

# Specific genetic markers for wheat, spelt, and four wild relatives: comparison of isozymes, RAPDs, and wheat microsatellites

Roberto Guadagnuolo, Dessislava Savova Bianchi, and François Felber

**Abstract:** Three types of markers—isozymes, RAPDs (random amplified polymorphic DNAs), and wheat microsatellites—were tested on wheat, spelt, and four wild wheat relatives (*Aegilops cylindrica*, *Elymus caninus*, *Hordeum marinum*, and *Agropyron junceum*). The aim was to evaluate their capability to provide specific markers for differentiation of the cultivated and wild species. The markers were set up for subsequent detection of hybrids and introgression of wheat DNA into wild relatives. All markers allowed differentiation of the cultivated from the wild species. Wheat microsatellites were not amplified in all the wild relatives, whereas RAPDs and isozymes exhibited polymorphism for all species. The dendrograms obtained with RAPD and isozyme data separated Swiss wheat cultivars from those collected in Austria and England, while no difference was found between Swiss spelt and wheat. RAPD data provided a weak discrimination between English and Austrian *E. caninus*. The microsatellite-based dendrogram discriminated populations of *Ae. cylindrica*, but no clear separation of *H. marinum* from *E. caninus* was revealed. The similarity matrices based on the three different sets of data were strongly correlated. The highest value was recorded between the matrices based on RAPDs and isozymes (Mantel's test,  $r = 0.93$ ). Correlations between the similarity matrix based on microsatellites and matrices based on RAPDs and isozymes were lower: 0.74 and 0.68, respectively. While microsatellites are very useful for comparisons of closely related accessions, they are less suitable for studies involving less-related taxa. Isozymes provide interesting markers for species differentiation, but their use seems less appropriate for studies of within-species genetic variation. RAPDs can produce a large set of markers, which can be used for the evaluation of both between- and within-species genetic variation, more rapidly and easily than isozymes and microsatellites.

**Key words:** Triticeae, isozymes, RAPDs, microsatellites, polymorphism.

**Résumé :** Trois types de marqueurs—les isozymes, les RAPDs (« random amplified polymorphic DNAs ») et les microsatellites du blé—ont été testés sur six espèces appartenant à la tribu des Triticeae, le blé, l'épeautre et quatre espèces sauvages apparentées (*Aegilops cylindrica*, *Elymus caninus*, *Hordeum marinum* et *Agropyron junceum*). Le but de l'étude était d'évaluer leur capacité de générer des marqueurs spécifiques pour la différenciation des espèces cultivées et sauvages. Les marqueurs ont été produits afin de pouvoir détecter ultérieurement de l'introgression d'ADN du blé dans les espèces apparentées. Tous les types de marqueurs ont permis de séparer les espèces cultivées des sauvages. Les microsatellites du blé n'ont pas été amplifiés dans toutes les espèces sauvages, alors que les RAPDs et les isozymes ont détecté du polymorphisme chez toutes les espèces. Les dendrogrammes obtenus à partir des données RAPD et isozymes distinguaient les variétés de blé autrichienne et anglaises des suisses, alors qu'aucune séparation entre blé et épeautre suisses n'y était observée. Une légère discrimination entre populations anglaises et autrichiennes d'*E. caninus* a été obtenue avec les marqueurs RAPD. Les populations d'*Ae. cylindrica* étaient séparées dans le dendrogramme obtenu avec les microsatellites du blé, alors que ce dernier groupait *H. marinum* et *E. caninus*. Les matrices de similarité basées sur les trois types de marqueurs étaient fortement corrélées. La valeur la plus élevée a été obtenue entre la matrice des RAPD et celle des isozymes (test de Mantel,  $r = 0,93$ ). Entre la matrice basée sur les microsatellites et celles basées sur les RAPD et les isozymes, la corrélation était plus faible, 0,74 et 0,68 respectivement. Alors que les microsatellites sont très utiles pour la comparaison de lignées fortement apparentées, ils sont moins appropriés pour l'étude de taxons relativement éloignés. Les isozymes peuvent générer suffisamment de marqueurs pour différencier les espèces, mais ils sont moins adaptés pour l'étude de la diversité génétique intraspécifique. Les RAPDs peuvent produire un grand nombre de marqueurs utiles pour l'étude de la diversité inter- et intra-spécifique, plus rapidement et facilement que les isozymes et les microsatellites.

**Mots clés :** Triticeae, isozymes, RAPDs, microsatellites, polymorphisme.

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## Introduction

The usefulness of molecular tools for identifying species and clarifying intergeneric relationships in the tribe Triticeae is well documented (e.g., Ørgaard and Heslop-Harrison 1994; Svitashv et al. 1996, 1998; Sun et al. 1997). Moreover, hybrids between wheat and many wild and cultivated Triticeae have an impact on crop breeding (Limin and Fowler 1990; Friebe et al. 1992; Seifers et al. 1995; Yuan et al. 1997). They are also important for the cultivation of transgenic wheat in regions where wild relatives are abundant and close to cultivated areas, because of the risks of transgene escape into the wild (Seefeldt et al. 1998; Zemetra et al. 1998). A set of specific markers is thus needed to easily identify species, hybrids, and cross-derived individuals.

Isozymes have largely been used in population genetic studies because of their codominance and their aptitude for detecting genetic variation among and within natural populations (Heun et al. 1994; Linhart and Grant 1996). In the past, they have also provided useful markers for selection in several crops (Ainsworth et al. 1984; Nielsen and Johansen 1986). More recently, PCR-based markers have allowed the characterisation of the highly variable noncoding sequences of the genome. They permitted the detection of high levels of genetic variation, even in taxa considered to be highly monomorphic, such as cultivars or autogamous species, which is the case for most of the Triticeae. RAPD (random amplified polymorphic DNA) and microsatellite (or simple sequence repeat (SSR)) markers have become two of the most useful tools in population genetic studies (Williams et al. 1990; Dhar et al. 1997; Bauert et al. 1998; Cao et al. 1998; Gabrielsen and Brochman 1998; Strelchenko et al. 1999) and marker-assisted selection (Yang et al. 1994; Plaschke et al. 1995; Sánchez de la Hoz et al. 1996; Akagi et al. 1997; Milbourne et al. 1998). In particular, microsatellite markers are presently considered to be the most powerful tool for detecting genetic variation between and within species (Morgante and Olivieri 1993; Röder et al. 1995; Sun et al. 1999).

In recent years, the efficiency and usefulness of some of the most-used genetic markers—isozymes, RAPDs, SSRs, AFLPs (amplified fragment length polymorphisms), and RFLPs (restriction fragment length polymorphisms)—have been compared and discussed in several papers (i.e., Powell et al. 1996; Chan and Sun 1997; Sun et al. 1999). Most of these comparisons were performed to study crop genetic variability (Bohn et al. 1999), to find markers for crop selection (Schachermayr et al. 1995, 1994; Lübberstedt et al. 1998), and to study relationships between and within closely related taxa (Sun et al. 1997; Svitashv et al. 1998) or within the same species (Sun et al. 1999). All these studies, performed mostly on very closely related taxa, searched for the maximum level of polymorphism between and within species. In the present study, our first priority was genetic variation between species, whereas variation between populations of the same species was of secondary relevance. Therefore, comparisons among the three different techniques used—isozymes, RAPDs, and microsatellites—focused on the ability of the technique to provide a large set of specific markers for interspecific differentiation rapidly and easily rather than on the possibility of detecting polymorphism at a low taxonomic level. The markers set up were necessary for subse-

quent studies related to concerns linked to the cultivation of genetically engineered crops and were aimed at detecting possible introgressions of wheat DNA into the genomes of wild species (Guadagnuolo et al. 2001a, 2001b). Thus, with regard to the choice of markers, particular emphasis was placed on the differentiation of wheat from the wild species.

For the same reasons expressed above, the choice of wild species was based on their aptitude to hybridise naturally with spelt and wheat, as inferred from the literature, and was restricted to species growing in the northern part of Europe: *Aegilops cylindrica* Host., *Elymus caninus* L., *Hordeum marinum* s.str. Hudson, and *Agropyron junceum* (L.) Beauv (Fedak 1985; Sharma and Baezinger 1986; Maan 1987; van Slageren 1994).

The objectives of this study were (i) to differentiate wheat from four wild relatives with specific genetic markers and (ii) to compare the efficiency of three different types of markers—isozymes, RAPDs, and microsatellites—for this differentiation.

## Materials and methods

### Plant material

Seeds of the four wild species (Table 1) were collected in Switzerland, Austria, and England. In each population sampled (Table 2), a representative sample of 20–40 spikes (1 spike/plant) was collected. Three populations of *Ae. cylindrica* (Switzerland), two of *A. junceum* (England), two of *H. marinum* (England), and six of *E. caninus* (England and Austria) were sampled. In Austria and England, wheat (*Triticum aestivum*) seeds were also collected in the immediate proximity of the wild-relative populations, to detect possible introgression of wheat DNA into wild genomes. In addition, 13 Swiss wheat varieties (seven winter wheats and six spring wheats) and three spelt varieties (*Triticum spelta*; seeds obtained from Eric Schweizer Samen AG (CH-3602 Thun) were included in the study. Seeds were sown and plants cultivated in the Botanical Garden of Neuchâtel.

### Isozyme analysis

After testing three different extraction buffers, protein extraction from all regenerated plants was carried out by grinding two young leaves per plant in 1 mL of 0.1 M sodium acetate solution (pH 7.2). The extracts were then centrifuged at 12 000 rpm and the supernatant stored at  $-80^{\circ}\text{C}$  for subsequent isozyme analysis. A representative sample of each species or variety was chosen to be tested with 22 enzyme systems on starch and polyacrylamide gels.

Polyacrylamide gels (2.5 mm thick) were prepared according to Gasquez and Compoin (1976), as modified by Lumaret (1981): sample gels were 9% acrylamide plus 0.165% bis-acrylamide; stacking gels were 2.5% acrylamide (1 cm long); separation gels were 9% acrylamide and 0.165% bis-acrylamide (7.5 cm long). Forty microlitres of each sample (mixed with 20  $\mu\text{L}$  of bromophenol blue dye) was electrophoresed in Tris-glycine buffer (pH 8.6) at  $4^{\circ}\text{C}$  under the following conditions: 10 min at 600 V and 6 pulsations/s (pps), 20 min at 230 V and 7 pps, and 2.5 h at 600 V and 7 pps.

Starch gels (12%) were prepared according to Pasteur et al. (1987) and Wendel and Weeden (1989). Two gel-migration buffer systems were tested: borate (pH 8.2) (Pasteur et al. 1987) and histidine-citrate (pH 6.5) (Poulik 1957).

Protein separation was performed within 2 days after extraction. Enzyme staining was carried out as described in Savova Bianchi (1996). Several 10s of plants of each species were analyzed in other studies (Guadagnuolo et al. 2001a, 2001b), but only those also analyzed with DNA markers were considered in the present study (Table 2).

**Table 1.** Species studied and their genetic characteristics.

Species	Common name	Genome formula	Ploidy level	Reference
<i>Triticum aestivum</i> L.	Common or bread wheat	AABBDD	2n = 6x = 42	Miller 1987
<i>Triticum spelta</i> L.	Spelt wheat	AABBDD	2n = 6x = 42	Miller 1987
<i>Aegilops cylindrica</i> Host.	Jointed goatgrass	CCDD	2n = 4x = 28	Miller 1987
<i>Elymus caninus</i> L.	Bearded wheatgrass or bearded couch	SSHH	2n = 4x = 28	Sun et al. 1997
<i>Agropyron junceum</i> (L.) Beauv.	Sea wheatgrass	JuJu	2n = 2x = 14	Sharma 1996
<i>Hordeum marinum</i> s.str. Hudson	Sea barley	XX	2n = 2x = 14	Baum and Johnson 1998

**Table 2.** Species and individuals sampled for genetic analyses (performed in 1998).

Species	Country	Populations/varieties	Code	No. of plants analyzed with isozyme and DNA markers	
<i>Agropyron junceum</i>	England (GB)	Wells-next-the-Sea (52°59'N, 0°51'E)	Aj.4GB	3	
		Old Hunstanton (52°58'N, 0°32'E)	Aj.5GB	2	
<i>Elymus caninus</i>	England (GB) and Austria (Aut)	Warboys Wood Nature Reserve (2 subpopulations) (52°25'N, 0°05'W)	Ec.2GB	10	
		Horncastle (53°9'N, 0°08'W)	Ec.6GB	5	
		Scottlehorpe (52°46'N, 0°26'W)	Ec.7GB	5	
		Sonthey Wood, Peterborough (52°35'N, 0°22'W)	Ec.8GB	5	
		Achleiten (2 subpopulations) (48°5'N, 14°11'E)	Ec.1Aut	10	
		Kaltenbach (2 subpopulations) (48°16'N, 13°53'E)	Ec.2Aut	10	
<i>Hordeum marinum</i>	England (GB)	Wolferton, Norfolk (2 subpopulations) (52°51'N, 0°27'E)	Hm.3GB	10	
		Sonthey Wood, Peterborough (52°35'N, 0°22'W)	Hm.9GB	5	
<i>Aegilops cylindrica</i>	Switzerland (CH)	Brig (46°19'N, 8°00'E)	Aec.1CH	11	
		Sierre (46°18'N, 7°33'E)	Aec.2CH	11	
		Saillon (46°11'N, 8°11'E)	Aec.3CH	10	
<i>Triticum aestivum</i>	Switzerland	13 Swiss varieties (Arina, Galaxie, Tamaro, Boval, Runal, Hessischer Landweizen, Probus, Golin, Balmi, Greina, Albis, Frisal, Lona)	Tae.CH.var.	13 bulks of 10	
		Austria	1 Austrian variety (Favorit)	Tae.Aut.var.	1 bulk of 10
		England	3 English varieties (unknown)	Tae.GB1-3	3 bulks of 10
<i>Triticum spelta</i>	Switzerland	3 Swiss varieties (Oberkulmer, Ostro, Sertel)	Ts. CH.var.	3 bulks of 10	

### DNA analysis

Total DNA extraction from a single leaf was carried out on 10 or 11 samples per population for *Ae. cylindrica*, on five samples per population (or subpopulation) for *H. marinum* and *E. caninus*, and on all five samples of *A. junceum* obtained. For each wheat and spelt variety, the extraction was performed on a bulk of 10 plants. A simple SDS – sodium acetate protocol was used. DNA was re-suspended in a TE solution (1× TE: 10 mM Tris-HCl, 1 mM EDTA, pH 8.0) at a concentration of 30 ng/mL and stored at –20°C.

PCR for both RAPD and microsatellite amplifications was performed in 25-µL volumes under the following conditions.

### RAPDs

In each PRC tube, the concentrates were 1× PCR buffer, 1.5 mM MgCl<sub>2</sub>, 0.4× Q-solution (Qiagen AG, Basel), 0.2 mM dNTP, 0.2 µM primer, 0.03 U/µL Taq polymerase (Qiagen AG, Basel), and 1 ng/µL template DNA. Amplifications were performed in a Biometra I thermocycler using the following profile: initial de-

naturation at 94°C for 5 min, followed by 35 cycles of 1 min at 93°C, 1 min at 45°C, and 1 min at 72°C. Final extension was 10 min at 72°C. PCR products were mixed with a 1/5 volume of loading buffer and separated on a 1.6% (w/v) agarose gel (containing 0.4 µg/mL ethidium bromide) in 0.5× TBE (1× TBE: 90 mM Tris-borate, 2 mM EDTA, pH 8.0) at 60 V for 2 h. DNA fragments were then visualized under UV light.

Thirty-five primers of the series OPB 1–20, OPP 1–20, and OPT 1–20 (Operon Technologies, Alameda, Calif.) were tested on three morphologically pure individuals per species; eight primers were selected (OPB 6, 8, and 10 and OPP 6, 7, 8, 9, and 14), as they amplified specific markers for each species. The selection was also based on the number of amplified fragments and on the reproducibility of the results (at least two PCRs for each sample and each primer). The selected primers were then used on the whole set of samples.

### Microsatellites

In each PRC tube, the concentrates were 1× PCR buffer, 1.5 mM

MgCl<sub>2</sub>, 0.4× Q-solution (Qiagen AG, Basel), 0.2 mM dNTP, 0.6 nM each primer, 0.03 U/μL Taq polymerase (Qiagen AG, Basel), and 1 ng/μL template DNA. Amplifications were performed in a Biometra I thermocycler using the following profile: initial denaturation at 93°C for 3 min, followed by 45 cycles of 1 min at 93°C, 1 min at 55°C, and 2 min at 72°C. Final extension was 10 min at 72°C. PCR products were mixed with a 1/5 volume of loading buffer and separated on a 6% polyacrylamide gel in 0.5× TBE at 100 V for 6 h. Gels were stained in a 0.4 μg/mL ethidium bromide bath and DNA fragments were visualized under UV light.

Six wheat microsatellite primers pairs (WMS 43, 44, 46, 47, 106, and 159) allowing amplification of the more polymorphic fragments were selected from 12 tested and publicly available wheat microsatellite primers pairs covering the A, B, and D genomes of wheat (Plaschke et al. 1995; Röder et al. 1995).

The microsatellites chosen were described as being present in a specific genome of *T. aestivum* and *T. spelta* (Plaschke et al. 1995; Röder et al. 1995). Moreover, only those situated in the D genome were expected to be amplified in *Ae. cylindrica*. Despite these considerations, we used the same primers for all the species, because some of them were able to amplify species-specific bands in wild relatives not necessarily possessing the D genome, as already demonstrated by Sun et al. (1997) for *Elymus* species.

#### Data analysis

The data obtained with the three techniques were scored in a binary form as presence or absence (1 and 0) of bands for each individual or bulk (in the case of the wheat DNA samples).

In a first step, the data were analyzed by cluster analysis using the CLUSTER package (<http://www.biology.ualberta.ca/jbrzusto>), to determine if species and populations formed groups according to their genetic characteristics. The asymmetrical Jaccard's coefficient ( $J_j$ ) was used to calculate similarity between samples:

$$J_j = a/(a + b + c)$$

where  $a$  is the number of fragments present in both individuals and  $b$  and  $c$  are the number of fragments present exclusively in individual 1 and exclusively in individual 2, respectively. This coefficient does not take into account the double absence of fragments, which is closer to reality, considering that the absence of a DNA fragment or isozyme band in two samples is an absence of information rather than an element of similarity.

The similarity matrices obtained with the three sets of data— isozyme, RAPD, and microsatellite—and with the pooled data were used to generate UPGMA (unweighted pair-group method with arithmetic averaging) dendrograms, visualized with TREEVIEW (Page 1996).

To test the statistical significance of groups determined by cluster analysis, as well as the discrimination of species and populations for each genetic marker, Mantel tests (999 permutations) were performed using the R4 (Beta version) package (P. Casgrain and P. Legendre, Département des sciences biologiques, Université de Montréal, Montréal, Québec). Similarity matrices obtained by genetic data were converted into distance matrices and compared pairwise (microsatellite–RAPD, microsatellite–isozyme, RAPD–isozyme, isozyme – combined data, RAPD – combined data, and microsatellite – combined data) and with a generated matrix in which a distance value of 0 was assigned between two samples of the same species and a value of 1 was assigned between two samples of different species. Mantel statistics calculate a linear correlation between the distance matrix and the generated matrix and test its significance.

## Results

### Isozymes

Among 22 isozyme systems tested on starch and polyacrylamide gels, four were monomorphic and 16 did not produce interpretable bands. Two enzyme systems, GOT (glutamate oxaloacetate transaminase) and PRX (peroxidase) on polyacrylamide gels, were selected to be used for the whole set of individuals, as they were polymorphic. Twenty-one isozyme bands were produced in total, of which 19 were polymorphic between species (Fig. 1). Eleven bands were polymorphic between as well as within species. The number of bands produced by the two systems was almost identical, 10 and 11 for the GOT and PRX systems, respectively. However, GOT showed less polymorphism within species than PRX, although it produced at least one specific band for each species. PRX was polymorphic both between and within species, but two bands were present in all samples of all species.

### RAPDs

The eight RAPD primers used on the whole set of plants produced a total of 126 polymorphic fragments (none of the fragments was present in all the species) (Fig. 2). For all individuals, a maximum of 20 fragments were amplified with primer OPB-08, while only 10 were obtained with primer OPP-07. An average of 42.7 fragments per individual were produced. Swiss varieties of wheat amplified between 49 and 55 fragments, while only 44 were amplified by Austrian and English varieties.

Primer OPB-10 amplified one fragment that was present only in individuals of the populations Aec.1 and Aec.3 of *Ae. cylindrica*, while primer OPP-08 amplified a band specific for population Aec.2.

### Microsatellites

Among all individuals, 27 polymorphic fragments (i.e., none of these fragments was present in all the species) were amplified using the six wheat microsatellite primer pairs. Three primer pairs (WMS 43, 44, and 159) amplified four fragments each, whereas one (WMS 47) produced five fragments (Fig. 3). The primer pair WMS 46 produced eight polymorphic markers, whereas only two were produced by WMS 106. In spite of its low polymorphism, this latter primer pair amplified one marker specific for populations Aec.1 and Aec.3 of *Ae. cylindrica* that was absent in all samples of population Aec.2 of the same species.

Primer pairs WMS 47, 106, and 159 did not amplify any fragments in *E. caninus*, *A. junceum*, or *H. marinum*.

### Genetic differentiation

Cluster analyses based on similarity matrices clearly separated wild species from cultivated ones, regardless of type of marker.

In the dendrogram obtained with isozymes, the species formed separated clusters, with the exception of *T. aestivum* and *T. spelta*, which grouped together (data not shown). Almost no polymorphism was detected within the populations of wild species. The ability to differentiate the species by this technique was confirmed by a high Mantel test correla-

Fig. 1. Zymogram of the species for two enzyme systems: glutamate oxaloacetate transaminase and peroxidase.

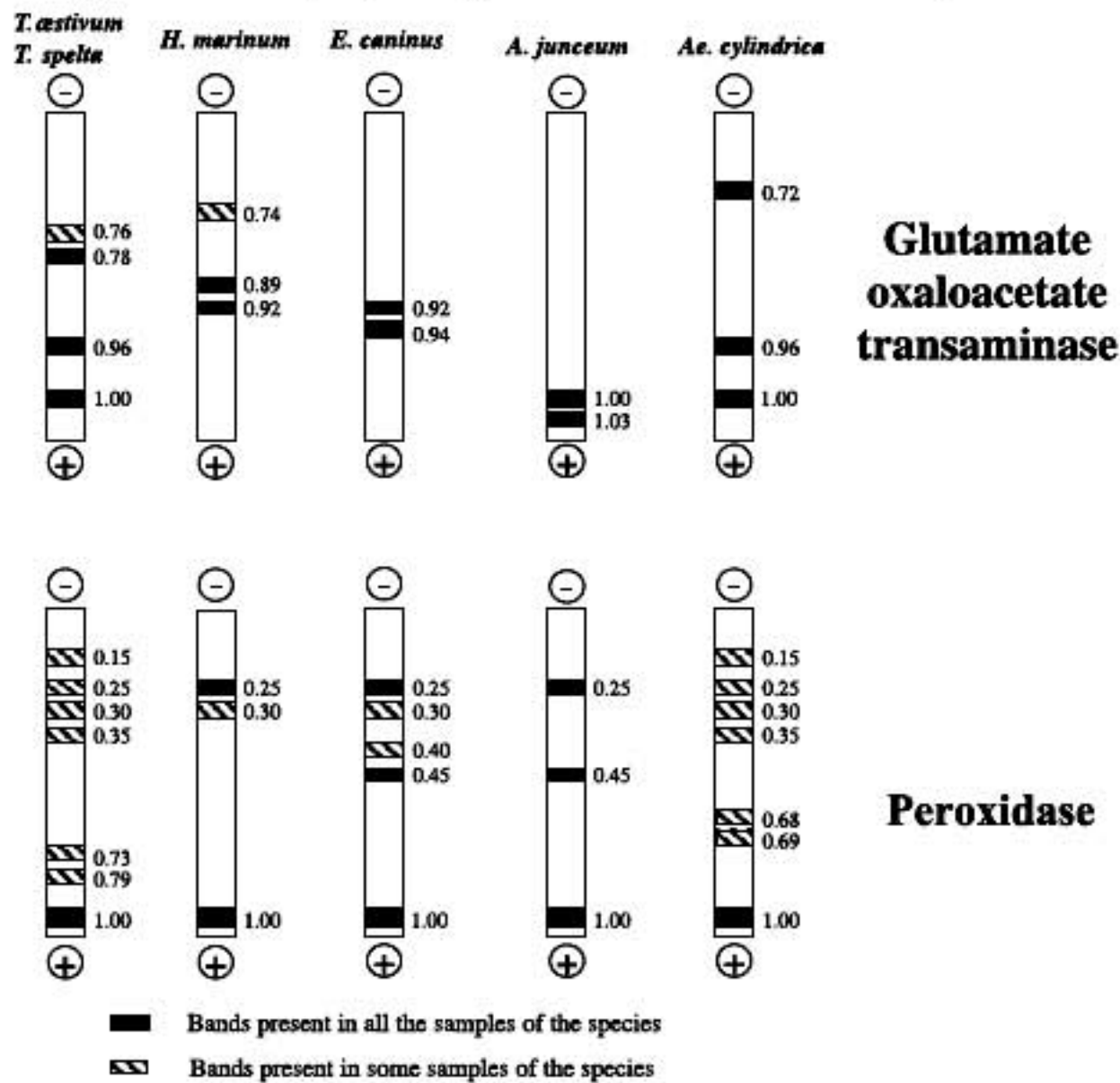
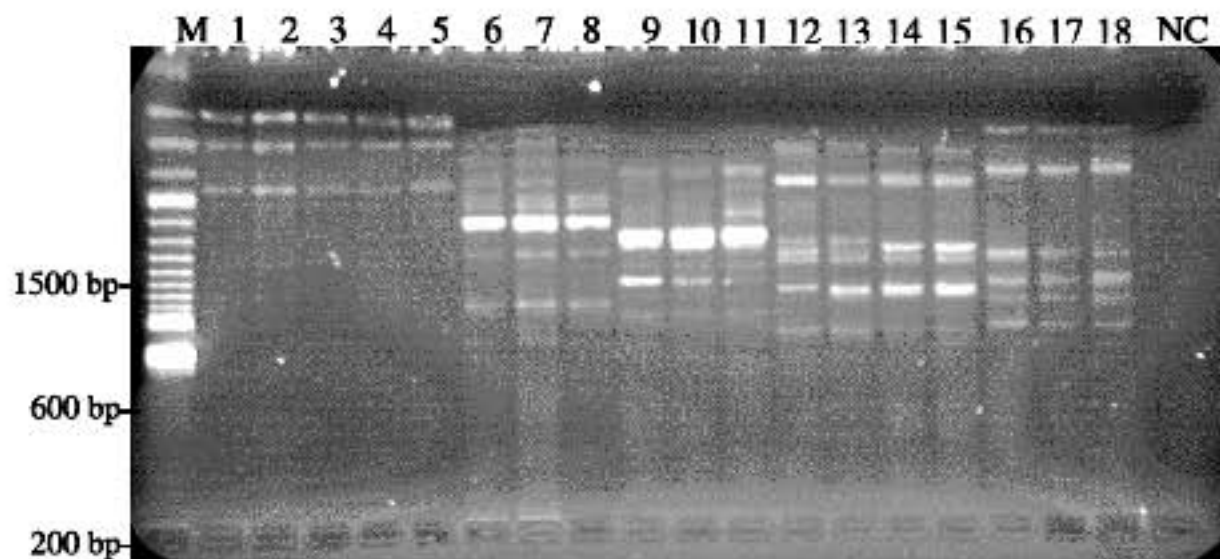


Fig. 2. Genetic polymorphism between species as revealed by RAPD primer OPB-08. Lanes: M, 100-bp DNA ladder (GibcoBRL®, Life Technologies); 1 and 2, Tae.CH; 3, Tae.GB; 4, Tae.Aut; 5, Ts.CH; 6-8, Aec.CH; 9-11, Hm.GB; 12 and 13, Ec.GB; 14 and 15, Ec.Aut; 16-18, Aj.GB; and NC, negative control.



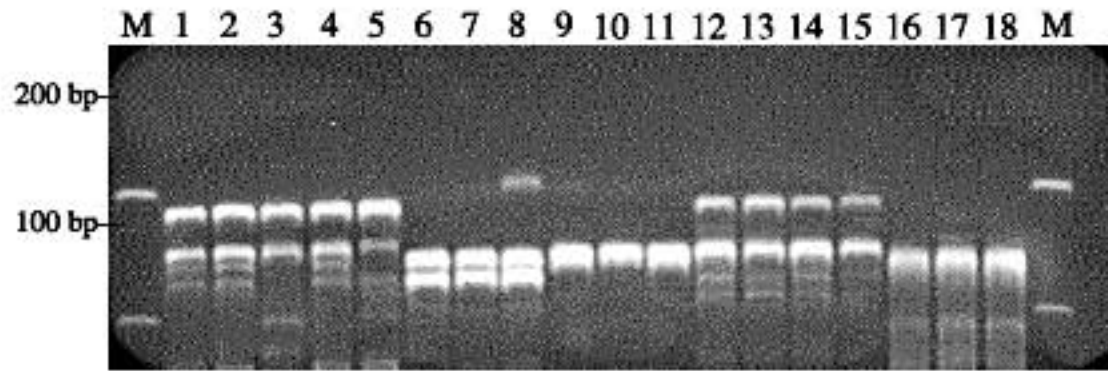
tion value ( $r = 0.90$ ) between isozyme and species distance matrices (Table 3).

With regard to the separation of the species, similar results were obtained with RAPD data (Fig. 4). In this case as well, individuals of different species, with the exception of wheat and spelt, clustered separately. The correlation calcu-

lated between RAPD and species matrices was even higher than for isozymes, namely,  $r = 0.94$ .

Both RAPD and isozyme data indicated that *Ae. cylindrica* was the closest relative of wheat among the species studied, with a mean distance ranging from 0.6 for isozyme data to 0.7 for RAPD data. In addition, little separa-

Fig. 3. Example of polymorphism between species as revealed by wheat microsatellite primer WMS-43. Lanes: M, 100-bp DNA ladder (GibcoBRL®, Life Technologies); 1 and 2, Tae.CH; 3, Tae.GB; 4, Tae.Aut; 5, Ts.CH; 6–8, Aec.CH; 9–11, Hm.GB; 12 and 13, Ec.GB; 14 and 15, Ec.Aut; and 16–18, Aj.GB.



tion of Austrian and English wheat varieties from Swiss ones was obtained with either data set. Moreover, the Mantel test correlation calculated between the isozyme and RAPD distance matrices ( $r = 0.93$ ) confirmed the congruence of the results obtained with these two types of markers.

The results obtained using microsatellite data differed considerably from the others (Fig. 5). The separation of the species was less clear, with some individuals of *H. marinum* clustering together with *E. caninus*. This led to a Mantel test correlation value for the separation of the species that was clearly lower than those for the RAPD and isozyme data: 0.72 versus 0.94 and 0.90, respectively (Table 3). *Aegilops cylindrica* clustered far from wheat, and the calculated distance between these two species (0.85) was even higher than those between wheat and the other wild relatives. In contrast, microsatellite data clearly separated one population of *Ae. cylindrica* (Aec.2, Sierre) from the other two, which were situated about 50 km east (Aec.1, Brig) and west (Aec.3, Saillon). Moreover, Mantel test correlation values between microsatellite and RAPD similarity matrices (0.74) and between microsatellite and isozyme matrices (0.68) were consistently lower than those between RAPD and isozyme matrices (0.93) (Table 3).

None of the markers showed significant genetic differences between English and Austrian populations of *E. caninus*. However, the RAPD-based dendrogram, as well as the one constructed using the combined data, separated the six populations into two groups: one composed of the Austrian populations and the English population of Wistow Wood Nature Reserve (Ec.2GB) and one that included the other three English populations. The separation of Swiss from Austrian and English wheat cultivars was clearer in the dendrogram based on the combined data (data not shown) than in those based only on RAPD or isozyme data.

The dendrogram obtained using the combined data showed a higher similarity with those based on RAPDs and isozymes than with the one based on microsatellites. This was confirmed by Mantel tests comparing the combined data similarity matrix with those obtained with the three separate data sets (Table 3).

## Discussion

All three methods used provided useful markers for species differentiation. The highest number of specific markers, which was clearly correlated with the large number of frag-

Table 3. Mantel tests on the distance matrices obtained with the three types of genetic data compared with a matrix generated for the species (where a distance of 1 was assigned between individuals of different species and 0 was assigned between individuals of the same species) (comparison 1), pairwise (comparison 2), and with a matrix obtained with the combined data set (obtained by grouping the three sets of data (isozyme, RAPD, and microsatellite)) (comparison 3).

	Correlation
<b>Comparison 1</b>	
Isozymes vs. species	0.90**
RAPDs vs. species	0.94**
Microsatellites vs. species	0.72**
Combined data vs. species	0.95**
<b>Comparison 2</b>	
Isozymes vs. RAPDs	0.93**
Isozymes vs. microsatellites	0.68**
RAPDs vs. microsatellites	0.74**
<b>Comparison 3</b>	
Combined data vs. isozymes	0.94**
Combined data vs. RAPDs	0.99**
Combined data vs. microsatellites	0.77**

Note: The  $r$  statistics were calculated from Mantel's permutation test. Bonferroni's correction was applied to adjust significance levels for multiple comparisons. All differences were highly significant (\*\*,  $P < 0.01$ ).

ments amplified with short primers, was obtained with the RAPD technique.

The number of polymorphic isozyme markers is limited and reflects only variation in the coding parts of the genome, which is by nature more conservative and thus less polymorphic. The screening of enzyme systems in this study confirmed these expectations. Indeed, only two among 22 enzyme systems tested provided markers useful for differentiating closely related and essentially autogamous species. In addition, within Triticeae, several amphiploids, and especially the hexaploid wheats, often produce complex electrophoretic patterns that are difficult to interpret because of the presence of multilocus isozymes (Hart 1983). Despite these disadvantages, which are specifically related to polyploid species, the two enzyme systems used produced clearly distinguishable specific markers.

The reliability of isozyme markers has been demonstrated (e.g., Sharp et al. 1988). However, the activity of several en-

Fig. 4. UPGMA dendrogram obtained using the RAPD similarity matrix (based on Jaccard's similarity coefficient).

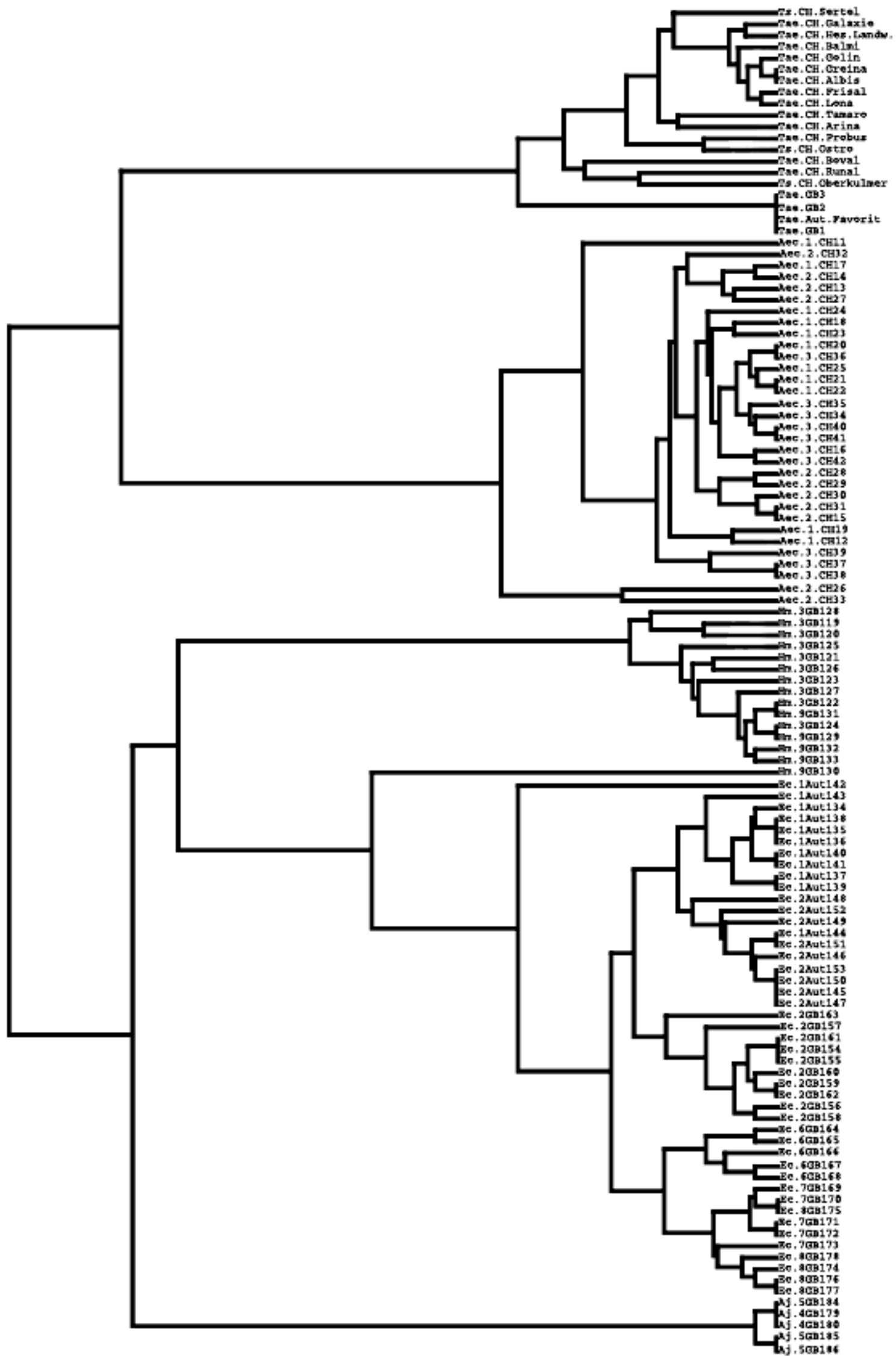
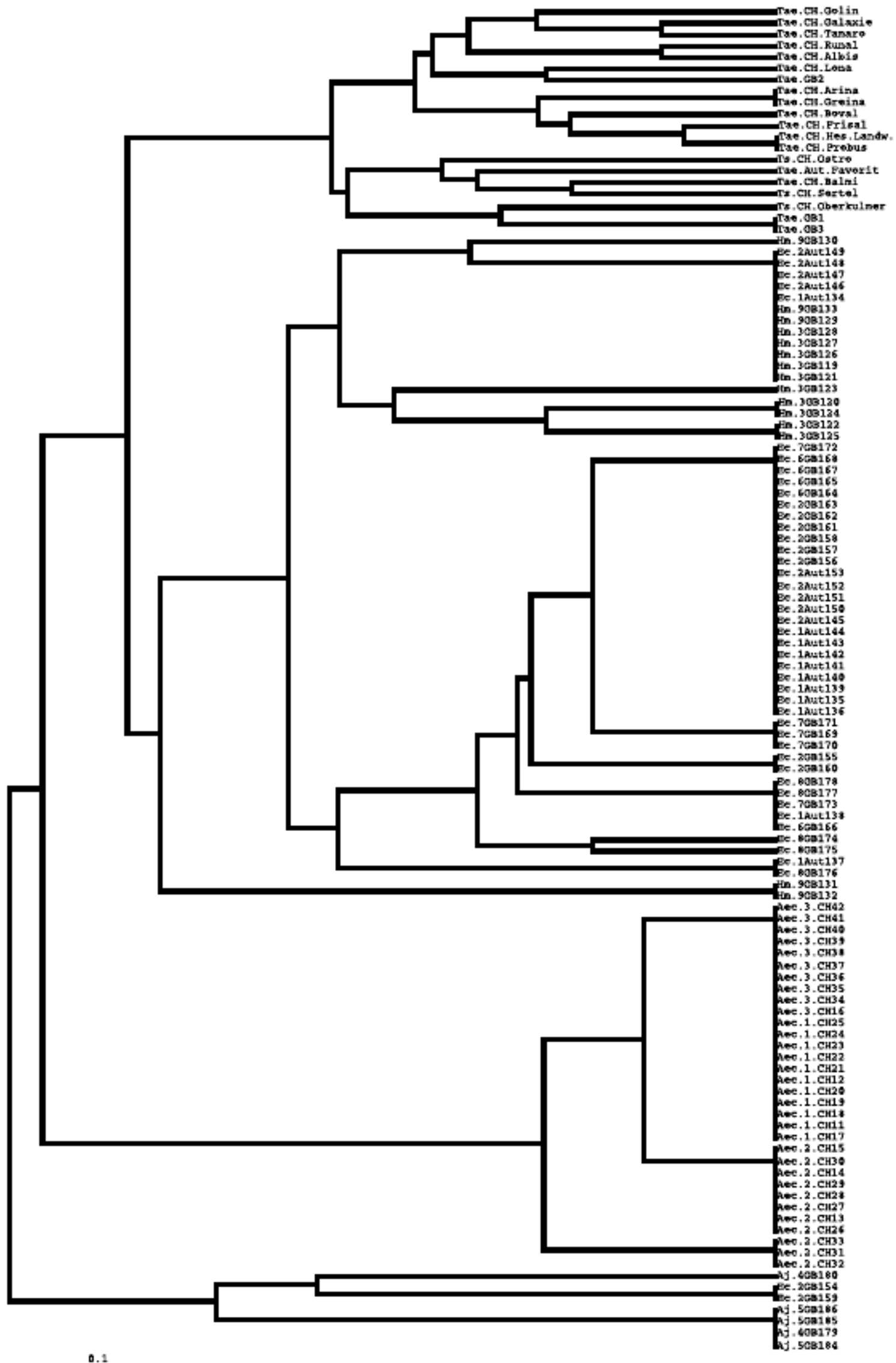


Fig. 5. UPGMA dendrogram obtained using the wheat microsatellite similarity matrix (based on Jaccard's similarity coefficient).



zyme systems (e.g., the peroxidases) may be modified, qualitatively and quantitatively, during a plant's life cycle. These modifications are related to several physiological and ecological factors, such as flowering, senescence, attack by pathogens, or extreme temperatures (e.g., Akatsu and Watanabe 1978). This may cause biases, if protein extractions are performed on samples of tissues collected at different stages of plant development. To avoid such biases, the leaves sampled were all collected at the same stage of development.

RAPD amplifications provided the largest set of polymorphic markers. This is not surprising, since RAPDs can detect variation in both coding and noncoding sequences, and the length of the primers allows the amplification of a large number of fragments with a single primer. Compared with the other two techniques used, it appeared to be the most suitable for this kind of study and had the lowest cost in time and money. Even if the dominance of RAPD markers remains a problem for population genetic studies, it is possible to overcome it, if specific fragments for each taxon or group of taxa compared are produced.

Advantages and disadvantages of each technique are determined by, among other factors, the ease of use, the cost in time and money, and the type of information produced. Notwithstanding the progress in population genetics and plant breeding due to PCR-based markers, several studies have questioned their reliability as well as their efficiency (Haymer 1994; Lynch and Milligan 1994). Criticism about the reproductibility of RAPD results is also often expressed. Nevertheless, accurate application of protocols allows possible artefacts due to random amplification with short primers to be avoided. Moreover, in the present study, most of the amplifications were performed at least twice and even three times for the samples used in the preliminary screening of the primers, to ascertain their effectiveness and reproductibility.

The number of fragments amplified with wheat microsatellite primers was relatively low compared with the number of RAPD fragments (27 vs. 126). The principal reason was probably the higher specificity of amplifications with microsatellite primers, which are longer than RAPD primers and allow the amplification of only a few fragments with a single primer pair. In addition, the amplified microsatellites were identified in wheat DNA, thus only a few were discovered in the wild species.

The hypervariability and codominant character of microsatellites, as well as their reliability, have already made them the ideal substitute for the robust but less polymorphic RFLPs in plant marker-assisted selection (Röder et al. 1995; Taramino et al. 1997; Milbourne et al. 1998; Senior et al. 1998). Their usefulness in population genetic studies is also well documented (Sun et al. 1998a, 1998b). However, their use as nonspecific primers could require extensive screening of primers to amplify enough specific markers.

Regarding the use of crop-specific microsatellite primers to differentiate one or more closely related species, the principal questions are (i) to define whether testing a great number of existing primers for a related species consumes less time and money than identifying specific sequences and primers and (ii) if the accuracy of the "random" amplification of fragments with nonspecific primers is reliable enough. The steps of identifying, cloning, and sequencing

prior to the synthesis of specific microsatellite primers for PCR amplification are laborious and expensive and have been well documented (e.g., Powell et al. 1996). Nevertheless, with regard to the results of the present study and those in the literature (Sun et al. 1997; Lübberstedt et al. 1998), we can conclude that only in the case of a large screening of non-species-specific primers would it be possible to avoid the steps necessary to identify microsatellites in the species studied. This is reinforced when such primers are used on remotely related species.

Nevertheless, if the goal is to differentiate crops from wild relatives, the use of crop-specific microsatellites can easily produce crop-specific markers, which can be extremely useful for detecting the introgression of crop DNA into wild relatives.

Nonspecific amplifications may cause difficulties in the genetic determination of the amplification products, which are avoided if bands are scored as presence-absence (Plaschke et al. 1995; Sun et al. 1997, 1999; the present study).

#### Data analysis and genetic differentiation

Isozyme data (not shown) separated most of the species clearly but showed almost no genetic variation within the species. Only two enzyme systems could be used and, consequently, a relatively small number of bands were produced, which explains these results. More unexpected was that, with these markers, English and Austrian cultivars of *T. aestivum* formed a separate cluster within that of the Swiss ones, while no clear separation between Swiss cultivars of *T. spelta* and *T. aestivum* was observed. This reflects the fact that each country started to develop its own cultivars of these two species a long time ago, which has probably led to a divergence in their genetic characteristics, while hybridisation between them (performed either in Switzerland or in other countries) for wheat improvement (Winzeler et al. 1993) has brought the two species closer genetically. These observations were confirmed by the RAPD-based dendrogram. This confirms that the ability of enzyme markers to generate polymorphism at a low taxonomic level is limited, as discussed above.

The best-resolved dendrogram was obtained using RAPD data (Fig. 4). Principal-coordinate analysis carried out on distance matrices based on the three types of genetic data (data not shown) confirmed the results obtained with the clustering calculations. Indeed, the best separation of the species, with the exception of the cultivated ones, was also obtained with RAPD data. As described above, this is certainly due to the much larger number of markers obtained for each species with this technique compared with the others.

High levels of genetic diversity have been detected by the RAPD technique, even for plants considered to be highly monomorphic (e.g., see Gabrielsen and Brochman 1998). The separation of the *E. caninus* populations into two groups (three English vs. one English and the two Austrian populations) using RAPD data agrees with these results. Contrasting with this, Sun et al. (1999) have already described *E. caninus* as a highly monomorphic species in a study that included the analysis of a large number of geographically distant Eurasian populations using isozymes, RAPDs, and microsatellites. Genetic diversity found within and between

these populations was very low, even with data obtained using specific microsatellites, which are considered to be the most polymorphic type of marker (Powell et al. 1996; Sun et al. 1999).

Sun et al. (1997, 1999) established the usefulness of microsatellite markers for finding polymorphism within and among populations of wild species. As well, the genetic diversity in several crops was assessed using microsatellite polymorphism (Plaschke et al. 1995; Sánchez de la Hoz et al. 1996; Akagi et al. 1997; Chan and Sun 1997; Senior et al. 1998; Bohn et al. 1999). It is important to point out that all these studies were performed to find polymorphism at a low taxonomic level (species, subspecies, or even varieties of crops) or in highly autogamous species. In all these cases, the genetic polymorphism was expected to be low, but microsatellites could generate specific markers because of the high mutation rate in repetitive DNA (slippage, etc.).

The use of crop microsatellites to characterize related species is probably not the most appropriate but its usefulness has already been demonstrated (Sun et al. 1997; Lübberstedt et al. 1998). Moreover, in our study, it allowed wheat to be separated clearly from the other species studied, which was our first priority. Compared with RAPDs and isozymes, microsatellite data showed a weaker separation of the species, especially for those more remotely related to wheat. This is certainly due to the small number of fragments amplified in these species using wheat microsatellite primers. As expected, the repetitive sequences situated on the D genome of wheat and spelt were successfully amplified in *Ae. cylindrica*, whereas none of the microsatellites situated in the other two genomes were identified in this wild relative. However, three of the wheat microsatellite primer pairs amplified fragments in all the wild relatives. The presence of these sequences in different species (or genera) is probably due to the reticulated relationships existing within tribe Triticeae.

Among the species studied, microsatellite analyses indicated that *Ae. cylindrica* is genetically the most distant from wheat, even though it is considered to be one of the most closely related species among all the Triticeae, with one parent in common (*Aegilops squarrosa* L.) supplying the D genome. This contradiction depends on the way the data were scored and on the type of analyses that were performed. In the present study, bands were scored for presence-absence and no inference on genetic determinism was carried out. Because most of the amplified microsatellites were situated in the D genome, more fragments were amplified in *Ae. cylindrica* than in the other wild relatives and most of these fragments were specific. Jaccard's similarity coefficient is negatively correlated with the number of specific markers. The large number of specific bands of *Ae. cylindrica* allowed good discrimination of this species from wheat and led to low Jaccard's similarity coefficients. Comparatively, the other wild relatives amplified fewer specific markers. This led, paradoxically, to higher Jaccard's similarity coefficients between unrelated species than between *Ae. cylindrica* and wheat. It should be noted that all binary coefficients, whether double absence is included or not (e.g., Sørensen's, simple matching, Kulczynski's, etc.), produce such biases.

In spite of a limited set of primers, only microsatellites allowed the separation of a newly discovered population of *Ae.*

*cylindrica* (Aec.2, Sierre) from two others (Aec.1, Brig and Aec.3, Saillon). Jointed goatgrass is an adventive species in Switzerland (Hess et al. 1967) and no previous indications for this population were found in the floristic literature and herbaria. The populations of Brig and Saillon, however, have been known to exist for at least 90 years (van Slageren 1994; J. Keller Senften, personal communication). Nevertheless, all population sites that were found are in very disturbed habitats (train stations and roadsides). Therefore, the genetic differences observed between populations could be due either to (i) a different origin for the new population (jointed goatgrass is supposed to have been introduced into Switzerland by road or rail transport from the Aosta Valley (Italy) (Hess et al. 1967) but it is possible that population Aec.2, Sierre, derives from another geographic region (e.g., France)) or, less probably, (ii) longer selective pressure operating on the two old populations (e.g., by the common and large use of herbicides in the types of habitat where these populations were found).

The results obtained using the combined set of data synthesized the results obtained with each technique considered separately. However, it is interesting to observe that, in the combined-data dendrogram, Swiss varieties of wheat were separated more clearly from the Austrian and English ones than in the dendrograms based on RAPDs and isozymes. This supports the results obtained by Sun et al. (1999) and suggests that the combination of different kinds of markers to detect genetic diversity could be more useful and perhaps less laborious than searching for the most polymorphic type of marker.

The extremely high similarity between the combined-data and RAPD similarity matrices can be easily explained by the fact that each descriptor (isozyme band, RAPD, or microsatellite fragment) is considered in the same manner and the number of RAPD descriptors (126) was much higher than the number of isozyme or microsatellite descriptors (21 and 27, respectively).

## Conclusions

In contrast with data in the literature, our results indicate that RAPDs and isozymes resolve the separation of the species more clearly than microsatellites and are probably more appropriate for differentiating taxa that are genetically relatively distant. However, microsatellites are particularly useful for differentiating very closely related or highly monomorphic taxa.

A combination of different kinds of markers can generate enough polymorphism to evaluate genetic relationships even within monomorphous species.

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