Genomic *in situ* hybridization identifies parental chromosomes in the wild grass hybrid × *Festulpia hubbardii*

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Genomic *in situ* hybridization (GISH) was used to discriminate between the parental chromosomes of the grass × *Festulpia hubbardii* ($2n = 5x = 35 \pm 2B$), a naturally occurring pentaploid hybrid between *Festuca rubra* ($2n = 6x = 42 \pm 2B$) and *Vulpia fasciculata* ($2n = 4x = 28$). Biotinylated total genomic DNA of *V. fasciculata*, mixed with an excess of unlabelled *F. rubra* genomic DNA as a block, hybridized preferentially *in situ* to mitotic *Vulpia* chromosomes. Unlike Giemsa C-banding, which provided a physical marker only for the terminal regions of the parental chromosomes, GISH unequivocally identified chromatin from all regions along every *Vulpia* chromosome. Clearly, GISH has the potential for testing the theory that *Vulpia* genetic material has been transferred into *Festuca rubra* populations by introgressive hybridization. Genomic probing also discriminated between homogenetic (*Vulpia–Vulpia* and *Festuca–Festuca*) and heterogenetic (*Vulpia–Festuca*) bivalents, showing that GISH can aid the interpretation of chromosome behaviour in meiotic nuclei of hybrids.

Keywords: *Festuca rubra*, × *Festulpia*, genomic *in situ* hybridization (GISH), grass hybrids, introgressive hybridization, *Vulpia fasciculata*.

Introduction

Interspecific hybridization is a major force in flowering plant evolution. The relatively high incidence of natural hybridization in the grasses is well known; 57 different interspecific combinations have been found in Britain alone (Stace, 1991).

Festuca L. is a widespread genus of perennial, outbreeding, temperate grasses with probably over 300 species. *Festuca rubra* L. is a very variable species which is important both ecologically (being a major constituent of North-temperate grassland) and economically (second in Britian only to *Lolium* in terms of tonnage of turf-grass seed production). It is part of a complex aggregate of fine-leaved taxa, chiefly of North-temperate Eurasia and America, and especially characteristic of maritime habitats, dominating large areas of sand-dune, cliff-top and salt-marsh vegetation.

Vulpia C. C. Gmelin is a closely related genus of about 20 mostly annual, inbreeding, species found in Europe, western Asia and temperate North and South America. In western and north-western Europe five species, including *V. fasciculata* (Forsskål) Fritsch, are characteristic of maritime sandy regions, where they commonly occur in close proximity to *Festuca rubra*. Four combinations of intergeneric hybrids between members of the *F. rubra* aggregate and species of *Vulpia* have been found in such habitats in England (Stace, 1991).

This paper concerns the hybrid *Festuca rubra × Vulpia fasciculata* = × *Festulpia hubbardii* Stace & Cotton, which is frequent in southern Britain as far north as S. Lancashire and in the Channel Islands (Ainscough *et al.*, 1986). *Vulpia fasciculata* is a semi-chasmogamous, self-compatible species; isolated plants produce a full seed-set and, in the wild, plants probably produce a negligible pollen-rain and are highly inbreeding. In contrast, *Festuca rubra* is highly (though not fully) self-incompatible (Auquier, 1977) and produces an abundant pollen-rain, so wild intergeneric hybrids are almost certainly derived from female *Vulpia* × male *Festuca*. The hybrid was produced artifi-

cially with great ease but only in the latter direction (Stace & Cotton, 1974). As *Vulpia fasciculata* is a tetraploid $(2n = 4x = 28)$ and the *Festuca rubra* involved in these crosses a hexaploid $(2n = 6x = 42)$, the hybrid × *F. hubbardii* genome $(2n = 5x = 35)$ consists of 14 *Vulpia* and 21 *Festuca* chromosomes in a *Vulpia* cytoplasm. Some plants of *F. rubra* and × *F. hubbardii* possess one or two B-chromosomes, as did some of the present material (Table 1).

Meiosis in the pentaploid wild × *Festulpia* is mostly very irregular and the plants are highly sterile. A very variable number of bivalents is formed at meiosis, ranging between the minimum (0) and theoretical maximum (14) (Barker & Stace, 1986). Since the karyotypes of *F. rubra* and *V. fasciculata* are indistinguishable by conventional staining techniques (e.g. Feulgen or orcein), all the chromosomes being similar in size and all metacentrics or submetacentrics, it is not possible to distinguish between homogenetic (V–V or F–F) and heterogenetic (V–F) pairing at meiosis (up to 14 bivalents could form from either or both types of pairing). Bailey & Stace (1992) investigated the Giemsa C-banded karyotypes and found that the two parental species presented different patterns. In *Festuca rubra* all the chromosomes have a conspicuous terminal heterochromatic block on one or both arms, such blocks being completely absent from *Vulpia fasciculata*. Weak interstitial or centromeric bands are present in most of the *Vulpia* chromosomes, but not in *Festuca*. This difference allows the parental origin of every chromosome of the F_1 hybrid × *Festulpia* to be determined at both mitosis and meiosis (Bailey & Stace, 1992), and demonstrates unequivocally that both homogenetic and heterogenetic pairing occurs, roughly in the ratio 3:1 (Bailey & Stace, 1992).

Although the F_1 hybrid is highly sterile, a small amount of seed is set (almost certainly via backcrossing to male *F. rubra*) and mature plants can be raised. One plant examined in detail by Stace & Ainscough (1984) proved to be hexaploid with a near-regular meiosis (19–21 bivalents) and a reasonably high seed-set. This plant resembles normal *F. rubra* closely, but differs in a few quantitative characters that are matched by some wild hexaploids growing in maritime sand near populations of *Vulpia fasciculata*. The existence of such plants demonstrates a mechanism for the introgression of *Vulpia fasciculata* into *Festuca rubra*, so increasing the variability of the latter. Presumably, the cytoplasm of these backcross derivatives is essentially that of *Vulpia*, and up to 14 of the chromosomes (but possibly many fewer) contain *Vulpia* genes. However, all of the 42 chromosomes have one or two terminal *Festuca*-derived bands (Bailey & Stace, 1992). Possibly, interstitial segments of *Vulpia* chromosomes are integrated

into the genome, but the pattern of C-banding in the two parents is inadequate to demonstrate it. Hence, a reliable marker for the entire length of each *Vulpia* chromosome is needed.

Genomic *in situ* hybridization (GISH) provides a direct, visual method of distinguishing entire parental genomes in both intergeneric (Schwarzacher *et al.*, 1989; Anamthawat-Jónsson *et al.*, 1990; Leitch *et al.*, 1991) and interspecific (Schwarzacher *et al.*, 1992a; Parokonny *et al.*, 1992a) man-made hybrids in plants. As a physical marker for whole genomes, GISH has been used to identify alien chromosomes or chromosome segments in wheat (Heslop-Harrison *et al.*, 1990; Mukai & Gill, 1991; Schwarzacher *et al.*, 1992b), to examine genome reorganization in asymmetric somatic hybrids (Parokonny *et al.*, 1992b), and to test genome homology for studying plant evolution and biosystematics (Bennett *et al.*, 1992; Kenton *et al.*, 1993).

We aim to use GISH to test the hypothesis that *Vulpia* chromatin has been transferred into *Festuca rubra* populations by introgressive hybridization. The present paper reports the results of experiments to assess whether GISH can reliably discriminate between *Vulpia* and *Festuca* chromosomes in both somatic and meiotic nuclei of × *F. hubbardii*.

Materials and methods

Plant material

Plants of *Festuca rubra*, *Vulpia fasciculata* and their F_1 hybrid × *Festulpia hubbardii*, were obtained from natural sources (Table 1). These have formed the basis of previous studies (Stace & Cotton, 1974; Stace & Ainscough, 1984; Ainscough *et al.*, 1986; Bailey & Stace, 1992), and have subsequently been cultivated at the Department of Botany, University of Leicester.

Actively growing root tips were pre-treated in saturated α-bromonaphthalene at 4°C for 22 h, and fixed and subsequently stored in 3:1 (v/v) absolute ethanol:glacial acetic acid at 4°C.

Preparation of chromosome spreads

Air-dried mitotic spreads were prepared by physically removing meristematic tissue from fixed root tips using tungsten needles and without prior enzyme hydrolysis. Meiotic spreads were made from the anther contents of fixed inflorescences as described by Bailey & Stace (1992). Care was taken to minimize exposure of fixed anthers to ambient conditions by storing florets awaiting dissection on solid carbon dioxide covered with ice. Anthers of the required size for meiosis were quickly stripped out and stored on the same cold plate in a

Table 1 Origin and chromosome numbers of the material used in this study

Taxon	Location	Grid reference	Habitat	Chromosome number (2n)
Vulpia fasciculata	Berrow, N. Somerset, England	ST/29.51	Sand-dunes	28
Festuca rubra	Nr. Ballymacarbry, Waterford, Eire	S/25.14	Bank of river Nier	42 + 2B
V. fasciculata × *F. rubra*	Pwllheli, Caernarvonshire, Wales	SH/37.34	Sand-dunes	35 + 2B
	Berrow, N. Somerset, England	ST/29.51	Sand-dunes	35
	Ainsdale, S. Lancashire, England	SD/28.11	Sand-dunes	35

sealed bijou bottle. Coverslips of both mitotic and meiotic preparations were removed using solid carbon dioxide (Conger & Fairchild, 1953). Slides were air-dried before being stored in a vacuum desiccator at 4°C for up to several weeks.

Prior to *in situ* hybridization, and usually the evening before, slides were re-fixed in 45 per cent acetic acid for 10 min, washed in absolute ethanol twice, 10 min each, and dried in an oven overnight at 42°C. Slides were then treated with 100 μg ml^{-1} of DNase-free RNase in 2 × SSC (0.3 M NaCl, 0.03 M sodium citrate, pH 7) for 1 h at 37°C in a moist chamber, washed thrice in 2 × SSC, 5 min each, dehydrated through an ethanol series (70 per cent, 80 per cent, absolute (twice), 5 min each) on ice, and dried in an oven at 42°C.

Genomic probe preparation and in situ hybridization

Genomic DNA was extracted from 0.5–1.0 g of fresh leaf material using the CTAB method of Doyle & Doyle (1987) and mechanically sheared to give fragments largely in the range of 10–12 kb, as assessed using gel electrophoresis. For use as a probe, 2–3 μg were labelled with biotin-14-dATP by nick translation using a modified version of the manufacturer's (GIBCO BRL BioNick Labeling System) protocol; essentially ethanol precipitation was performed only once to reduce DNA loss, and the DNA pellet was washed in 70 per cent ethanol prior to drying before resuspension in TE (10 mM Tris-HCl, 1 mM EDTA, pH 8.0). Total genomic DNA from each of the parental species, *Festuca rubra* and *Vulpia fasciculata*, was autoclaved for 5 min to give DNA fragments of about

100–250 bp long to be used as blocking DNA (cf Anamthawat-Jónsson *et al.*, 1990).

Genomic *in situ* hybridization was as described by Bennett *et al.* (1992) with only minor modifications. Chromosomal DNA on the slides was denatured by immersion in 70 per cent de-ionized formamide (Fisons, electrophoresis grade), 30 per cent 2 × SSC at 68–72°C for 2 min. Slides were then rapidly dehydrated in an ethanol series (70 per cent, 80 per cent, absolute (twice), 2 min each) at −20°C (from Mukai *et al.*, 1991) and air-dried. The probe mix containing 5–10 μg ml^{-1} biotinylated genomic DNA, 50 per cent (v/v) de-ionized formamide, 10 per cent (w/v) dextran sulphate, 900 μg ml^{-1} sonicated salmon sperm DNA, 50–100 μg ml^{-1} blocking DNA (cf. Anamthawat-Jónsson *et al.*, 1990), and 0.3 M NaCl, was denatured at 76°C for 15 min, and then immediately quenched on ice for 2–5 min. Twenty microlitres were applied to each slide and covered with a plastic coverslip. DNA–DNA *in situ* hybridization was carried out overnight in a moist chamber at 37°C. Following hybridization the slides were washed in 2 × SSC at 42°C, 50 per cent (v/v) de-ionized formamide in 2 × SSC at 42°C, 2 × SSC at 42°C, and 2 × SSC at room temperature, 10 min each. According to Schwarzacher *et al.* (1989), these conditions allow hybridization between DNAs sharing at least 80–85 per cent sequence identity, assuming that the parameters affecting nucleic acid hybridization in solution can be applied to chromosomal DNA *in situ* (Meinkoth & Wahl, 1984). Biotinylated DNA was detected with 5 μg ml^{-1} fluoresceinated (FITC) avidin (Vector Laboratories) and amplified once with 25 μg ml^{-1} biotinylated anti-avidin D followed by 10 μg ml^{-1} fluoresceinated

avidin as described by Schwarzacher *et al.* (1989). Chromatin was counterstained using 1–2 μg ml^{-1} of the non-specific fluorochrome propidium iodide, and the differentiating fluorochrome DAPI (4′, 6-diami-dino-2-phenylindole), which highlights AT-rich hetero-chromatin (Schweizer, 1976). The *in situ* hybridization signal and propidium iodide were excited with blue light (450–490 nm), and detected by their yellow and red fluorescence, respectively. DAPI fluorescence was visualized after excitation with ultraviolet light (near 365 nm). Photographs were taken with Kodak Ektach-rome P800/1600 Colour Reversal Film, processed at 800 ASA.

Results

DAPI-stained chromosome spreads of hybrid mitotic metaphases after *in situ* hybridization (Fig. 1a) showed that 21 of the 35 chromosomes had brightly fluores-cing, DAPI-positive bands located distally on either one or both chromosome arms. The other 14 chromo-somes, and the two B chromosomes when present, each had largely uniform DAPI fluorescence, although in the former some small, faintly fluorescing bands were seen occasionally about the centromeric region of some chromosomes. DAPI staining in × *F. hubbardii*, therefore, gave a very similar chromosome banding profile to that found with Giemsa C-banding by Bailey & Stace (1992). Thus, in the present experiments, the 21 chromosomes with brightly fluorescing, terminal, DAPI-positive bands were derived from *F. rubra*. Of the chromosomes lacking conspicuous DAPI-positive bands, the two smallest, when present, were B chromo-somes probably from *F. rubra*, and the 14 larger were derived from *V. fasciculata*.

Figure 1(a) and (b) shows fluorescent micrographs of the same somatic root tip spread of × *Festulpia*

hubbardii. Biotinylated genomic DNA from *V. fascicu-lata*, mixed with an excess of unlabelled DNA from *F. rubra*, hybridized preferentially *in situ* to practically the entire length of the *Vulpia* chromosomes, as revealed by their yellow fluorescence (Fig. 1b, c and e). This GISH probe also hybridized partially along the euchromatic regions of the *Festuca* chromosomes, which fluoresced a dull orange-yellow (Fig. 1b and c). The red fluorescence seen at those regions of the *Festuca* chromosomes coinciding with the DAPI-posi-tive blocks (*cf.* Fig. 1c and d), indicated little or no hyb-ridization of the *Vulpia* genomic probe to such AT-rich heterochromatic segments. This pattern of hybridization occurred at all stages of mitosis (e.g. Fig. 1e), and also at interphase. In a reciprocal GISH (Fig. 1f), using labelled *F. rubra* DNA as a probe with an excess of unlabelled *V. fasciculata* DNA block, the reverse result was obtained. This time, the *Festuca* probe hybridized more strongly to heterochromatic than to euchromatic regions of the *Festuca* chromo-somes, implying that the euchromatic regions were preferentially blocked by the unlabelled *Vulpia* DNA (*cf.* Anamthawat-Jónsson *et al.*, 1990). *Vulpia* chromo-somes fluoresced red owing to the lack of hybridization signal, coupled with the relative increase in the amount of propidium iodide staining in this particular experi-ment.

The *Vulpia* probe was used in a subsequent GISH experiment with a view to discriminating between homogenetic (*Vulpia–Vulpia* and *Festuca–Festuca*) and heterogenetic (*Vulpia–Festuca*) chromosome associa-tions at first meiotic metaphase in × *Festulpia hubbardii* (Fig. 1g–j). *Vulpia* and *Festuca* chromo-somes, as at mitosis, fluoresced yellow and red, respectively. Homogenetic bivalents were identified unequivocally by their colour. Those involving only *Festuca* chromosomes were red (Fig. 1g and i), while

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Fig. 1 (opposite) Fluorescent micrographs of root tip (a–f) and meiotic (g–j) chromosome preparations of × *Festulpia hubbardii* after GISH. Sites of probe hybridization fluoresce yellow, while unprobed sites fluoresce red. (a–e and g–j) Pwllheli hybrid, (f) Ainsdale hybrid (see Table 1). (a and b) The same mitotic metaphase cell stained with DAPI (a) and probed with biotinylated genomic DNA from *V. fasciculata* mixed with excess unlabelled total genomic DNA from *F. rubra* (b). B chromosomes are arrowed. (c and d) Individual chromosomes derived from *V. fasciculata* and *F. rubra* showing the characteristic hybridization pattern of the *Vulpia* genomic probe (c), and stained with DAPI (d). Note uniform labelling of the *Vulpia* chromosome, except for at the centromeric region coinciding with a faint DAPI-positive segment (arrowed in d), and the relative absence of probe hybridization signal at the terminal region of the *Festuca* chromosome (arrowed in c) coinciding with DAPI-positive hetero-chromatin (arrowed in d). (e) Prometaphase cell probed with the same *Vulpia* genomic probe with 14 *Vulpia* chromosomes labelled. (f) Metaphase cell probed with biotinylated total genomic DNA from *F. rubra* mixed with an excess of unlabelled total genomic DNA from *V. fasciculata*. Note preferential probe hybridization to the terminal regions of the *Festuca* chromosomes. (g–j) Two meiotic first metaphase spreads probed with a *Vulpia* genomic probe (g and i), and after GISH stained with DAPI (h and j), showing homogenetic (V–V and F–F) and heterogenetic (V–F) bivalents, labelled accordingly. Scale = 10 μm (a and b, e–j), and 5μm (c and d).
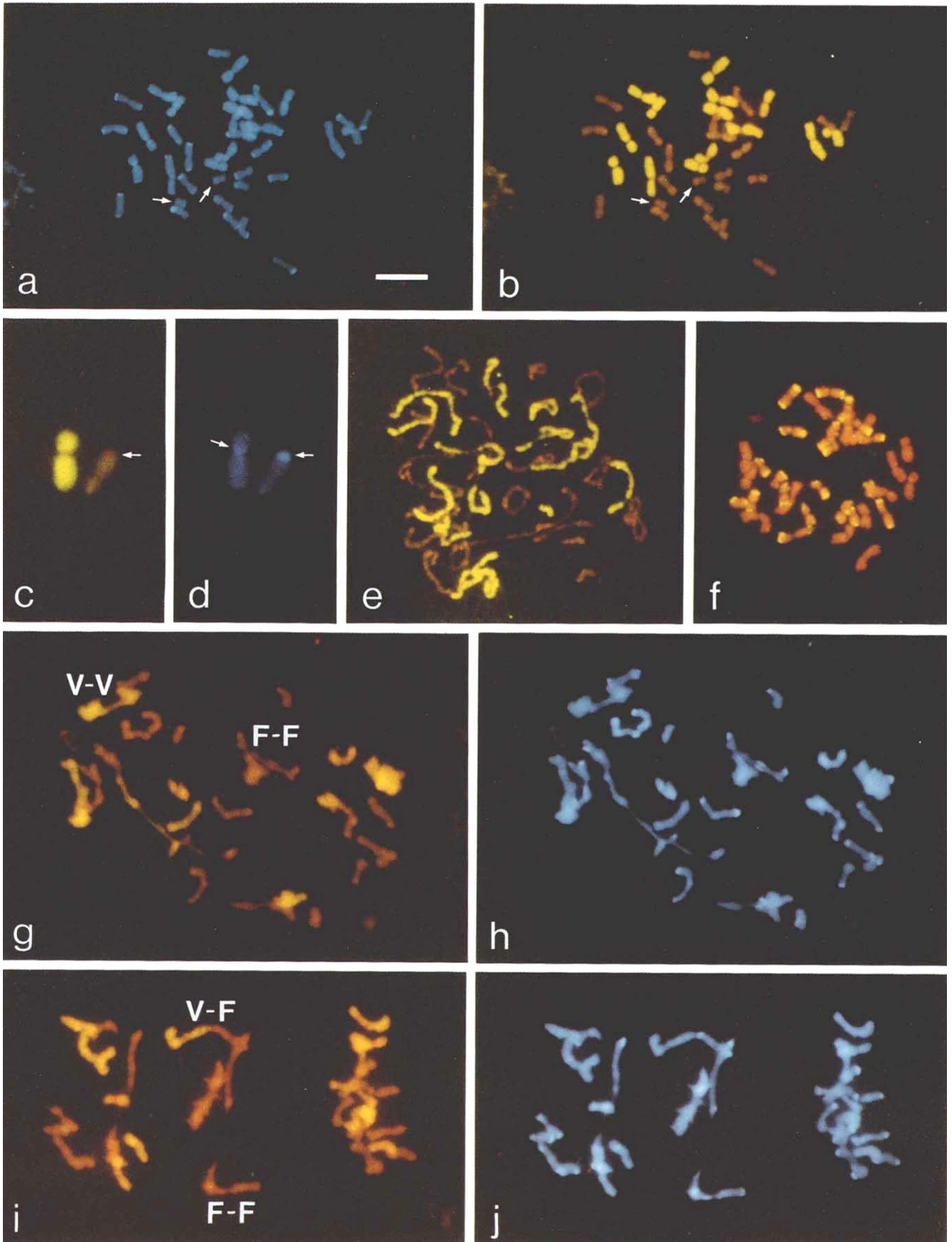
Vulpia–Vulpia bivalents were yellow (Fig. 1g). Heterogenetic bivalents were bi-coloured with red (*Festuca*) and yellow (*Vulpia*) components (Fig. 1i). Unlike the case with mitotic spreads, the DAPI-positive bands on the *Festuca* chromosomes were sometimes not visible at first meiotic metaphase (Fig. 1h and j). In such cases the parental chromosomes could be identified only by GISH.

Discussion

Our results show that GISH offers a reliable means of discriminating between chromosomes derived from *Vulpia fasciculata* and *Festuca rubra* in their naturally occurring F_1 hybrid × *Festulpia hubbardii*. Moreover, *Vulpia* chromatin could be unambiguously identified by its yellow fluorescence in both somatic and meiotic nuclei. Unlike Giemsa C-banding, where the *Vulpia* and *Festuca* chromosomes could be distinguished only by the respective absence or presence of terminal heterochromatic blocks on at least one chromosome arm (Bailey & Stace, 1992), genomic probing provides a colour-coded, physical marker for virtually the entire length of every *V. fasciculata* chromosome. Despite the use of excess unlabelled *F. rubra* genomic DNA to suppress non-specific binding, there was some hybridization of the *Vulpia* probe to the *Festuca* chromosomes. This implies the occurrence of DNA sequences common to *V. fasciculata* dispersed throughout, and in particular along euchromatic regions of, the *Festuca* genome. Given that these species produce sexual hybrids in nature, in which up to the theoretical maximum of 14 bivalents can occur at meiosis (Stace & Cotton, 1974) (albeit usually far fewer), a considerable degree of genomic homology is not surprising. However, there was apparently little hybridization of the *Vulpia* probe to the terminal heterochromatic regions of the *Festuca* chromosomes. This may be owing to either the physical inaccessibility of the *Vulpia* probe at these sites of highly condensed DNA, or sequence dissimilarity between the *V. fasciculata* genomic probe and the *F. rubra* terminal heterochromatin. However, as genomic DNA from *F. rubra* hybridized preferentially to the terminal heterochromatic blocks of the *Festuca* chromosomes in a reciprocal GISH, the physical inaccessibility explanation seems highly unlikely. Instead, the most feasible interpretation is that these terminal regions of the *Festuca* chromosomes comprise localized, *Festuca*-specific repeated DNA sequences, similar to the species-specific repeats found near telomeres in other grasses such as *Secale cereale* (e.g. McIntyre *et al.*, 1990). So, GISH can provide not only physical markers for identifying any point along the length of the *Vulpia* genome in × *Festulpia hubbardii*,

but also an insight into the physical organization of both the *Vulpia* and *Festuca* chromosomes.

A direct method of assessing inter-genomic homologies is to analyse hybrid meiotic chromosome behaviour in terms of the frequency of heterogenetic bivalents (i.e. V–F pairing in our material). However, where at least one of the parents is polyploid and where the chromosomes are all similar in size and morphology, and lack distinctive markers, this may be difficult or even impossible. The occurrence of both homogenetic and heterogenetic pairing can be sometimes safely inferred because the number of bivalents formed exceeds the number that could result from either form of pairing alone. In tetraploid hybrids between *Lolium perenne* ($2n = 2x = 14$) and *Festuca rubra* or *F. arundinacea* ($2n = 6x = 42$), only seven bivalents could form from either homogenetic or heterogenetic pairing. Jauhar (1975) and Evans & Taing Aung (1986) found numbers of bivalents closer to 14 than to 7 in *L. perenne* × *F. rubra* and in *L. perenne* × *F. arundinacea* respectively, proving the occurrence of both forms of pairing. However, in triploid hybrids between diploids and tetraploids, or in pentaploid hybrids between tetraploids and hexaploids, no such inference can be made, because the maximum number of bivalents possible (x and $2x$, respectively) can be accounted for by one form of pairing alone. In such cases, the parental origin of *each* chromosome needs to be determined. Previously, this was impossible. However, as we have shown, GISH provides the necessary discrimination. In × *Festulpia hubbardii* GISH permitted the precise characterization of bivalents at first meiotic metaphase. Although the bivalent frequency in the present material was low, with only one or two bivalents per cell, both homogenetic and heterogenetic bivalents were clearly recognized. Thus, GISH has clear potential scope in aiding chromosome recognition in meiotic as well as mitotic nuclei.

Despite being closely related, there appear to be enough different dispersed DNA sequences in *V. fasciculata* and *F. rubra* genomes to permit their discrimination in their naturally occurring F_1 hybrid. Previously, genomic probing has distinguished genetically similar genomes in *Hordeum vulgare* × *H. bulbosum* (Schwarzacher *et al.*, 1992a) and in *Gibasis karwinskyana* × *G. consubrina* (Parokonny *et al.*, 1992a). Thus, our results provide a further successful example of GISH for identifying parental chromosomes, here in a wild hybrid, between closely related plant species.

Our ultimate aim is to test whether *Vulpia* chromatin has been transferred into *Festuca rubra* populations by introgressive hybridization, through backcrossing of the pentaploid F_1 × *F. hubbardii* to male hexaploid *F.*

rubra. The present results show that GISH will probably provide the necessary means to carry out this investigation. However, what will be an important question, noted previously by both Schwarzacher *et al.* (1992b) and Parokonny *et al.* (1992a), concerns the limitations of GISH. Is GISH sensitive enough to detect small introgressed segments of *Vulpia* chromatin and, if so, down to what size limit? Although sufficiently sensitive to detect alien chromosomes or chromosome segments in wheat (Heslop-Harrison *et al.*, 1990; Mukai & Gill, 1991; Schwarzacher *et al.*, 1992b), 'relatively small' regions of homology between the genomes of closely related species (Parokonny *et al.*, 1992a; Kenton *et al.*, 1993), and intergenomic translocations in asymmetric somatic hybrids (Parokonny *et al.*, 1992b), the finer limits of genomic probing remain unresolved. Further experiments with both × *Festulpia* and other wild hybrids should provide important new insights into the process of introgressive hybridization in nature and its importance in plant genome evolution and speciation.

Acknowledgements

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