Theor Appl Genet (2005) 111: 980–992
DOI 10.1007/s00122-005-0038-8

ORIGINAL PAPER

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An expressed sequence tag SSR map of tetraploid alfalfa (*Medicago sativa* L.)

Received: 17 February 2005 / Accepted: 2 July 2005 / Published online: 2 August 2005

Abstract A genetic map constructed from a population segregating for a trait of interest is required for QTL identification. The goal of this study was to construct a molecular map of tetraploid alfalfa (*Medicago sativa*.) using simple sequence repeat (SSR) markers derived primarily from expressed sequence tags (ESTs) and bacterial artificial chromosome (BAC) inserts of *M. truncatula*. This map will be used for the identification of drought tolerance QTL in alfalfa. Two first generation backcross populations were constructed from a cross between a water-use efficient, *M. sativa subsp. falcata* genotype and a low water-use efficient *M. sativa* subsp. *sativa* genotype. The two parents and their F_1 were screened with 1680 primer pairs designed to amplify SSRs, and 605 single dose alleles (SDAs) were amplified. In the F_1, 351 SDAs from 256 loci were mapped to 41 linkage groups. SDAs not inherited by the F_1, but transmitted through the recurrent parents and segregating in the backcross populations, were mapped to 43 linkage groups, and 44 of these loci were incorporated into the composite maps. Homologous linkage groups were joined to form eight composite linkage groups representing the eight chromosomes of *M. sativa*. The composite maps consist of eight composite linkage groups with 243 SDAs from *M. truncatula* EST sequences, 38 SDAs from *M. truncatula* BAC clone sequences, and five SDAs from alfalfa genomic SSRs. The total composite map length is 624 cM, with average marker density per composite linkage group ranging from 1.5 to 4.4 cM, and an overall average density of 2.2 cM. Segregation distortion was 10%, and distorted loci tended to cluster on individual homologues of several linkage groups.

Electronic Supplementary Material Supplementary material is available for this article at http://dx.doi.org/10.1007/s00122-005-0038-8

Communicated by F. J. Muehlbauer

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Introduction

Cultivated alfalfa is an autotetraploid species ($2n = 4x = 32$). Due to the complexity of tetrasomic inheritance, most genetic maps of alfalfa have been constructed in diploids (Brummer et al. 1993; Kiss et al. 1993; Echt et al. 1994; Tavoletti et al. 1996; Kalo et al. 2000). Two genetic maps have been constructed in tetraploid populations (Brouwer and Osborn 1999; Julier et al. 2003). Mapping of QTL in tetraploids may be desirable, however, as differences in combining ability (Groose et al. 1988) and forage yield (Kidwell et al. 1994) have been documented between isogenic diploid and tetraploid alfalfa populations.

The difficulty of mapping in autotetraploids using available marker technologies such as RFLPs and SSRs, lies in the inability to distinguish between different classes of heterozygous genotypes that may possess one, two, or three doses of a fragment representing an allele. For example, a heterozygous locus with two copies of each of two alleles cannot be distinguished from a genotype with one copy of one allele, and three copies of the other allele—both genotypes result in the amplification of two pools of fragments. One strategy to circumvent such complexities is to utilize single dose fragments, or single dose alleles (SDAs; Wu et al. 1992), which possess a defined allelic state. When mapping SDAs, the segregation ratios and linkage equations are equivalent to diploids for coupling phase linkages, which allows the use of mapping software designed for diploids. Single dose alleles are abundant in autopolyploid plants, where up to 70% of all polymorphic loci are expected to behave as SDAs (da Silva et al. 1993), and

should provide an adequate number of markers for the construction of a genetic linkage map. When mapping SDAs, a linkage group must be constructed for each homologue of each chromosome. The homologues are then aligned and the markers are combined into a single composite linkage group based on the presence of homologous SDAs amplified by the same primer pair (PP).

The disadvantage of using only SDAs for map construction is that a large number of markers are needed. Expressed sequence tagged (EST)-derived simple sequence repeat (SSR) PPs can identify a large pool of SDAs on multiple homologues to address this challenge (Scott et al. 2000; da Silva 2001). The growing number of available EST sequences in public databases facilitates the development of EST-SSR markers. There are currently 226,923 ESTs in The Institute for Genome Research (TIGR) *Medicago truncatula* Gene Index (MtGI 8.0) (http://www.tigr.org/tdb/tgi/mtgi/). *Medicago truncatula* based ESTs contain 3% SSRs, and PPs designed from these sequences frequently amplify ESTs containing SSRs in alfalfa as well as other closely related legumes (Eujayl et al. 2004). EST–SSRs have several advantages over other marker systems. EST–SSRs are PCR-based markers, which makes them more efficient than hybridization-based RFLP markers. SSR PPs designed from EST databases are both inexpensive and efficient to generate, since cDNA library construction and sequencing of clones has already been accomplished. EST–SSRs are derived from gene sequences; therefore, they should be more informative than genomic SSRs, which may be derived from non-coding DNA sequences (Cordeiro et al. 2001; Eujayl et al. 2002). Furthermore, a higher percentage of EST–SSR PPs will amplify sequences across species compared to genomic SSRs (Cordeiro et al. 2001; Eujayl et al. 2002) making them more useful for comparative genetic studies.

The various alfalfa maps constructed to date have few markers in common and there is no single map that unifies them. EST–SSR markers provide a set of easily shared markers that can be used to unify and cross reference established genetic maps (Tang et al. 2002). The EST–SSRs developed in this study will provide an important resource for genetic mapping and marker-assisted selection in alfalfa, as well as for comparative genetic studies between *M. sativa* and other species, such as the model legume *M. truncatula*, or other pasture legumes such as white clover (*Trifolium repens*).

The goal of the present research was to construct an EST–SSR map of tetraploid alfalfa and to anchor this genetic map to an *M. truncatula* physical map that is currently under construction (http://www.genome.ou.edu/medicago.html). EST–SSRs were derived from publicly available *M. truncatula* sequence data (http://mtgenome.ucdavis.edu/index.html) (Eujayl et al. 2004). Mapping was conducted in two first generation backcross populations derived from a cross between *M. sativa* subsp. *sativa* and *M. sativa* subsp. *falcata*.

Materials and methods

Plant materials

Two first generation backcross populations of tetraploid alfalfa were constructed from a cross between a water-use efficient, fall-dormant, winter hardy *M. sativa* subsp. *falcata* genotype var. Wisfal (Bingham 1993), and a low water-use efficient, non-fall dormant, non-winter hardy *M. sativa* subsp. *sativa* var. Chilean genotype (Ray et al. 2004). A single F_1 plant from the cross was used as the female parent to create the Chilean BC_1 and Wisfal *M. falcata* BC_1 backcross populations. The backcross nature of each progeny was verified through the presence of AFLP markers derived from the F_1 parent, as well as markers derived from its respective recurrent parent that were not present in the F_1 donor genotype. For SSR analysis, 93 BC_1 individuals from each population were analyzed.

Origin and detection of SSR fragments

Approximately 147,000 ESTs from NCBI's dbEST (ftp://ftp.ncbi.nih.gov/blast/db/) were searched for the presence of di-, tri-, tetra- and pentanucleotide motifs with at least 10, 7, 5, and 4 repeats, respectively using the PERL program, simple sequence repeat identification tool (SSRIT) (http://www.gramene.org/gramene/searches/ssrtool). Those SSRs with start and end positions at least 50 bp from the 5′ and 3′ EST ends were selected for primer development. Primers were designed using Primer3 software (http://www.genome.wi.mit.edu/genome_software/other/primer3.html) according to Eujayl et al. (2004) to amplify fragments ranging in size from 125 to 300 bases, and that also contained the SSR. Eighty-four SSRs previously mapped in alfalfa by Julier et al. (2003), 148 *M. truncatula* EST-SSRs obtained from Dr. Nevin Young (University of Minnesota, personal communication), ten alfalfa genomic SSRs identified by Diwan et al. (2000) were also used. An additional 488 BAC-SSRs, identified through the *M. truncatula* Genome Sequence SSR index (http://mtgenome.ucdavis.edu/), that were distributed over all eight *M. truncatula* chromosomes, were also screened for polymorphism between the *M.sativa* and *M. falcata* parents.

Forward and reverse primers were synthesized by Qiagen/Operon Biotechnologies (Los Angeles, CA, USA) with an additional 18 nucleotides from the M13 universal primer appended to the 5′ end of the forward primer (Schuelke 2000). PCR reactions were prepared in a reaction volume of 10 µl which contained 20 ng of template DNA, 2.5 mM $MgCl_2$, 1× PCR buffer II (Applied Biosystems, Foster City, CA, USA), 0.15 mM dNTPs, 1.0 pmol of each reverse and M13 (−21) universal primer, 0.25 pmol of the forward primer, 0.5 U

AmpliTaq Gold DNA polymerase (Applied Biosystems). The M13 universal primer was labeled either with blue (6-FAM), green (HEX), or yellow (NED) fluorescent tags. PCR products with different fluorescent labels and with different fragment sizes were pooled for detection. Typically, four PCR products were pooled. PCR products (1.6 μl) were combined with 12 μl of deionized formamide and 0.5 μl of GeneScan-500 ROX internal size standard and analyzed on the ABI PRISM 3100 Genetic Analyzer (Applied Biosystems). The total reactions were visualized with GeneScan 3.7 software, and manually scored.

Single-dose alleles

EST–SSR markers were named by taking the first two letters of the Genbank accession name, followed by an arbitrary number. The EST–SSR name, Genbank accession (when available) name, size of fragment amplified, and forward and reverse primer sequences of SDAs identified in this study are available as electronic supplementary material.

For each PP, individual fragments were scored as dominant markers. PPs with fragments that were polymorphic between the two parents were screened with ten backcross progeny to identify potential single dose markers. SSR primer pairs with fragments that segregated 5:5 or 6:4 were used to genotype both backcross populations. Following the method of Brouwer and Osborn (1999), fragments segregating at a presence to absence ratio of less than 2.24:1 in the backcross populations were considered to be single-dose alleles. The segregation ratio at a χ^2 of 3.84, the upper limit for accepting a fragment as segregating 1:1, is approximately 1.5:1. The segregation ratio at the same χ^2 for a 5:1 segregation ratio is approximately 3.2:1. The point where the segregation ratios have an equal χ^2 for both hypotheses, which occurs at a 2.24:1 presence to absence ratio, was used to distinguish an allele as being either a single dose allele (below this ratio) or a double dose allele (above this ratio). We recognize that a 5:1, or double dose locus, with segregation distortion, deviating toward presence of a fragment, could have a presence to absence ratio that falls into this interval, as could a single dose allele with segregation distortion. Therefore, segregation data from selected PPs were subjected to a χ^2 test to identify alleles that showed a significant ($P < 0.05$) segregation distortion, with deviation toward presence or absence of a fragment.

SSR linkage analysis

Linkage groups were constructed for the F_1 genotype using JoinMap 3.0 software (Van Ooijen 2001) to map SDAs that were present in one parent, absent in the other parent, present in the F_1 and segregating in one or the other backcross population. The SDAs were analyzed as population type BC_1, using the Kosambi mapping function and a minimum LOD of 5. Allelic fragments of differing sizes that were amplified by the same PPs were used to identify and join F_1 homologous linkage groups into composite linkage groups using Joinmap's "Combine Groups for Map Integration" function. SDAs absent in the F_1, but present in the recurrent parent and segregating 1:1 in the backcross progeny, were mapped to linkage groups representing recurrent parent linkage blocks that were not inherited by the F_1. These recurrent parent markers were manually added to the composite map when they occurred between flanking loci common to the recurrent parent linkage group and the F_1 composite group. These recurrent parent markers were added to the composite group at a distance that was proportional to the distances between the common flanking markers on the two groups. *M. truncatula* BAC SSRs were used to anchor the alfalfa genetic map to the *M. truncatula* physical and genetic maps. Linkage maps were drawn using MapChart 2.1 (Voorips 2002).

Results and discussion

A total of 1,680 PPs designed to amplify SSRs were screened against the two parents and the F_1, including 1,182 PPs from *M. truncatula* ESTs, 488 PPs from *M. truncatula* BAC clones, and 10 PPs from alfalfa genomic DNA clones (Table 1). Approximately 25% of all PPs from *M. truncatula* ESTs amplified SDAs, with each PP amplifying an average of 1.8 SDAs. Approximately 11.5% of PPs from *M. truncatula* BAC clones amplified SDAs, with an average of 1.5 SDAs amplified per PP. Approximately 50% of alfalfa

Table 1 Number of SSR fragments amplified by primer pairs (*PPs*) designed from *M. truncatula* EST and BAC sequences and alfalfa genomic DNA

Origin of PPs	PPs screened (no.)	PPs amplifying SSR fragments (no.)	PPs polymorphic between the two parents (no.)	PPs amplifying SDAs (no.)	Total SDAs amplified (no.)
M. truncatula ESTs	1,182	761	518	285	511
M. truncatula BAC clones	488	233	112	56	82
Alfalfa genomic DNA clones	10	10	5	5	12
Total	1,680	1,004	635	346	605

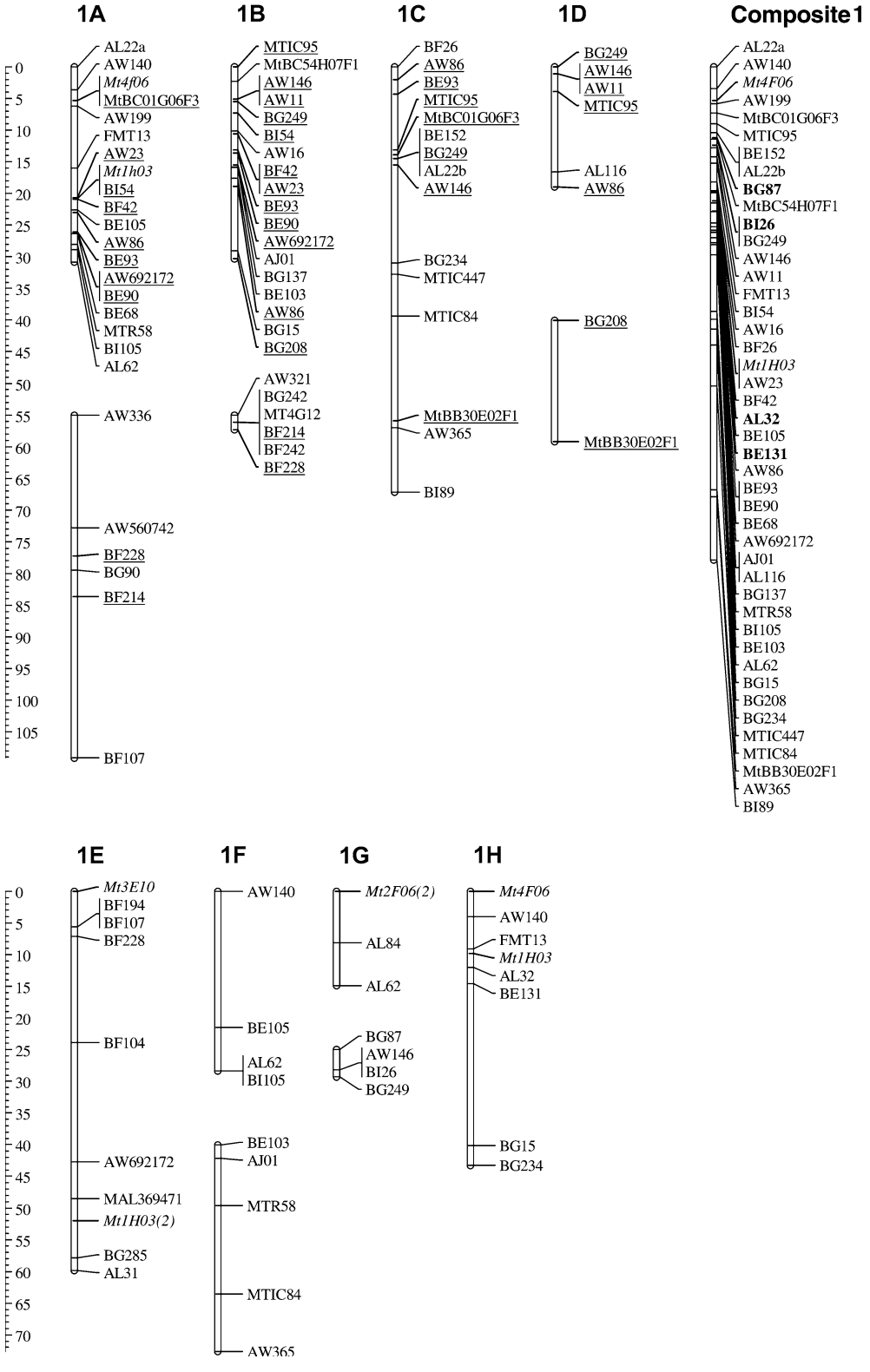

Fig. 1 Simple sequence repeat linkage map of tetraploid alfalfa (*M. sativa* L.) from a cross between *M. sativa* subsp. *falcata* and *M. sativa* subsp. *sativa*. Linkage maps are shown for each of the eight chromosomes of alfalfa. For each chromosome, linkage groups a–d represent the F_1 homologous linkage groups. Markers in linkage groups A and B were inherited from the *M. falcata* parent. Markers in linkage groups C and D were inherited from the *M. sativa* parent. The F_1 linkage groups were combined to form the consensus groups. Linkage groups E–H represent recurrent parent linkage blocks not inherited by the F_1. Markers in linkage groups E and F were inherited from the *M. falcata* parent. Markers in linkage groups G and H were inherited from the *M. sativa* parent. *Asterisk* indicates loci showing segregation distortion. *Underlined marker* loci occur on more than one linkage group, and were the loci used by JoinMap 3.0 to form the consensus linkage groups. Marker loci in *italics* are SSRs amplified from *M. truncatula* BAC clone sequences. Marker loci in *boldface* were markers in the parental linkage blocks not inherited by the F_1, and were manually added to the map based on the genetic distance between flanking markers that were common to the parental linkage blocks and the consensus maps

genomic DNA PPs amplified SDAs, with an average of 2.4 SDAs per PP. From among these PPs, a total of 605 polymorphic SDAs were identified. The number of PP amplifying polymorphic loci was 635, and 346 of these polymorphic loci (55%) amplified one or more SDAs. This is lower than the expected 70%, and may be due to the prescreening of markers. By screening markers on ten BC_1 progeny, and selecting for mapping only those markers that segregated 5:5 or 6:4, we identified a total of 374 PPs amplifying potential

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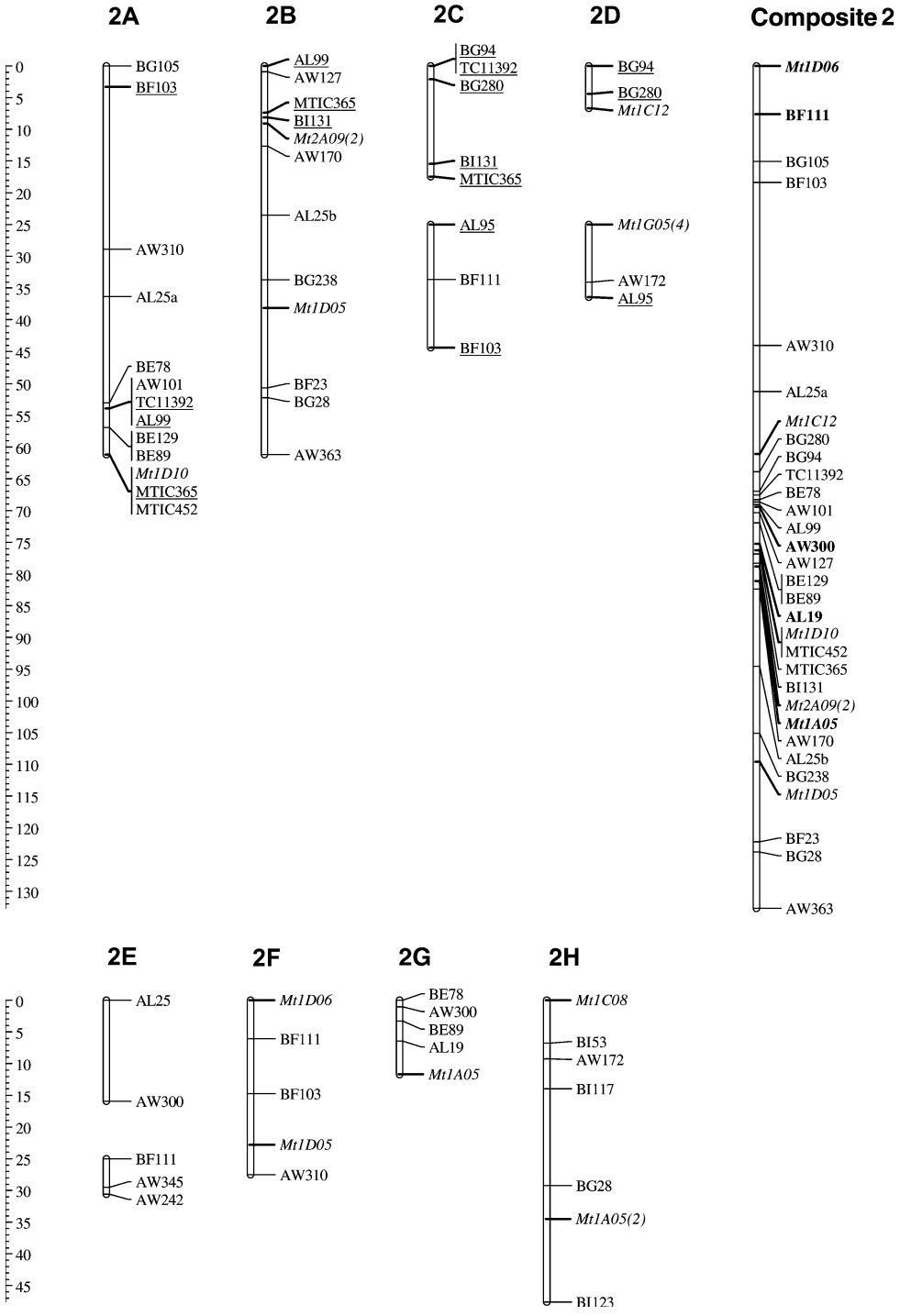

SDAs. Of these, 28 (8%) turned out to amplify double dose markers.

F_1 linkage groups

In the F_1, 351 SDAs from 256 loci were mapped, forming 41 linkage groups at LOD 5 or higher (Fig. 1). Twenty-five SDAs (3.6%) and 13 loci (4.8%) were unlinked. Four PPs detected duplicate loci and are designated by an "a" and "b" appended to the locus name: AL22 on composite group 1, AL 25 on composite group 2, AW249 on composite groups 4 and 8, and AL 47 on composite group 8. In the two backcross populations, 42% of all PPs amplified multiple SDAs. One PP amplified four SDAs, 14 PPs amplified three SDAs, and 67 PPs amplified two SDAs. F_1 linkage groups with at least two homologous loci were joined to form composite linkage groups for seven of eight homologous linkage groups. Linkage group five had only a single

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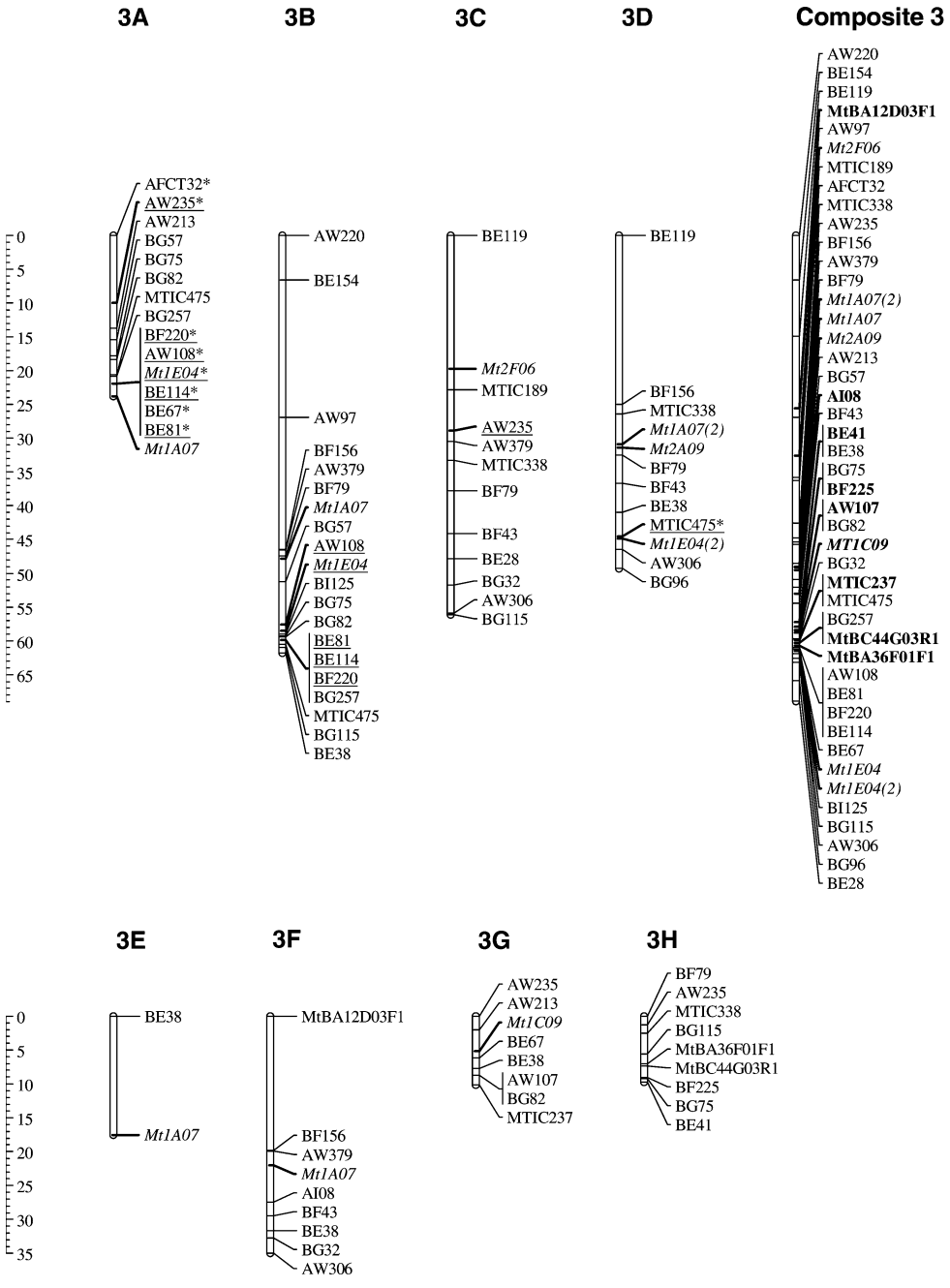

common homologous locus, and was integrated manually. Linkage groups that did not have two markers in common with another group were not incorporated into the composite linkage groups; therefore, the number of markers in the composite maps is less than the total number of markers mapped in the homologous linkage groups (Fig. 1).

Recurrent parent linkage groups

There were 201 recurrent parent alleles from 197 loci that were not inherited by the F_1, but that segregated in the backcross progeny, and were mapped to 43 linkage groups. Twenty-eight SDAs and 20 loci segregating in the recurrent parents were unlinked. These recurrent parent linkage groups are shown because they provide further evidence for aligning F_1 homologues. For example, while F_1 linkage group 1A lower and 1B lower have markers in common, neither have markers in common with the other F_1 homologues in this group. The recurrent parent linkage group 1E, however, has markers common to both the 1A upper and 1A lower linkage groups (e.g. BF107, BF228, and AW692172), providing evidence for placing these homologues in the same group. When the composite group and the recurrent parent linkage blocks had common flanking markers, markers within these re-

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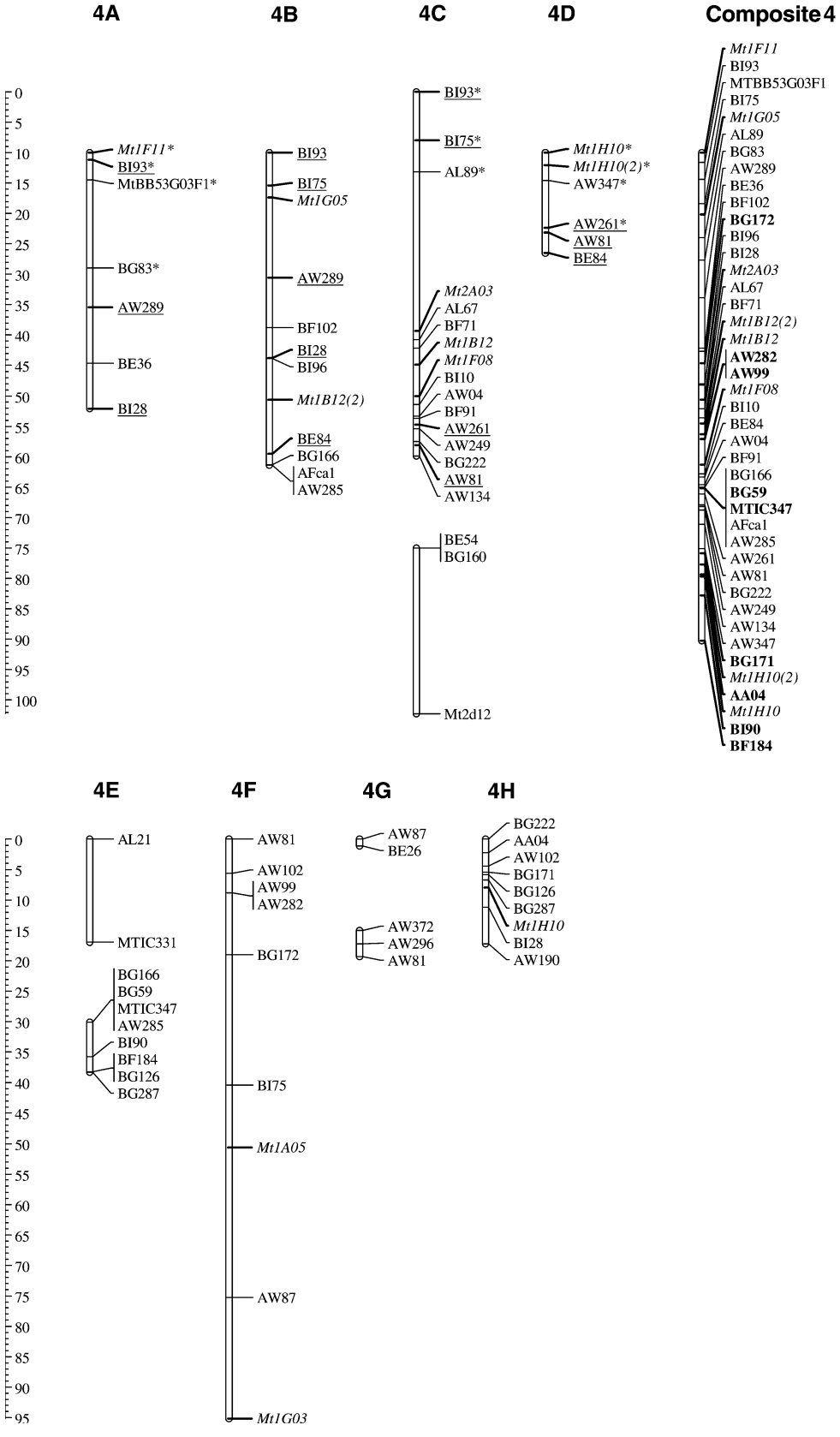

gions were manually incorporated into the composite linkage groups. In this way, 44 loci from the recurrent parent linkage blocks were added to the composite linkage map, and are indicated in bold (Fig. 1). In some instances, markers from F_1 linkage groups that could not be incorporated into the composite maps

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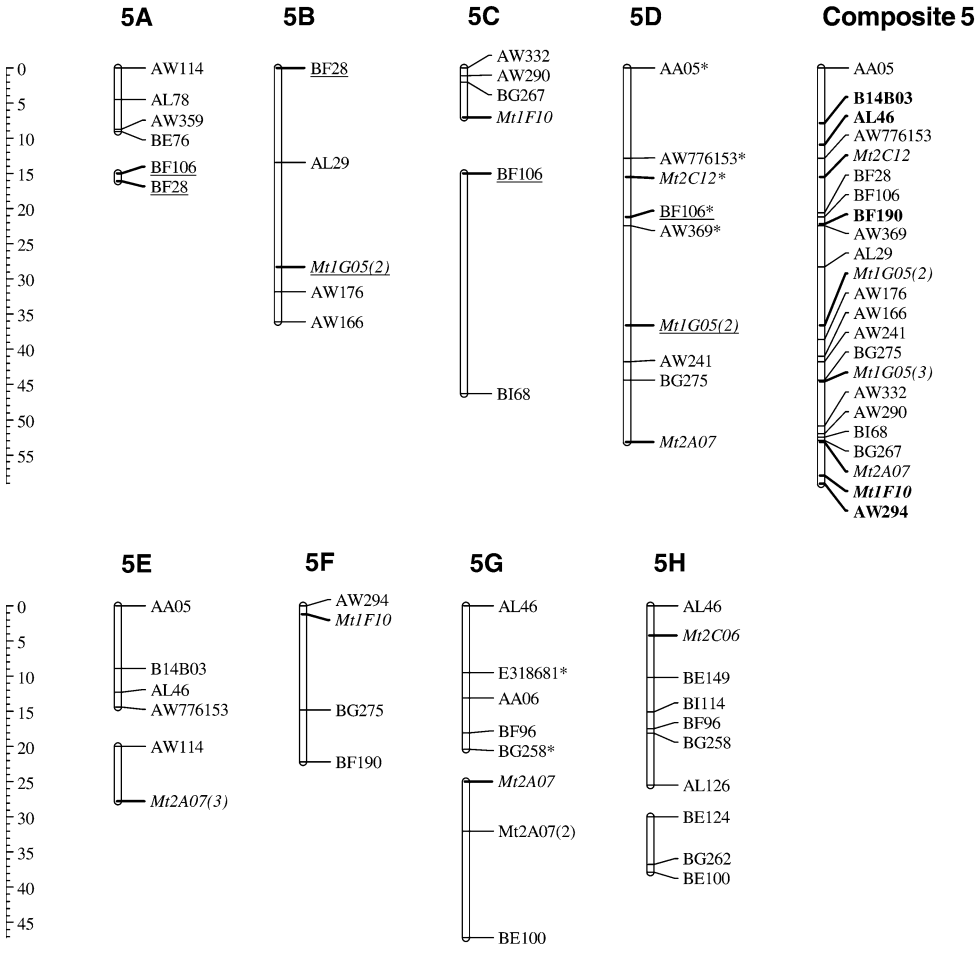

using JoinMap 3.0, due to a lack of two or more common markers, were incorporated based on the recurrent parent linkage group information. Thus, markers in bold may appear in both F_1 and recurrent parent linkage groups, but were incorporated into the composite groups based on flanking markers in the recurrent parent linkage groups.

Composite linkage groups

The composite map consists of 286 SDAs: 243 *Medicago truncatula* EST–SSRs including 21 EST–SSRs from Julier et al. (2003), and 23 SSRs from Dr. Nevin Young (personal communication); 38 BAC–SSRs from 29 *M. truncatula* BAC clones; and five genomic SSRs (Fig. 1). The markers mapped by Julier et al. (2003), and the BAC–SSRs were used to number the composite linkage groups such that they correspond to *M. truncatula* linkage groups. The composite map length is 624 cM, with the average marker density per group ranging from 1.5 to 4.4 cM, and an overall average density of 2.2 cM. Diploid maps of alfalfa range from 234 cM (Tavoletti et al. 1996) to 754 cM (Kalo et al. 2000). The composite tetraploid maps range from 452 cM (Brouwer and Osborn 1999) to 709 cM (Julier et al. 2003). A comparison of the recombination distance of the linkage groups and marker density in the current study to the tetraploid linkage groups of Brouwer and Osborn, (1999) and Julier et al. (2003) is given in Table 2. In the current map, the marker density of composite groups 1, 3, 4, 7, and 8 is <2 cM. Composite groups 2, 5, and 6 have marker densities >2.0. While 10 cM intervals are considered sufficient for detecting QTL (Piepho 2000), the number of SDAs required to coalesce linkage groups into composite linkage groups resulted in a higher density. This map, therefore, will be adequate for detecting QTL for traits segregating in these backcross populations.

Other alfalfa maps

There are five previously published diploid maps of alfalfa (Brummer et al. 1993; Kiss et al. 1993; Echt et al. 1994; Tavoletti et al. 1996; Kalo et al. 2000), and two previously published tetraploid maps (Brouwer and Osborn 1999; Julier et al. 2003). The map of Brummer et al. (1993) was based on 86 F_2 individuals from a cross between a cultivated alfalfa at the diploid level (CADL) (Bingham and McCoy 1979) parent and a diploid *M. sativa* subsp. *coerulea* plant introduction. It consisted of

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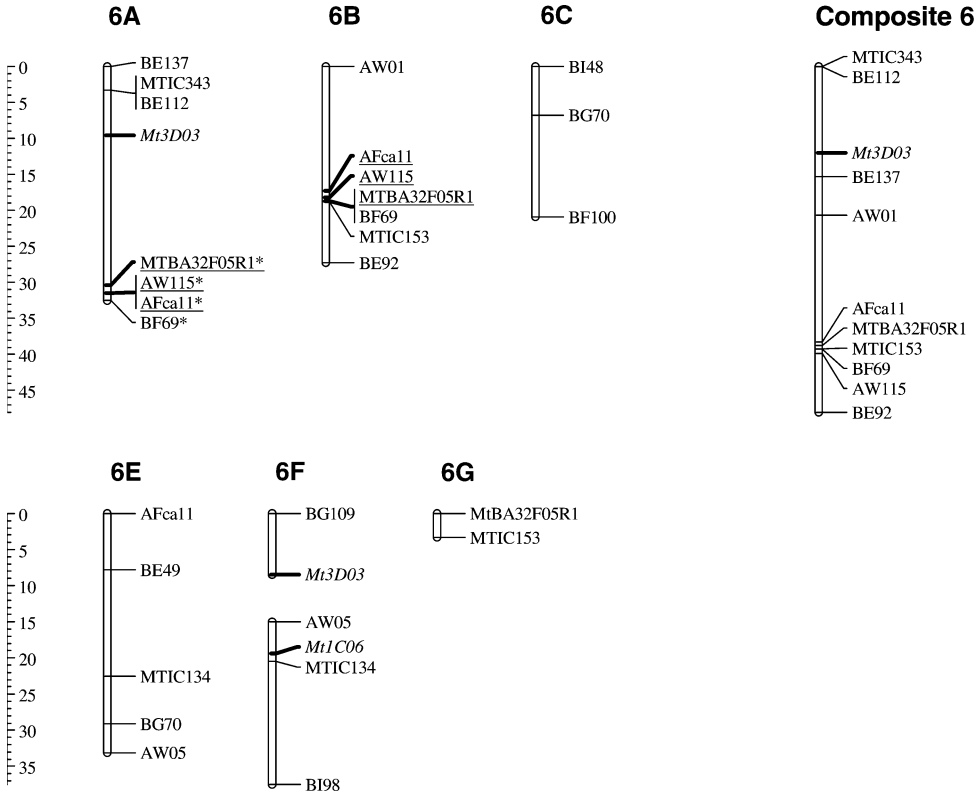

108 RFLP markers mapped to ten linkage groups, and spans 465.7 cM. Segregation distortion in this population was 50%. A revised map of Kiss et al. (1993) was published by Kalo et al. (2000) who mapped additional markers in the same F_2 population of 138 genotypes, that originated from a cross between diploid *M. sativa* subsp. *quasifalcata* and *M sativa* subsp. *coerulea*. The final map consisted of 868 markers (608 RAPDs, 216 RFLPs, 26 seed protein, 12 isozyme, 4 morphological, and 2 specific PCR markers) mapped to eight linkage groups, and covers 754 cM. This map exhibited up to 63% segregation distortion. Echt et al. (1994) used 87 BC_1 individuals from a cross between two heterozygous CADL parents. A map was constructed for the F_1 parent and one recurrent parent, and the two maps were connected by 16 common markers. The interconnected maps consisted of 130 loci, both RFLPs and RAPDs, mapped to 8 linkage groups. The F_1 parent linkage map was 603 cM, while the recurrent parent map was 553 cM. Segregation distortion was 34%. Tavoletti et al. produced the only diploid F_1 map, which consisted of 55 F_1 individuals from a cross between a mutant producing a high frequency of 2*n* eggs, and a CADL parent. A pseudo test cross strategy (Grattapaglia et al. 1995) was employed, and a map of each parent was produced. The CADL map was constructed from 55 RFLP markers, and was 261 cM long. The 2*n* egg parent map was constructed from 50 RFLP markers, and was 234 cM long. Segregation distortion was 8.8%. In general, each of the mapping populations derived by selfing or backcrossing demonstrated high levels of segregation distortion, with distorted markers clustered on specific linkage groups.

Brouwer and Osborn (1999) produced the first tetraploid alfalfa map. They used two BC_1 populations, each of which consisted of 101 individuals each. They used a single dose marker approach (Wu et al. 1992), and mapped 88 RFLP loci on seven composite linkage groups, with a recombination length of 443 cM. They produced four homologue maps for each linkage group, and joined them into consensus groups using Map+ (Collins et al., 1996). Segregation distortion in this population was 5.5%. Julier et al. (2003) produced tetraploid maps for a single F_1 population of 168 genotypes using 589 AFLPs and 109 SSRs. Homologue maps were constructed for each parent, but were not joined into composite maps. Based on their AFLP data one parental map was 2,649 cM, and the other was 3,045 cM. Of the 589 mapped AFLPs, 389 were simplex, or single dose alleles. The remainder consisted primarily of duplex and double simplex markers. Segregation distortion of AFLP markers was 35%, and was distributed randomly across the genome. They also constructed a separate map using 107 SSRs, with a recombination length of 709 cM. Segregation distortion of SSR markers was 25%, again with distorted markers distributed randomly across the genome. A combination of TetraploidMap (Hackett and Luo 2003) and Joinmap (Van Ooijen 2001) was used to construct these maps. Our current map is most similar to the map produced by Bouwer and Osborn (1999). In both studies, two backcross populations were used, and a map representing the

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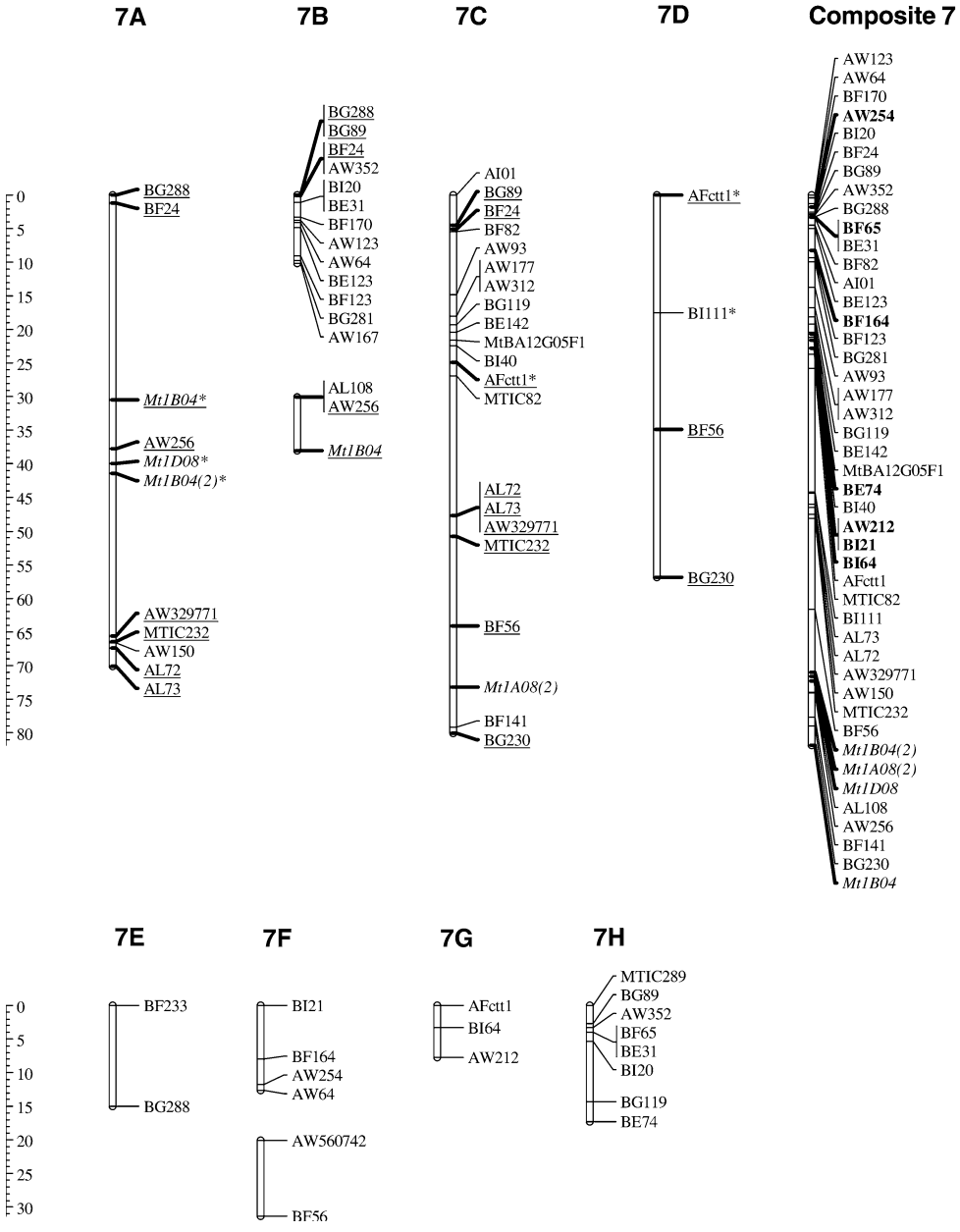

F_1 was developed. The current map, however, uses SSR markers rather than RFLP markers, and is more highly saturated, with 246 loci versus 88 loci for Brouwer and Osborn (1999).

Segregation distortion

In the current study, 34 of 351 F_1 SDAs (10%) exhibited segregation distortion (Fig. 1). This is similar to the 5.5% segregation distortion observed by Brouwer and Osborn (1999) in two tetraploid alfalfa backcross populations, and the 9% segregation distortion observed by Tavoletti et al. (1996) in a diploid F_1 population. Julier et al. (2003) hypothesized that single-dose mapping may underestimate the detection of segregation distortion, since only alleles that segregate 1:1 are selected for mapping. In our study, the criteria for considering an allele to be a SDA (having a segregation ratio of less than 2.24:1, presence to absence), allowed for the inclusion of SDAs that fell outside of the 1:1 ratio, thereby including some alleles with segregation distortion. We acknowledge that this method could underestimate segregation distortion when the direction of distortion favors presence, rather than absence of a fragment. Such a scenario could have occurred when a SDA, due to segregation distortion, had a segregation ratio greater than 2.24:1, and was falsely declared to be a double dose allele. In our study, 21 of 34 distorted loci exhibited deviation toward absence of a fragment, whereas only 13 of 34 distorted loci exhibited deviation toward presence of a fragment. Thus, if the number of

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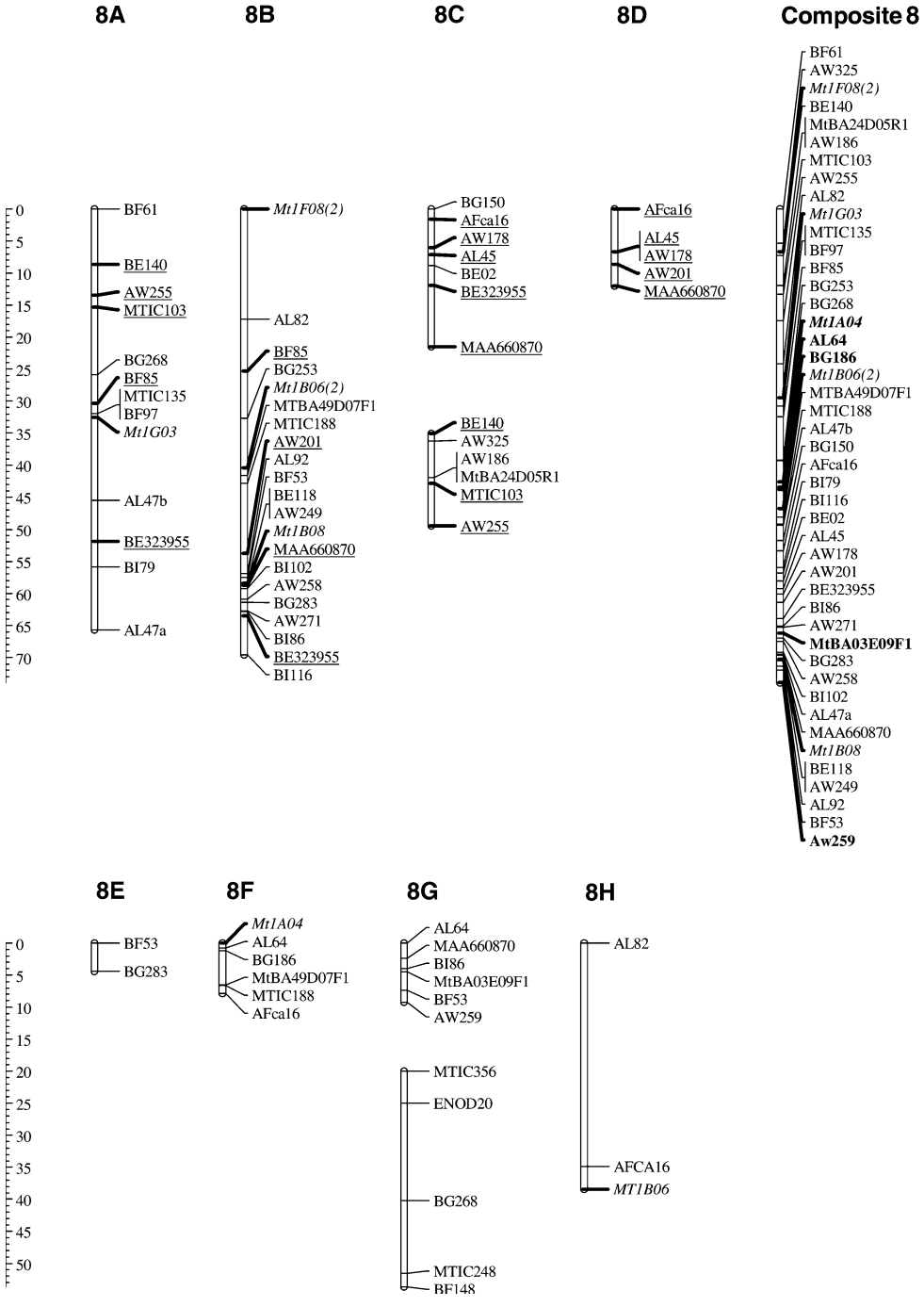

loci with deviation in either direction were equal, segregation distortion could have been as high as 42 out of 351 SDAs (i.e. 12%) in this population.

In the current study, distorted loci clustered on individual homologues of linkage groups 3, 4, 5, 6 and 7. For example, linkage group three had eight distorted loci on one homologue inherited from the *M. falcata* parent, and one distorted locus on a homologue inherited from the *M. sativa* parent. An SSR locus with a distorted allele will frequently show normal segregation for the homologous allele(s). For example, six distorted loci on linkage group three have alleles on other homologues, none of which show distorted segregation. It is also interesting to note that deviation toward presence or absence of a fragment depended on which parent donated the SDA. SDAs with segregation distortion from the *M. falcata* parent exhibited deviation toward absence of the fragment, and were clustered on linkage groups 3A, 4A, 6A, and 7A. SDAs with segregation distortion from the *M. sativa* parent exhibited deviation towards presence of the fragment, and occurred on linkage groups 4C, 4D, and 5D. There were two exceptions, in which a SDA derived from the *M. sativa* parent exhibited deviation toward absence of the

Table 2 Comparison of genetic distance and average marker density of the eight major linkage groups of alfalfa from three tetraploid alfalfa linkage maps

Genetic distance, cM/ marker density, cM			
Linkage group	Current study	Brouwer and Osborn (1999)	Julier et al. (2003)
1	78/1.8	69/8.6	96/6
2	133/3.8	82/6.9	102/4.9
3	70/1.5	64/3.8	89/5.9
4	80/1.9	15/3.1	58/6.4
5	60/2.6	83/5.5	105/11.7
6	48/4.4	43/8.7	117/13
7	82/1.9	–	55/4.6
8	73/1.6	96/8.7	87/7.9
Total	624/2.4	452/6.5	709/7.6

fragment, and these were restricted to single loci associated with alleles of genes located on linkage groups 3D and 5G. The ability to observe the level of marker saturation and nature of segregation distortion on homologues is an advantage of the single-dose mapping method, in which individual homologues are mapped prior to formation of composite linkage groups.

In diploid maps of alfalfa (Brummer et al. 1993; Kiss et al. 1993; Echt et al. 1994; and Kalo et al. 2000), 34 to 63% of mapped loci exhibited segregation distortion. Distorted loci on these maps, as well as on a *M. truncatula* map (Thouquet et al. 2002), were clustered, probably indicating biological significance. The explanation most often given for segregation distortion is the exposure of deleterious alleles upon inbreeding, which negatively affect fitness. In diploids, individuals with homozygous deleterious alleles do not survive, and thus segregation ratios favor heterozygous loci. Under this hypothesis, segregation distortion should be lower in a diploid F_1 population than in an F_2 diploid population, since F_1 individuals from crosses between divergent parents are expected to be heterozygous at the majority of the loci. Heterozygote frequency is even higher in an autotetraploid, in which homozygous loci occur at an extremely low frequency; therefore, segregation distortion in an autotetraploid would be expected to be lower than in a diploid. In our study, distorted alleles were observed to cluster on individual homologues. Thus, segregation distortion may be due to deleterious effects associated with alleles of loci located in the same genomic region as the distorted marker alleles. This hypothesis is supported by the observation that the direction in which distorted alleles exhibited deviation (i.e. presence or absence) depended upon their parental origin. Distorted loci from the *M. falcata* parent exhibited deviation toward absence of a SDA, indicating that the SDA could have been linked in coupling to one or more deleterious loci, resulting in reduced fitness. Death of these individuals could result in fewer individuals carrying the SDA, and deviation towards absence of the fragment. Distorted loci from the *M. sativa* parent most often exhibited distortion toward presence of the SDA suggesting that they may be linked in coupling with favorable alleles. These *M. sativa* SDAs could also be linked in repulsion to one or more deleterious alleles. Reduced fitness could then result in elimination of individuals that did not possess the *M. sativa* SDA, resulting in deviation toward presence of the SDA. In our populations, such repulsion phase linkages involving deleterious alleles are twice as likely to have originated from the *M. falcata* parent than from the *M. sativa* parent. The behavior of the distorted alleles seems to agree with the breeding history of *M. sativa* and *M. falcata*. Although *M. falcata* possesses a number of desirable characteristics including winter-hardiness and high water-use efficiency, it is also slow growing, low in seed production, and susceptible to numerous pests and pathogens (Barnes et al. 1977; Ray et al. 2004). The *M. sativa* 'Chilean' germplasm has been characterized as possessing good general combining ability (Busbice and Rawlings 1974; Segovia-Lerma et al. 2004), and has contributed significantly to alfalfa cultivars development in the Southwest and Great Plains regions of the United States.

Conclusions

A total of 605 SDAs were identified in this study and used to develop a genetic map that represents the highest density tetraploid alfalfa map yet produced (Table 3). The PPs described in this study constitute a large group of easily shared, PCR-based markers that are useful for genetic mapping in alfalfa and other legumes, and could also be used to unify and cross-refer the existing diploid and tetraploid alfalfa maps. Alignment of the markers used in our study, with the emerging *M. truncatula* genome sequence, is also in progress to confirm the placement and order of markers on this alfalfa genetic map. Results of that work will be reported elsewhere. Efforts are now underway to use this map, and the segregating progeny from which it was derived, to detect QTL for drought tolerance in tetraploid alfalfa.

Acknowledgements We thank Drs. Malay Saha and Mark Sorrells for critical reading of the manuscript. This research was supported by The Samuel Roberts Noble Foundation, Inc.

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