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Segregation distortion in *Lolium*: evidence for genetic effects

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Abstract Segregation distortion (SD) is the deviation of genetic segregation ratios from their expected Mendelian fraction and is a common phenomenon found in most genetic mapping studies. In this study two segregating *Lolium perenne* populations were used to construct two genetic maps: an 'F_2 biomass' consisting of 360 genotypes and an 'F_1 late flowering' sibling based population consisting of 182 genotypes. Additionally two parental maps were generated for the 'F_1 late flowering' population. SD was detected and *p*-values for SD were calculated for each marker locus. The 'F_1 late flowering' map had only half of the extent of SD (32%) compared to the map based on the 'F_2 biomass' population (63%). Molecular marker data have been supplemented with genomic in situ hybridization (GISH) data to show non major non-recombined segments of *Fescue* chromosomes within the parental inbred ryegrass lines with a *Festuca* × *Lolium* pedigree. We conclude that SD in our study is more likely caused by genetic effects rather than by population structure and marker types. Two new *L. perenne* mapping populations including their genetic maps are introduced; one of them is the largest reported *Lolium* mapping population consisting of 360 individuals.

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Introduction

Segregation distortion (SD) is defined as the deviation of genetic segregation ratios from their expected Mendelian fraction (Lyttle 1991). The phenomenon of SD and its causes are poorly understood (Jenczewski et al. 1997). A single or a combination of different mechanisms may be responsible for SD in any particular case. Events leading to SD can be initiated in different developmental stages including sporogenesis, spore function, seed development and seed germination (Zamir and Tadmor 1986) and can arise from a dysfunction of the gametes in pollen, megaspores or both (Lyttle 1991). Hartl (1980) described a well characterized genetic SD mechanism in *Drosophila melanogaster*: four genetic loci, *Sd*, Rsp^{ins}, *E*(*SD*) and *M*(*SD*) were found on linkage group (LG) 2 in *Drosophila*. Loci *Sd* and *Rsp* were mainly involved in SD. The loci *E*(*SD*) and *M*(*SD*) enhanced SD further.

SD has been frequently detected in *Lolium perenne* mapping populations. In the 'VrnA' F_2 mapping population (Jensen et al. 2005a) 60% of the marker loci showed SD. A two way pseudo-testcross mapping population was displayed with two parental maps: 24% of marker loci in 'NA_6' showed distortion while 15% of the loci were distorted in 'AU_6' (Faville et al. 2004). Cogan et al. (2006) used the same population and reported similar SD values of 16% distorted loci. The two *Lolium* mapping populations which have been most widely used for many mapping studies are the 'ILGI' 'p150/112' population derived from a cross between a di-haploid plant and a hybrid F_1 plant as parents (Bert et al. 1999; Jones et al. 2002a, b; Armstead et al. 2002; Jensen et al. 2005b; Cogan et al. 2005) and the 'RASP' 'WSC F_2' mapping population derived from self-pollinating a single hybrid plant, obtained by crossing individuals from partially inbred lines (Armstead et al. 2002,

2004; Gill et al. 2006; Turner et al. 2006). Maps of these two populations were constructed using different marker types and sets. The marker density of genetic maps for the same populations increased over the time the populations were used. In studies using the 'ILGI' mapping population, SD ranged between 12% (Bert et al. 1999) and 34% (Jones et al. 2002a). In the 'RASP' population SD ranged between 18% (Gill et al. 2006) and 40% (Armstead et al. 2004). In most of the studies mentioned above it was assumed that SD was caused either by self-incompatibility loci (Bert et al. 1999; Jones et al. 2002b; Armstead et al. 2002; Faville et al. 2004; Jensen et al. 2005a; Gill et al. 2006) or by differences in gametophytic and sporophytic viability (Jones et al. 2002b; Armstead et al. 2002; Jensen et al. 2005a; Gill et al. 2006). In perennial ryegrass to date no comprehensive study exists if marker types and population structure influence SD.

SD is a natural phenomenon which cannot be prevented, but needs to be taken into account in the choice of breeding strategies. SD can be used to identify specific target regions or loci closely linked to a distorted marker. Grini et al. (1999) employed the multiple marker chromosome *mm1* as a tool to screen for ethyl methansulfonate (EMS) induced gametophytic mutants in *Arabidopsis thaliana.* This assay screened for developmental and gametophytic functionally distorted mutants. Harbord et al. (2000) used SD as a method in *Petunia* to identify transgenes that are linked to the pollen gene of the *S* gametophytic self-incompatibility locus. This approach provides a method for identifying transgenes linked to gametophytic self-incompatibility loci and for transposon tagging of the *S*-locus in *Petunia.* It is important to have a good knowledge of the occurrence and nature of SD to estimate which genes will be held together or will segregate by SD. On chromosome 6 in a substitution line of *Lycopersicon esculentum* × *Lycopersicon pennellii* a segment of *L. pennellii* was successfully introgressed in *L. esculentum* where SD was instrumental in the maintenance of the desired trait (Weide et al. 1993). These are examples to use SD directed in further genetic basic research and in applied genetic ryegrass studies. To date no such approach has been used in perennial ryegrass experimental programmes, but could be useful in future.

Conventional breeding has already been successful in generating commercial varieties of forage grasses with traits for enhanced agricultural sustainability. Breeding objectives focus on stress resistance against drought, cold and pathogens, and on agronomic traits like nutrient use efficiency, carbohydrate content, fatty acid content, winter survival, flowering time and biomass yield (Humphreys et al. 2005). To develop successful varieties it is important to have freely segregating breeding populations and stable breeding lines. Knowledge about SD will benefit the breeding programmes and could be very helpful in molecular breeding programmes. The identification of a homologue of the *Sd* gene in perennial ryegrass as previously demonstrated in *Drosophila* (McLean et al. 1994) will contribute to control the phenomenon SD.

The objectives of this study were to (1) compare SD of an extensive F_2 population of *Lolium* derived from two inbred grandparents and of a F_1 sibling based population of *L. perenne* using a similar set of molecular markers and to compare the findings of these two mapping studies with SD in other *Lolium* maps and SD in other species. (2) To identify specific distorted regions using in situ fluorescent hybridization and molecular markers and consider possible reasons for SD, and (3) to discuss these findings in relation to applications in practical and molecular plant breeding. This manuscript provides suggestions how SD can be directed used in breeding research and investigates the phenomenon SD from different perspectives.

Materials and methods

Plant material

The 'F_2 biomass' mapping population was constructed from a cross between two inbred *Lolium* lines. Both parents of the F_1 genotype were developed by Dr. V. Connolly as part of a cytoplasmic male sterility (CMS) programme in Teagasc, Oak Park. They were maintainer lines in this CMS programme (Connolly and Wright-Turner 1984) and originated from an inter-specific cross between meadow *fescue* (*Festuca pratensis*) and perennial ryegrass (*L. perenne*). The initial interspecific hybrid was backcrossed for several generations to the ryegrass parent and selfed for nine or ten generations. For the maternal parent of the inbred lines the ryegrass cultivar 'S24' (IGER) was used in the pedigree while for the paternal parent the ryegrass cultivar 'Premo' (Mommersteeg International BV) was chosen. The maternal parent of the F_2 population was emasculated under a binocular microscope and stigmas were pollinated with pollen from the paternal plant. Pollinated florets were bagged in cellophane bags, individual F_1 seed was harvested, and single F_1 plants were raised and self-pollinated by bagging in cellophane pollination bags to generate independent F_2 populations. One of these independent F_2 populations was used for the genetic map construction and 360 viable F_2 individuals of this population were randomly chosen. F_2 plants were raised in the greenhouse.

The 'F_1 late flowering' sibling based mapping population was constructed from a reciprocal cross between two highly heterozygous (see "Results" section) sibling perennial ryegrass lines 'J43' and 'J51' which were developed in the Teagasc Oak Park breeding programme and selected to differ for heading date. One hundred and eighty two plants

from this cross were raised and used for the construction of the genetic F_1 map.

DNA extraction, AFLP, SSR, CAPS marker analysis

Genomic DNA was extracted using a cetyltrimethylammonium bromide (CTAB) method after Doyle and Doyle (1987).

The amplified fragment length polymorphism (AFLP) marker procedure was carried out following the Applied Biosystems protocol for AFLP™ Plant Mapping with a modification in the sample dilution of the preselective amplification product to a 1:2 dilution ($TE_{0.1}$ buffer:product). The *Eco*RI and *Mse*I enzymes were used for the enzyme primer combinations *Eco*ACA*Mse*CAC, *Eco*AGC-*Mse*CTA, *Eco*ACA*Mse*CTA. The AFLP forward primer was fluorescently 5′-labelled with FAM™, JOE™ or NED™ and the internal sizing standard GeneScan™500 ROX was used.

Simple sequence repeat (SSR) markers were chosen from a number of public and non-public sources (Gill et al. 2006; Jensen et al. 2005b; Jones et al. 2001; Kubik et al. 2001; Lauvergeat et al. 2005; Studer et al. 2007; Warnke et al. 2004). Licensed tall *fescue* SSRs (NFFa017, NFFa036, NFFa136, NFFa142 and NFFa155) from the Robert Samuel Noble Foundation, OK, USA were optimised for amplification of *L. perenne* DNA, and cross-species amplifying and polymorphic SSRs used for further mapping work. In total 267 SSR markers were screened for polymorphism in the parental lines of the ‘F_2 biomass’ and for the parents of the ‘F_1 late flowering’ population. PCR reactions were carried out in a total volume of 10 µl containing 25 ng total genomic DNA as template, 2.5 µM forward and reverse primer (Applied Biosystems, Warrington, UK), 0.3 U of DNA Taq polymerase (New England Biolabs, Ipswich) and 2 mM dNTPs in a Biometra Thermocycler. SSR markers from the Samuel Robert Noble Foundation, USA had the following PCR programme profile: (1) 95°C for 5 min, (2) 35 cycles of: 95°C for 1 min, specific annealing temperature (AT) for 1 min and 72°C for 1 min, (3) 72°C for 10 min. For licensed SSR markers from CRC/Australia (LpSSRH02F01, LpSSRH11G05, LpSSRK12E06 and LpSSRK14F12) the following touch down PCR programme was used: (1) 10 cycles (touch down −1°C) of: 95°C for 1 min, AT for 30 s and 72°C for 1 min, (2) 30 cycles of: 94°C for 1 min, AT for 30 s and 72°C for 30 s. For licensed IGER/UK SSR markers (LpACA8A8a, LpACT15H3, LpACT44A7, LpACT13H1, LpACT13H2, LpACT43C6, LpACTR1C5, LpHCA18A2b, LpHCA16B2 and LpHCA18B12) the following PCR programme profile was applied: (1) 96°C for 5 min, (2) 35 cycles of: 96°C for 15 s, AT for 30 s and 72°C for 30 s, (3) 72°C for 4 min. For licensed ViaLactia/New Zealand SSR markers (Gill et al. 2006; Lp13Ca1 and all markers starting with ‘rv’) the following touch down PCR programme profile was utilised: (1) 95°C for 10 min (2) 10 cycles (touch down −1°C) of: 94°C for 1 min, AT for 30 s and 72°C for 1 min, (3) 25 cycles of: 94°C for 30 s, AT for 30 s and 72°C for 30 s, (4) 72°C for 10 min. For SSR markers from Jensen et al. (2005b) the PCR programme of Kubik et al. (2001) was used. Conditions for SSR markers described in Lauvergeat et al. (2005) and Studer et al. (2007) were used as given by the authors, respectively. The SSR forward primers were fluorescently 5′-labelled with 6FAM™, VIC®, NED™ or PET™ and GeneScan™500 LIZ® was applied as internal sizing standard. SSR and AFLP genotyping were performed on an ABI Prism 3100 genetic analyser (Applied Biosystems, Warrington, UK) with POP-4 polymer and 36 cm capillaries. Amplification patterns were scored using GeneMapper® V3.7 software (Applied Biosystems, Warrington, UK). Cleaved amplified polymorphic sequence (CAPS) markers (Ck2B3, AGO4, MYO, Cullin, BHLH) were developed (S. Byrne, unpublished data) and were used only for mapping of the ‘F_1 late flowering’ population.

Genetic-map construction

SSR markers for the ‘F_2 biomass’ population were scored as co-dominant markers and for each allele a letter was assigned. AFLP markers were scored as dominant markers and recorded in a zero/one format for absence and presence of a band. For the ‘F_1 late flowering’ population SSR and CAPS markers were scored for the consensus map co-dominantly and for the two parental maps dominantly. Markers were classified in two segregation types. Co-dominant markers had bands present in both parents and were expected to segregate in a 1:2:1 pattern; dominant markers were expected to segregate in a 3:1 pattern. SSR and AFLP marker data were inserted into the linkage map construction software package JoinMap® V 3.0 (Van Ooijen and Vorrips 2001) using the F_2 segregation type. Expected Mendelian segregation ratios of SSR and AFLP markers for the ‘F_2 biomass’ population and the ‘F_1 late flowering’ population were analysed using χ^2-square tests in Join Map® V3.0 software. For LG calculations and determination, a log of odds (LOD) threshold of not lower than 4.0 was utilised. For both populations the calculation of the map LOD threshold larger than 1.0 and a jump threshold in goodness-of-fit of 5.0 were used. Kosambi’s mapping function was applied to estimate genetic distances in cM. Markers on LGs were positioned with JoinMap® V3.0. The genetic maps were drawn using MapChart V2.2 software (Voorrips 2002).

In situ hybridisation

To test for substantial segments of *Fescue* chromosomes within the parental inbred ryegrass lines which had a

Fescue × *Lolium* cross in their parentage and to analyse aspects of the organisation of the perennial ryegrass genome, we used DNA molecular in situ hybridization on the F_1 chromosomes following the protocol of Schwarzacher and Heslop-Harrison (2000) with some modifications. For the preparation of mitotic root tip spreads, root tips were treated for 24 h in ice-cold water and metaphases were fixed in 3:1 (v/v) 100% ethanol:glacial acetic acid solution. The root tips were enzymatically digested (pectinase and cellulase, Sigma-Aldrich, Taufkirchen, Germany) and meristematic cells were squashed in 75% acetic acid on a glass slide. Genomic DNA of the parental lines, 18-26S-rDNA (pTa71) and 5S-rDNA (pTa794) genes were labelled with digoxigenin-16-dUTP (Roche, Basel, Switzerland) and biotin-11-dUTP (Roche, Basel, Switzerland). The hybridisation mixture contained 4 µl unlabelled genomic DNA (200 ng; blocking 1:40) and 2 µl labelled pTa71 or pTa794 probe in 40 µl total hybridization solution with 50% (v/v) formamide, 2x saline sodium citrate (SSC), 10% (w/v) dextran sulphate, 1 µg/µl salmon sperm DNA, 0.125 mM EDTA and 0.125% SDS. Chromosomes and probes were denatured together for 8 min at 75°C and hybridization was carried out overnight at 37°C. After the hybridization, slides were washed under stringent conditions at 42°C in 20% formamide and 0.1× SSC. For the detection of signals the slides were incubated in 0.1 µg/ml Alexa 594 streptavidin (Roche, Basel, Switzerland) and FAB antidig (Roche, Basel, Switzerland) in 5% (w/v) bovine serum albumin (BSA) in 4× SSC solution (containing 0.2% (v/v) Tween20), counterstained with 4′,6-diamidino-2-phenylindole (DAPI) (Fluka, Sigma-Aldrich, Buchs, Switzerland) and mounted in antifade AF1 (Citifluor, London, UK). Hybridisation signals were visualised with an epifluorescence microscope with filter blocks for DAPI, FITC and Alexa 594.

Results

AFLP, SSR and CAPS marker detection

Thirty polymorphic AFLP markers were selected from the enzyme-primer combinations *Eco*ACA*Mse*CAC, *Eco*AGC-*Mse*CTA, *Eco*ACA*Mse*CTA and *Eco*ACT*Mse*CTA. Only ten of the AFLP markers were used for the construction of the 'F_2 biomass' population genetic map. Out of the 267 tested, 70 SSR markers were polymorphic. Sixty-five out of the 70 polymorphic SSR markers were used for the construction of the genetic linkage map. The 25 non-mapped AFLP and SSR markers had either a too high degree of SD or were positioned too distantly in relation to the next markers assigned to LGs, presumably on the distal ends of LG 1 and 6. AFLP markers had their mapping positions in general on the distal ends of the LGs in the 'F_2 biomass' population and SSR markers were clustered around the centromeric regions (Fig. 1a).

In the 'F_1 late flowering' population, out of 151 SSR markers 72 were polymorphic. Fifty-five out of 72 SSR markers and the five CAPS markers were used to construct the genetic linkage map with 182 F_1 individuals (Fig. 1b).

Seven LGs were calculated for the genetic linkage maps of both populations using the software package Join Map V3.0 (Fig 1). The polymorphism degree was 23% for the 'F_2 biomass' population and 55% for the 'F_1 late flowering' population.

Segregation distortion

In the 'F_2 biomass' population, 47 (63%) out of 75 mapped loci (SSR and AFLP) showed significant ($p < 0.05$) SD (Supplementary material Table 1). LGs 3, 6 and 7 had the highest amount of SD (Fig. 1a). LG 6 was completely distorted and alleles of the maternal line were favoured. LG 2 and 4 showed the lowest SD. LG 5 was distorted on both ends containing a centromeric non-distorted part. LG 2 was distorted only on one end of the LG.

In the 'F_1 late flowering' population segregation ratios of only 19 (32%) out of 60 SSR markers were significantly distorted (Fig. 1b; Supplementary material Table 2). All loci on LG 1 on the consensus map were skewing towards the 'J51'parent and loci on LG 7 towards the 'J43' parent (Fig. 1b).

In general, the 'F_2 biomass' population genetic map had the double amount of SD compared to the 'F_1 late flowering' population map. LG 1 was severely distorted in all the maps except for the 'J43' map, and LG 7 was severely distorted across all maps except for the parental map 'J51'. Comparing across all maps and the two mapping populations LG 2 and LG 4 showed the least amount of SD.

In situ hybridisation

Genomic in situ hybridisation (GISH) on the F_1 hybrid chromosomes of the 'F_2 biomass' population with both parental DNA as probes could not identify distinct regions pointing towards bigger non-recombined blocks of one parent. The entire chromosomes were evenly hybridised and no distinct differences could be found (Fig. 2: 2b, 3a, 3b, 3c). The 18-26S-rDNA landmark (pTa71) (Fig. 2: 1c, 2c) showed on the F_1 hybrid seven bands and the 5S-rDNA (pTa794) landmark showed two hybridisation signals on the chromosomes (Fig. 2: 1a). The 5S-rDNA landmarks were located on the more distal end of the chromosome together with one of seven 18-26S-rDNA landmarks on the same chromosomes (Fig. 2: 3a).

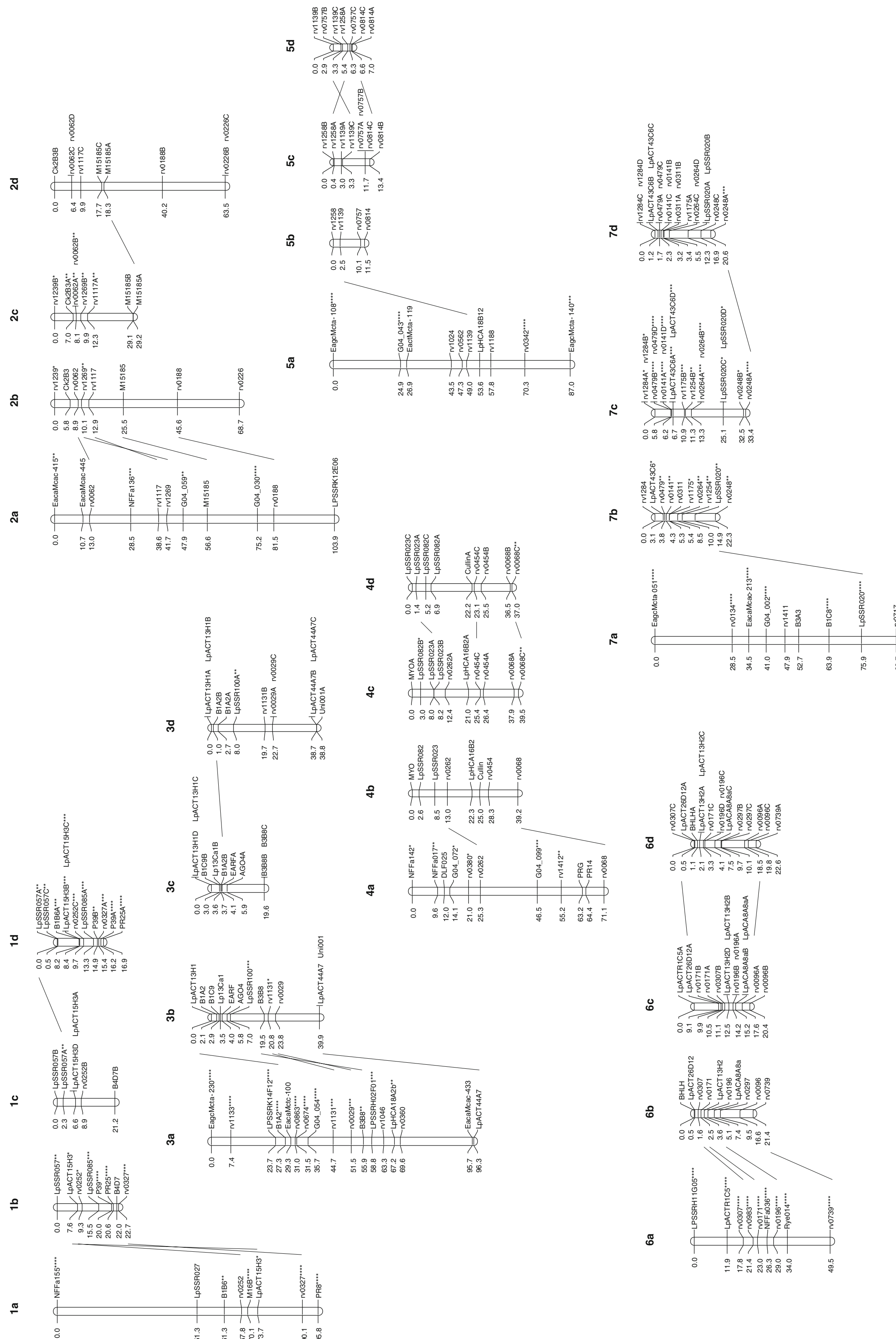

Fig. 1 **a** 'F_2 biomass' population genetic map, **b** 'F_1 late flowering' consensus population map, **c** 'J43' parental map, **d** 'J51' parental map generated in Join Map V3.0 using Kosambi's mapping function. Distances are given in cM. *Asterisks* indicate segregation distortion ($^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$)

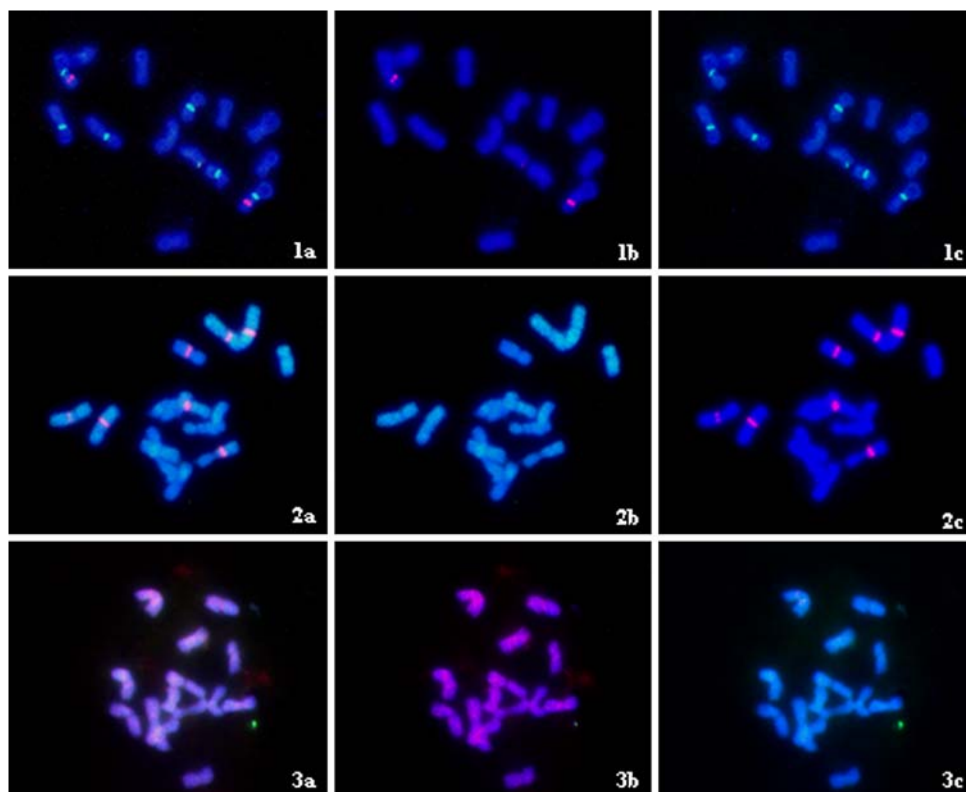

Fig. 2 In situ hybridisation of F_1 chromosomes of the 'F_2 biomass' population labelled with DAPI (*blue*), biotin-11-dUTP (*red*) and digoxigenin-16-dUTP (*green*). Pictures show: **1a** 5S (*red*) and 18–26S rDNA (*green*); **1b** 5S rDNA (*red*); **1c**: 18–26S rDNA (*green*); **2a** 18–26S rDNA (*red*) and paternal genomic DNA (*green*); **2b** paternal genomic DNA (*green*); **2c** 18–26S rDNA (*red*); **3a** paternal (*red*) and maternal genomic DNA (*green*); **3b** paternal genomic DNA (*red*); **3c** maternal genomic DNA (*green*)

Discussion

Segregation distortion across linkage maps

Additionally to the five previously published mapping populations for *L. perenne* (Bert et al. 1999; Muylle et al. 2001; Armstead et al. 2002; Faville et al. 2004; Jensen et al. 2005a) two new mapping populations are introduced in this study with four genetic maps. The four genetic linkage genetic maps using a set of common markers presented in this study enabled us to compare SD across populations. Population structure seems to be an important factor for SD and can lead to variation in the proportion of distorted markers. In our study, the 'F_1 late flowering' population showed a small percentage of distorted marker loci compared to the 'F_2 biomass' population. Sixty-three percentage of the markers in the 'F_2 biomass' population did not fit the expected Mendelian ratios, which is similar to the findings of Jensen et al. (2005a). Xu et al. (1997) published a study based on six genetic maps of rice with different population structures; the recombinant inbred lines (RIL) population had the highest frequency of marker SD compared to populations derived from other population structures. Lu et al. (2002) made a similar observation and reported on higher SD in RIL populations than in doubled haploid (DH), backcross (BC) and F_2 populations. F_2 populations based on non inbred lines had the lowest frequency of SD. An explanation for SD in RIL populations could be inbreeding depression because of an increase of homozygote genotypes over heterozygotes. All these studies indicate that SD most likely accumulates along with additional generations of meiosis in an inbreeding context. This could explain the higher rate of SD in the 'F_2 biomass' population compared to the 'F_1 late flowering' population. Different population structures in perennial ryegrass showed also different regions with distorted loci ('p150/112' ('ILGI'): Bert et al. 1999; Jones et al. 2002a, b; Armstead et al. 2002; 'AU_6'/'NA_6': Faville et al. 2004; 'WSC F_2' ('RASP'): Armstead et al. 2004; Gill et al. 2006; Turner et al. 2006; 'VrnA': Jensen et al. 2005a). According to Xu et al. (1997) SD can differ between specific populations based on a range of genetic, physiological and environmental factors.

Xu et al. (1997) found in rice that for SD in all analysed populations either the alleles of one parent or the other parent was favoured. In male gametes, pollen killers or pollen abortion result more frequently in SD as compared to disturbances in female gametes (Taylor and Ingvarsson 2003). Gamete selection eliminating gametes of either sex has been previously reported (Sano 1990). SD during female meiosis can lead to genomic disorders (Jenczewski et al. 1997). In our study, results from the map construction of the 'F_2 biomass' population pointed towards maternal or paternal favouring of alleles. In the 'F_1 late flowering' population, clusters of SD were identified being unique to an individual parent. Therefore, LG 1 was skewed towards the 'J51' parent and LG 7 towards the 'J43' parent. A similar

situation was seen in another study using an F_1 population of perennial ryegrass (Faville et al. 2004), where the individual parental maps 'AU_6' and 'NA_6' displayed 15% and 24% distorted loci, respectively. It is reasonable that the results may reflect the nature of the former parental genotypes, with a higher proportion of recessive sub-lethal mutations capable of expression during gametogenesis or in the gametophyte (Faville et al. 2004).

Chromosomal rearrangements and genomic disorders have been postulated as a further cause of SD (Jenczewski et al. 1997). These events occur at the interchromosomal, intrachromosomal or intrachromatid levels. Interchromosomal and intrachromosomal misalignments are caused by deletion and duplication or inversion and duplication. Intrachromatid loops can result from deletion, separating a single acentric fragment or from inversion (Stankiewicz and Lupski 2002). This could be a reason that certain areas on LGs in our study favoured alleles of one parental line, e.g. all loci on LG 6 in the 'F_2 biomass' population favoured maternal alleles. Additionally it could be argued that the high extent of SD in the 'F_2 biomass' population is derived from a larger not recombined *Festuca* contingent in the inbred parental lines. Hybrids between *Lolium* and *Festuca* have been regarded as relatively genetically unstable often favouring genome segments of one of the parental lines. Canter et al. (1999) found this favouring especially the *Lolium* parent and related it to dysfunctional intergeneric chromosome pairing. In a tomato BC_1 population of (*Lycopersicon esculentum*) × *Solanum lycopersicoides* homozygote and heterozygote alleles were favoured by SD on different chromosomes. SD was suggested to be linked to a small number of loci on the affected chromosomes (Chetelat et al. 2000). In a complementary study using restriction fragment length polymorphism (RFLP) markers the tomato alleles and introgressed homozygote segments were more frequently associated with SD (Chetelat and Meglic 2000). GISH and fluorescent in situ hybridisation (FISH) could identify in some cases depending on specific landrace lines small introgressed rye chromosomal segments in *Triticum aestivum* (Ribeiro-Carvalho et al. 1997). In situ hybridization can also detected alien chromosomes in late breeding lines, e.g. in a BC_6 line of *Pennisetum squamulatum* with a *P. glaucum* introgression (Goel et al. 2003). Based on these approaches we deemed in situ hybridization suitable in our study to identify alien segments of *Festuca* in the *Lolium* lines which could be responsible for SD. In our study after several generations of backcrossing to *L. perenne* during the line development, the *Festuca* proportion in the lines diminished as confirmed by GISH (Fig. 2: 2b, 3a, 3b, 3c) and thus the proportion introgressed from *Festuca* in the parental inbred line is most likely not responsible for SD. This underlines our assumption that through the procedure of backcrossing and selfing during the development of the inbred parental lines the *Festuca* proportions became negligible. Humphreys et al. (1998) identified *Festuca* fragments using GISH in only 0.6% of the individuals of a BC_2 population (*Festulolium* backcrossed to *L. perenne*). This demonstrates that only very small introgressed *Festuca* chromosome segments, not detectable with GISH, remain in the 'F_2 biomass' population after several generations of backcrossing and selfing of the parental lines. In addition, our study tried to distinguish between parental DNA on the F_1 chromosomes of the 'F_2 biomass' population with GISH to explain why SD loci favoured alleles of one parent. But no particular parental fragment was found on the chromosomes in the GISH study although, as in the tomato study, the influence of an intergeneric background on SD, with alien chromosome segments below the detection limit, cannot be completely ruled out. Generally FISH approaches to study SD events have been successful in the past (De Martino et al. 2000) and thus GISH/FISH approaches are applicable for SD studies.

Another observation in our study was the significant difference in the distance between markers common to both mapping populations. In general, the distance between markers was much greater in the 'F_2 biomass' population compared to the 'F_1 late flowering' population. This finding was consistent even when the F_1 map was compared with the 'framework' map developed by Gill et al. (2005) using a common set of markers. SSR markers were scored as dominant markers for the F_1 parental maps which could have lead to a lower marker density in the parental maps.

Technical reasons can contribute as well to SD, e.g. errors during genotypic analysis or mutations within the binding site of a DNA marker. These mutations would affect only certain marker loci (Sibov et al. 2003) and are independent of population structure and species. Missing data and genotyping errors might occur and can lead to SD. Missing values can result as well in shorter genetic maps (Hackett and Broadfoot 2003). False marker order can originate from SD (Lorieux et al. 1995). However, the majority of SD loci in the maps presented in this study occurred in clusters and therefore, technical reasons are unlikely to be of major significance, except for changes in marker order of single markers in our study.

We are concluding from our study that SD is more likely caused by genetic effects rather then by population structure and marker types.

Specific distorted regions and possible causes of segregation distortion

We examined existing genetic maps in order to identify and describe specific SD regions in *L. perenne*. Faville et al. (2004) found 71% distorted loci on the 'NA_6' map on LGs 2, 3, 4. On the 'AU_6' map 38% of the distorted loci were

located on LG 5, which was comparable to the findings of Bert et al. (1999) reporting on highly skewed markers on LG 5 in the 'ILGI' population. In three studies which used the same F_2 population LG 5 and LG 7 were reported with the highest amount of distorted marker loci using sets of different markers (Armstead et al. 2004; Gill et al. 2006; Turner et al. 2006). Armstead et al. (2002) found other SD affected regions in a BC_1 population using the same set of markers compared to the studies with the F_2 population of Armstead et al. (2004) and Turner et al. (2006). In the BC_1 population LG 3 and LG 4 had severe extents of SD. Markers distorted in the BC_1 population were not distorted in the F_2 population. Jones et al. (2002a, b) used in two studies the 'ILGI' F_1 population with a multiple heterozygous parent and a double haploid parent in the pedigree. One genetic map of Jones et al. (2002b) was constructed with RFLPs, AFLPs, ESTs and isoenzyme markers; their second map (Jones et al. 2002a) was extended with additional SSR markers but had the previously used markers in common. Both maps showed severe SD on LG 3, but the first map of Jones et al. (2002b) featured an additional segregation distorted region on LG 4. All these previously reported findings share little similarities with regard to the extent of SD on both parental maps of the 'F_1 late flowering' population, except for LG 2 which had a larger amount of SD on the 'NA_6' and 'J43' maps. On the 'J43' parental map 88% of the distorted loci were located on LGs 2 and 7 and on the 'J51' parental map 65% of the distorted loci were located on LG 1. Jensen et al. (2005a) reported on SD on all LGs, but the highest amount of distorted loci was found on LG 1 and 3. As well the 'F_2 biomass' population showed on all LGs SD with the largest number of distorted loci on LG 1, 3, 6 and 7. Concluding, all studies showed similar distorted regions in the same populations. No specific common hotspot regions for SD were found; although some regions on LGs with a higher frequency of SD could be identified (LG 3, 4, 5, 7). We assume that SD has largely genetic root causes.

It was reported that self-incompatibility (SI) and self-compatible loci could cause SD (Thorogood et al. 2002 and 2005). Regions on the genome with consistent distorted marker ratios in the homozygous genotypes can be associated with loci segregating to self-compatibility (Thorogood et al. 2005). Thorogood et al. (2002) found in the 'ILGI' mapping population segregation of particular alleles mapping to the *S* SI locus region on LG 1 and a particular locus linked to SI on LG 3 which resulted in significant SD on both LGs. The *Z* SI locus was mapped on LG 2 (Thorogood et al. 2002). Additionally, distorted segregation ratios of markers on LG 5 were found by Thorogood et al. (2005) indicating the possibility of the presence of a gametophytic self-compatibility (*T*) locus on LG 5. These findings could be an explanation for SD on LG 1, 3 and 5 caused by the SI loci regions. However for SD affected regions on other chromosomes additional loci must be involved in the genetic causes of SD. Fine mapping and further characterization of the identified SD genes on LG 3 and LG 5 are the next steps towards the cloning of these genes which is a prerequisite to determine the allelic series of SD genes causing SD in certain genotypes.

SD research and its applications in practical and molecular plant breeding

The use of SD as a directed tool to identify regions or genetic loci was previously described by Grini et al. (1999) which used SD to screen for EMS-induced gametophytic mutants in *Arabidopsis*. Harbord et al. (2000) used a SD assay to identify transgenes linked to the pollen genes of the *Petunia* S gametophytic SI. Approaches like these could be interesting in the future for *L. perenne* studies for the screening of induced tilling populations or T-DNA tagged *Lolium* lines. However, a better knowledge of the influences of SD in mapping populations and breeding programmes is important. A breeding programme which takes often more than ten years to develop lines carrying the traits of interest would progress much faster by the use of molecular breeding techniques. SD can impede the selection process when not being recognised as a factor in the population structure. Therefore, a deeper understanding of the causes of SD for the breeding context is required. SD might have an impact on the order of markers on a map or the length of the map (Hackett and Broadfoot 2003), but SD might have even a bigger impact on the evolution of the genetic structure of a population as alleles favoured by distortion may tend to spread throughout a population (Jenczewski et al. 1997). Therefore, it is questionable if segregation distorted markers in a mapping population can be ignored for further work or can be eliminated from further calculations. These markers distort distances of genetic markers on a map and can lead to an underestimation of the required marker numbers for fine mapping studies. Two strategies can be followed to reduce negative impacts of SD in plant breeding. If a target is linked to a SD locus and the favoured alleles are underrepresented in a desired population, the frequency of the favourable allele can be increased by using molecular markers for selection of recombinants in the region of interest (Xu et al. 1997). Such a proceeding in the breeding method would offer more opportunities for favourable recombination in later generations (Xu et al. 1997, Lu et al. 2002) and would speed up the selection of stable breeding lines. A second strategy to reduce negative impacts of SD is to decrease the number of generations required for stabilizing breeding lines (Xu et al. 1997). Further studies should be carried out in breeding programmes by measuring SD in different

generations of a breeding programme to see if the genetic structure of the breeding lines changes. Breeding programmes would truly benefit from knowledge on the genetic and non-genetic causes of SD to control the phenomenon SD and to avoid strategic errors in progressing the selection of the trait of interest.

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