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ORIGINAL PAPER

Genomic constitution of *Festuca* × *Lolium* hybrids revealed by the DArTFest array

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Abstract Complementary attributes of *Festuca* and *Lolium* grasses can be combined in hybrid cultivars called Festuloliums, which are becoming increasingly popular fodder crops and amenity plants. Genomic constitution of commercially available Festuloliums was reported to vary from almost equal representation of parental genomes to apparent lack of one of them based on molecular cytogenetic analyses and screening with a small set of DNA markers, both approaches with limited resolution. Here, we describe the use of the DArTFest array comprising 3,884 polymorphic DArT markers for characterization of genomes in five Festulolium cultivars. In any of the cultivars, the minimum number of informative markers, which discriminated the parental *Lolium* and *Festuca* genomes was 361 and 171, respectively. Using the DArTFest array, it was possible to determine hybrid genome constitution at resolution which has never been achieved before and the analysis of a set of randomly selected plants from each cultivar provided information on genetic structure of outcrossing Festulolium cultivars. In addition to a core set of markers typical for each hybrid cultivar, markers occurring at low frequency among the plants within each cultivar were identified. Biological significance of genomic loci associated with the rare markers is yet to be determined. Finally, with the aim to simplify the use of DArTFest arrays to characterize *Festuca* × *Lolium* hybrids, various bulking strategies were compared. While all bulks were suitable for identification of hybrids, only bulks of few plants have been found to reveal the rare markers.

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Introduction

Lolium species, namely Italian ryegrass (*Lolium multiflorum* Lam.) and perennial ryegrass (*L. perenne* L.) are important fodder crops and amenity plants, especially in temperate regions. They are widely used for turf, pasture and high-quality animal feed. However, their broader use is limited by low survival under abiotic stress conditions. On the other hand, *Festuca* species, particularly *F. pratensis* Huds. and *F. arundinacea* Schreb. are known for winter hardiness and drought tolerance (Rognli et al. 2010). As members of the two genera can be successfully crossed under certain conditions (reviewed by Jauhar 1993), several hybrid cultivars have been released (Lewis et al. 1973; Buckner et al. 1977; Fojtik 1994). These so called Festuloliums combine agronomically valuable attributes of both parental species and are characterized by high yield, rapid development, deep green color, uniformity and palatability of ryegrasses and stress tolerance of fescues (Jauhar 1993).

Despite the increasing economic importance and widespread use of Festulolium cultivars, the knowledge on their nuclear genomes remains limited. Yet, the ability to

determine genomic constitution is important for breeders and seed distributors who often face a difficulty in discriminating between Festulolium and single-species cultivars. A reliable genotyping method and a possibility to follow the evolution of hybrid genome(s) would facilitate faster and cost-effective development of improved Festulolium cultivars. Last, but not least, the availability of suitable genotyping tools could make Festuloliums an attractive model to study the evolution of hybrid species.

Because of the outcrossing nature of the parental species and Festulolium itself, each plant of a cultivar is genetically unique and may possess a particular combination of parental genomes. Hence, the Festulolium cultivars are not genetically homogenous. In our previous work, we analyzed 25 plants per cultivar in a set of commercially available Festuloliums using genomic in situ hybridization (GISH), a cytogenetic method which facilitates identification of parental chromosomes (Kopecký et al. 2005, 2006). A variety of combinations of the fescue and ryegrass genomes was observed in the hybrids, ranging from equal proportion of the parental genomes to an apparent absence of one of them, and including exchanges of chromosome segments between both parental species. This work demonstrated a considerable variation in genomic makeup within and between Festulolium cultivars and pointed to a need for more thorough examination of the genome constitution of hybrid cultivars.

Although GISH has been used in a number of studies to analyze genomic constitution of wide hybrids (Thomas et al. 1994; Cai and Jones 1997; Snowdon et al. 1997), the sensitivity of GISH to detect chromatin introgressions is limited to large megabase-sized chromosome segments (Lukaszewski et al. 2005). This limits cytological detection of exchanges and introgressions to relatively large genome parts and may explain the apparent lack of one of the parental genomes in some Festuloliums (Kopecký et al. 2005, 2006). In addition to problems with detecting small chromosome segments, GISH procedure is time consuming, laborious and low throughput and hence not suitable for large-scale screening. This may limit its use in breeding programs to follow genomic constitution in hybrid progenies.

A viable alternative to GISH in screening for chromatin introgressions in Festulolium hybrids is to employ DNA markers. Among the parental species of Festulolium, genetic maps have been developed for *L. perenne* and *L. multiflorum* (Jones et al. 2002; Inoue et al. 2004) and in both fescue species, *F. pratensis* (Alm et al. 2003) and *F. arundinacea* (Saha et al. 2005). These maps were constructed using isozyme and DNA markers including AFLP, RFLP and SSR. The availability of DNA markers facilitated the analysis of genetic diversity among fescues and ryegrasses (Kolliker et al. 1999; Guthridge et al. 2001; Fjellheim and Rognli 2005). Momotaz et al. (2004) used 40 SSR markers to reveal relationships among *Festuca* and *Lolium* species and their hybrids. However, the use of SSR, AFLP, RFLP and other molecular markers in Festulolium breeding is highly limited, mainly due to their low throughput.

Diversity Arrays Technology (DArT) represents another type of DNA markers, which recently became available for *Festuca* and *Lolium* (Kopecký et al. 2009). DArT relies on DNA polymorphisms at (SNPs, InDels and methylation differences) and between (InDels) restriction-enzyme sites and excels in throughput and cost effectiveness (Jaccoud et al. 2001; Wenzl et al. 2004; http://www.DiversityArrays.com). Its current version is based on hybridization, which does not require sequence information and uses microarray technology to identify and type thousands of dominant markers in parallel (Wenzl et al. 2004). A possibility of performing high-throughput screening of *Festuca* × *Lolium* hybrids and their progenies at affordable cost is especially attractive for Festulolium breeders.

In our previous study, we developed DArTFest array for the *Festuca–Lolium* complex containing 7,680 probes (Kopecký et al. 2009). We have shown that hundreds of these markers distinguish parental genomes of various *Festuca* × *Lolium* hybrids. In case of the widely used *L. multiflorum* × *F. pratensis* hybrids, the parental genomes could be distinguished by up to 1,203 and 699 markers, respectively (Kopecký et al. 2009). A number of genome-specific DArT markers have already been mapped genetically in both species and sequencing DNA fragments on the DArTFest array is in progress (Bartoš et al., in preparation).

In this study, we set out to explore a possibility of using a DNA array as a tool to identify Festulolium cultivars and characterize their genomic constitution. We describe the performance of the DArTFest array and compare the impact of analyzing individual plants and bulked samples. We show that the analysis of bulked DNA from a specific number of individuals is sensitive enough to discriminate between Festulolium cultivars. The availability of the array will make it possible to follow changes in the genome composition of *Festuca* × *Lolium* hybrids in successive generations as well identify markers associated with traits of interest in the hybrids.

Materials and methods

Plant material and DNA extraction

Five commercial Festulolium cultivars were used in this study. The same cultivars were analyzed previously for their genomic constitution using GISH (Kopecký et al. 2005, 2006). Two of them were derived from *L. multiflorum* × *F. arundinacea* hybrids: tetraploid cv. 'Lofa',

developed by backcrossing an F1 hybrid to a tetraploid *L. multiflorum*, and hexaploid cv. 'Hykor', which was developed by backcrossing an F1 hybrid to *F. arundinacea*. The remaining three cultivars were derived from *L. multiflorum* × *F. pratensis* hybrids and they differed in genomic constitutions, as shown by GISH (Kopecký et al. 2006). Cv. 'Elmet' had almost equal proportions of parental genomes; cv. 'Perun' had a slight prevalence of the *L. multiflorum* genome and cv. 'Spring Green' was predominantly *L. multiflorum* with just a few chromatin segments of *F. pratensis* (Kopecký et al. 2006).

Plant genomic DNA was extracted from young leaves using the Invisorb Spin Plant Mini Kit (Invitek, Berlin, Germany) according to manufacturer's instructions. Each population (Festulolium cultivar) was represented by 100 randomly selected plants. Leaf samples for DNA extraction were collected in several ways. First, leaves from 20 individual plants were collected and their DNA was extracted separately. In addition, bulks of leaves from 5, 10, 15, 20, 25, 50 and 100 plants in each cultivar were collected and DNA was extracted separately in each bulk. We collected an equal amount of leaves (by weight), mixed them and extracted the DNA from the mixture.

DArTFest screening

The hybridization on the DArTFest array was done as described previously (Kopecký et al. 2009). Arrays were hybridized with fluorescently labeled genomic representation of individual plants and bulked samples. The image processing and marker classification were performed using DArTsoft version 7.3 (DArT P/L, unpublished), a software package developed at DArT P/L (Yarralumla, Australia). The markers reported in the present study were selected with a call rate >80% and with technical reproducibility of above 99.0%.

We used the DArTsoft-generated 0–1 scores as input for the RESTDIST and NEIGHBOR programs of the PHYLIP 3.6 software package to construct dendrograms based on the Unweighted Pair Group Method with Algorithmic Mean (UPGMA) and Felsenstein's modification of the Nei/Li restriction fragment distance (Felsenstein 1989, 2004). For the dendrograms, we used the hybridization 0–1 scores from this particular experiment and the scores of 40 accessions of *L. multiflorum*, 40 accessions of *F. arundinacea* and 39 accessions of *F. pratensis* obtained in our previous study (Kopecký et al. 2009).

Sequence analysis of selected DArT markers

Based on the results of hybridization, we choose to sequence 100 randomly selected "rare" DArT markers. These markers were 'species-specific' and in hybrid plants occurred in low frequency (≤25%). A total of 50 sequenced markers were specific for *F. arundinacea* and were selected based on scoring individual plants of cv. 'Lofa'. The remaining 50 sequenced markers were *L. multiflorum*-specific and were selected based on scoring individual plants of cv. 'Hykor'. Reaction mix for cycle sequencing was prepared using standard BigDye chemistry (BigDye® Terminator v3.1 Cycle Sequencing Kit, Applied Biosystems). The dilution of reaction components was scaled to the final volume of 10 µl containing 3.2 pmol of universal M13 (forward or reverse) primer and 20 ng of sequence-ready template. The reaction products were purified by CleanSEQ kit (Agencourt Bioscience Corp., Beckman Coulter Comp., Beverly, MA, USA) and analyzed on ABI 3730xl DNA analyzer (Applied Biosystems, Foster City, CA, USA). Raw sequence data were assembled and edited by DNA Baser software v.2 (Heracle-Software, http://www.DnaBaser.com). Vector and adaptor sequences were removed prior to further analysis.

All sequences were blasted to each other to determine extent of redundancy in DArT clones. Stand alone blastn software was used for the search with E-value set to e−10. Only reciprocal blast hits were taken into account. In total, 82 sequences (after reduction of duplicated and multiplicative sequences) were further compared to known plant repeats. Repeat content was analyzed using TREP Release 10 (http://wheat.pw.usda.gov/ITMI/Repeats/) and TIGR Plant Repeat Databases for Brassicaceae, Fabaceae, Gramineae and Solanaceae (Ouyang and Buell 2004). The repeat analysis was performed by RepeatMasker software (http://repeatmasker.org) with CrossMatch search engine (http://www.phrap.org/phredphrapconsed.html) and default settings. To estimate gene content, 82 DArT sequences were compared with non-human, non-mouse ESTs database (est_others) and non-redundant protein sequences (nr) at GenBank. The protein search (blastx) was performed using blastcl3 with default settings and BLOSUM62 scoring matrix. Nucleotide search (blastn) was also performed by blastcl3 with default settings, but reward for a nucleotide match was set to 2. Only the best blast hits (with lowest E-value) were taken into account.

Results

Analysis of genomic constitution

Identification of 'species-specific' markers

To determine genomic constitution in individual plants and bulked samples, we first identified 'species-specific' markers based on the results of our previous study (Kopecky et al. 2009). A marker was considered 'species-specific' if it had a

negative score (0) in all accessions of one species and positive score (1) in at least one accession of other species. For each plant and bulk, we classified markers into three groups: (a) specific for one species; (b) specific for the other species; and (c) shared by both species. The number of scored 'species-specific' markers for each cultivar is given in Fig. 1.

Individual plants

The first experiment involved the analysis of 20 single plants selected randomly from each hybrid cultivar. The scores for 'species-specific' markers were classified into five categories according to their frequency of occurrence among the plants: (a) 76–100%; (b) 51–75%; (c) 26–50%; (d) 1–25%; and (e) absent. Among the three *L. multiflorum* × *F. pratensis* cultivars ('Elmet', 'Perun' and 'Spring Green'), the lowest amount of the *Festuca* genome was found in 'Spring Green'; it was considerably higher in 'Perun', but still lower than in 'Elmet'. The proportion of the *Lolium* genome was inverse to that of *Festuca* (Fig. 1a). The analysis revealed a slight predominance of *F. pratensis* genome over that of *L. multiflorum* in cv. 'Elmet' and prevalence of *L. multiflorum* genome over that of *F. pratensis* in cv. 'Spring Green'. Almost equal proportion of parental genomes was found in cv. 'Perun'. Among *L. multiflorum* × *F. arundinacea* cultivars, 'Hykor' showed a clear prevalence of the *F. arundinacea* genome over that of *L. multiflorum* and 'Lofa' showed prevalence of *L. multiflorum* genome over that of *F. arundinacea* (Fig. 1a).

Bulked samples

One of the main goals of this study was to evaluate the efficiency of marker detection in bulked samples. All species-specific markers were divided into five groups based on their presence in individual plants. Only small differences in the ability to detect DArT markers were observed between bulks prepared from a different number of plants (Fig. 2). However, it became obvious that the ability to detect a marker using bulks depends on the frequency of particular marker in the population. Markers present in more than 50% of plants could be detected using bulks. However, bulks did not permit efficient detection of 'rare' markers, which were present in <25% plants of a cultivar. Out of the different bulked samples, bulks made with the smallest number of plants (B5) appeared to be most efficient to detect rare markers (Fig. 2).

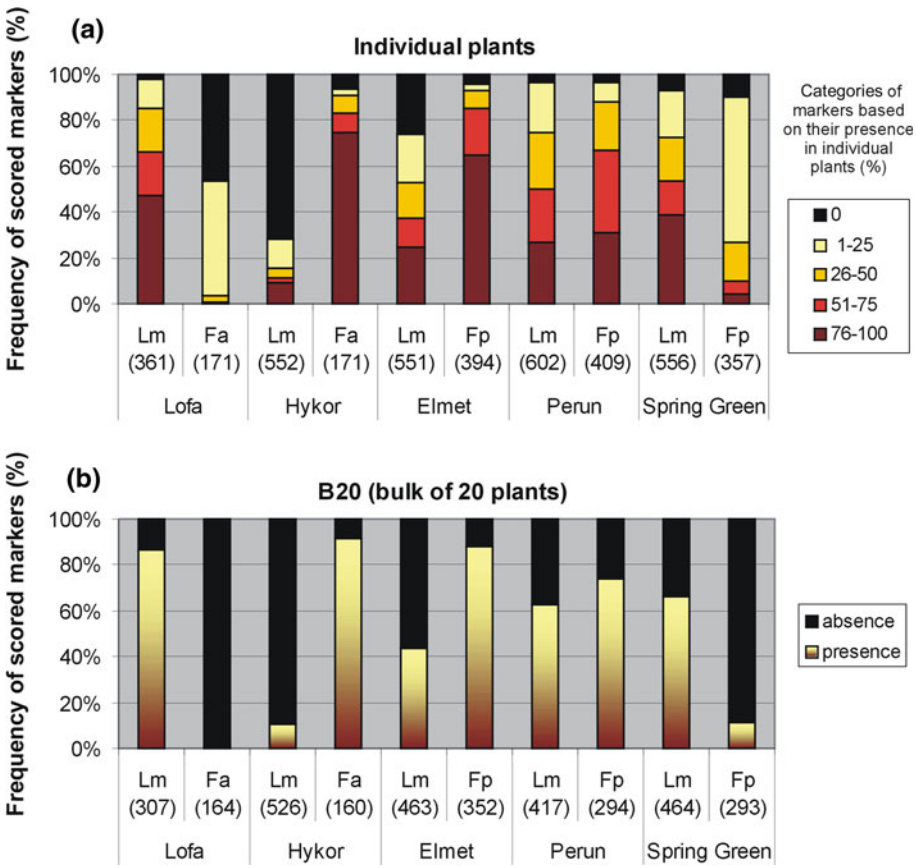

Fig. 1 Frequency of *Festuca*- and *Lolium*-specific markers determined in hybrid cultivars using individual plants (**a**) and bulks of 20 plants—B20 (**b**). For the analysis of single plants, markers were classified according their frequency of occurrence into five groups: absent (*black*), present in 1–25% of plants (*yellow*), present in 26–50% of plants (*orange*), present in 51–75% of plants (*red*) and present in 76–100% of plants (*brown*). Number of 'species-specific' markers used is given in *brackets*

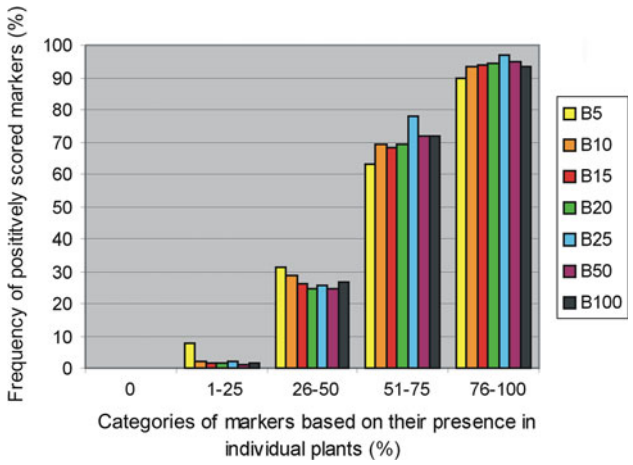

Fig. 2 Frequency of DArT markers in various types of bulks differing by the number of plants (5, 10, 15, 20, 25, 50 and 100). All markers were classified into five groups based on their frequency in individual plants: absent (0), present in 1–25% of plants (1–25), present in 26–50% of plants (26–50), present in 51–75% of plants (51–75) and present in 76–100% of plants (76–100). Note the negligible differences in the frequency of markers in various bulks. Only bulk of 5 plants (B5) detected rare markers with a slightly higher efficiency

Table 1 Frequency of 'species-specific' markers in bulks of five hybrid cultivars

Cultivar	Frequency of *Lolium*-specific markers (%)	Frequency of *Festuca*-specific markers (%)
Elmet	43.5–46.4	86.6–90.1
Perun	61.6–66.0	70.6–76.6
Spring Green	63.1–67.2	10.9–14.4
Lofa	81.1–87.0	0.6–1.9
Hykor	9.7–11.6	89.0–91.3

Range observed in different types of bulks

In general, the results of bulked samples agreed with those obtained after analyzing the sets of 20 single plants (Fig. 1b). Among the *L. multiflorum* × *F. pratensis* hybrid cultivars, cv. 'Elmet' had the highest frequency of *F. pratensis*-specific markers and cv. 'Spring Green' had lower frequency of such markers than cv. 'Perun' (Table 1). There was a slight predominance of *F. pratensis*-specific markers compared to markers specific for *L. multiflorum* in cv. 'Elmet'. In cv. 'Perun', there was almost equal number of parental species-specific markers positively scored. Bulked samples of cv. 'Spring Green' showed predominance of *L. multiflorum* genome. The low frequency of markers specific for *F. arundinacea* in cv. 'Lofa' and markers specific for *L. multiflorum* in cv. 'Hykor' reflected their introgression origin.

Analysis of genetic diversity in Festulolium cultivars

Of the 3,884 polymorphic markers identified on the DArTFest array (Kopecký et al. 2009), 1,471 were informative and gave unequivocal scores in accessions of three parental species and five hybrid cultivars, each represented by 20 plants and by seven bulks differing in the number of plants. Two samples—a single plant of cv. 'Elmet' (xE 08) and a bulk of 25 plants (B25) of cv. 'Perun'—produced very divergent scores from the related accessions. They were probably contaminants by other accessions, similar to a divergent accession of *F. pratensis* detected in our previous study (Kopecký et al. 2009). We excluded these two putative contaminants from further analyses.

As with the analysis of genomic constitution, we analyzed genetic diversity of the Festulolium cultivars using single plants and bulked samples. The first analysis involved 99 individually sampled plants from the five hybrid cultivars and all accessions of *L. multiflorum*, *F. pratensis* and *F. arundinacea*, which were used to develop the DArTFest array (see Kopecký et al. 2009 for the complete list of species and accessions). The second analysis included 33 bulks (seven bulks per cultivar, except that of 'Perun', where bulk B25 was excluded, and bulk B5 of 'Elmet', which failed to hybridize on DArTFest array) and all accessions of *L. multiflorum*, *F. pratensis* and *F. arundinacea* from which the array was developed. Both approaches, i.e. the analysis of single plants and bulks, gave very similar results. Samples from each cultivar formed a tight cluster and all five cultivars could be easily discriminated (Fig. 3a, b).

The analysis allowed us to estimate the level of variation within and among individual cultivars. The level of intervarietal variation was always higher than intravarietal variation. In general, the degree of variation within cultivars reflected the origin and age of cultivars. The lowest level of intravarietal variation was found among the plants of cv. 'Hykor' (Fig. 3a). This cultivar originated from multiple backcross of F1 *L. multiflorum* × *F arundinacea* hybrid to *F. arundinacea*, which was found to have the lowest intraspecific variation (Kopecký et al. 2009). The intravarietal diversity was higher in cv. 'Spring Green' than in cvs. 'Perun' and 'Elmet', which were released much earlier and went through more cycles of seed multiplication and selection.

This analysis also revealed the relationship of individual cultivars to their parental species. Among the *L. multiflorum* × *F. pratensis* cultivars, 'Spring Green' appeared close to *L. multiflorum*, 'Elmet' and 'Perun' appeared to be closer to *F. pratensis* than to *L. multiflorum*. Among the *L. multiflorum* × *F. arundinacea* hybrids, cv. 'Lofa' was close to *L. multiflorum*, while cv. 'Hykor' was close to *F. arundinacea*.

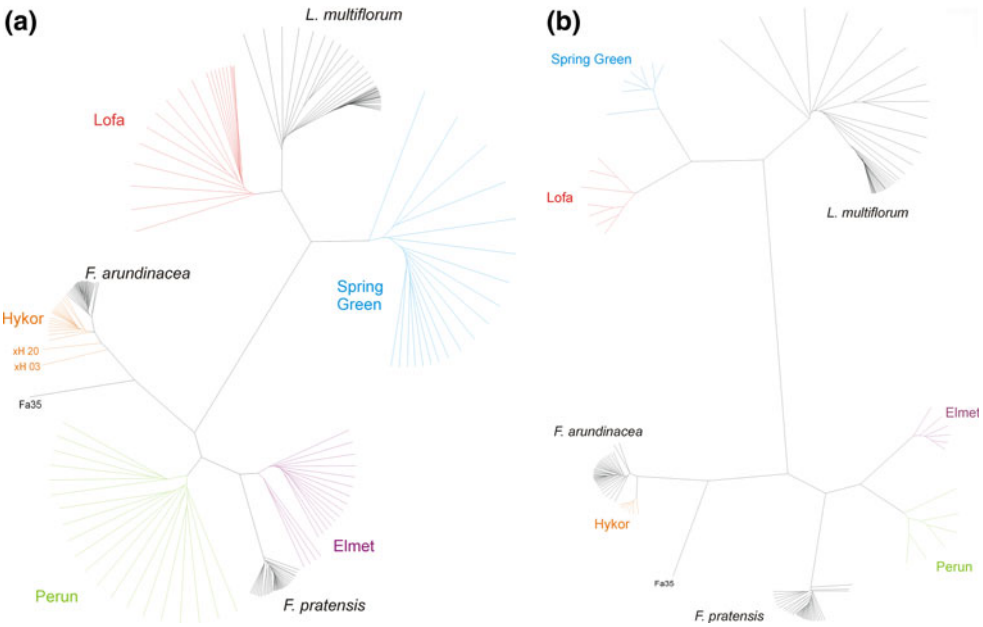

Fig. 3 UPGMA dendrogram (shown as Radial tree) based on the analysis of 40 *Lolium multiflorum*, 40 *Festuca arundinacea* and 39 *F. pratensis* accessions and five hybrid cultivars ('Elmet', 'Perun', 'Spring Green', 'Hykor' and 'Lofa') using 1471 DArT markers and Felsenstein's modified Nei/Li restriction fragment distance. Each cultivar is represented by either 20 individual plants (**a**) or by seven bulks with various numbers of plants (**b**). It is evident that this type of analysis facilitates discrimination between the Festulolium cultivars and reveals their hybrid origin

Sequencing of 'rare' markers

Sequencing of 100 randomly selected "rare" DArT markers resulted in 94 sequences with average length 524 bp. The sequences were deposited in Genbank (Genbank Acc HN150588-HN150681; dbGSS ID 28497707-28497800). The sequence analysis revealed that 21.3% of markers (20) were redundant (duplications or multiplications). Two pairs of such duplications were probably caused by contaminations of clones from adjacent wells. Comparison to in-house built plant repeat database revealed repetitive elements in 10 out of 82 (12.2%) sequenced markers (after duplication/multiplication reduction). To estimate the frequency of genes or expressed sequences among DArT markers, we used blastn search against GenBank "est_others" database. We identified 41 DArT markers (50.0%) with significant homology to expressed sequences. A blastx search against non-redundant protein sequences (nr) identified 13 DArT markers (15.9%) with significant homology to known and hypothetical proteins. Out of these, sequences of 11 markers were in parallel identified as expressed sequences using blastn.

Discussion

This work describes a novel and efficient approach to assess genomic constitution of *Festuca* × *Lolium* hybrids using the Diversity Array Technology. Previous attempts to characterize genomes of *Festuca* × *Lolium* hybrids relied on cytogenetic analyses using GISH (Thomas et al. 1994; Kopecký et al. 2006), which suffers from low sensitivity and resolution that may preclude detection of small chromosome introgressions, deletions and translocations. Moreover, the procedure is laborious and hence unsuitable for high-throughput analyses. Another possibility to reveal genomic constitution of hybrids and to analyze intra- and intervarietal variation is to use PCR-based markers. Unfortunately, a limited number of such markers is available, especially in *Festuca*. Moreover, the ability to distinguish individual cultivars from each other using a limited set of molecular markers could be compromised (Pharmawati et al. 2005; Rojas et al. 2008).

On the other hand, the DArT technology can screen thousands of markers in a single pass. In our previous work, we have developed a total of 7,680 DArT markers from *Festuca* and *Lolium* species (Kopecký et al. 2009). Out of them, 148 and 529 markers were mapped genetically in *F. pratensis* and *L. multiflorum*, respectively (Bartoš et al., in preparation) with even distribution among all linkage groups. A considerable number of DArT markers were found to be specific for parental genomes of Festuloliums and, depending on the parental species combination, their numbers ranged from 448 to 1,203 (Kopecký et al. 2009). In this study, the number of scored 'species-specific' markers was lower than expected as some markers were uninformative (no scores in particular hybrid plant/bulk due to technical limitation). Furthermore, a more detailed estimation of hybrid genomic constitution was hampered by dominant character of DArT markers. Thus, the absence of a particular marker in a hybrid (score '0') did not necessarily indicate the absence of the corresponding genome region. However, as one may expect that this was true in both groups of 'species-specific' markers, our estimates were probably not biased to a large extent.

Dominant markers (e.g. AFLPs) have been used to characterize the genomic constitution of several interspecific and intergeneric hybrids (Iovene et al. 2004; Tu et al. 2009). In spite of the dominant character of these markers, they clearly identified interspecific introgressions. Similarly, DArT markers identified hybridity in all Festulolium cultivars used in this study. In contrast, Kopecký et al. (2005) were not able to detect *Festuca arundinacea* chromatin in 23 (79%) plants of cv. 'Lofa' by GISH. In the case of DArT markers, the lowest number of informative markers in any combination of the parental genomes was 532 (171 plus 361) and both parental genomes were identified by at least one marker in all hybrid plants. Number of markers used in this study was more than five times higher than the number of markers used in previous attempts to analyze genetic diversity in the *Lolium–Festuca* complex (Momotaz et al. 2004; Tamura et al. 2009). Theoretically, higher number of markers should allow identification of smaller introgressions and increase the chance to identify hybridity of plants. This is of high importance especially if one or more backcrosses were used during the breeding process.

In some cases, our findings with DArT do not fully agree with the previous results obtained using GISH (Kopecký et al. 2005, 2006) and with the known pedigree of the selected Festuloliums. The cultivars 'Lofa' and 'Hykor' were developed after several backcrosses of F1 *L. multiflorum* × *F. arundinacea* hybrids to one of the parental species, which was reflected by their position on the dendrogram in a close vicinity of the parental backcross species. On the other hand, the location of 'Elmet' and 'Perun' closer to *F. pratensis* than to *L. multiflorum* was an unexpected observation. According to GISH, both cultivars should have a high proportion of *L. multiflorum* genome ('Elmet' slightly over 50% and 'Perun' about 70%; Kopecký et al. 2006). Although a reason for the discrepancy is not obvious, the close relationship of the two cultivars to *F. pratensis* may be due to higher intraspecific diversity of *L. multiflorum* compared to *F. pratensis* (Fjellheim et al. 2006; Kopecký et al. 2009) resulting in lower scoring of *L. multiflorum* markers in hybrids. It may also be caused by the clustering of DArT markers in particular chromosome regions and redundancy of the markers. However, based on the sequencing of DArT markers (Bartoš et al., in preparation), redundancy is ~20% with no more than six markers in a single cluster.

The use of bulks to characterize *Festuca* × *Lolium* hybrid populations would further increase the throughput and decrease the cost of DArT analysis. However, different sampling methods (bulked samples vs. individual plants) resulted in slightly different positions of Festulolium cultivars on the dendrograms (Fig. 3a, b). For example, cv. 'Elmet' appeared more closely related to *F. pratensis* if individual plants were analyzed as compared to the bulks. Similarly, cv. 'Lofa' seems more closely related to *L. multiflorum* if individual plants are used. The differences were probably due to uneven scoring of rare markers, which are present in a small number of plants and hence difficult to detect in bulked samples.

The identification of "rare" markers is an interesting outcome of this study and raises questions on the nature of these genome loci and their biological role including their effect on phenotype and agronomic performance of hybrid cultivars. As noted by Maccaferri et al. (2007), the rare alleles can be particularly useful in identifying small distinct breeding lineages within elite materials with low polymorphism levels. At this point, it is unclear if the occurrence of "rare" markers is a consequence of selective pressure during the breeding process or is a manifestation of the evolution of hybrid genomes (Adams and Wendel 2005). The absence of "rare" markers in most of plants could be due to the loss of specific genomic loci, its mutation or due to modification of DNA in hybrids as DArT is sensitive to methylation status of restriction sites. In order to reveal the nature of this class of markers, we selected a random set for sequencing. However, the analysis did not identify a significant difference in sequence type between rare markers and other DArT markers. The frequency of "rare" markers containing repetitive elements was similar to that of 621 sequenced DArT markers, which were placed in genetic maps of *F. pratensis* and *L. multiflorum* (Bartoš et al., in preparation). Similar results were also found after blastn and blastx sequence homology search against expressed sequence tags and non-redundant protein sequences (nr). Nevertheless, the sequences of "rare" markers could be used to convert DArTs to other types of markers and thus provide more options for identification of traits associated with "rare" markers.

Despite the differences between the results of analysis of genetic diversity using single plants and bulks, the examination of bulked samples with the DArTFest array unambiguously discriminated individual Festulolium cultivars from each other and also from parental species. These observations raise a question as to which markers should be used for hybrid cultivar identification. In principle, markers present in most or even all plants of a cultivar should be employed. However, in cultivars developed by repeated backcrosses to one of the parental species, majority of markers from other parent may be present in a small number of plants as 'rare' markers or even absent. Nevertheless, despite the apparent absence of the "rare" markers in bulked samples, it was possible to discriminate hybrid cultivars from pure species.

In conclusion, we have demonstrated the utility of DArT markers to assess the genetic constitution of Festulolium hybrids at sensitivity and resolution that have not been

achieved before. While at present it is only possible to quantify the proportion of parental genomes, mapping of DArT markers in *Festuca* and *Lolium* should make it possible to identify specific segments of the parental genomes, assess their frequency of occurrence in the population and possibly link them to particular agronomic traits. The observation on the ability to identify particular Festulolium cultivars using bulked samples provides a basis for a low-cost and rapid cultivar characterization. While we have developed this system for Festulolium cultivars, it should be generally applicable to any interspecific hybrids for which a DArT array will be available.

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