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Discrimination between closely related *Triticeae* species using genomic DNA as a probe

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Summary. Labelled total genomic DNA was used as a probe in combination with blocking DNA to discriminate between taxonomically closely related species in the genera *Hordeum* and *Secale*. Discrimination was possible both by Southern hybridization to size-fractionated restriction enzyme digests of genomic DNA and by in situ hybridization to chromosome preparations. To distinguish between two species (e.g. *H. vulgare* and *H. bulbosum*), genomic DNA from one species was used as the labelled probe, while unlabelled DNA from the other species was applied at a much higher concentration as a block. The blocking DNA presumably hybridized to sequences in common between the block and the labelled probe, and between the block and DNA sequences on the membrane or chromosomes in situ. If so, mainly species-specific sequences would remain as sites for probe hybridization. These species-specific sequences are dispersed and represent a substantial proportion of the genome (unlike many cloned, species-specific sequences). Consequently, rapid nonradioactive methods detected probe hybridization sites satisfactorily. The method was able to confirm the parentage of hybrid plants. It has potentially wide application in plant breeding for the detection of alien DNA transfer, and it can be easily adapted to many species.

Key words: Genomic probe – Blocking DNA – Chemiluminescence – Species identification – Cereals

Introduction

There is often a need to investigate whether a plant in the Triticeae includes chromosome or chromosomes segments that originate from more than one species (Law 1981; Gale and Miller 1987). Such discrimination requires analysis of species-specific characteristics, e.g. phenotypic examination (Sears 1954), chromosome morphology (Gill et al. 1988) or isozyme analysis (Koebner et al. 1988; Hart and Tuleen 1983). Cloned probes of repetitive or single-copy DNA sequences can be used to detect the origin of genetic material by probe hybridization to isolated DNA (Metzlaff et al. 1986; Sharp et al. 1989), or to chromosomes in situ (Lapitan et al. 1986). It is normally a laborious process to isolate species-specific clones because there are large homologies in DNA sequences between species, particularly in the tribe Triticeae (Flavell et al. 1977), and in some cases the probes may be localized to only specific chromosomes or chromosome regions (Bedbrook et al. 1980).

An alternative approach is to use total genomic DNA directly as a probe in DNA hybridization experiments where the parental origin of chromosomes in sexual intergeneric plant hybrids can be differentiated in spread chromosome preparations with total genomic DNA probe (Schwarzacher et al. 1989; Le et al. 1989). For example, nonradioactive dot-blot hybridization and colorimetric detection using total genomic DNA from a wild rye, *Secale africanum*, as a probe showed that probe DNA hybridized more strongly to *S. africanum* DNA than to DNA from a wild barley species, *Hordeum chilense* (Schwarzacher et al. 1989). The present paper describes experiments aimed at increasing the specificity of the genomic probing system to detect the species origin of DNA in plants.

Materials and methods

Triticeae species and an intergeneric hybrid

Table 1 shows the species and an intergeneric hybrid used in the experiments. All plants were grown under glasshouse condi-

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Table 1. The Triticeae species and the hybrid used in the experiments (all 2n = 2x = 14)

Barley (*Hordeum*):	*H. vulgare* L. cv Tuleen 346
	H. chilense Roem. & Schult., Line 1
	H. bulbosum Nevski, clone Lange 6, Line 6R45
Rye (*Secale*):	*S. cereale* L. cv Petkus Spring
	S. africanum Stapf., Line R102
Intergeneric hybrid:	*H. chilense* Roem. & Schult. × *S. africanum* Stapf.

tions. For chromosome preparations, the hybrid was transferred to a hydroponic medium in a controlled environment cabinet for 2–5 days before roots were collected.

DNA extraction, digestion and transfer

Methods for DNA extraction, restriction enzyme digestion, agarose gel electrophoresis and alkaline "Southern" transfer of DNA to nylon membranes were as described by Sharp et al. (1988), with minor modifications such as the use of Hybond N^+ membranes (Amersham). Total genomic DNA was digested to completion using EcoRI and DraI restriction endonucleases (Gibco – BRL), and lambda HindIII DNA was used as a size marker. The agarose gels were stained with ethidium bromide and only gels where all tracks of genomic DNA had approximately equal amounts of DNA were used for transfer.

Probe labelling and Southern hybridization

The nonradioactive chemiluminescence method, ECL (Amersham), was used for probe labelling, hybridization and the detection of hybridization sites, following the manufacturer's instructions. Total genomic DNA was mechanically sheared, denatured by boiling for 5 min and labelled by linking horseradish peroxidase to the DNA with glutaraldehyde at 37 °C for 10 min. The length of ECL-labelled probes was estimated by gel electrophoresis to be about 500 bp. Lambda DNA was labelled in the same way as the probe. The membrane was incubated for at least 15 min at 42 °C in ECL hybridization buffer containing 6 *M* urea, with the addition of between 0.1 and 0.5 *M* sodium chloride to control stringency before the addition of DNA.

For experiments involving genomic blocking, DNA fragments of 100–200 bp length were obtained by autoclaving the total genomic DNA for 5 min. The required amount of blocking DNA, 1–10 μg ml^{-1}, was denatured by boiling for 7 min, added to the hybridization buffer surrounding the membrane and incubated at 37°–39 °C for at least 30 min. The labelled probe (10–20 ng ml^{-1}), with labelled lambda DNA (3–6 ng ml^{-1}), was then added and the incubation continued for 8–16 h at 42 °C in a shaking water bath.

The stringency of hybridization and washing was calculated using the formula of Meinkoth and Wahl (1984). In cereal plants, 45.5% of the bases are guanine or cytosine (Swanson and Webster 1975); 6 *M* urea in the hybridization and wash buffer is equivalent to 50% formamide (Amersham).

Washing and chemiluminescent detection

After hybridization, weakly hybridized and unhybridized probe was removed by two washes of 20 min each at 37°–39 °C in 6 *M* urea and 0.4% SDS (sodium dodecyl sulphate) in 0.1 × to 0.5 × SSC (20 × SSC: 3 *M* sodium chloride, 0.3 *M* sodium citrate, pH 7), to raise the stringency approximately 8% above that used for hybridization. This was followed by two rinses of 5 min each in 2 × SSC at room temperature.

Hybridization sites were detected using ECL detection reagents that contained the chemiluminescent compound luminol and hydrogen peroxide. Oxidation of the luminol was catalyzed by horseradish peroxidase linked to the hybridized probe, and the light emitted was recorded directly on X-ray film. Exposure times for the luminographs varied from 1 min to 1 h. After detection, the membranes were stored moist at 4 °C and all were reprobed several times without removal of probe or blocking DNA.

The blocked and unblocked pairs of Southern blots shown in the figures were probed at the same time with samples from the same batch of labelled probe. After hybridization under similar conditions except for the blocking DNA, the pairs of membranes were washed together and the sites of hybridization were detected on a single piece of film. Therefore, differences in hybridization that are visible could not be accounted for by probe batches, exposure or development times.

Signal quantification

Probe hybridization and DNA amounts were measured semi-quantitatively with a microcomputer-based image digitizing system. Films with short exposure time (where the most intense hybridization areas were not completely black, such as Fig. 2b and c) were digitized, and the average grey level of each track was calculated. The background level from an unhybridized track was substracted from each track of interest, and relative amounts of signal in the relevant tracks were calculated.

Chromosome preparations

Chromosome spreads of root-tip meristem cells of the intergeneric hybrid *H. chilense* × *S. africanum* were prepared and in situ hybridization was performed using techniques modified from Schwarzacher et al. (1989). The hybridization probe mixture consisted of 5 μg ml^{-1} biotinylated total genomic DNA from *S. africanum*, 50 μg ml^{-1} unlabelled autoclaved total genomic DNA from *H. chilense*, 50% (v/v) deionized formamide and 0.1% (w/v) SDS in 2 × SSC. Hybridization was carried out at 37 °C overnight, followed by stringent washing in 50% formamide in 2 × SSC at 40 °C for 10 min. For the detection of hybridized probe, Texas Red conjugated avidin (Vector Laboratories) and signal amplification was used. The preparations were stained with 1–2 μg ml^{-1} DAPI (4',6-diamidino-2-phenylindole) in McIlvaine citric acid buffer (pH 7) before examination by epifluorescence light microscopy.

Results

Differentiation of closely related species

Experiments were designed to test the effect of blocking DNA in combination with various hybridization and washing stringencies. The luminographs in Fig. 1 show the hybridization of labelled genomic *S. cereale* (rye) probe to EcoRI digests of *S. cereale* and *S. africanum* genomic DNA. In Fig. 1a, strong probe hybridization to DNA tracks from both species is visible, and bands of restriction fragments from highly repeated DNA families are very similar. When the membrane was blocked with unlabelled DNA from *S. africanum* under more stringent conditions (Fig. 1b), the hybridization of *S. cereale* probe to the *S. africanum* track was greatly decreased,

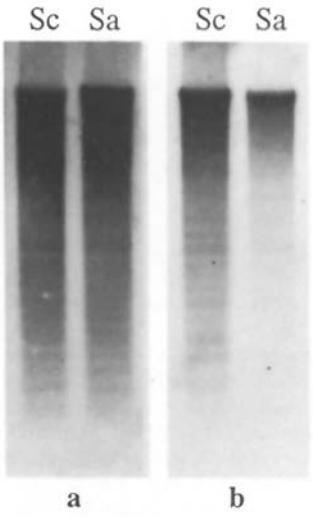

Fig. 1 a and b. Luminographs showing Southern hybridization of labelled total genomic DNA from *Secale cereale* to size-fractionated EcoRI-digested genomic DNA of *S. cereale* (Sc) and *S. africanum* (Sa); 1 μg DNA per track, probe concentration 20 ng ml^{-1}, 25 min exposure. **a** Hybridization stringency 78%, no blocking DNA. **b** Hybridization stringency 90%, membrane blocked with 8 μg ml^{-1} *S. africanum* genomic DNA

while the amount of hybridization to the *S. cereale* track was much less decreased. In addition, a different pattern of bands on the *S. cereale* track was apparent, suggesting that certain restriction fragments in common with *S. africanum* were blocked, leaving the *S. cereale*-specific bands available for probing.

Similar results were obtained from experiments with another pair of related plant species, *H. vulgare* (barley) and *H. bulbosum* (Fig. 2). A photograph of the stained gel confirms that all genomic tracks were loaded with similar amounts of DNA (Fig. 2a). Results from two experiments where the same membranes were probed at different stringencies and detected at different exposure levels are shown. The higher stringency and lower exposure shows the improvement of species differentiation by blocking, while the higher exposure shows interspecific differences in probing restriction fragment bands of highly repeated DNA families. When the genomic DNA from *H. bulbosum* was used as a probe, it hybridized to DNA from both species (Fig. 2b and d), with reduced hybridization to the *H. vulgare* track at a higher stringency level (Fig. 2b). Blocking with unlabelled genomic DNA from *H. vulgare* greatly reduced cross-hybridization between the *H. bulbosum* probe and *H. vulgare* tracks. Probe hybridization to the *H. bulbosum* tracks remained strong. Thus, the two species could be easily distinguished (Fig. 2c and e). When digested with two different restriction enzymes, the genomic DNAs gave different fragment length distributions (Fig. 2a). However, the strength of probe hybridization and the effect of blocking were similar with both enzymes.

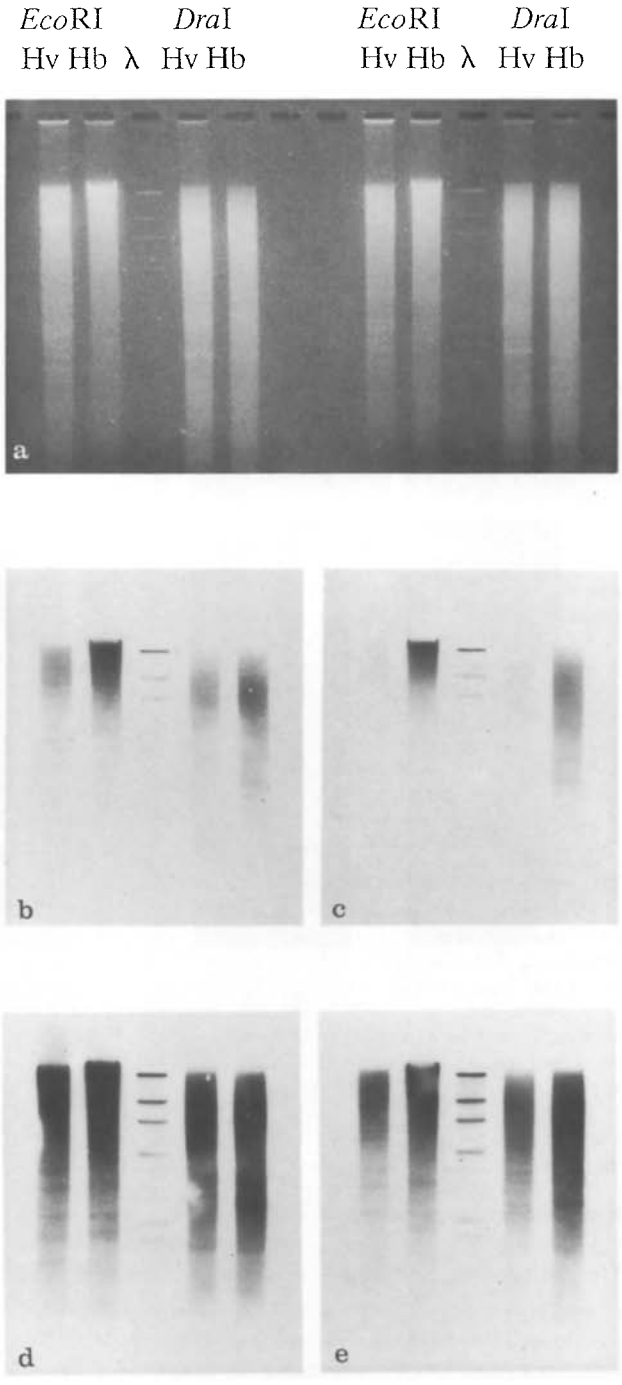

Fig. 2 a–e. Southern hybridization using total genomic DNA from *Hordeum bulbosum* as a probe to discriminate between two *Hordeum* species, *H. vulgare* (Hv) and *H. bulbosum* (Hb). **a** Ethidium-bromide-stained gel showing size-fractionated EcoRI- and DraI-digested genomic DNA of the two species; 1 μg DNA per track, lambda HindIII size marker (top to bottom, 23.1, 9.4, 6.6, 4.4, 2.3 and 2.1 kb). **b** and **c** Luminographs showing hybridization with probe concentration 12.5 ng ml^{-1}, hybridization stringency 90%, 10 min exposure. **b** No blocking DNA. **c** Blocked with 6 μg ml^{-1} *H. vulgare* genomic DNA. **d** and **e** The same membranes after reprobing; probe concentration 16 ng ml^{-1}, hybridization stringency 82%, 15 min exposure. **d** No blocking DNA. **e** Blocked with 5 μg ml^{-1} DNA

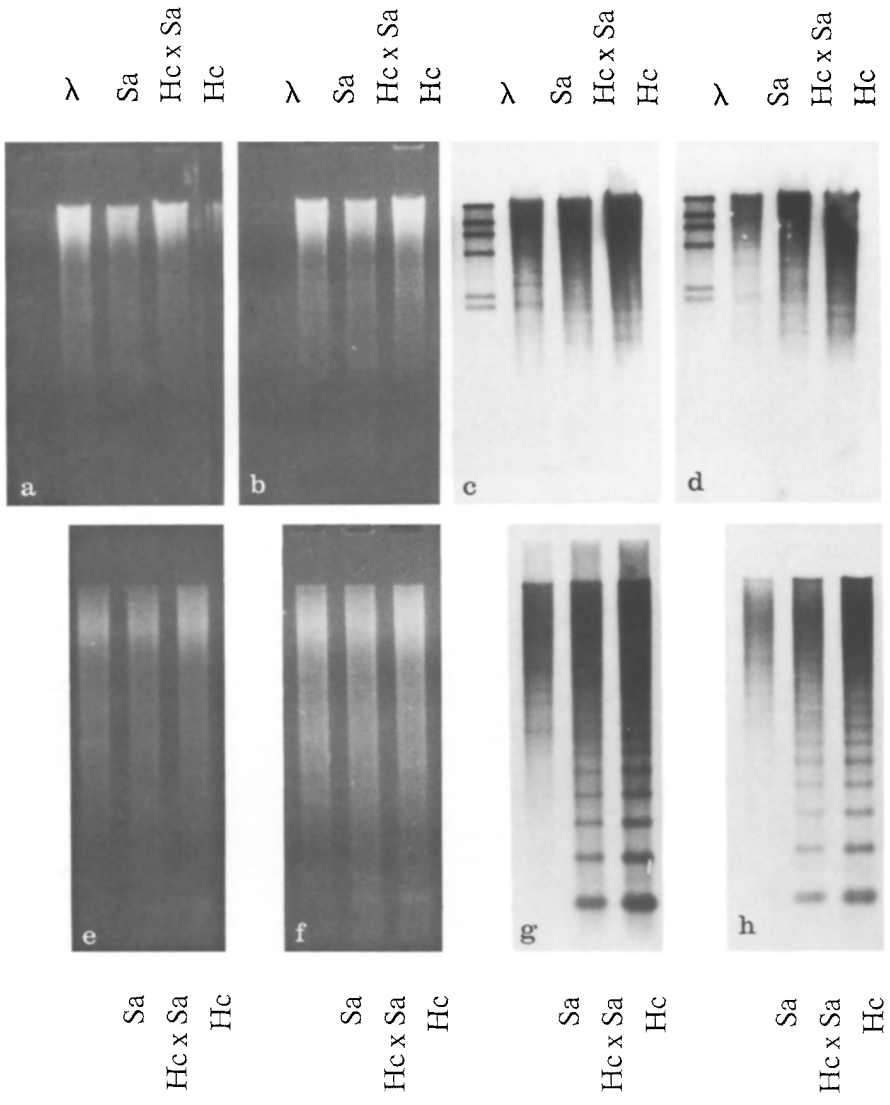

Fig. 3a–h. Southern hybridization using total genomic DNA from *Hordeum chilense* as a probe to confirm the parental origin of the hybrid *H. chilense* × *Secale africanum* and to differentiate between the parental species. **a** and **b** Ethidium-bromide-stained gels showing size-fractionated EcoRI genomic digests of *S. africanum* (Sa), the hybrid (Hc × Sa) and *H. chilense* (Hc); 1 μg DNA per track, lambda size marker as in Fig. 2. **c** and **d** Luminographs showing hybridization with probe concentration 12.5 ng ml^{-1}, hybridization stringency 82%, 30 min exposure. **c** No blocking DNA. **d** Blocked with 6 μg ml^{-1} *S. africanum* genomic DNA. **e** and **f** Stained gels showing size-fractionated DraI genomic digests of the same species and hybrid, 1 μg DNA per track. **g** and **h** Luminographs showing hybridization of *H. chilense* probe to DraI-digested genomic DNA; probe concentration 12.5 ng ml^{-1}, hybridization stringency 79%, 3 min exposure. **g** No blocking DNA. **h** Blocked with 10 μg ml^{-1} *S. africanum* DNA

Confirmation of parents in a hybrid

The total genomic probe was used to confirm the parents in an intergeneric hybrid. Figure 3 shows the results of Southern hybridization of labelled genomic probe from a wild barley species, *H. chilense*, to the EcoRI (Fig. 3a–d) and DraI (Fig. 3e–h) digests of genomic DNA from the hybrid *H. chilense* × *S. africanum* and its parental species. The relative amount of DNA from each species and the hybrid can be compared in the ethidium-bromide-stained gel (Fig. 3a and b, e and f). Strong hybridization was detected on the *H. chilense* track but less on the hybrid track that contains DNA from both *H. chilense* and *S. africanum* (Fig. 3c and g). Low-molecular-weight restriction fragments, characteristic of the *H. chilense* genome, are also visible. Cross-hybridization between the *H. chilense* probe and the *S. africanum* track was reduced by blocking the membrane with unlabelled genomic DNA from *S. africanum* (Fig. 3d and h).

The amount of hybridization to the tracks from the blots shown in Fig. 3 (g and h) was quantified (Table 2). The results showed that the hybridization to the *S. africanum* track was reduced to 15% (4% out of 26%) of the unblocked level, while the blocking only reduced the hybridization to the *H. chilense* track to 64% of the unblocked level.

Based on the hybridization to the two parental tracks, the expected amount of probe hybridization to the hybrid can be estimated. The DNA content of a prophase (4C) nucleus of *S. africanum* is 29.7 pg, while that of *H. chilense* is 21.8 pg (Bennett and Smith 1976). Since the hybrid plants contain one copy of each genome per cell, the relative intensities of the three tracks from *H. chilense*, the hybrid and *S. africanum* probed with a

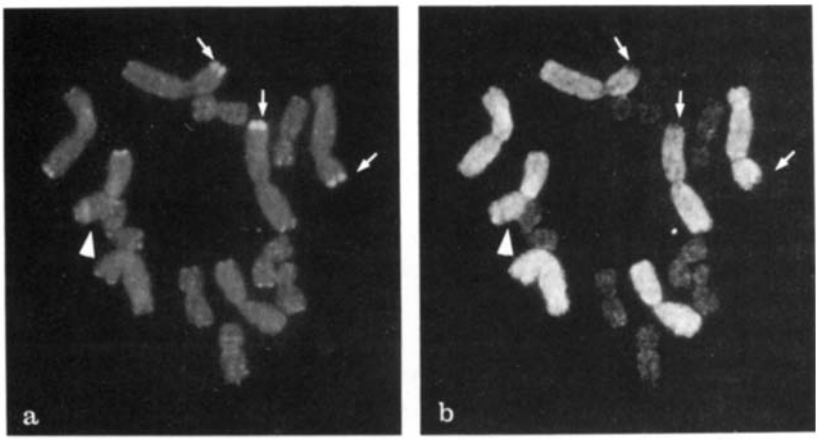

Fig. 4a and b. Epifluorescent photomicrographs showing metaphase chromosomes from a root-tip cell of the hybrid *Hordeum chilense* × *Secale africanum* after in situ hybridization using biotinylated total genomic DNA from *S. africanum* as probe and unlabelled DNA from *H. chilense* as block. **a** DAPI staining of DNA shows fluorescence of all 14 chromosomes. **b** The same metaphase shows Texas Red fluorescence at sites where the probe hybridized. Nucleolus organizer region: *arrowheads;* telomeric heterochromatin: *arrows;* magnification ×1,250

Table 2. The amounts of hybridization of *H. chilense* genomic probe to the DNA tracks shown in Fig. 3 (g and h) calculated relative to the hybridization to the unblocked and blocked *H. chilense* tracks. A luminograph with a shorter exposure time than that shown in Fig. 3 (where the film was saturated) was used. Two replicates of each track on the same blot were averaged to give the results shown

	Signal relative to *H. chilense* tracks (%)	
	unblocked	blocked
Unblocked tracks:		
H. chilense	100	–
H. chilense × *S. africanum*	58	–
S. africanum	26	–
Tracks blocked with *S. africanum:*		
H. chilense	64	100
H. chilense × *S. africanum*	27	42
S. africanum	4	6

H. chilense specific probe would be expected to be 1.00:0.42:0.00. The actual ratio is 1.00:0.58:0.26 (unblocked) or 1.00:0.42:0.06 (blocked).

Sites of hybridization in chromosome spreads

Figure 4 shows fluorescent micrographs of a metaphase from a root-tip chromosome spread of the hybrid *H. chilense* × *S. africanum*. After DAPI staining, the euchromatic DNA of all 14 chromosomes fluoresced under UV excitation, while the telomeric and some intercalary heterochromatic bands were characterized by more intense fluorescence (Fig. 4a).

Biotinylated total genomic DNA from *S. africanum* was used as a probe together with a tenfold excess of unlabelled genomic DNA from *H. chilense* for in situ hybridization (Fig. 4b). Sites of probe hybridization were visualized by bright Texas Red fluorescence under green light. The chromosomes of the hybrid were distinguished into two groups by the fluorescence intensity and hence probe hybridization. Strong probe hybridization was detected to the seven larger chromosomes, which are of *S. africanum* origin in this hybrid (Schwarzacher-Robinson et al. 1987). The label was almost uniform along all euchromatic DNA, while some segments of the telomeric heterochromatin (Fig. 4, arrows), the nucleolus organizer region (Fig. 4, arrowhead) and centromeric regions showed little or no label. The seven smaller chromosomes of *H. chilense* origin were almost unlabelled.

Discussion and conclusions

The use of genomic DNA as a probe

The discrimination between species using total genomic DNA was substantially improved, or in some cases became possible, by using blocking DNA. This involves the addition of relatively high concentrations of unlabelled genomic DNA from a related species not used as the probe. The use of total genomic DNA in combination with blocking as a species-specific probe has several advantages over the use of cloned species-specific probes. The genomic DNA is used directly as a probe, without the need for cloning or the time-consuming and uncertain procedure of screening clones for species specificity. It has a particular advantage when a range of different species and genera are used in a research program. The genomic probing procedure is simple and straightforward in application. Genomic DNA includes a wide range of multiple-copy sequences, so is it unlikely to be chromosome- or chromosome-region-specific, an advantage in situations where a broad specificity is required. A species-specific cloned probe may be homologous to only some chromosomes or chromosome segments.

Total genomic DNA has previously been used as a probe for in situ hybridization to identify human chro-

mosomes in human-rodent cell fusion hybrids,where the chromosomes originated from evolutionarily distant species (Manuelidis 1985; Schardin et al. 1985; Pinkel et al. 1986). In plants, total genomic probes have been used to detect rye chromosomes in wheat × rye hybrids (Le et al. 1989) and in barley × rye hybrids (Schwarzacher et al. 1989) by in situ hybridization.

The species used in this work are related grasses in the tribe Triticeae that have evolved from a common ancestor (Miller 1987). The cereal genome consists of less than 1% highly conserved coding sequences, while 55%–70% of the cereal genome consists of interspersed or tandem repeats (Flavell et al. 1981). It is these repeated sequences that are likely to be detected by the hybridization of total genomic DNA. The Southern blots show that the genomic probe hybridized both to highly repeated DNA families, which appeared as bands on the membrane, and to less highly repeated sequences, which gave rise to the continuous smear of restriction fragment lengths. When blocking DNA was added, species-specific bands became more visible, and other bands reduced in relative intensity. The level of the smear was also reduced. On this basis, it seems that the probe and block hybridized to both highly repeated DNA families and less-repeated families.

The uniform labelling of the *S. africanum* chromosomes after in situ hybridization (Fig. 4b) indicated that the genomic DNA probe hybridized to sequences that are dispersed throughout the genome, and the strength of hybridization indicated that the hybridization involved middle and highly repetitive DNA sequence families. This interpretation agrees with the results from the Southern hybridization experiments. The lack of hybridization to some segments of heterochromatic bands (Fig. 4b, arrows), which show hybridization if no blocking DNA is used (Schwarzacher et al. 1989), is probably due to the blocking of highly repeated tandem sequences common to *H. chilense* and *S. africanum*.

Some of the Southern membranes were reprobed with the highly repetitive rDNA clone pTa71 (Gerlach and Bedbrook 1979), which labelled sharply defined restriction fragment bands. No corresponding strongly defined bands were visible when the membrane was probed with genomic DNA.

In Southern and in situ hybridizations, using genomic probes with the control of stringency alone was often able to distinguish genera because the DNA sequences were sufficiently nonhomologous that the amount of probe hybridization to the species was different (Fig. 3; Schwarzacher et al. 1989). Distantly related species within a genus (e.g. *H. vulgare* and *H. chilense*; von Bothmer and Jacobsen 1985) could also be differentiated at high stringencies without blocking. Increasing the stringency of hybridization reduced the overall amount of hybridization because the sequences had to match more closely. The effect was emphasized because the efficiency of hybridization of short DNA fragments was decreased substantially at higher stringencies (Flavell et al. 1977; Meinkoth and Wahl 1984). Closely related species within a genus (e.g. *S. cereale* and *S. africanum*; Fig. 1) could not be differentiated by the control of stringency alone. In order to differentiate the species, and where increased resolution was essential (e.g. to identify alien chromosomes or chromosome segments, or the parents of hybrids), blocking DNA as well as stringency control was required.

Use of blocking DNA

Unlabelled genomic DNA used as a block increases the specificity of probing (Figs. 1–4). Such blocking not only increases the differentiation between the probed species and the species whose DNA is used as a block, but also reduces cross-hybridization to other species (results not shown), presumably because many sequences are shared between all the plants in the family under study. The major effect of the blocking in the present experiments may be because of (a) hybridization between probe DNA and common sequences in the block, (b) hybridization between the block and common sequences on the membrane-immobilized DNA or the chromosomes in situ, or (c) a combination of both. Additional experimentation is required to elucidate the main effect of the blocking; probes and block DNA could be multiply labelled (e.g. with ^{32}P, biotin and ECL methods) and the amount and positions of hybridization of each could be analyzed. Steric hindrance of probe hybridization after block hybridization may enhance the blocking effect, because hybridization of probe may be inhibited in the vicinity of block. The data in Table 2 can be used to show that the steric hindrance effect is unlikely to be large, but cannot be ruled out completely. Based on the actual hybridization to the species tracks in the unblocked membrane, the hybrid (containing 2:3 ratio of DNA from *H. chilense* and *S. africanum*) would be expected to show 57% of the level of hybridization to the *H. chilense* track, similar to the actual value of 58%. In the blocked tracks, the prediction would be 29%, again similar to the actual value of 27%. Perhaps the use of longer sequences for blocking would increase steric hindrance and hence increase probe specificity further.

Alternative blocking approaches have been reported for use with cloned probes, both to DNA in situ (Lichter et al. 1988; Pinkel et al. 1988) and on Southern blots (Sealey et al. 1985). Preannealing of a denatured mixture of human total genomic DNA and human chromosome-specific probes allowed the genomic DNA to block sequences in the probes that were common to many chromosomes. The method proved useful in the detection of chromosome aberrations such as trisomies (Cremer et al. 1988). Arnold (1988) reported the use of cloned or syn-

thesized DNA sequences as a block. With highly repetitive sequences, such an approach may prove useful in the cereals.

Application of the technique

In plant breeding, it is important to test for interspecific hybrid origin or the incorporation of alien chromosomes or chromosome segments carrying desirable traits. Often, wide hybrids need to be examined to see whether they contain the genomes from both parents, since one genome may have been eliminated (e.g. Bennett et al. 1976) or the progeny may have arisen by parthenogenetic development (Heslop-Harrison 1972). The parentage of any hybrid plant must also be confirmed, since stray pollen may fertilize an egg. The method using genomic probing and blocking allows such detection and it is applicable in different situations involving a wide range of species.

Because the total genomic DNA includes sequences homologous to many or all sequences of the DNA under study, highly sensitive detection of hybridization is not required. Thus, both the chemiluminescent method on Southern blots and the biotin method for nonradioactive in situ hybridization detection are satisfactory. For screening of recombinant lines it would be possible to use dot blots, or even squash dots, where tissue (e.g. root tips) is directly squashed onto nitrocellulose membrane (Hutchinson et al. 1985). Hybridization times may also be reduced to allow the procedure to be carried out within 1 day. Chromosome preparations for in situ hybridization are technically more difficult than Southern blotting, but in situ hybridization is necessary to show which chromosomes or chromosome segments are probed, and it enables identification of chromosome segments involved in translocations.

Species-specific probes consisting of dispersed repeats can be used to identify chromosomes (Lapitan et al. 1986; Appels et al. 1986) but they may be chromosome or chromosome-segment specific, as well as genome specific, and hence cannot detect all possible translocations. The effort to isolate and characterize such clones is considerable and may not be commercially worthwhile in species of small economic importance. The genomic probing method reported here may be of wide application in the introduction of alien genetic variation for crop improvement.

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