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Gene flow in selected crops and related weeds. Risk assessment for the field release of GMOs: case of wheat (*Triticum æstivum* L.) in Northern and Middle Europe

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IMPRIMATUR POUR LA THESE

Gene Flow in selected crops and related weeds. Risk assessment for the field release of GMOs: case of wheat (*Triticum aestivum* L.) in Northern and Middle Europe

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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; Svitashev 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; Svitashev 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 subsequent 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. 2001*a*, 2001*b*). 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 æstivum*) 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°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 Compoint (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 μL of bromophenol blue dye) was electrophoresed in Tris-glycine buffer (pH 8.6) at 4°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. 2001*a*, 2001*b*), 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
Triticum æstivum L.	Common or bread wheat	AABBDD	$2n = 6x = 42$	Miller 1987
Triticum spelta L.	Spelt wheat	AABBDD	$2n = 6x = 42$	Miller 1987
Aegilops cylindrica Host.	Jointed goatgrass	CCDD	$2n = 4x = 28$	Miller 1987
Elymus caninus L.	Bearded wheatgrass or bearded couch	SSHH	$2n = 4x = 28$	Sun et al. 1997
Agropyron junceum (L.) Beauv.	Sea wheatgrass	JuJu	$2n = 2x = 14$	Sharma 1996
Hordeum marinum 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
Agropyron junceum	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
Elymus caninus	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
Hordeum marinum	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
Aegilops cylindrica	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
Triticum æstivum	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
Triticum spelta	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 resuspended 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 $MgCl_2$, 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 1 thermocycler using the following profile: initial denaturation 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 vizualized 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

$MgCl_2$, 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 1 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 vizualized 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. æstivum* 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 (I_j) was used to calculate similarity between samples:

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

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. æstivum* 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.

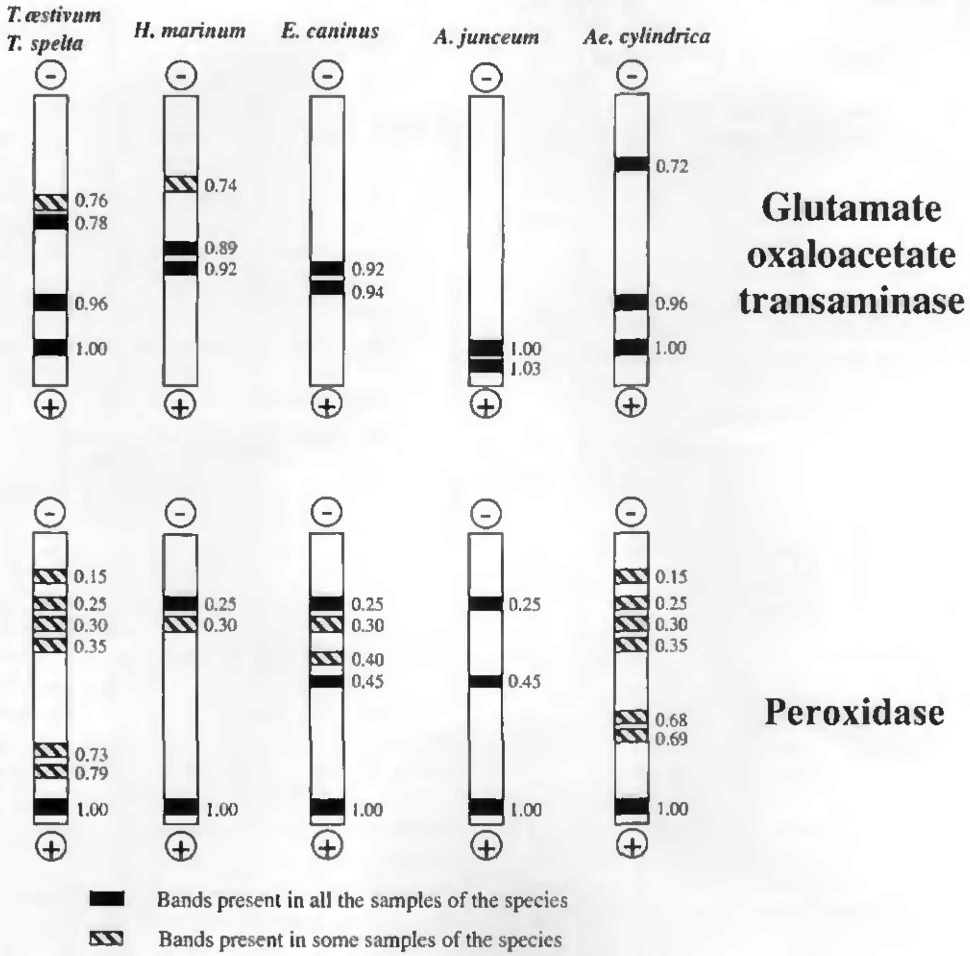
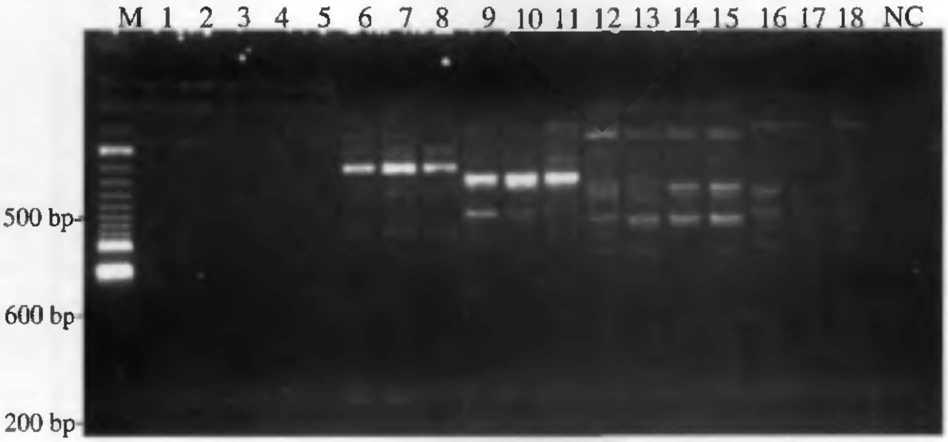


Fig. 2. Genetic polymorphism between species as revealed by RAPD primer OPB-08. Lanes: M, 100-bp DNA ladder (GibcoBRL®, Life Technologies); 1 and 2, Tae.CH; 3, Tae.GB; 4, Tae.Aut; 5, Ts.CH; 6–8, Aec.CH; 9–11, Hm.GB; 12 and 13, Ec.GB; 14 and 15, Ec.Aut; 16–18, Aj.GB; and NC, negative control.

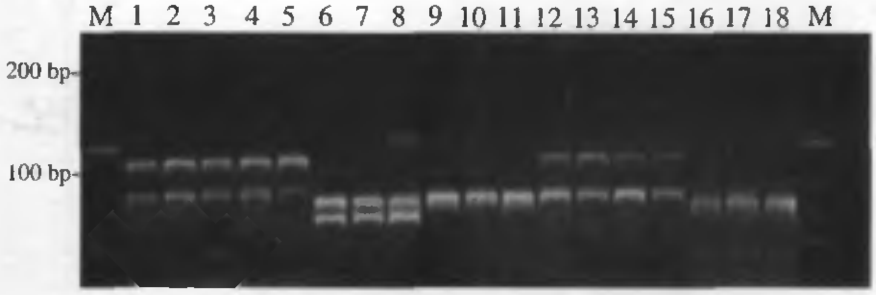


tion value ($r = 0.90$) between isozyme and species distance matrices (Table 3).

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 calculated 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 fragments amplified with short primers, was obtained with the RAPD technique.

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) (comparison1), 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
Comparison 1	
Isozymes vs. species	0.90**
RAPDs vs. species	0.94**
Microsatellites vs. species	0.72**
Combined data vs. species	0.95**
Comparison 2	
Isozymes vs. RAPDs	0.93**
Isozymes vs. microsatellites	0.68**
RAPDs vs. microsatellites	0.74**
Comparison 3	
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 (**, $P < 0.01$).

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).

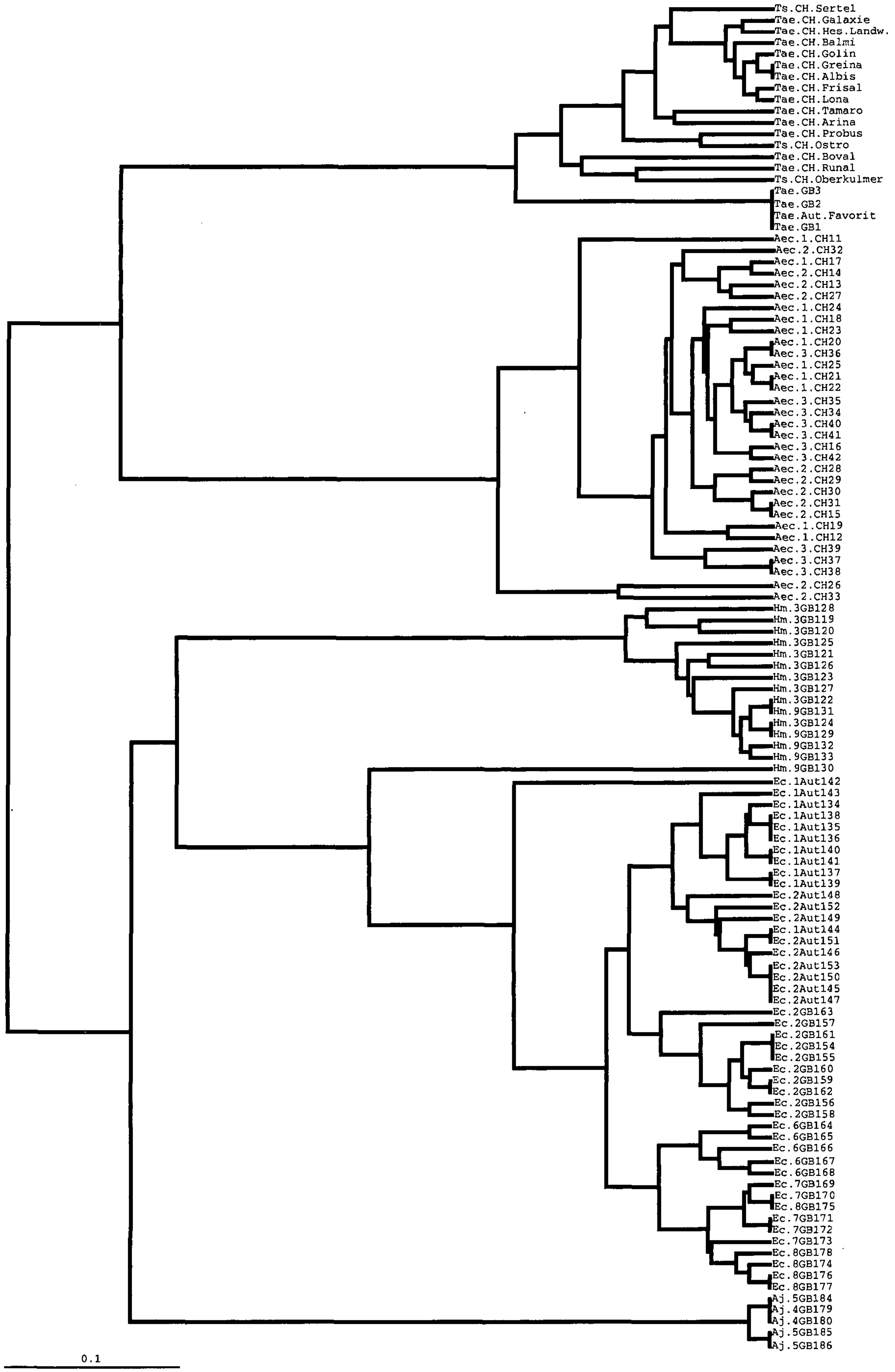
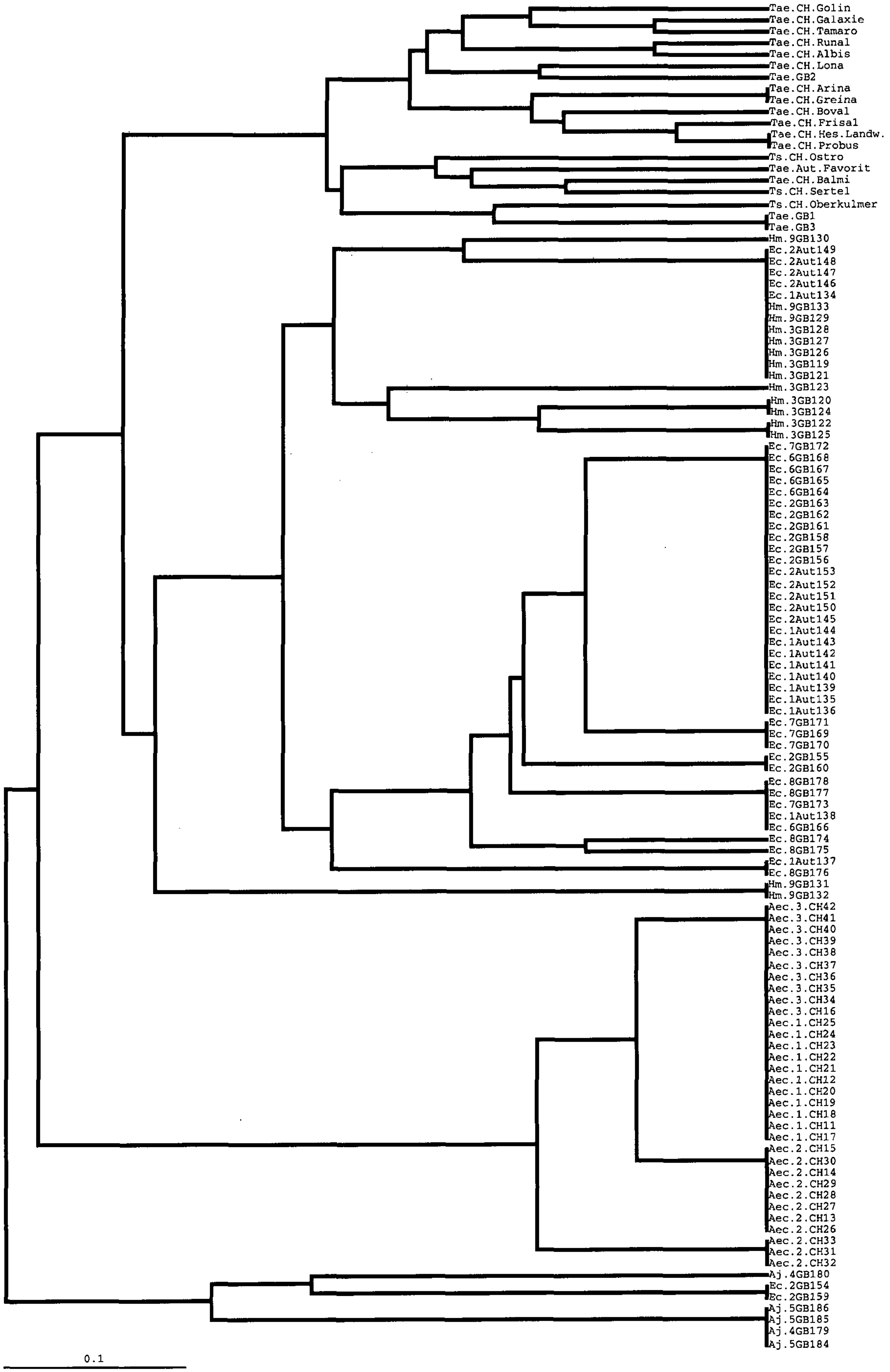


Fig. 5. UPGMA dendrogram obtained using the wheat microsatellite similarity matrix (based on Jaccard's similarity coefficient).



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 it 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. 1998*a*, 1998*b*). 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 determinism 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. æstivum* formed a separate cluster within that of the Swiss ones, while no clear separation between Swiss cultivars of *T. spelta* and *T. æstivum* 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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Gene flow from wheat (*Triticum aestivum* L.) to jointed goatgrass (*Aegilops cylindrica* Host.), as revealed by RAPD and microsatellite markers

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Abstract In order to estimate the potential of gene flow between wheat (*Triticum æstivum* L.) and jointed goatgrass (*Aegilops cylindrica* Host.), we carried out mixed pollinations in experimental and natural conditions. A set of species-specific RAPD (random amplified polymorphic DNA) and microsatellite markers were used to detect the presence of parental markers in the progeny of the plants used in these experiments. No hybrids were found within the offsprings of the plants used for the greenhouse experiments, while 85 *Ae. cylindrica*×*T. æstivum* hybrids were found within 2400 analyzed F_1 plants resulting from the field pollinations. The hybridization rates for individuals of different populations of the wild species differed considerably: 1% for two populations known for more than 90 years versus 7% for a newly discovered population. Most of the hybrids were completely sterile, but five of them produced 13 seeds (BC_1) by backcross with *Ae. cylindrica*. Twelve seeds germinated and generated viable and partly fertile plants. About 25% of the wheat specific RAPD markers were found in the BC_1 plants, indicating that introgression of wheat DNA into *Ae. cylindrica* is possible. In addition, one microsatellite marker, known to be situated on the D genome (a genome shared by both species), was also found in the BC_1 plants.

Keywords Gene flow · Risk assessment · Introgression · Wheat · Jointed goatgrass

Introduction

One of the most important concerns related to the field cultivation of transgenic plants is the possibility of gene transfer from crops to their wild relatives. The advantages that a wild plant would acquire in receiving resistance genes could lead to an increase of its weediness, with possible damage for agriculture and for the ecological equilibrium, e.g. increased competitiveness against other species and a consequent loss of biodiversity (Williamson 1994).

With regard to these concerns, the evaluation of the capacity of cultivated species to cross with wild relatives and to produce viable seeds is of the highest importance (Ellstrand et al. 1999). Several cases of intergeneric hybrids between wheat and wild relatives have been reported during the last few years, obtained by forced crossings (emasculation of flowers of one species) and embryo rescue (e.g. Limin and Fowler 1990; Chen et al. 1992; Sharma 1996). Such studies have focused generally on crop improvement; for example the introduction of resistances to diseases into crops. More rare and recent are studies providing evidence of this type of crossing under natural conditions (Zemetra et al. 1998). Thus, in order to determine the real range of the ecological risks related to crop-to-wild gene flow, it is necessary to determine under which conditions and at what frequency these hybridizations can occur in nature.

Most kinds of crop plants are cultivated worldwide, independently of their geographic origin. Therefore, risks existing in one region because of the presence of wild relatives may be negligible in other regions. Moreover, the genetic diversity of a wild relative itself may change completely the range of the risks, even within a relatively small region. One example of this is the case reported by Savova et al. (1996) of a wild relative (*M. falcata* L.) of tetraploid alfalfa (*Medicago sativa* L.) in Switzerland. Both species are either diploid or tetraploid, and crosses are possible and frequent only between cytotypes of the same chromosome number. In Engadin, a region in the east of Switzerland, only diploid *M. falcata* is present and, therefore, crosses with tetraploid crop alfalfa are not possible. Risk assessment studies on crop-to-wild gene flow on a regional scale are thus required.

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Several wild relatives are known to cross-fertilize with wheat (*Triticum æstivum* L.), which is one of the most important crops in the world. Its production is about 21 million tons per year worldwide, with a cultivation area of about 230 million hectares (reviewed in Ellstrand et al. 1999). Most of the hybrids are believed to be sterile (almost completely male-sterile), and this could greatly reduce the impact of genetically engineered wheat. However, some recent studies have demonstrated that some of the hybrids with wheat can produce some seeds, mostly by backcrossing with one of the parents (Li and Dang 1993; van Slageren 1994; Seefeldt et al. 1998; Zemetra et al. 1998) and only rarely by self-pollination (Li and Dong 1991).

In the study reported here, we focused on the closest relative of wheat growing in Switzerland, the tetraploid jointed goatgrass (*Aegilops cylindrica* Host.). Like hexaploid wheat (genome AABBDD) and most of the polyploid Triticeae, *Ae. cylindrica* (genome formula: CCDD) is an amphiploid, resulting from hybridization between the diploids *Ae. caudata* (CC) and *Ae. squarrosa* (DD). The presence of the D genome in both species is supposed to make the introgression of wheat genes into the jointed goatgrass genome easier after hybridization. *Ae. cylindrica* occurs as an adventive in Switzerland, which is its northwestern limit of distribution in Eurasia. This species is, however, frequent in the Mediterranean area and in the Middle East, which is its center of distribution. In North America, *Ae. cylindrica* is an introduced species. It grows within wheat fields and is considered to be a major weed, infesting about 3 million hectares (Dewey 1996) and causing significant yield losses (Ogg and Seefeldt 1999). Moreover, the control of jointed goatgrass in wheat fields using herbicides is impossible, because of its genetic similarity to wheat (Donald and Ogg 1991).

The objectives of our study were (1) to determine the hybridization rate between wheat and *Ae. cylindrica*, (2) to estimate the possibilities of introgression of wheat DNA into *Ae. cylindrica* and consequently, (3) to evaluate the relevance of the results of the present study within the framework of risk assessment of field release of transgenic wheat.

Material and methods

Plant material

Screening for wild populations of *Aegilops cylindrica* (Aec) was carried out by field excursions, on the basis of literature data, in the following regions of Switzerland: Valais, Ticino (south of the Alps), Geneva, Bern, Vaud and Zürich (Hess et al. 1967; van Slageren 1994). Only three populations, all in Valais, were found at this time (Table 1). All of the populations occupied disturbed habitats; for example, train stations and roadsides. Two populations were close to vineyards, but none were in immediate proximity of fields cultivated with cereals. In each population, a representative sample of 20–40 spikes (1 spike/plant) was collected. In addition, seeds of 13 Swiss wheat varieties [7 winter wheats and 6 spring wheats (Table 1)], obtained from Eric Schweizer, Samen AG, Thun, were included in the study. Seeds were sown and plants grown in individual pots in the Botanical Garden of Neuchâtel.

Mixed pollination experiments

Two types of mixed pollination experiments were carried out: under experimental conditions in the greenhouse and under natural conditions in the field.

Greenhouse pollinations

A total of 32 plants of *Ae. cylindrica* were used for crosses with four Swiss wheat varieties (Table 2). Experiments were performed by combining two spikes, one of each species, under a pollination bag. In order to estimate the possibility of hybridization in both directions, we did not emasculate the flowers. The bags were left on the spikes until the end of the flowering period, during which time they were shaken regularly, in order to mix the pollens. All of the

Table 1 Sampling of the species and of the individuals for genetic analyses

Species	Populations/varieties	Identification numbers of populations and species	Number of analyzed plants
Aegilops cylindrica	Brig (46°19′N; 8°00′E)	Aec1	30
	Sierre (46°18′N; 7°33′E)	Aec2	30
	Saillon (46°11′N; 8°11′E)	Aec3	20
Triticum æstivum	4 Swiss varieties		
	Arina	T.ae	1 bulk of 10
	Galaxie	T.ae	1 bulk of 10
	Boval	T.ae	1 bulk of 10
	Runal	T.ae	1 bulk of 10

Table 2 Experimental crosses in the greenhouse (*T. ae*=*T. aestivum*)

Crosses	Number of individuals *Ae. cylindrica* (1 spike/individual)	Number of individuals *T. æstivum* (1 spike/individual)
T. ae. var. Arina×*Ae. cylindrica*	8	8
T. ae. var. Galaxie×*Ae. cylindrica*	8	8
T. ae. var. Boval×*Ae. cylindrica*	8	8
T. ae. var. Runal×*Ae. cylindrica*	8	8

seeds obtained from these crosses (of both species) were collected and sown.

Field pollinations

Eighty individuals of *Ae. cylindrica* (30 individuals of populations Aec1 and Aec2; 20 of population Aec3) were planted in the middle of a cultivated field of *T. aestivum* var. Arina. This is the most cultivated variety in Switzerland, grown in 40–50% of the area cultivated with wheat (Valenghi 1998; Rüegger 2000). The plants were placed in four rows, each 45 m long, with 5 m between rows. In order to avoid a bias in the hybridization rate, due to possible differences in the quantity of wheat pollen in the field, individuals of each Aec population were distributed over the entire field area. This experiment was performed at the Swiss Federal Research Station for Plant Production of Changins (RAC, Nyon).

All seeds produced by *Ae. cylindrica* plants were collected. A first subset of ten seeds per individual was sown in the Botanical Garden and grown under open pollination conditions. Subsequently, in order to confirm differences in the hybridization rates of the populations observed in the first subset, we sowed an additional series of 20 seeds per mother plant. Thirteen seeds produced by the hybrids were also sown and grown in the Botanical Garden.

Hybrid detection

The different ploidy levels of *T. æstivum* (2n=42) and *Ae. cylindrica* (2n =28) enabled us to detect hybrid plants by analyzing the DNA content. The F_1 plants resulting from all of the crosses were thus analyzed by flow cytometry for quick detection of hybrids. For each individual, a small (1 cm^2) piece of fresh leaf was taken and sliced with a razor blade in a petri dish containing 0.5 ml of ice-cold PBS buffer in order to release the cell nuclei. Twenty microliters of staining solution [50% propidium iodide and 50% Triton X-100 (10%)] was then added to the solution, which was filtered through a 30-µm mesh nylon screen. Finally, the fluorescence of stained nuclei was measured with a Becton Dickinson FACStrak flow cytometer and visualized as a histogram. Samples of known ploidy (tetra- and hexaploid) were used to calibrate the flow cytometer at the beginning of each measurement session.

A morphological survey of all hybrids detected was carried out to confirm the results of the cytometry analyses. Chromosome counts of the plants produced by the hybrids were performed on root tips as described in Savova et al. (1996).

Genetic marker detection

Specific genetic markers for wheat and jointed goatgrass, obtained with random amplified polymorphic DNA (RAPD) and microsatellite (simple sequence repeats; SSRs) techniques were set up as described in Guadagnuolo (2000) and used to analyze the F_1 plants. Eight RAPD primers (OPB 6, 8, 10 and OPP 6, 7, 8, 9, 14; Operon Technologies, Alameda, Calif.) and six publicly available wheat microsatellite primers pairs (WMS 43, 44, 46, 47, 106 and 159; Plaschke et al. 1995, Röder et al. 1995) were used, as they amplified specific markers for the species. Four plants, produced by hybrids as mother plants, were analyzed with the same markers.

DNA analyses

Total DNA extraction from a single leaf was carried out on 11 samples per population for *Ae.cylindrica*. The extraction was performed on a bulk of ten plants for each wheat and spelt variety. We used a simple SDS-Na-acetate protocol (Savova-Bianchi 1996). DNA was resuspended in a TE (pH 8) solution at a concentration of 30 ng/ml and stored at –20°C.

Subsequently, DNA extraction was performed on 23 F_1 hybrids (generated by the *Ae. cylindrica* plants involved in the crosses in the field) and on the progeny (4 plants) of these hybrids.

Polymerase chain reactions (PCR) for both RAPD and SSR amplifications were performed in a volume of 25 µl under the following final concentrations:

RAPD

The amplification reaction contained 1×PCR mix, 1.5 m*M* $MgCl_2$, 0.4×Q-solution, 0.2 m*M* dNTP, 0.2 µ*M* primer, 0.03 U/µl *Taq* polymerase and 1 ng/µl template DNA. Amplifications were performed in a Biometra I thermocycler with the cycling following profile: an initial denaturation 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, with a final extension for 10 min at 72°C. PCR products were mixed with a 1/5 vol of loading buffer and separated on a 1.6% (w/v) agarose gel, containing 0.4 µg/ml ethidium bromide, in 0.5×TBE at 60 V for 2 h. The DNA fragment were then vizualized under UV light.

Microsatellites

The amplification reaction contained 1×PCR mix, 1.5 m*M* $MgCl_2$, 0.4×Q-sol, 0.2 m*M* dNTP, 0.6 n*M* of each primer, 0.03 U/µl *Taq* polymerase and 1 ng/µl template DNA. Amplifications were performed in a Biometra I thermocycler with the following cycling profile: an 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, with a final extension for 10 min at 72°C. PCR products were mixed with 1/5 vol of loading buffer and separated on a 6% polyacrylamide gel in 0.5×TBE at 100 V for 6 h. The gels were then stained in a 0.4 µg/ml ethidium bromide bath and the DNA fragments vizualized under UV light.

Data analysis

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

We used the R4 (Beta version) package (P. Casgrain and P. Legendre, Université de Montréal) to calculate Jaccard's similarity coefficient between samples for the two sets of data: RAPD and microsatellites. Jaccard's similarity measure does not take into account double absence. This is closer to the biological reality, considering that the absence of a DNA fragment in two samples is an absence of information rather than an element of similarity.

The similarity matrices were converted into distances matrices (D=1-S) and used to perform principal coordinates analysis (PCoA) (Gower 1966).

Results

Greenhouse pollinations

The 36 *Ae. cylindrica* spikes involved in the mixed pollinations with four different wheat varieties produced 492 seeds in total (average of about 14 seeds/spike), while 853 seeds were produced by the 36 wheat spikes (an average of about 24 seeds/spike). No hybrids were detected within this progeny, neither with flow cytometry nor by observation of morphological traits.

RAPD and microsatellite analyses performed on one individual per cross from these F_1 plants (data not shown) confirmed their breeding by self-pollination because their DNA profiles corresponded exactly to those of their mother plants.

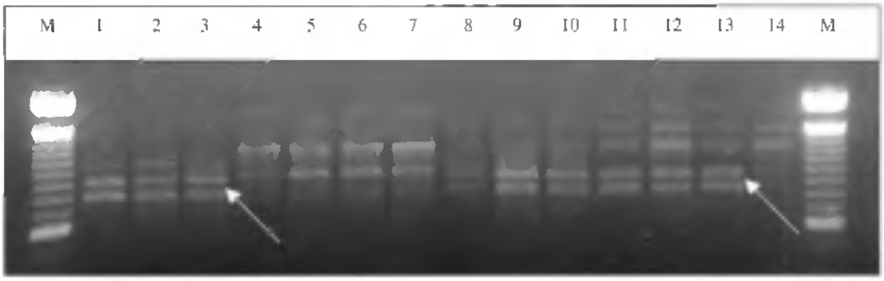

Fig. 1 Example of RAPD amplification in *T. æstivum* (*lanes 1–3*), *Ae. cylindrica* (*lanes 4–7*), hybrids *Aegilops cyl.×Triticum æst* (*lanes 8–10*) and BC_1 plants (*lanes 11–14*) with primer OPB-10. *M* 100 bp DNA ladder (GibcoBRL®, Life Technologies); *arrows* indicate two specific fragments of wheat present in three BC_1 individuals

Field pollinations

Among the 2,400 grown offsprings of *Ae. cylindrica*, 85 hybrids (2n=35) were detected. Ten were produced by plants of population Aec1 (Brig), five by plants of population Aec3 (Saillon) and 70 by plants of the newly discovered population of Sierre (Aec2). On average, the hybridization rate was 1% for the individuals of populations Aec1 and Aec3, while individuals of population Aec2 showed a hybridization rate of 7%. The morphology of the hybrids was intermediate between that of the parental species.

As expected, the hybrids were completely male-sterile and almost completely female-sterile. However, 5 of the 85 detected hybrids produced 13 seeds, of which 12 germinated. The mother plants of four from the five partially fertile hybrids were from population Aec2 and one was from population Aec3.

The DNA content of the offsprings of the hybrids was identical to that of pure *Ae. cylindrica*. This indicates that they were produced by a backross of the hybrid mother plant with *Ae. cylindrica*. Moreover, their morphology was identical to that of *Ae. cylindrica* and their chromosome numbers ranged from 28 to 29. These BC_1 plants were grown under open-pollination conditions within blocks of pure *Ae. cylindrica* individuals and produced fertile flowers and seeds.

Marker detection

The selection of molecular markers for the differentiation of the species was discussed in Guadagnuolo (2000).

RAPDs markers

The RAPD profiles of the hybrids presented most of the specific bands of both parents (Fig. 1). As expected, fragments present in both parent species were also amplified in the hybrids. Sixteen out of thirty species-specific markers of wheat (var. Arina), and 16 out of 22 species-specific markers of *Ae. cylindrica* were amplified in the hybrids as well. In addition, one fragment specific for population Aec2 of *Ae. cylindrica* was only amplified in the hybrids produced by individuals of this population.

Of the 16 specific fragments of *T. æstivum* inherited by the hybrids, 9 were amplified in the BC_1 plants as well, indicating introgression of wheat DNA into the wild species (Fig. 1). Of these fragments, five were present in all four BC_1 individuals analyzed, three were amplified in two individuals and one fragment was inherited by only one individual. On the contrary, only 1 of the 16 specific fragments of *Ae. cylindrica* was not amplified in the BC_1 plants.

Wheat microsatellite markers

All seven specific markers of *T. æstivum* were amplified in the hybrids, independently of their location on the different genomes, while this was the case for only three of the five specific bands of *Ae. cylindrica*. One fragment, only present in jointed goatgrass of populations Aec1 and Aec3, was only amplified in the hybrids produced by plants of these populations.

Five fragments in total were amplified in BC_1 plants, all of them present in the hybrids as well. One of these fragments was amplified with primer WMS 159 and was originally a specific marker of *T. aestivum*, being situated there in the D genome. Two primers of microsatellites supposed to be located in the B genome (WMS 43 and 46) amplified fragments in BC_1 plants, which, however, were already present in pure *Ae. cylindrica* and the hybrids as well. Among all the other specific fragments of wheat, neither those amplified with primers WMS 44 and 106 (supposed to be located in the D genome) nor those located in the B genome (primers WMS 43, 46 and 47) were amplified in BC_1 plants.

Species differentiation

The Principal Coordinates Analyses (PCoA) performed on both kinds of data showed a clear separation of the parental species, the hybrids and the BC_1 plants (Figs. 2 and 3). The position of the hybrids in the PCoA performed on RAPD data was perfectly intermediate between those of the parental species. In addition, the posi-

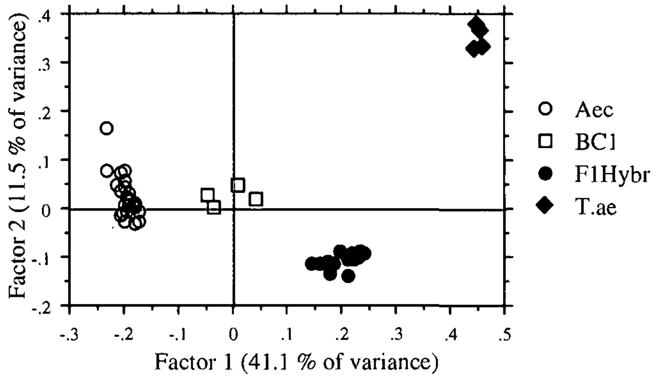

Fig. 2 Principal Coordinates Analysis (PCoA) performed on the similarity matrix obtained with RAPD data (based on Jaccard's similarity coefficient)

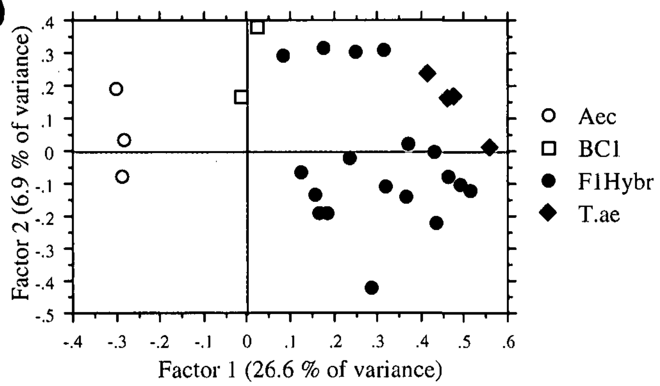

Fig. 3 PCoA performed on the similarity matrix obtained with microsatellite data (based on Jaccard's similarity coefficient)

tion of BC_1 plants was intermediate between those of hybrids and jointed goatgrass (Fig. 2). In the case of the analyses performed on wheat microsatellite data, the hybrids were dispersed and closer to wheat than to *Ae. cylindrica*. The BC_1 plants were also grouped closer to the hybrids than to *Ae. cylindrica* (Fig. 3).

The relative position of BC_1 plants, placed between hybrids and *Ae. cylindrica* by PCoA on both kinds of data, confirmed that the seeds produced by the hybrids were obtained by backcrosses with this parental species.

Discussion

Hybridization

Ae. cylindrica×wheat hybrids have already been observed, but these were believed to be sterile (van Slageren 1994). The results of the present study confirm that spontaneous hybridization between conventional (i.e. non-genetically engineered) wheat and *Ae. cylindrica* under natural conditions is possible. However, the hybrids are partly fertile, which confirms the recent results of Seefeldt et al. (1998) and Zemetra et al. (1998).

The absence of hybrids in the progeny of the experimental mixed pollinations, compared with the results of those under natural conditions, indicates that a large source of external pollen is probably necessary to induce *Ae. cylindrica* to produce hybrids. A huge amount of pollen was available in the field pollinations, where *Ae. cylindrica* was planted intermixed with wheat, but not in the greenhouse experiments, where the pollination bag limited the wheat pollen source to that of only one spike. Moreover, as shown by Fritz and Lukaszewski (1989) for wheat cultivars crosses, several pollination events are necessary to stimulate the recipient flower to allow for pollen growth on the stigma. The probability of such repeated events is certainly higher within a field with thousands of flowers producing pollen than in a pollination bag.

The spikes involved in the experimental crosses produced seeds of good quality, a result of self-fertilization, and in normal quantities. Therefore, the absence of hybrids in the progeny of these crosses was probably not caused by unfavorable environmental conditions inside the bags, which could have lowered the pollen fertility (temperature, moisture etc).

Because the hybrids were grown within a large number of pure *Ae. cylindrica* plants, all of the BC_1 plants were produced by backcrossing with the wild species and not with *T. æstivum*. Indeed, the relative DNA quantity of the BC_1 plants measured by flow cytometry was exactly the same as for pure *Ae. cylindrica* plants. Moreover, chromosome counts confirmed these results, indicating a ploidy level that was almost completely restored. This situation corresponds to the typical agricultural conditions in Europe, where crop rotation is generally applied. Therefore, hybrids produced by jointed goatgrass growing in the middle of wheat fields would grow the following year surrounded by more *Ae. cylindrica* than by wheat plants.

The BC_1 plants produced numerous seeds, indicating at least a partially restored female fertility. However, BC_1 plants were also grown within pure jointed goatgras, and thus within a large amount of pollen. Further analyses could tell whether the seeds produced by the BC_1 resulted from self pollination, thereby representing BC_1S_1 plants, or from pollination by pure *Aegilops*, consequently being BC_2 plants.

One of the most surprising results of the present study was the big difference in the hybridization rate between individuals of different populations. The plants of population Aec2 used in the crosses in the field showed an hybridization rate of 7%, while those of the two other populations (Aec1 and Aec3) hybridized with wheat at a rate of 1%. Zemetra et al. (1998) observed an hybridization rate closer to this latter, i.e. of about 2%, between wheat and *Ae. cylindrica* infesting wheat fields in USA. It is presently impossible to speculate on the reasons of these results (lower male fertility of individuals of population Aec2, partial incompatibility with own pollen etc.), but they are probably correlated with the genetic differences between this population and the two others, as observed with RAPD and microsatellite markers (Guadagnuolo 2000). Although *Ae. cylindrica* is an adventive species in Switzerland, populations Aec1 and Aec3 have been known since at least 90 years (van Slageren 1994), while Aec2 is a newly discovered one. The important differences in the hybridization rate for populations of the same species demonstrate the need for risk assessment studies on a regional scale, especially given the fact that the three populations are located at a maximum of 100 km from each other.

Marker inheritance

The morphology of the jointed goatgrass×wheat hybrids was intermediate between those of the parental species. This was also reflected by the amplification, in hybrids DNA, of an equal number of specific RAPD markers of both species. However, a higher rate of *Ae. cylindrica*-specific RAPD markers (16 out of 22), compared to those of wheat (16 out of 30), were inherited by hybrids. This could indicate that some of these fragments originated from the cytoplasmic DNA. Indeed, the female parent of the hybrids was *Ae. cylindrica*, and in Poaceae paternal chloroplast inheritance has never been observed. Moreover, it is well-known that RAPD fragments can originate from nuclear, chloroplast and mitochondrial DNA (Lorenz et al. 1997).

The use of both RAPD and microsatellites markers allowed us to demonstrate the transfer of wheat DNA into the BC_1 plants by natural hybridization. The large number of wheat-specific RAPD markers inherited by the BC_1 plants suggests that most of them were located in the D genome, but this was not proven. Nevertheless, one fragment known to be located in the D genome, wheat microsatellite WMS 159 (Plaschke et al. 1995), was amplified in the BC_1. While these results demonstrate that introgression of wheat DNA fragments located in the D genome into *Ae. cylindrica* is possible, they can not yet prove that this is also possible from the A or B genomes. Therefore, the question of whether translocations between the A or B and C or D genomes can occur during meiosis in hybrids or subsequent backcrosses remains open, as already noticed by Seefeldt et al. (1998) and Zemetra et al. (1998).

The natural maintenance of part or the entire foreign genomes in the progeny of forced hybrids between wheat and several *Triticeae* has been recorded (Limin and Fowler 1990; Chen et al. 1992; Sharma 1996). Moreover, the genomes of the species involved in these studies were much more remotely related to those of wheat (e.g. genomes P or Ju of several *Agropyron* species) than the genomes of *Ae. cylindrica*.

The data analyses clearly separated the parental species, the hybrids and the BC_1 plants. Moreover, the persistence of parental traits in the progeny of the crosses was reflected by the relative position of each class of samples on the PCoA scattergrams. The selected DNA markers combined with such analyses would therefore be useful in detecting the putative introgression of wheat DNA into jointed goatgrass under natural conditions.

Relevance of the results for the risk assessment of transgenic wheat cultivation

In order to correctly evaluate the risks of gene or transgene escape from a crop plant to a wild relative, investigators must take several factors into account. The genetic compatibility, *i.e.* the capacity of hybridization and the fertility of the hybrids and successive backcrosses, has to be investigated. The possibility of hybridization with a wild relative and the subsequent persistence of crop genes in the wild species have already been demonstrated for several crops; for example sunflower (Arias and Rieseberg 1994; Whitton et al. 1998), sorghum (Arriola and Ellstrand 1996, 1997), radish (Lee and Snow 1998) and alfalfa (Jenczewski et al. 1999).

The results of the present study, combined with those of Zemetra et al. (1998) and Seefeldt et al. (1998), show that (1) natural hybridization between wheat and jointed goatgrass is possible with both species as female parent, (2) hybrids can produce at least some seeds; and (3) wheat traits can be transferred into the *Ae. cylindrica* genome after only two backcrosses. Therefore, the possibility of gene transfer from wheat to jointed goatgrass under natural conditions (e.g. agroecosystems) is likely.

We used here conventional instead of transgenic wheat varieties. However, the presence of a transgene is supposed to have no impact on the pollen production of wheat and thus on the hybridization rate with jointed goatgrass. Therefore, this should not have an impact on the evaluation of gene flow between the species. On the contrary, studies using transgenic crops will be necessary to evaluate the consequences of that gene flow.

Because crop-to-wild gene flow is mostly pollen-mediated (Ellstrand and Hoffman 1990), proximity and overlapping in the flowering period of both kind of species are also required (Klinger et al. 1992). Gene dispersal from crop to wild relatives growing tens or hundreds meters away from the cultivated source has been reported for insect-pollinated species as radish (Klinger et al. 1992), sunflower (Arias and Rieseberg 1994) or potato (Conner and Dale 1996). However, it is likely that for wind-pollinated species, like most Poaceae, hybridizations are restricted to a much narrower zone. Indeed, most of pollen migration of Poaceae occurs within few meters (Pedersen 1994), although pollination over 20 m (Doll 1987) and even 60 m (Wagner and Allard 1991) has been reported for crop barley. Kertesz et al. (1995) found that durum wheat pollen migrated up to 20 m but that open pollination between durum wheat varieties occured only within 3 m. In the case of wheat and jointed goatgrass in Switzerland, an overlapping of flowering periods was observed. However, the populations of the wild species were situated at least at few kilometers from fields cultivated with cereals, and all of them were in Valais, a region with a moderate cereal cultivation. It is thus unlikely that pollination by wheat could occur in these sites. Nevertheless, as noted above, *Ae. cylindrica* is an adventive species in Switzerland, and seeds are supposed to be transportated by train or trucks from neighboring regions south of the Alps (Italy, France). Moreover, the seeds of this species exhibited a germination rate almost equal to those of the wheat varieties. The establishment of new populations in the direct proximity of wheat fields is thus not unlikely. In addition, the more recent population (Aec2) showed an hybridization rate with wheat that was much higher than those of the old ones. Therefore, the risk of gene escape from transgenic wheat to *Ae. cylindrica* in Switzerland exists but is moderate. It could be higher if new populations, with a crossing rate with wheat similar to that of population Aec2, established close to wheat cultivation areas.

Nonetheless, this risk could be dramatically higher in other countries. The geographic distribution of *Ae. cylindrica* in Europe and the Middle East, where it grows in the vicinity of or within wheat fields (van Slageren 1994), makes this species the ideal recipient for crop genes. The likelihood of this gene transfer is even higher in the USA. Jointed goatgrass is not native there but has been introduced and already infests between 2 and 3 million hectares of wheat fields, causing important yield losses (Dewey 1996; Ogg and Seefeldt 1999). Despite the low fertility of the hybrids, risks must not be underestimated. The large area occupied in parapatry by both species could lead to a great number of hybridization events and thus to a relevant number of cases of introgression. The weediness of jointed goatgrass could consequently become even higher. The extent of these hazards will be greatly influenced by the type of transgene transferred. In the case of jointed goatgrass, even a small number of plants receiving an herbicide resistance gene could have important consequences on wheat cultivation in regions where both species grow intermixed.

Conclusion

The results of the present study demonstrate that gene flow between *T. æstivum* and *Ae. cylindrica* under natural conditions is possible. The discovery that *Ae. cylindrica*×*T. æstivum* hybrids are at least partly fertile is of great importance, because it proves that introgression of wheat traits into a wild relative, and thus transgene escape, is possible. In Switzerland, jointed goatgrass has not been found in direct proximity of wheat fields and grows only in Valais, a region with a moderate cereal cultivation; the risks are thus modest. However, the high hybridization rate of one population could lead to higher risks, especially considering that jointed goatgrass is an adventive species, able to establish new populations. In the Middle East and in regions where this species has been introduced (e.g. USA), the risk is much higher, because it often grows intermixed with wheat.

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Search for evidence of introgression of wheat (*Triticum aestivum* L.) traits into sea barley (*Hordeum marinum s.str.* Huds.) and bearded wheatgrass (*Elymus caninus* L.) in central and northern Europe, using isozymes, RAPD and microsatellite markers

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Abstract Seeds of English and Austrian populations of bearded wheatgrass (*Elymus caninus* L.) and sea barley (*Hordeum marinum* Huds.) growing in the vicinity of wheat (*Triticum aestivum* L.) fields were collected in order to search for evidence of the introgression of wheat traits into these wild relatives. Seeds were sown and plants grown for subsequent analyses using morphological and genetic (isozymes, RAPD and wheat microsatellites) markers. No F_1 hybrids were found within the individuals of the two species grown, neither with morphological nor with genetic markers. Also, no evidence of introgression of wheat traits into *E. caninus* was observed. However, in one individual of *H. marinum* which had the typical morphology of this species, numerous species-specific DNA markers of wheat were amplified, thereby demonstrating previous hybridization. Consequently, the hybridization between wheat and *H. marinum* under natural conditions and the introgression of wheat traits into this wild relative seems to be possible. Our results contribute to the risk assessment of transgenic wheat cultivation.

Keywords Wheat · Wild relative · Gene flow · Introgression · Genetic marker

Introduction

Among the concerns related to genetic engineered crops, the risk of gene escape toward wild flora is one of the most discussed. The potential for such gene flow is directly proportional to the potential of crop-wild hybridization (Ellstrand and Hoffman 1990). For numerous crops, wild relatives are known that can hybridize with them somewhere in the world. This is the case, among others, of maize (Doebley 1990), oilseed rape (Klinger et al. 1992), sunflower (Whitton et al. 1997) and sugar beet (Bartsch and Pohl-Orf 1996).

For wheat, the center of distribution is assumed to be the Middle East and the Mediterranean area. Many wild species closely related to wheat exist in these regions; most belong to genus *Aegilops* and grow close or within wheat fields, frequently hybridizing with this crop (van Slageren 1994). More generally, the *Triticeae* tribe is distributed worldwide throughout the temperate regions of both hemispheres (Miller 1987). Many wild relatives of wheat have been introduced as adventive in other regions and even on other continents, as is the case for *Ae. cylindrica* in North America (Donald and Ogg 1991).

In northern Europe two wild relatives of wheat, sea barley (*Hordeum marinum* Huds.) and bearded wheatgrass (*Elymus caninus* L.), occasionally grow in direct proximity or even within wheat fields and have been described as being not strictly autogamous (Sun et al. 1997; De Bustos et al 1998). Several studies have described the production of viable and partially fertile hybrids between these wild species and wheat for agronomic purposes (Sharma and Baezinger 1986; Fedak 1991). Considering their breeding system, their belonging to the same tribe and the fact that they often grow within a huge amount of wheat pollen, the risk of spontaneous hybridization with wheat cannot be excluded.

In order to estimate the ecological risks involved with field trials or wide cultivation of transgenic crops, it is essential to determine the potential for such hybridizations to occur under natural conditions. A compilation of the literature shows that there is no evidence that the pollen production of transgenic and conventional crops differs essentially. Therefore, the search for hybrids between conventional wheat cultivars and wild relatives will be a good measure of the risk of gene escape from

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transgenic wheat. Indeed, any hybridization will depend on the outcrossing rate of the recipient species rather than on the pollen production of the crop. This kind of study can be considered as a necessary step prior to field tests with genetically engineered crops.

The aim of the study presented here was to estimate the potential of spontaneous hybridization between wheat and two wild relatives, *Hordeum marinum* s.str. Huds (*H. marinum* subsp. *marinum*) and *E. caninus* L. and to search for evidence of the introgression of wheat DNA into these wild species. Morphological, DNA [randomly amplified polymorphic DNA (RAPD) and microsatellite] and enzyme markers were used for this purpose.

Materials and methods

Plant material

Seeds of the two wild species were collected in Austria and England (Fig. 1, Table 1). In each sampled population (Table 2), a representative sample of 20–40 spikes (1 spike/plant) was collected. Two populations of *Hordeum marinum* subsp. *marinum* (England) and six populations of *Elymus caninus* (England and Austria) were sampled.

Wheat (*Triticum æstivum*) seeds were collected in the immediate proximity of the wild relatives populations in order to obtain specific markers and detect their possible introgression into wild genomes. In addition, we included in the study five Swiss wheat varieties (seeds obtained from Eric Schweizer Samen AG, (Thun). Seeds were sown and plants cultivated in the Botanical Garden of Neuchâtel.

Morphological markers

Specific morphological markers were determined on pure plants of each species (Table 3) and used to analyze the offspring of the sampled populations.

Genetic markers

Specific genetic markers, obtained with isozymes, RAPD and microsatellite techniques, were set up as described in a previous paper (Guadagnuolo et al. 2001a) and used to analyze the offspring of the sampled populations. Two enzyme systems, glutamate oxaloacetate transaminase (got) and peroxidase (prx), eight RAPD primers (OPB 6, 8, 10 and OPP 6, 7, 8, 9, 14; Operon Technologies, Alameda, Cal.) and six publicly available wheat microsatellite primers pairs (WMS 43, 44, 46, 47, 106 and 159; Plaschke et al. 1995; Röder et al. 1995) were used as they produced species-specific markers. The amplified microsatellites were described being present in a specific genome of *T. æstivum* (Plaschke et al. 1995; Röder et al 1995). However, some of them amplified specific bands in the wild relatives, as already demonstrated by Sun et al. (1997) for *Elymus* species.

Isozyme analyses

Protein extraction from 451 *E. caninus*, 88 *H. marinum* and 35 *T. æstivum* plants (Table 2) was carried out by grinding two young leaves per plant in 1 ml 0.1 *M* sodium acetate solution (pH 7.2). The extracts were then centrifuged at 12000 rpm and the supernatant stored at –80 °C for subsequent isozymes analyses.

Polyacrylamide gel electrophoreses (PAGE) (2.5 mm thick) were prepared according to Gasquez and Compoint (1976), using the modifications of Lumaret (1981): sample gel (9% acrylamide and 0.165% bis-acrylamide); stacking gel (2.5% acrylamide, 1 cm long) and separation gel (9% acrylamide and 0.165% bis-acryl-

Table 1 The species studied and their genetic characteristics (Miller 1987; Sun et al. 1997; Baum and Johnson 1998)

Species	Genome formula	Ploidy level
Triticum æstivum L. (common or bread wheat)	AABBDD	2n=6X=42
Elymus caninus L. (bearded wheatgrass or b. couch)	SSHH	2n=4X=28
Hordeum marinum Hudson *s.str.* (sea barley)	XX	2n=2X=14

Table 2 Sampling of the species and of the individuals for genetic analyses

Species	Populations/varieties	Identification names (Fig 1)	Number of plants analyzed with DNA markers	Number of plants analyzed with morphological and enzyme markers
E. caninus	England (GB) and Austria (Aut)			
	Warboys Wood Nature reserve (2 subpops) (52°25′N; 0°05′W)	Ec.2GB	10	26
	Horncastle (53°9′N; 0°08′W)	Ec.6GB	5	54
	Scottlehorpe (52°46′N; 0°26′W)	Ec.7GB	5	46
	Sonthey Wood, Peterborough (52°35′N; 0°22′W)	Ec.8GB	5	93
	Achleiten (2 subpops) (48°5′N; 14°11′E)	Ec.1Aut	10	133
	Kaltenbach (2 subpops) (48°16′N; 13°53′E)	Ec.2Aut	10	97
H. marinum	England (GB)			
	Wolferton, Norfolk (2 subpops) (52°51′N; 0°27′E)	Hm.3GB	10	65
	Sonthey Wood, Peterborough (52°35′N; 0°22′W)	Hm.9GB	5	23
T. æstivum	1 Austrian variety (Favorit)	Tae.Aut.var	1 bulk of 10	10
	3 English varieties (unknown)	Tae.GB1–3	3 bulks of 10	30
	5 Swiss varieties (Arina, Galaxie, Tamaro, Boval, Runal)	Tae.CH.var	5 bulks of 10	50

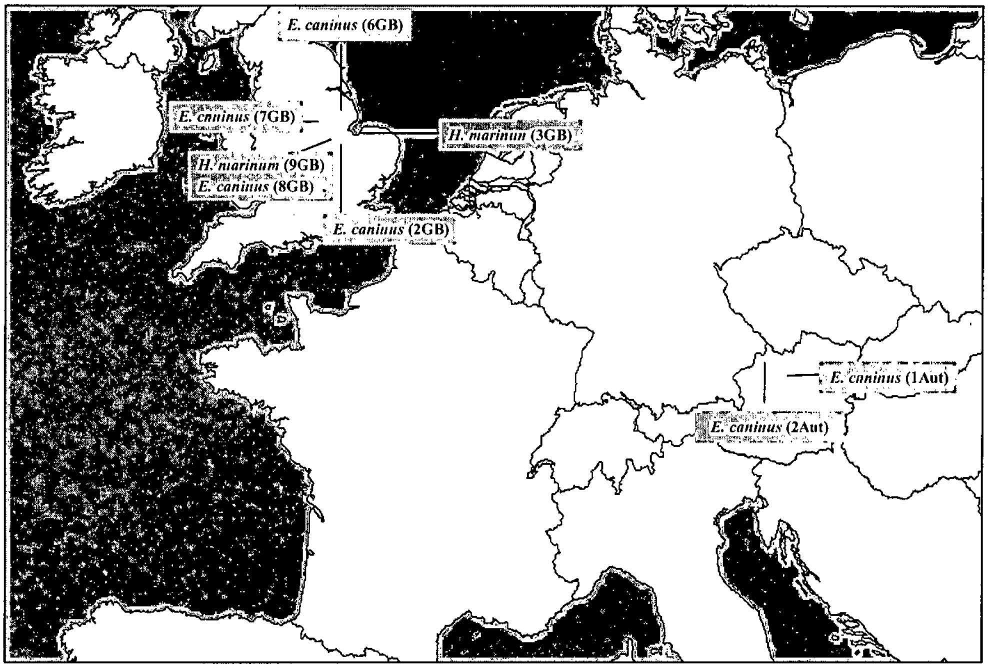

Fig. 1 Sampling sites of the wild relatives of wheat (identification numbers of populations in *brackets*)

Table 3 Main morphological characteristics of the species studied

Traits	*Triticum aestivum*	*Elymus caninus*	*Hordeum marinum*
Culm	100–150 cm	30–110 cm	10–40 cm
Leaves	Long, broad	Shorter, smaller, bluish-green	Leaf blade sometimes rolled up, inside with few hairs
Spike	Thick, compact; whitish, yellow, reddish color	Thin, drooping to one side	Compact, short
Spikelets	2 to 4 in a row, thick	Mostly 3 in a row, thin	Always 3, middle spikelet fertile, side spikelets sterile
Awns	Depends on varieties	Awns longer than glumes, wavy	Glumes of side and middle spikelets awned, palea of middle spikelet awned

amide, 7.5 cm long). Forty microliters of each sample (mixed with 20 µl of dye bromophenol blue) migrated in a TRIS-glycine (pH 8.6) buffer at 4°C under the following conditions: 10 min at 600 V and 6 pps (pulsations/s), 20 min at 230 V and 7 pps and 2.5 h at 600 V and 7 pps.

Protein separation was performed within 2 days after the extraction. Enzyme staining was carried out as described in Savova Bianchi (1996).

DNA analyses

Total DNA extraction from a single leaf was carried out on five samples per population (or subpopulation) for *H. marinum* and *A. caninum*. For each of the wheat varieties, the extraction was performed on a bulk of leaves of ten plants using a simple SDS-na-acetate protocol (Savova Bianchi 1996), the DNA was resuspended in TE (TRIS-EDTA, pH 8) at a concentration of 30 ng/ml and stored at –20°C. In total, 15 *H. marinum*, 45 *E. caninus* and nine *T. æstivum* DNA samples were analyzed.

Polymerase chain reactions (PCR) for both RAPD and microsatellite amplifications were performed in a volume of 25µl under the following final conditions:

RAPD

The reaction volume consisted of 1× PCR buffer, 1.5 m*M* $MgCl_2$, 0.4× Q-solution (Qiagen AG, Basel), 0.2 m*M* dNTP, 0.2 µ*M* primer, 0.03 U/µl *Taq*-pol (Qiagen AG, Basel) and 1 ng/µl template DNA. Amplifications were performed in a Biometra 1 thermocycler using the following profile: initial denaturation 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; a final extension was for 10 min at 72°C. PCR products were mixed with 1/5 vol loading buffer and separated on a 1.6% (w/v) agarose gel containing 0.4 µg/ml ethidium bromide in 0.5× TBE at 60 V for 2 h. The DNA fragment were then visualized under UV light.

Microsatellites

The reaction volume consisted of 1× PCR buffer, 1.5 m*M* $MgCl_2$, 0.4× Q-sol. (Qiagen AG, Basel), 0.2 m*M* dNTP, 0.6 n*M* each primer, 0.03 U/µl *Taq*-pol (Qiagen AG, Basel) and 1 ng/µl template DNA. Amplifications were performed in a Biometra 1 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; a final extension was for 10 min at 72°C. PCR products were mixed with 1/5 vol 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 μ g/ml ethidium bromide solution, and DNA fragments were vizualized under UV light.

Data analysis

The data obtained with the three techniques were scored in a binary form as the presence or absence (1 and 0) of bands for each individual or bulk (in the case of wheat DNA samples). We used the R4 (Beta version) package (Philippe Casgrain and Pierre Legendre, Université de Montréal) to calculate Jaccard's similarity coefficient and, after conversion of the similarity matrices into distance matrices, to perform Principal Coordinates Analyses (PCoA).

Results

Elymus caninus

None of the 450 offsprings showed intermediate morphology between the two parental species and detailed morphological analyses (data not shown) confirmed general observations. In addition, none of the specific DNA or enzyme markers of wheat was found in any of the progeny of wild plants. Therefore, no evidence of introgression of wheat DNA into bearded wheatgrass was found.

Principal Coordinates Analyses performed on the distance matrices obtained with the three different types of markers confirmed these observations. Indeed, in all cases, wheat and *E. caninus* individuals grouped separately (e.g. Fig. 2).

Hordeum marinum

In *H. marinum*, indications of hybridization with wheat were found in the analyzed plants. None of these individuals had a morphological appearence intermediate between those of wheat and sea barley (data not shown). However, despite its typical *H. marinum* morphology, one offspring of the population of Sonthey Wood (H.m. 9GB-130, Fig. 1) amplified one out of eight microsatellites (Fig. 3) and 8 out of 32 RAPDs specific markers of wheat, while it exhibited none of the enzyme markers of wheat. These nine DNA fragments were also absent in all *E. caninus* individuals, including those harvested 5 m away from this sample (E.c. 8 GB, Fig. 1). None of the other *H. marinum* individuals possessed wheat DNA- or enzyme-specific markers.

PCoA performed on the isozyme based distance matrix showed that all the *H. marinum* samples were well separated from wheat. The samples of the two species

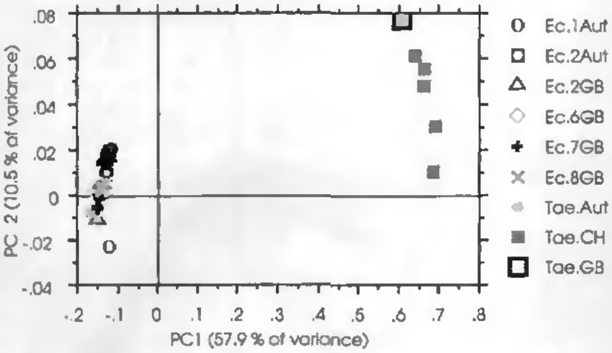

Fig. 2 Principal Coordinates Analysis (PcoA) based on RAPDs data (Jaccard's similarity coefficient). *Ec Elymus caninus*, *Tae Triticum æstivum*

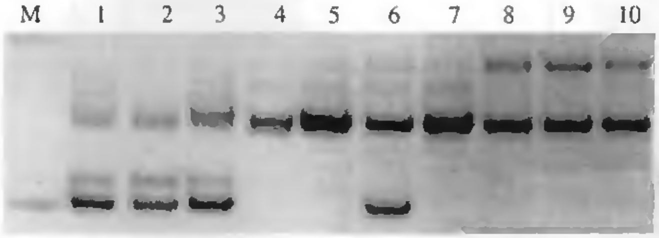

Fig. 3 Example of microsatellite amplification in *T. æstivum* (*lanes 1–3*) and *Hordeum marinum* (*lanes 4–10*) with primer WMS-46. *M* 100-bp DNA ladder (GibcoBRL, Life Technologies), showing one fragment specific for wheat present in one individual of *H. marinum* (H.m. 9GB-130)

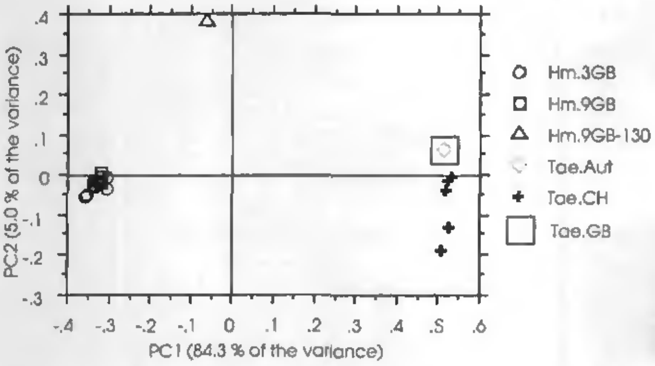

Fig. 4 Principal Coordinates Analysis (PCoA) based on RAPDs data (Jaccard's similarity coefficient), *Hm H. marinum*, *Tae* T. *æstivum*

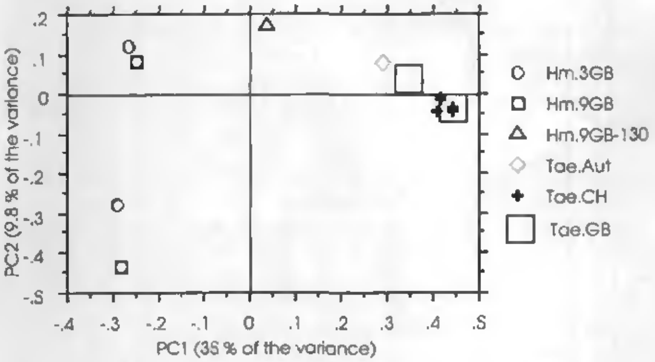

Fig. 5 Principal Coordinates Analysis (PCoA) based on microsatellites data (Jaccard's similarity coefficient), *Hm H. marinum*, *Tae T. æstivum*

were also well separated by the analyses performed on the RAPD and microsatellite distance matrices, with the exception of the individual that amplified specific DNA markers of wheat, which had an intermediate position (Figs. 4 and 5).

Discussion

Hybridization

Spontaneous crop-wild relative hybridization has been shown to be more frequent than previously expected (e.g. Ellstrand et al. 1996). However, because most hybrids are almost completely sterile or the inherited crop traits are maladaptive, conventional crop traits generally do not persist in wild populations. On the other hand, the persistence of foreign traits in one species is possible if hybrids can backcross with one of the parental species and produce viable seeds (Zemetra et al. 1998).

Data obtained with all different types of markers and based on the analysis of 450 individuals showed no evidence of the introgression of wheat traits into *E. caninus* growing within or in the direct proximity of wheat fields. With bearded wheatgrass, even a single hybridization event could have long-term effects. Indeed, the likely partial sterility of such a hybrid would be counterbalanced by its perennial habit, allowing several years to obtain some reproductive success (Ellstrand et al. 1996).

On the contrary, in one sample of *H. marinum* numerous DNA-specific markers of wheat were amplified, that were absent in all the other samples of the same species, including the progeny of population H.m. 3 GB (Wolferton) collected 20–50 m away from the cultivation area. A trait shared by a crop and a wild relative can be the result of either hybridization or the inheritance from a common ancestor (Doebley 1990). However, the presence of crop traits in a population of a wild species growing in agroecosystems, which are absent in populations of the same species isolated from the influence of agriculture, will be an indication of introgression. Because such hybridizations are generally rare events, the presence of only few individuals that possess crop traits within a population of a wild species also indicates introgression. Our results could indicate that at least one hybridization event between the two species has occurred in previous generations. Subsequent backcrosses of the hybrid with pure *H. marinum* could then have led to the introgression of wheat DNA into sea barley. Notwithstanding this species was previously considered to be essentially autogamous, our findings indicate that outbreeding cannot be excluded. This confirms recent results obtained by De Bustos et al. (1998) describing at least partial outbreeding in Spanish populations of the same species.

Whether if the putative hybridization described above is a recent or ancient event can not be determined by only looking at the morphological appearance. Indeed, in a previous study (Guadagnuolo et al. 2000b) we observed that BC_1 plants resulting from backcrosses between *Aegilops cylindrica*×wheat hybrids, and *Ae. cylindrica* had the same morphology as the latter. This indicates that probably one or two backcrosses are sufficient to restore the main morphological traits.

Only DNA markers detected crop traits in the sample H.m. 9GB-130, while the expression of two enzyme systems was identical to that of the other sea barley individuals. Isozymes reflect variation only in the coding parts of the genome, which are probably eliminated during the introgression process if they do not confer adaptative advantages. On the contrary, non-coding DNA (amplified with RAPD and microsatellite primers) can be integrated without affecting the fitness of a plant and thus persist more easily.

Relevance for risk assessment of transgenic wheat cultivation

There is general agreement for considering hybridization between crops and wild relatives as a danger for biodiversity. However, traits introduced in crops by conventional breeding are generally already present in wild plants. Therefore, the consequences of eventual crop-to-wild gene flow should be minor. Moreover, the traits commonly selected in conventional – *i.e.* non-transgenic – crops, like dwarfing or absence of dormancy, do not represent competitive advantages for a wild species (Ellstrand and Hoffman 1990). Even if hybridization occurs, the persistence of this kind of crop gene in the wild is hence unlikely. In addition, because crops and wild relatives are often of different ploidy levels, putative hybrids are mostly sterile and generally eliminated by selection. It is consequently difficult to detect evidence of past introgressions of conventional crop traits into wild species if they do not confer ecological advantages.

The absence of hybrids and introgressants in the progeny of *E. caninus* growing close to wheat fields indicates a very low risk of crop-to-wild gene flow. Therefore, gene escape from transgenic wheat to this wild relative seems unlikely. On the contrary, relatively strong indications of the introgression of wheat DNA into *H. marinum* growing on the border of wheat fields was found. Sea barley generally grows at seasides or inland on saline soil, and its presence in the vicinity of areas of wheat cultivation is not frequent. In order to avoid gene escape from transgenic wheat fields, an isolation distance is thus required, the extent of which will depend on the distance that wheat pollen can cover but also on its longevity. We have found no report in the literature in which the pollination of wild species by wheat was investigated as a function of distance. Several studies, however, have estimated the maximum distance for effective cross pollination (*i.e.* resulting in seed set) between wheat cultivars (de Vries 1974; Kertesz et al. 1995). The results of these studies indicated that cross fertilization decreased to 10% at 3 m and was just detectable at 20 m from the source. Moreover, repeated pollination events were necessary to activate the pollen growth on the stigma (de Vries 1974; Fritz and Lukaszewski 1989), which indicates that few pollen grains will not necessarily fertilize the recipient species. It is thus likely that, independently of the target species, maximum pollination distance will be a few tens of meters. In addition, Poaceae pollen is generally short lived (Zandonella 1984). Fritz and Lukaszewski (1989) found a maximum longevity of 1 hour for wheat (cv. Chinese Spring) pollen, which could decrease severely in the case of hot and dry conditions, as is the case with strong wind in summer. Therefore, an isolation distance of 20–30 m should avoid or strongly reduce the risk of gene escape from transgenic wheat fields to sea barley.

Conclusion

Spontaneous hybridization between wheat and *E. caninus* has not been shown and, thus, introgression of wheat traits into this wild species seems unlikely, regardless of the fact that it often grows in the direct vicinity of wheat fields.

Despite limited contact zones and distant phylogenetic relationships, we found evidence that wheat traits can be transferred into *H. marinum*. As a precautionary principle, an isolation distance of at least 20–30 meters should be required to avoid the risk of gene escape from transgenic wheat fields to sea barley.

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