraces, molecular diversity, principal coordinates analysis

Introduction

The assessment of genetic diversity is an essential component in the characterisation and conservation of germplasm (Wenguang *et al*. 1998). Various collection centres throughout the world maintain germplasm of crop species for utilization by breeding programs, and play an important role in the conservation of species diversity. Within the collections, some of the resources may be genetically redundant or duplicated and therefore not of significance. As conservation and maintenance of the germplasm is an expensive exercise, it is important to accurately characterise and catalogue germplasm. The Australian Winter Cereals Collection (AWCC) at Tamworth (New South Wales, Australia) houses more than 33000 accessions of wheat, including over 5000 accessions classified as bread wheat landraces. Genetic diversity in cultivated wheat has been narrowed due to extensive modern breeding approaches utilising a narrow genetic base, while in wheat landraces, diversity has been lost by the reduction in population size (Brown 1978; Doebley 1989). To assist with enlarging the genetic base in breeding and for conservation, it is important to assess the diversity present within these wheat landraces.

Different methods such as pedigree data, morphological traits (DeLarcy *et al.* 2000), isozymes (Guadagnoulo *et al*. 2001), storage proteins (Caballero *et al*. 2004), and molecular markers can be used for characterisation and cataloguing of germplasm (Roder *et al.* 2002). Pedigree information is not an accurate predictor of ancestral contributions to an artificially bred line (Culp 1998). Often, expression of morphological traits is influenced by a combination of genetics and the environment. Isozymes and protein markers are influenced by maturity of tissue, may be stage-

specific, and are not amenable for high throughput genetic analysis. However, DNA based markers are more reliable and better suited to high throughput characterisation of genotypes and eliminate the inherent limitations associated with morphological and biochemical methods. Furthermore, these markers measure diversity directly at the DNA level, provide an accurate estimate of phenotypic diversity (Burr *et al*. 1983), and hence are a good indication of potentially valuable germplasm held in *ex situ* collections. Different molecular markers based upon restriction fragment length polymorphism (RFLP), amplified fragment length polymorphism (AFLP), and simple sequence repeat (SSR/microsatellite) have been used to measure qualitative and quantitative variations displayed by genes in crop plants, including wheat (Lubbers *et al*. 1991; Plaschke *et al*. 1995; Paull *et al*. 1998; Wenguang *et al*. 1998; Bertin *et al*. 2001; Eujayl *et al*. 2001; Manifesto *et al*. 2001; Ahmed, 2002; Soleimani *et al*. 2002; Van Cutsem *et al*. 2003; Roussel *et al*. 2004). Unlike most of the marker systems, SSR markers are multiallelic, highly polymorphic, chromosome-specific and evenly distributed along chromosomes of wheat (Röder *et al* 1998). However, the use of a combination of techniques can increase the information content gained to a point where large amounts of beneficial data may be acquired for populations containing many individuals. SSR and AFLP markers offer a complimentary marker system, whereby SSRs offer high resolution in population structure (Neigel 1997) and AFLPs offer high resolution in genetic relationships at the individual level (Van der Wurff *et al*. 2003). Both marker technologies are highly reproducible and are suitable for high throughput analysis using automated systems. These attributes make DNA markers attractive candidates for the development of genotyping tools capable of distinguishing diverse germplasm. In addition, molecular characterisation of genetic diversity may also assist in the identification of geographic

regions containing high diversity. These regions may include germplasm with novel genes for desirable traits, such as disease resistances and quality attributes.

The objectives of this study were to 1) identify a core set of molecular markers suitable for the detection of genetic diversity in landraces of bread wheat, and 2) identify differences in genetic diversity between geographic regions.

Materials and methods

Germplasm selection and DNA extraction

Forty-four accessions of bread wheat landraces from the AWCC were selected from three geographic regions for use in identifying a set of polymorphic molecular markers (Table 1). All accessions were previously grown in field plots at the AWCC. Seed from each plot was taken to be largely self-fertilised and thus used to represent the accession. To obtain material for DNA extraction, five seeds of each accession were germinated and allowed to develop for three weeks under glasshouse conditions. Five centimetre leaf segments were taken from each of the five seedlings and used to create a pooled leaf sample. The leaf tissue was used to extract DNA following the method of Guidet *et al* (1991). The final DNA pellet was suspended in 50 µL TE buffer (10 mM Tris-HCl, pH 8.0; 1 mM EDTA).

AFLP analysis

The AFLP analysis was conducted as previously described (Vos *et al*. 1995). Approximately 1 µg of DNA was digested with either *Eco*RI and *Mse*I or *Pst*I and *Mse*I, in a 60 µL volume. Pre-amplification of restriction fragments was carried out using the primers *Eco* (5'-GACTGCGTACCAATTC-3') and *Mse* (5'-

GATGAGTCCTGAGTAA-3') or *Pst* (5'-GATGGATCCAGTGCAG-3') and *Mse*, in 25µL reactions.

Selective amplifications were performed using 16 primer combinations (Table 2). Standard Keygenes AFLP® primer nomenclature was adopted (http://wheat.pw.usda.gov/ggpages/keygene.AFLPs.html). Reactions (12 µL) contained 0.04µM WellRED (Beckman-Coulter, California, USA) fluorescently labelled forward primer (*Eco* or *Pst*), and 0.2 µM *Mse* primer, and 3 µL of a 1/20 dilution of pre-amplified template. All amplifications were performed on an AB Geneamp 2700 cycler (Applied Biosystems California, USA).

SSR analysis

The primers used in this investigation (Table 3) have been previously published (Röder *et al*. 1998; Gupta *et al*. 2002; Leigh *et al*. 2003) and were synthesised by Proligo Australia Pty. Ltd. (New South Wales, Australia). Sixty-three primers were chosen from wheat linkage and composite maps (Roder *et al*. 1998; http://www.shigen.nig.ac.jp/wheat/komugi/maps/) to cover the proximal, interstitial and distal region of each wheat chromosome. For each primer pair, the 5' end of the forward primer was synthesised with the addition of a 19 nucleotide long M13 core sequence (Rampling *et al*. 2001). The M13 core sequence was also synthesised as a separate primer with the addition of a fluorescent label (WellRED D3 or D4, Beckman-Coulter, USA) at the 5' end. Amplifications were carried out in a total reaction volume of 12 µL, containing 0.1 µM forward primer, 0.5 µM reverse primer, and 0.5 µM M13 primer. Amplifications were conducted using an AB Geneamp 2700 cycler (Applied Biosystems), using a touchdown cycle of 65°C to 55°C.

Fragment analysis

Both AFLP and SSR fragments were separated and detected using a CEQ Genetic Analysis System 8000 following manufacturers procedures (Beckman Coulter, USA). For AFLP, the amplification product (0.5 µL) was added to 27 µL sample loading solution and 0.25 µL of internal size standard. For SSR markers, 0.6 µL of D3 labelled amplification product and 0.4µL of D4 labelled amplification product were mixed with 27 µL sample loading solution and 0.25 µL of internal size standard to create a pooled sample. Sample plates were loaded onto the CEQ8000 system and then denatured at 90°C for 120sec before being separated through linear acrylamide containing 0.7 M urea, in a 33 cm, 75 µm capillary array, at 6.0 kV for 35 or 60 min (SSR and AFLP, respectively). Relative fluorescence was identified at 650 and 750 nm, using two diode lasers.

Fragments were analysed using the fragment analysis module of the CEQ8000 software, version 8.0. Peak criteria were set at 5% slope threshold, 5% relative peak height and a size estimation confidence level of 98%. A dye mobility calibration coefficient was used to correct for the addition of the WellRED labels (Beckman-Coulter).

Data analysis

AFLP fragments were binned into two nucleotide differences. A binary matrix was obtained from the fragment size data using the CEQ 8000 Genetic Analysis System software, version 8, with the Fragment Algorithm version 2.2.1 (Beckman-Coulter) to give a genotype for each accession. Only fragments with a frequency of at least 10% were used for analysis to ensure fragment artefacts were not analysed as polymorphism. The number of polymorphic fragments and Nei's measure of gene diversity (*Hz*, Nei

1987) were calculated using the program AFLP_Surv 1.0 (Vekemans 2002), The polymorphic information content (PIC) was calculated according to De Riek *et al* (2001).

For the SSR data, fragments with the highest peak (relative fluorescence) were scored in base pairs for each marker. The number of alleles, percent polymorphism and Nei's measure of gene diversity were calculated using the program GenAlEX ver5 (Peakall and Smouse 2001). The PIC for SSR markers was calculated according to Röder *et al* (1995).

A binary data set was created by converting the SSR allele data to a presence/absence (1/0) matrix, using the program LecPCR (Jarraud *et al*. 2002) and combining this with the binary data set from the AFLP analysis. Estimates of pair-wise genetic distances between individuals were calculated for the 44 core accessions according to Lynch and Milligan (1994). A principal co-ordinate (PCO) analysis was performed on the resulting matrices using MVSP ver3.13 (Kovach Computing Services, Wales, UK) and the first two axes plotted

Results

Genetic diversity at AFLP loci in wheat landraces:

Forty-four landrace accessions of bread wheat were used to determine the efficacy of 16 AFLP primer combinations in identifying polymorphism and hence genetic diversity between accessions. The setting of relative peak height and slope thresholds ensured that only reproducible peaks were analysed. A total of 2349 fragments were detected with an average of 146.8 (s.e. 19.6) fragments / primer combination and a mean of 496 fragments per accession. The *Pst* / *Mse* primer combinations identified a mean of 94.7% (s.e. 0.06) polymorphic fragments, while the *Eco* /*Mse* combinations identified a mean

of 85.1% (s.e. 1.6). For the 16 AFLP primer combinations, PIC ranged from 0.22 to 0.38 (Table 2), with the *Pst* / *Mse* and *Eco* /*Mse* primer combinations having means of 0.28 (s.e. 0.014) and 0.34 (s.e. 0.008), respectively.

Bread wheat landrace accessions were placed into groups based on the geographic region from which they originated, and analysed for diversity within and between regions. Of the 2349 fragments amplified, 1869 occurred with a frequency of 10% or greater and were used for diversity analysis Accessions from North Africa and Southern Europe contained the most number of polymorphic fragments (89.9%, Table 4), with accessions from the Middle East showing the least percentage of polymorphism (73.5%). However, Nei's measure of gene diversity (*Hz*) suggests that all regions contain a similar level of diversity at the AFLP loci examined, with accessions from the Middle East being least diverse (*Hz* = 0.23, Table 4).

Genetic diversity at SSR loci of wheat landraces

The 44-landrace accessions were used further to assess the efficacy of 63 SSR markers in identifying genetic polymorphism. The number of SSR alleles amplified by each marker ranged from 3 to 29, with a mean of 10.29 alleles amplified per primer pair. PIC values ranged between 0.24 and 0.94 (Table 3). Approximately 83% of SSR markers covering the seven chromosomes of the A, B and D genomes had a PIC value of greater than 0.5, suggesting that the majority of markers were able to detect high levels of polymorphism in the collection. GWM47 (2B), WMC25 (2D), GWM617(5A), and GWM46 (7B) identified ≥25 alleles (Table 3), indicating that these markers are highly polymorphic and suitable for molecular characterisation of wheat germplasm. The markers located on the B genome appeared to be most informative with a mean PIC of

0.72 (s.e. 0.03), compared to 0.65 (s.e. 0.04) and 0.64 (s.e. 0.05) for the A and D genome, respectively.

When the 44 accessions were separated into three geographic regions and analysed for diversity within regions, microsatellite markers indicated that accessions from the Middle East were most diverse with an *Hz* of 0.69, unlike the AFLP data which suggested that the Middle East was least diverse. Each geographic region displayed greater than 98% polymorphic loci (Table 4).

Data sets for the AFLP analysis and the SSR analysis were combined. Geographic based statistics suggest that accessions from North Africa and Southern Europe, and Southern and Eastern Asia have similar levels of allelic diversity (*Hz* = 0.24), while accessions from the Middle East had lower levels of diversity (*Hz* = 0.21). Principal co-ordinate analysis showed that the majority of accessions from Southern and Central Asia and those from the Middle East were restricted to the right half of the PCO plot and had minimal overlap with accessions from North Africa and Southern Europe (Fig.1).

Discussion

Both types of molecular markers used in this study were found to be suitable for revealing genetic diversity in landraces of bread wheat and were able to distinguish all 44 accessions examined. For the AFLP markers, *Pst* / *Mse* primer combinations amplified more fragments with a greater percentage of polymorphism compared to the *Eco* / *Mse* primers. However, the *Eco* / *Mse* primers display a higher mean PIC value. This suggests that a combination of the two sets may be more useful in assessing landrace germplasm in future experiments. All of the 63 SSR loci showed polymorphism within the collection of 44 genotypes. Fifty-four of the SSR markers

were considered to be highly polymorphic based on PIC value, and would be suited to diversity analysis of wheat germplasm collections. When the 44-landrace accession were analysed for diversity, the SSR markers detected approximately 10 alleles per locus with mean gene diversity (*Hz*) of 0.63, whereas AFLP primers identified approximately 147 fragments per primer with mean gene diversity of 0.25. While both marker types were suitable for assessing diversity within landraces accessions, there were some differences among the markers. The AFLP primers suggested that accessions from the Middle East were least diverse, having both lower percentage of polymorphic fragments and a lower *Hz* score than the other two regions. Conversely, SSR primers indicated that the accessions from the Middle East had the greatest diversity, with 100% polymorphism and the greatest *Hz* score. This is due to the different marker systems themselves. SSR markers are more efficient at resolving population structure (Neigel 1997), while AFLP markers are better suited to resolving genetic relationships at the individual level (Van der Wurff *et al*. 2003). Thus, the use of SSR markers may result in accessions from origins of diversity, such as the Middle East, having a greater number of alleles due to recombination events, and increases or decreases in microsatellite repeat number being maintained within the population. AFLP markers would be associated with individual diversity, whereby the adaptation of a population to a particular environment would decrease the amount of random diversity detected by AFLP primers. Thus regions such as Europe, which may have experienced many introductions of wheat cultivars to areas for agriculture as humankind spread from the Middle East, will have the greater AFLP diversity due to less adaptation time and a reduction in endemic species. This has been demonstrated in wild relatives of domesticated wheat by Heun *et al* (1997), who suggested that the site of Einkorn wheat domestication was associated with low diversity at AFLP loci.

The combination of marker types resulted in the majority of the North Africa and Southern European accessions being separated from those originating from the Middle East, while accessions from Southern and Eastern Asia were distributed across the PCO plot. The use of combined data based on AFLP and SSR markers is useful in the analysis of genetic diversity and population structure. Traditionally, SSR markers have been used in population studies. However, in investigations where the population structure is not fully known or is weak, SSRs are constrained by the low number of loci per individual (Cornuet *et al*. 1999). Increasing the number of loci examined is a possible way to overcome such issues (Bernatchez and Duchesne 2000). Thus, the use of AFLP in these situations is a useful way to improve the validity of studies in which population structure is unknown, such as with the study of germplasm collections. Further investigations are underway to determine the effectiveness of diversity arrays (DArT; Jaccoud *et al*. 2001) in determining the relationship between accessions collected from proposed regions of diversity.

The use of molecular markers for DNA profiling may allow for the identification of accessions that contain unique, agronomically important traits for future use in breeding programs. In recent years, a number of trait-marker associations have been identified in wheat, using SSR markers (Gupta *et al*. 1999; Langridge *et al*. 2001). SSR markers can be used to identify novel alleles linked to traits of importance to various breeding programs. Such marker-based assessment of novel genes eliminates the need for extensive test crossing, and instead relies upon the conservation of the linkage between the marker and gene of interest (Raman *et al*. 2003). Molecular markers can be employed for this purpose, as has been demonstrated for discriminating among barley disease resistance loci that confer indistinguishable resistance phenotypes (Garvin *et al.* 1997). In addition, markers such as expressed sequence tags, derived from the

transcribed portion of the genome, have been shown to be informative in diversity analysis (Bandopadhyay *et al*. 2004; Gao *et al*. 2004). These markers would add significant benefits when used in conjunction with AFLP and traditional SSR markers by allowing scrutiny of gene diversity. By examining accessions from geographic regions with distinct environmental indicators with DNA markers, it may be possible to identify fine scale eco-regions from which to collect and maintain valuable germplasm. Furthermore, integration of molecular marker data with morphological data on height, disease resistance, tolerance to abiotic stress and adaptability to specific environments will allow us to establish genetic disequilibrium between landraces collected from diverse agro-climatic conditions.

In conclusion, identification of polymorphic AFLP and SSR marker sets will facilitate in cataloguing of landraces held within the AWCC. These markers are currently being used to catalogue wheat germplasm from ecologically important regions. Availability of information on genetic diversity will allow wheat breeders to exploit genetic variation for their breeding program and allow for the collection of germplasm from specific geographic regions containing high genetic diversity.

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Table 1. Information on the forty-four wheat landraces used in this study. Origin of the accessions are given for each of the three regions.

Region	Origin	Number of accessions
North Africa and Southern Europe	Algeria, Morocco, Tunisia, Bulgaria, Croatia, Spain, Portugal, Italy, Greece, Bosnia & Herzegovina	18
The Middle East	Iran, Iraq, Syria, Turkey, Afghanistan, Jordan	12
Southern and Central Asia	China, Nepal, India, Pakistan	14

Table 2. Selected primer combinations and polymorphism levels for AFLP analysis of 44 landrace accessions of wheat. Polymorphic information content (PIC) was calculated according to De Riek *et al* (2001).

Primer Combination	Number of fragments	% Polymorphic fragments	PIC
P 32 / M 50	295	95.3	0.29
P 32 / M 51	289	92.4	0.31
P 32 / M 55	245	94.3	0.30
P 32 / M 60	90	92.2	0.34
P 32 / M 54	162	95.1	0.27
P 32 / M 48	157	96.2	0.24
P 32 / M 58	202	95.0	0.29
P 32 / M 49	126	96.8	0.22
E 40 / M 59	115	80.0	0.34
E 40 / M 48	81	82.7	0.31
E 40 / M 61	159	79.9	0.35
E 40 / M 62	160	81.9	0.34
E 38 / M 48	91	87.9	0.34
E 38 / M 59	39	87.2	0.31
E 38 / M 62	82	90.2	0.38
E 38 / M 61	56	91.1	0.33

Table 3: 63 SSR markers and their polymorphism in 44 landrace accessions of bread wheat used in this study. Polymorphic information content (PIC) was calculated according to Röder *et al* (1995).

Marker	Location	Alleles	PIC	Marker	Location	Alleles	PIC
BARC 213	1A	17	0.86	*GWM 192*	4D	3	0.32
GWM 666	1A	11	0.82	*WMC 048*	4D	9	0.65
WMC 304	1A	8	0.55	*WMC 285*	4D	6	0.69
GWM 011	1B	10	0.82	*GWM 617*	5A	29	0.94
GWM 413	1B	10	0.81	*GWM 291*	5A	8	0.80
GWM 498	1B	7	0.42	*GWM 415*	5A	5	0.64
GDM 111	1D	7	0.82	*GWM 118*	5B	4	0.55
WMC 036	1D	3	0.29	*GWM 604*	5B	4	0.47
WMC 147	1D	7	0.49	*GWM 499*	5B	13	0.85
GWM 636	2A	10	0.69	*WMC 096*	5D	6	0.27
GWM 388	2A	4	0.24	*WMC 097*	5D	15	0.86
WMC 198	2A	21	0.93	*WMC 190*	6A	3	0.33
GWM 047.1	2B	25	0.92	*WMC 233*	6A	11	0.45
GWM 210	2B	6	0.56	*GWM 639*	6A	8	0.55
BARC 159	2D	7	0.29	*BARC 003*	6A	12	0.80
WMC 170	2D	6	0.65	*GWM 459*	6A	10	0.84
WMC 025	2D	25	0.83	*GWM 626*	6B	5	0.65
DuPw 227	3A	8	0.81	*GWM 219*	6B	8	0.65
WMC 050	3A	8	0.74	*GWM 132*	6B	19	0.82
BARC 057	3A	6	0.59	*BARC 021*	6D	23	0.89
GWM 285	3B	10	0.72	*GWM 325*	6D	15	0.88
GWM 533	3B	5	0.62	*BARC 183*	6D	12	0.76
GWM 389	3B	13	0.84	*DuPw 254*	7A	7	0.58
BARC 270	3D	5	0.24	*WMC 247*	7A	7	0.63
BARC 042	3D	13	0.85	*GWM 060*	7A	10	0.39
GWM 114	3D	14	0.78	*GWM 333*	7B	8	0.75
DuPw 004	4A	6	0.54	*GWM 046*	7B	29	0.89
BARC 184	4A	19	0.82	*GWM 611*	7B	20	0.87
GWM 006	4B	12	0.87	*GDM 150*	7D	6	0.61
GWM 495	4B	9	0.77	*GWM 044*	7D	15	0.81
GWM 113	4B	7	0.66	*GWM 295*	7D	12	0.83
GWM 149	4B	7	0.67				

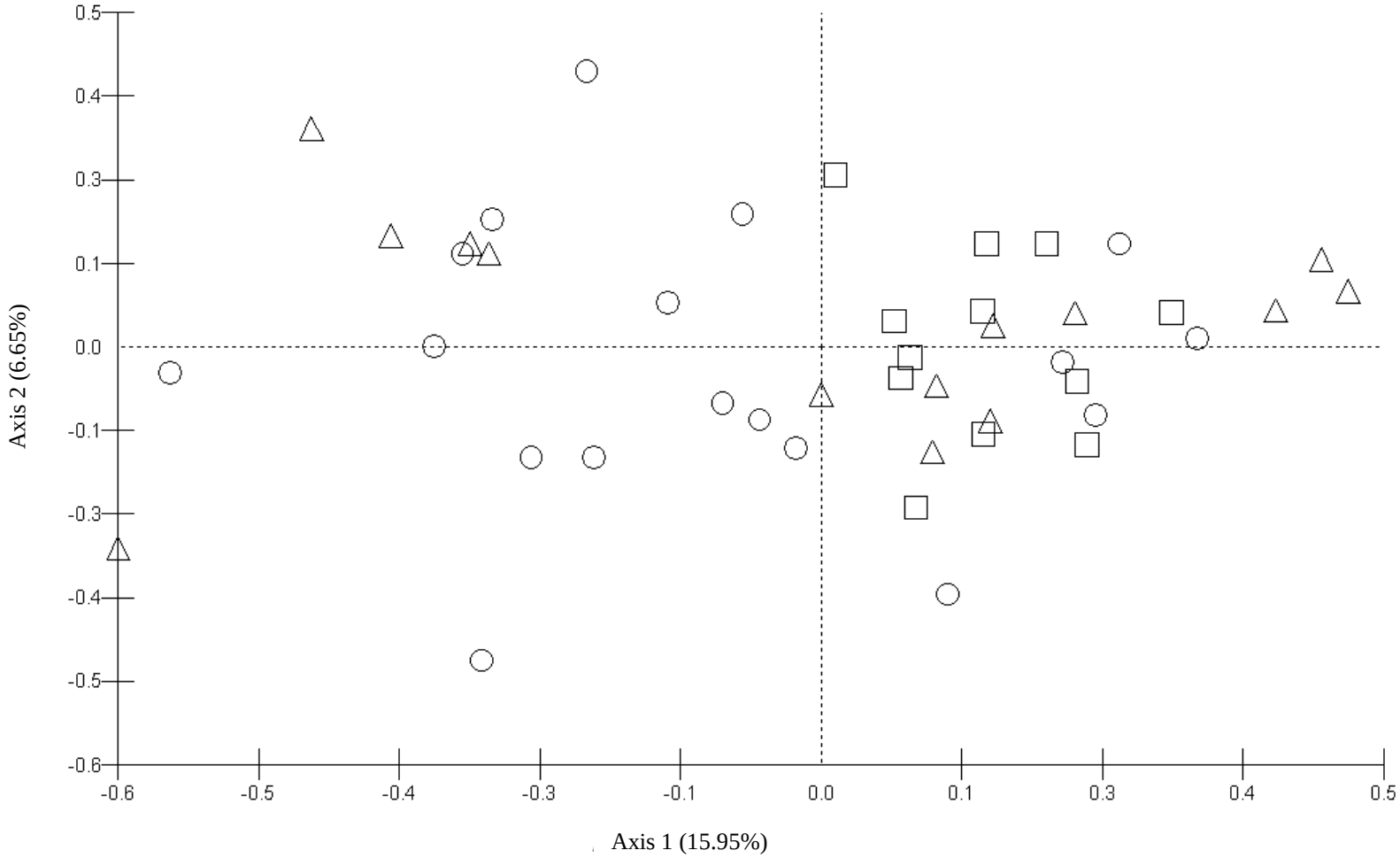
Table 4. AFLP and SSR polymorphisms and genetic diversity revealed with 16 AFLP primer combinations and 63 SSR primers for the 44 landrace accessions of wheat from 3 regions. Calculations were performed using the statistical software *AFLP-Surv* 1.0 (Vekemans, 2002) and the Microsatellite tool kit (Park, 2001). *Hz* = Nei's measure of gene diversity. Standard errors are in parenthesis.

		AFLP markers		SSR markers	
Region	Number of Accessions	Polymorphic fragments (%)	*Hz*	Polymorphic loci (%)	*Hz*
North Africa / Southern Europe	18	89.9	0.24 (0.003)	98.4	0.67 (0.03)
Middle East	12	73.5	0.23 (0.003)	100.0	0.69 (0.03)
Southern/Eastern Asia	14	83.9	0.24 (0.003)	98.4	0.68 (0.03)

Figure legends

Fig 1 Principal co-ordinates analysis revealing the relationship between the AFLP and SSR profiles of 44 wheat accessions and their geographic region. Pair-wise genetic distances were estimated by the method of Lynch and Milligan (1994), after the SSR data was converted to a binary matrix.

◯ North Africa / Southern Europe □ Middle East △ Southern/Eastern Asia


0.5
0.4
0.3
0.1
0.0
-0.1
-0.3
-0.4
-0.5
-0.6
-0.6
-0.5
-0.4
-0.3
-0.1
0.0
0.1
0.3
0.4
0.5
Axis 2 (6.65%)
Axis 1 (15.95%)

1

2 **Fig.1**