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A framework linkage map of bermudagrass (*Cynodon dactylon* × *transvaalensis*) based on single-dose restriction fragments

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Abstract This study describes the first detailed linkage maps of two bermudagrass species, *Cynodon dactylon* (T89) and *Cynodon transvaalensis* (T574), based on single-dose restriction fragments (SDRFs). The mapping population consisted of 113 F1 progeny of a cross between the two parents. Loci were generated using 179 bermudagrass genomic clones and 50 heterologous cDNAs from *Pennisetum* and rice. The map of T89 is based on 155 SDRFs and 17 double-dose restriction fragments on 35 linkage groups, with an average marker spacing of 15.3 cM. The map of T574 is based on 77 SDRF loci on 18 linkage groups with an average marker spacing of 16.5 cM. About 16 T89 linkage groups were arranged into four complete and eight into four incomplete homologous sets, while 15 T574 linkage groups were arranged into seven complete homologous sets, all on the basis of multi-locus probes and repulsion linkages. Eleven T89 and three T574 linkage groups remain unassigned. In each parent consensus maps were built based on alignments of homologous linkage groups. Four ancestral chromosomes were inferred after aligning T89 and T574 parental consensus maps using multi-locus probes. The inferred ancestral marker orders were used in comparisons to a detailed *Sorghum* linkage map using 40 common probes, and to the rice genome sequence using 98 significant BLAST hits, to find regions of colinearity. Using these maps we have estimated the recombinational length of the T89 and T574 genomes at 3,012 and 1,569 cM, respectively, which are 61 and 62% covered by our maps.

Keywords *Cynodon* · Bermudagrass · Autopolyploid · Single-dose restriction fragments · Genome · Linkage

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Introduction

Bermudagrass (*Cynodon* sp.) is a resilient, perennial grass native to the warmer temperate and tropical regions of the world that is found throughout the southern United States. Its many uses are directly related to the favorable traits it possesses. For instance, it is used for forage because of its ability to maintain active growth through the warm summer months when most other forage grasses temporarily decline. Also, it is widely used in landscaping because of its ability to grow well in a wide range of soil conditions (Casler and Duncan 2003) and its high growth rate and extreme invasiveness. Seeded bermudagrass can spread to provide full coverage of 1,000 ft^2 within 4–6 weeks after planting (CTAHR 1998). Bermudagrass is a popular grass used in the golf and turfgrass industries, owing to its ability to generate a variety of textures, rapid recovery, and its low growing nature, which allows it to tolerate very close mowing.

Cynodon species are members of the *Cynodonteae* tribe and the *Chloridoideae* sub-family, within the grass (*Poaceae*) family (Clayton and Renvoize 1986). While the grasses are among the better studied plant families at the level of comparative mapping (Feuillet and Keller 2002), the chloridoids are underexplored. Based on its location in the phylogeny of the grasses (Kellogg 1998), a bermudagrass map is a beneficial new tool for inclusion in grass genome comparisons, improving knowledge of genome structural diversity within the family.

Bermudagrass species have an average 1X nuclear DNA content of 0.547 pg (derived from Taliaferro and Lamle 1997) equating to a 1X genome size of ~540 Mbp. They have a base chromosome number of 9 (Advulow 1931; Burton 1947; Darlington and Wylie 1956), and are mostly tetraploid (Bogdon 1977), but can range from diploid to hexaploid (Forbes and Burton 1963; Malik and Tripathi 1968). Triploid plants are the natural or synthetic products of intra- and interspecific hybridizations of diploid and tetraploid species (Burton 1951; Harlan and de Wet 1969). Most leading turf genotypes of today are sterile triploids, resulting from artificial crosses between tetraploid *Cynodon dactylon* and diploid *Cynodon transvaalensis*.

In comparison to diploids, genetic mapping of polyploids has advanced at a much slower pace due to major obstacles, including more possible genotypes as a result of multiple genomes, the comigration of similar size fragments during electrophoresis, and the complexity of unclear genome constitution (auto vs. allo) for many species hinder the progress of mapping in polyploids. Single-dose restriction fragment (SDRF) mapping addresses these difficulties (Wu et al. 1992). The strategy evaluates the presence or absence of a restriction fragment that is only present in one parent, and in the progeny at a 1:1 ratio. In this way, the genetics is similar to that of diploids and mapping can proceed efficiently. Also, SDRF mapping permits the detection of both coupling and repulsion phase linkages, which can lead to the clarification of the genome constitution of many species. Finally, the multiple segregating loci that may be produced from a single probe serve as a means to group homologous chromosomes. This approach has been successfully applied to genetic mapping of sugarcane (Da Silva et al. 1993) and strawberry (*Fragaria* sp.; Lerceteau-Kohler et al. 2003).

Here we present a linkage map of the bermudagrass genome (*Cynodon* sp.) constructed using the SDRF technique. Mapping of triploid progeny from an artificial cross between tetraploid *C. dactylon* and diploid *C. transvaalensis* made the map representative of the genome organization of leading turfgrass genotypes. The use of low-copy DNA markers and high polymorphism rate was expected to lead to the mapping of multiple loci for many markers, permitting identification of homologous chromosomes within each parent and corresponding chromosomes between the parents. Based on this information, we sought to infer consensus gene (marker) orders across much of the *Cynodon* genome and begin exploration of conserved synteny with model cereal genomes.

Materials and methods

Mapping population

The mapping population consisted of 113 progeny of a synthetic cross of T89 (*C. dactylon*, 4*x*) and T574 (*C. transvaalensis*, 2*x*). Similar crosses between other members of these two species have led to the production of numerous leading bermudagrass cultivars, including Tifway. Triploid ($2n = 3x = 27$) F1 progeny were used for mapping, based on heterozygosity within each parent. The cross was made by Wayne Hanna, and the progeny are maintained in the field, in Tifton, GA.

RFLP probes

Four sets of probes, either bermudagrass-derived or heterologous, were used in this mapping experiment. Probes labeled T574 were hypo-methylated (*Pst*-I digested) genomic clones made from *C. transvaalensis* parental DNA following the protocol detailed by Chittenden et al. (1994), except that fragments were isolated from a 0.8% agarose gel using the Geneclean Kit (Bio-101). PCD probes were hypo-methylated (*Pst*-I digested) genomic clones from *C. dactylon* cultivar 'Arizona Common'. pPAP probes were cDNA clones isolated from apomictic pistils of *Pennisetum ciliare* for use in a previous map (Jessup et al. 2003), and RZ probes were cDNA clones from *Oryza sativa* cultivar IR36 (Causse et al. 1994).

Laboratory procedures

The molecular methods used are as previously described by Chittenden et al. (1994), with minor adjustments. Young leaf tissue was harvested from both parents and each of 113 progeny were lyophilized, ground in a tissue mill, and stored at −80°C until further use. DNA was extracted from each, following the protocol for CTAB extraction outlined by Chittenden et al. (1994). Approximately 5 µg of DNA from each sample was digested with either *Eco*R1 or *Hin*dIII, and run overnight at 22 V in a 1% agarose gel in 1× TAE buffer. Gels were then blotted onto Hybond N+ (Amersham), using 0.4 N NaOH, overnight, after which blots were rinsed in 2× SSC and stored at 4°C until use. About 20–50 ng of each probe was labeled with [^{32}P]dCTP and hybridized to a blot. Following this the blots were washed of excess probe, exposed to X-ray film, and the films were developed. The same conditions were used for both homologous and heterologous probes.

Identification of SDRFs and linkage analysis

The presence or absence of each polymorphic fragment was visually determined and recorded for each individual in the mapping population. SDRF mapping necessitates the building of separate maps for each of the parental genomes. Therefore, the parental origin of each polymorphic fragment was ascertained based on comparison to parental DNA. Next, a 1:1 ratio of presence or absence in the progeny was verified for each polymorphic fragment by a Chi-square test with 99% con-

fidence. Coupling linkage was analyzed separately for loci from each parent using MAPMAKER/EXP v. 3.0b, with error detection (Lincoln and Lander 1992). Map distances, in centiMorgans, were calculated using the Kosambi function. Initial linkage groups were constructed using the 'group' command with a LOD score of 4.0 and a recombination fraction of 0.4 as the linkage thresholds. Previously unlinked markers were individually added to linkage groups at the most likely interval using the 'try' command with a threshold of LOD≥2.0. Local maximum likelihood orders of markers were confirmed using the 'ripple' command.

To find repulsion linkages, a new data set was generated for each parent in which allelic designations were reversed . Then, each 'reversed' locus was considered individually for non-random association with correctly scored loci (Al-Janabi et al. 1994; Grivet et al. 1996; Ming et al. 1998).

Double dose restriction fragment mapping in T89

Forty-four RFLP loci were identified as double dose restriction fragment (DDRFs) by a Chi-square test to confirm a 5:1 segregation ratio in the progeny, as described by Ripol et al. (1999). In order to integrate DDRF markers with the SDRF framework map, a maximum likelihood estimation (MLE) approach was implemented in the R statistical programming language (R Core Team 2005) using the Bhat package (Luebeck 2003). The MLE used the Davidon–Fletch–Powell optimization algorithm to minimize the calculated value of the negative log-likelihood of Ripol et al.'s (1999) likelihood

$$L(p(r)|x) \propto (p_{\mathrm{AB}}(r))^{X_{\mathrm{AB}}}(p_{\mathrm{A}}(r))^{X_{\mathrm{A}}}(p_{\mathrm{B}}(r))^{X_{\mathrm{B}}}(p_0(r))^{X_0}$$

where r is recombination fraction and X_{AB}, X_{A}, X_{B}, and X_0 are the observed number of phenotypes of the offspring in the four possible phenotypic classes. The four phenotypic probabilities p_{AB}, p_{A}, p_{B}, and p_0 are defined differently for each of the possible genotypic configurations (Ripol et al. 1999). Linked pairs of SDRF:DDRF markers can exist in an asymmetric coupling (ac12) configuration or a repulsion configuration (r12). Since a priori knowledge of genotypic configurations is not possible, all 44 DDRF markers were tested for both asymmetric coupling and repulsion against all SSRF markers.

To determine if the estimated MLE recombination fraction was significantly different from the model of no linkage, the likelihood ratio test statistic

$$\lambda(x) = -2\log\left(\frac{L(p(\hat{r})|x)}{L(p(0.5)|x)}\right)$$

was compared to a Bonferroni corrected critical value of a one-tailed Chi-square test. Pairs of markers were declared linked if $\lambda(x) > \mathrm{X}^2_{[1,\alpha=0.001]}$. All significant linkages that were identified under this methodology were manually integrated with the SDRF framework map.

Estimated recombinational length and percent genome coverage

The approximate length of each parental genome was estimated using the method-of-moment formula (Hulbert et al. 1988), as modified in method 3 of Chakravarti et al. (1991).

$$E(G) = [n(n-1)2d]/2k;$$

where $E(G)$ represents the estimated genome length in cM, n the number of markers on the map, d the largest distance between two loci at a given LOD score, and k the actual number of pairs of loci linked at the specified LOD score or greater.

The percent of genome coverage, $E(C_n)$ was estimated using the method of Bishop et al. (1983).

$$E(C_n) = 1 - P_{1,n};$$

where

$$P_{1,n} = (2r/n+1) * [(1-d/2g)^{n+1} - (1-d/g)^{n+1}] + (1-rd/g)(1-d/g)^n.$$

In this case, r is the number of linkage groups, n the number of markers on the map, d the maximum distance used to detect linkage at the same preset LOD score, and g the estimated genome length in cM.

Homology between linkage groups

Wherever possible, linkage groups were assigned to homologous sets on the basis of 32 shared multi-locus probes and on shared repulsion linkages. Homologous groups were then merged to infer a consensus for each homologous group.

Inferred ancestral chromosomes

Inferred ancestral chromosomes were constructed by comparing the consensus groups and unassigned linkage groups of the two parents, aligning individual linkage groups on the basis of sharing two or more probes that either (a) showed segregation at multiple loci, (b) showed repulsion linkage, or (c) showed both. To assemble consensus maps, the total marker contents of the respective linkage groups were interleaved, based on relative recombinational distances from the common anchor points. The consensus maps were drawn to the same scale as the corresponding linkage groups; however, no recombination distances were presented on the consensus since recombination had not been directly measured between the consecutive points on the consensus map. This method of constructing consensus maps, together with software to perform it, has been described in detail elsewhere (Rong et al. 2005).

Table 1 Summary of probes used in this study

Probes	Source	Screened	Polymorphic	Successful	Mapped SDRF loci	
					T89	T574
T574	*Cynodon transvaalensis*	298	155	143	92	53
pCD	*Cynodon dactylon* (A.C.)	121	51	36	26	10
pPAP	*Pennisetum ciliare*	122	53	15	9	5
RZ	*Oryza sativa*	125	71	35	28	9
Total		666	330	229	155	77

'Successful' refers to probes that generated at least one SDRF locus mapped on a linkage group in either parent

Comparative mapping

Bermudagrass–rice

The sequences of marker generating probes from both maps were BLASTed against the TIGR rice pseudomolecule v. 2.0 (http://www.tigr.org) at a significance threshold $\leq 1 \times 10^{-10}$. Colinearity was inferred by comparing the chromosomal locations of the best rice hits to the orders of the corresponding markers on the inferred *Cynodon* ancestral chromosomes.

Bermudagrass–Sorghum

Eighty-three of the probes used in this study were also applied to the *Sorghum* map of Bowers et al. (2003), and consequently 66 new markers were added to the already

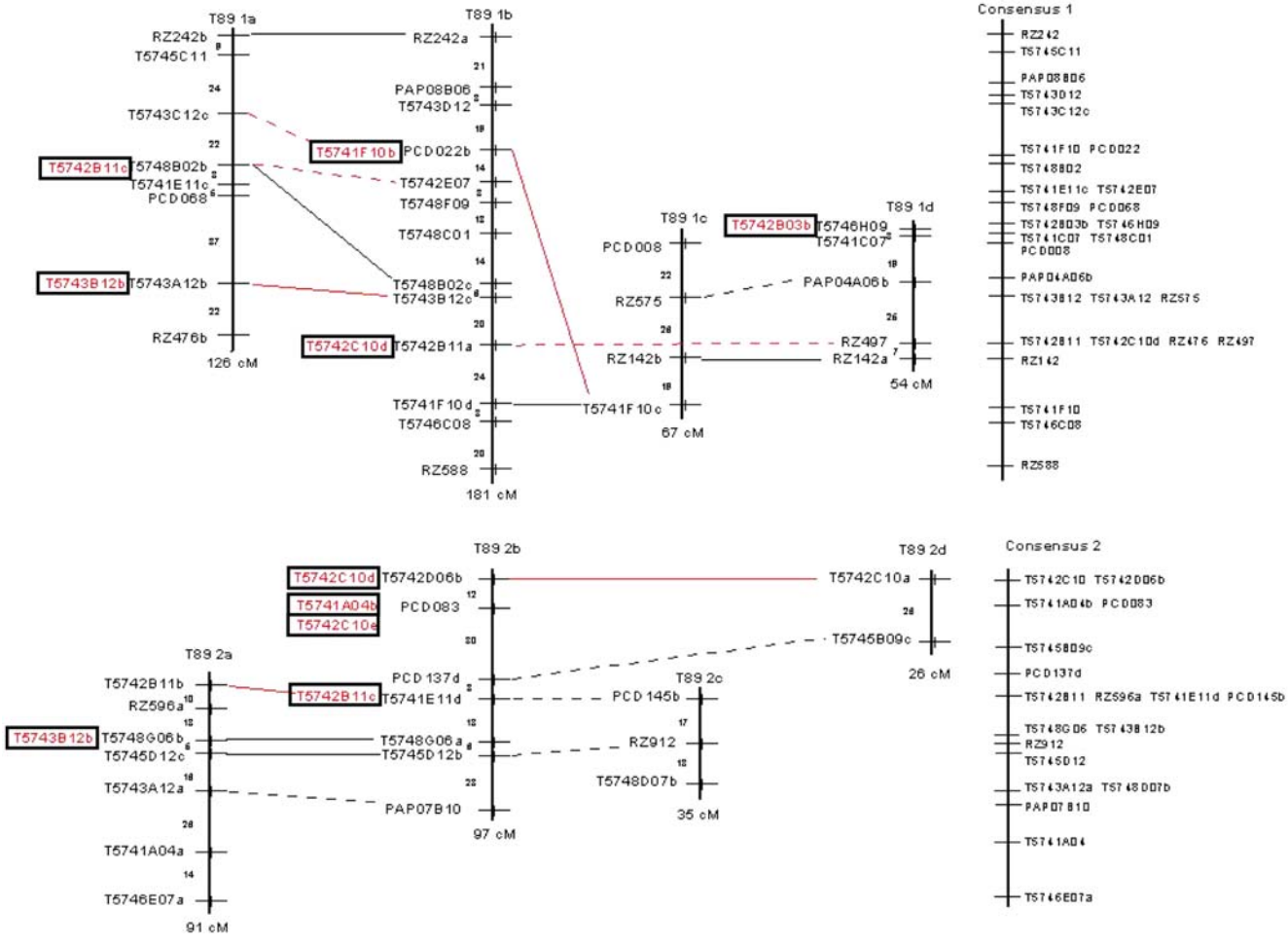
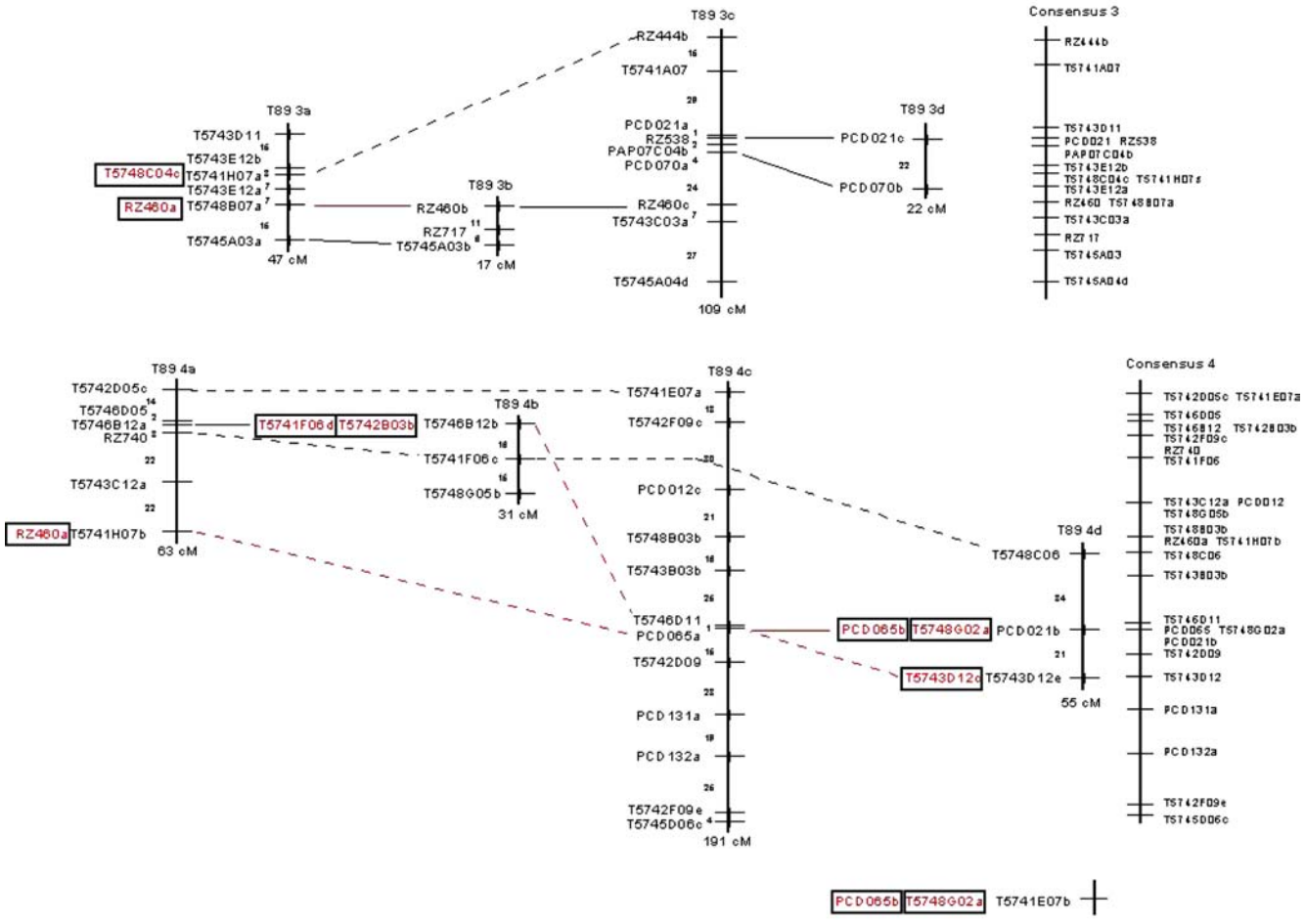


Fig. 1 Linkage maps of *Cynodon dactylon* ($4x = 36$) linkage groups. Map distances are in cM. *Boxed markers* are DDRF loci superimposed on the SDRF framework map. Where possible, homologous groups are aligned on the basis of multi-locus probes (*solid lines*), and repulsion linkages (*dashed lines*) to infer a consensus. In both cases, *red lines* involve DDRF loci. Shown is linkage between DDRFs PCD065b and T5748G02a and unlinked SDRF T5741E07b



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dense genetic map. Twenty-eight of these markers were also mapped in bermudagrass, and were added to the 12 common markers that already existed on the *Sorghum* map. On this basis, the *Sorghum* genetic map was compared to the inferred *Cynodon* ancestral chromosomes to find regions of conserved marker order.

Mode of inheritance

The proportion of pairs of markers linked in repulsion versus coupling phase was calculated for each testable linkage group for both genomes (Wu et al. 1992). Linkage groups for which no repulsion linkages were found were considered non-testable and not included. A Chi-square test with 95% confidence was used to determine if the calculated ratios fit the 1:1 or 0.25:1 models expected for disomic and polysomic inheritance, respectively.

Results

Marker screening and mapping

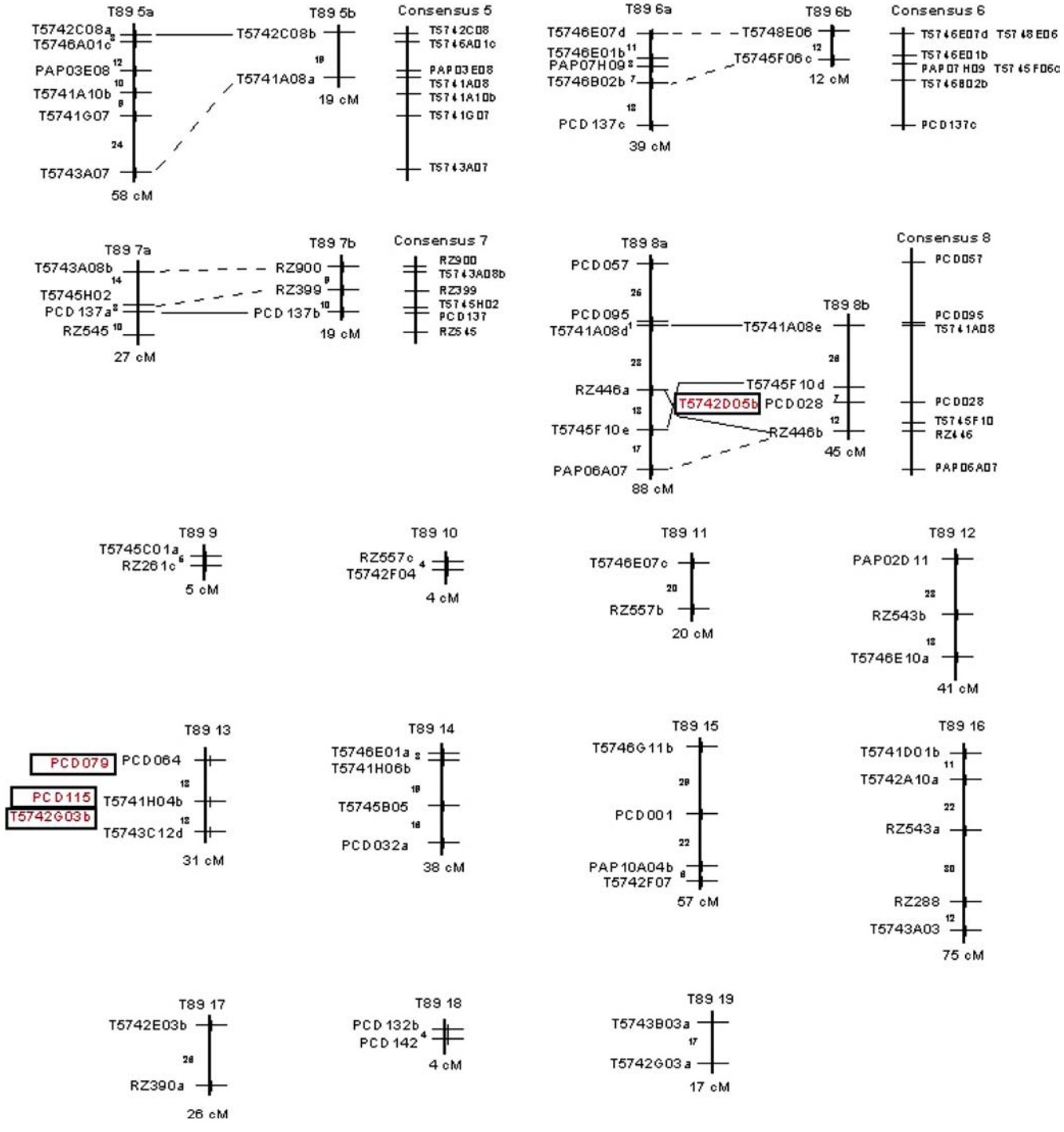
A total of 666 probes were screened, of which 330 detected at least one polymorphic fragment in the parents, and 229 produced at least one SDRF that was mapped to a bermudagrass linkage group. These data are summarized in Table 1. A total of 440 segregating loci were scored, but the Chi-square test confirmed only 299 as SDRFs.

Map construction

T89–C. dactylon (2n = 4x = 36)

Construction of the map for T89 was based on the analysis of 202 SDRF loci. The map is composed of 35 linkage groups, with 16 organized into four complete homologous groups where all four chromosomes are represented, eight into four incomplete homologous groups with only two chromosomes, and 11 unassigned. The map covers a total of 1837.3 cM, with 155 markers separated by an average distance of 15.3 cM (Fig. 1). Forty-seven markers remain unlinked. On average, linkage groups are defined by about four markers and cover ~52.5 cM (Table 2).

By applying the method of Ripol et al. (1999), we analyzed 44 candidate double-dose fragments, and were able to find linkages involving 17 (39%) of them. In



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principle, these detect 34 segregating loci (by definition), however, we could only link 31 of them to SDRF markers. Twenty-five of the 31 were already on our linkage groups, and the remaining six were previously unlinked. Since this approach does not allow for the calculation of map distances, or the identification of specific map locations for the DDRFs, these loci were super-imposed onto the SDRF maps, in alignment with the SDRF to which they showed the tightest linkage (Fig. 1). In addition, these DDRF loci were involved in numerous repulsion linkages that are also shown in Fig. 1.

Table 2 Summary of statistics for the T89 and T574 genetic maps

	Length		No of SDRF loci		cM/pair	
	T89	T574	T89	T574	T89	T574
Total	1837.3	973.4	155	77		
Average	52.5	54	4.4	4.3	15.3	16.5
Largest group	191.2	149	12	7	17.4	24.8
Unlinked			47	20		

Map lengths are in cM

T574–C. transvaalensis (2n = 2x = 18)

The T574 genetic map was constructed on the basis of 97 SDRF loci. It is composed of 18 linkage groups, with 15 of them organized into seven homologous groups. The map covers a total of 973.4 cM with 77 markers separated by an average of 16.5 cM (Fig. 2). Twenty markers remain unlinked. On average, linkage groups are defined by about four markers and cover ~54 cM (Table 2).

Estimated recombinational length and percent genome coverage

For each parent, the LOD scores used for recombinational length and genome coverage estimates were consistent with the LOD score threshold used for map construction. The total recombinational length of the *C. dactylon* genome was estimated to be 3,012 cM, which is ~61% covered by the map of T89. Meanwhile, the *C. transvaalensis* genome was estimated to be 1569 cM,

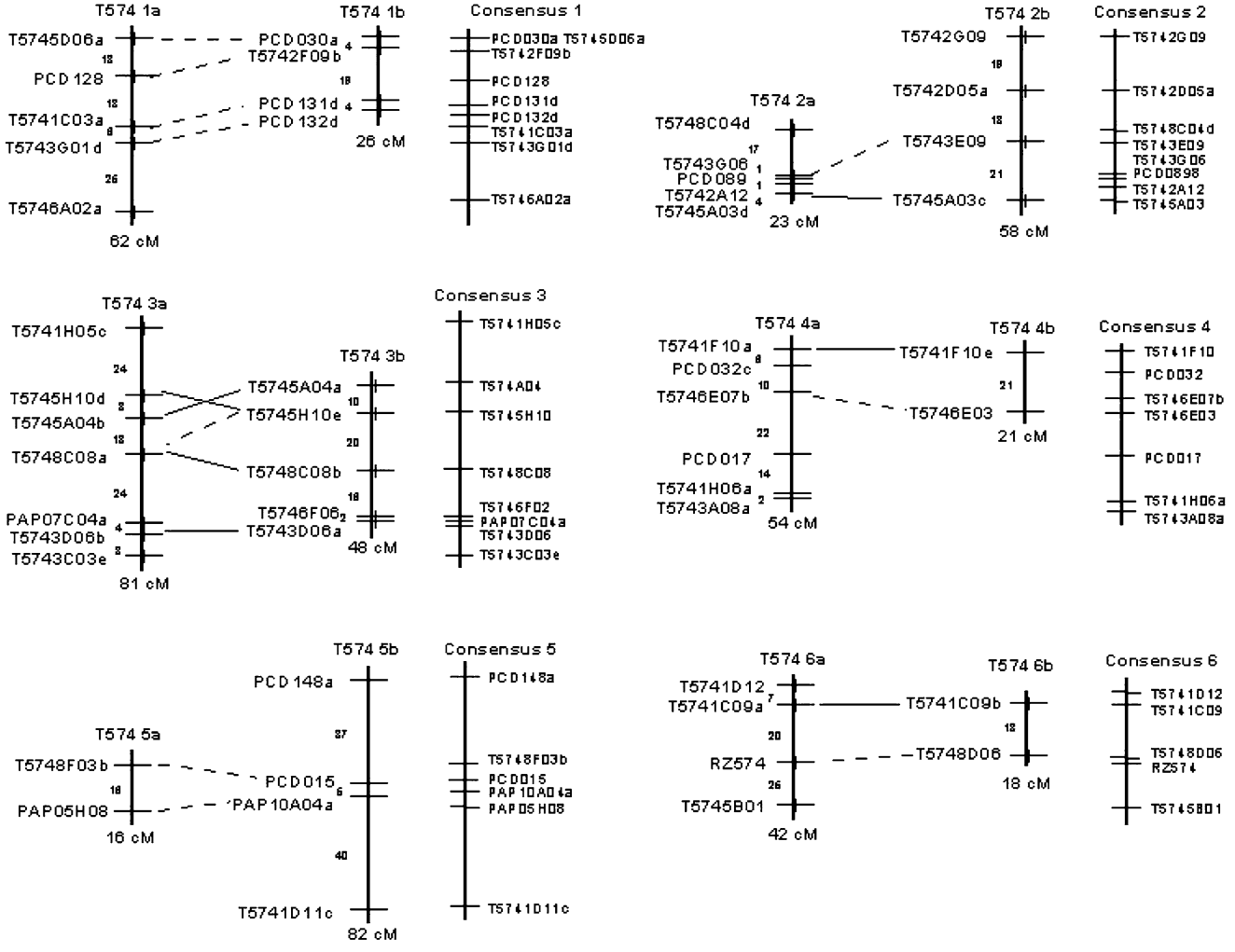
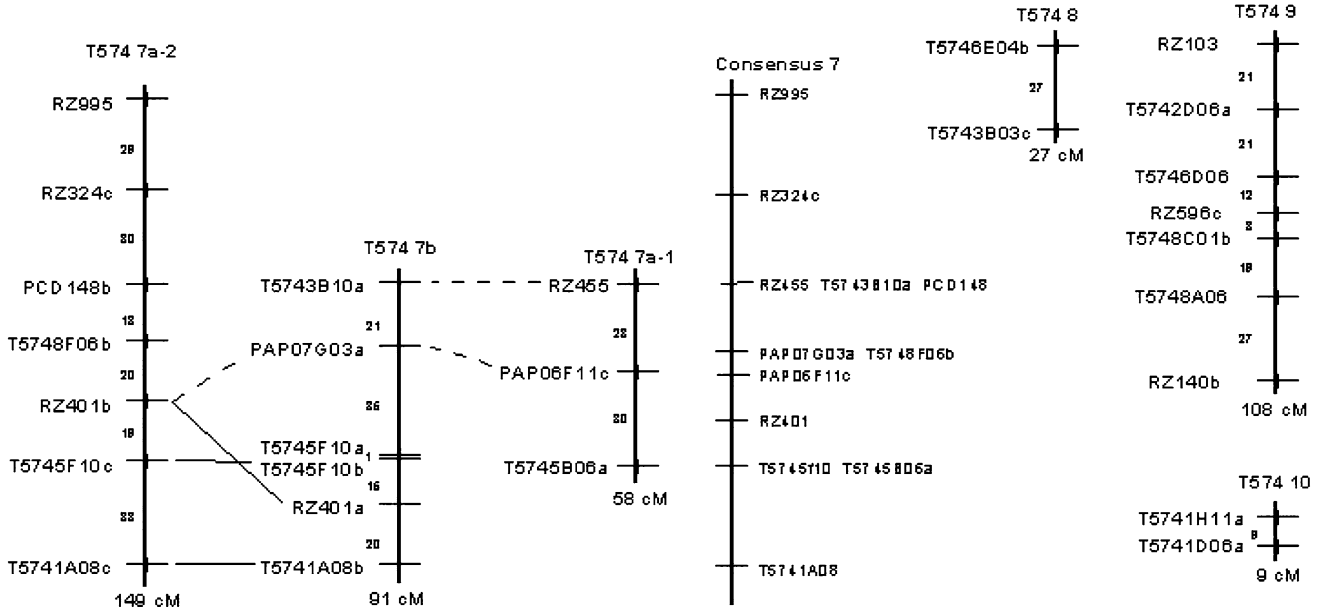


Fig. 2 Linkage maps of *Cynodon transvaalensis* (2x = 18) linkage groups. Map distances are in cM. Where possible, homologous groups are aligned on the basis of multi-locus probes (*solid lines*), and repulsion linkages (*dashed lines*) to infer a consensus



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which is ~62% covered by the map of T574. The estimated recombinational length of the T89 genome is about twice that of T574, which is consistent with their ploidy levels.

Inferred ancestral chromosomes

After constructing consensus groups of homology sets in each parent, in three cases T89 consensus groups were aligned with T574 consensus groups, and in one case a T89 consensus group was aligned with the unassigned T574 linkage group 9, on the basis of shared multi-locus probes. Markers on each pair of groups were interleaved to construct four inferred *Cynodon* ancestral chromosomes (Fig. 3).

Comparative mapping

Bermudagrass–rice

The sequences of the 71 probes that generated markers on the T89 map, and 36 from the T574 map produced 98 significant hits when BLASTed against the rice genome sequence data. With these hits, inferred *Cynodon* ancestral chromosomes and homology consensus groups were compared to the rice genome. In three separate comparisons, inferred *Cynodon* ancestral chromosomes shared at least three significant BLAST hits with regions of rice chromosomes 1, 2, and 9 (Fig. 3). In another comparison, an inferred ancestral chromosome from an alignment of T89 consensus group 4 and T574 consensus 1, shared three significant hits each to regions of rice chromosomes 1 and 7 (Fig. 3). Further, using the numbered base pair location of the 50 kb block of rice sequence that gave each hit, it was possible to determine that in four of five comparisons at least three of the significant hits between bermudagrass and rice showed conserved order in the two genomes (Fig. 3).

Bermudagrass–Sorghum

The bermudagrass-*Sorghum* comparison relied on 40 common probes. An inferred *Cynodon* ancestral chromosome from the alignment of T89 consensus group 3 and T574 consensus group 3 showed significant colinearity with *Sorghum* linkage group A (Fig. 3; Bowers et al. 2003). Also, in separate comparisons, the three other inferred ancestral chromosomes shared two common probes each with *Sorghum* linkage groups A, B, and D (Supplementary Table 1). The *Sorghum* comparisons relied on substantially less data points than the bermudagrass–rice, and only produced one alignment of a bermudagrass and a *Sorghum* linkage group with at least three conserved loci, yet this finding is noteworthy because it demonstrates the potential for comparisons using a more saturated bermudagrass map to detect more regions of colinearity between the two genomes.

Mode of inheritance

Coupling-phase linkages were much more frequent than repulsion-phase linkages. In the tetraploid T89 map, we

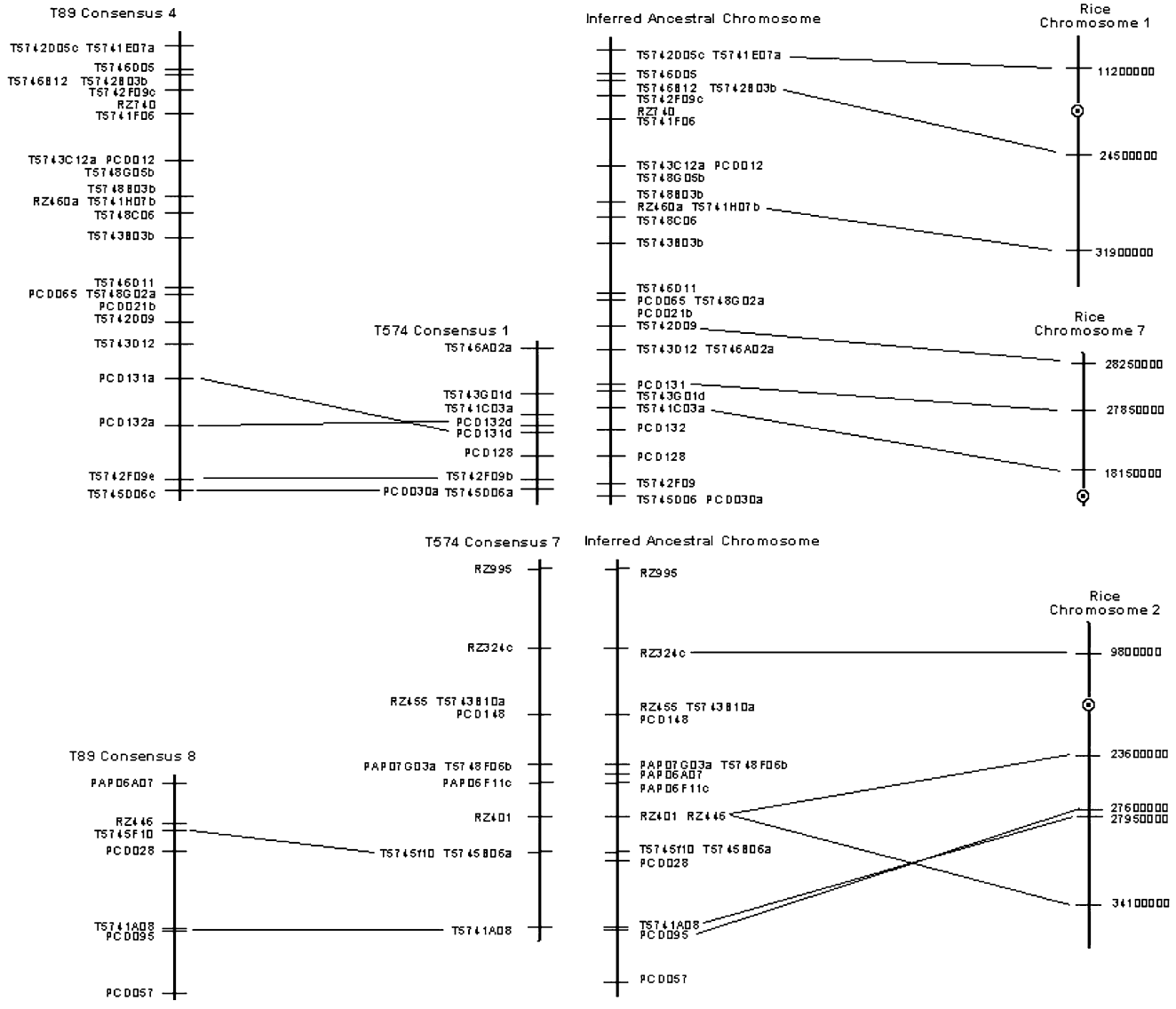


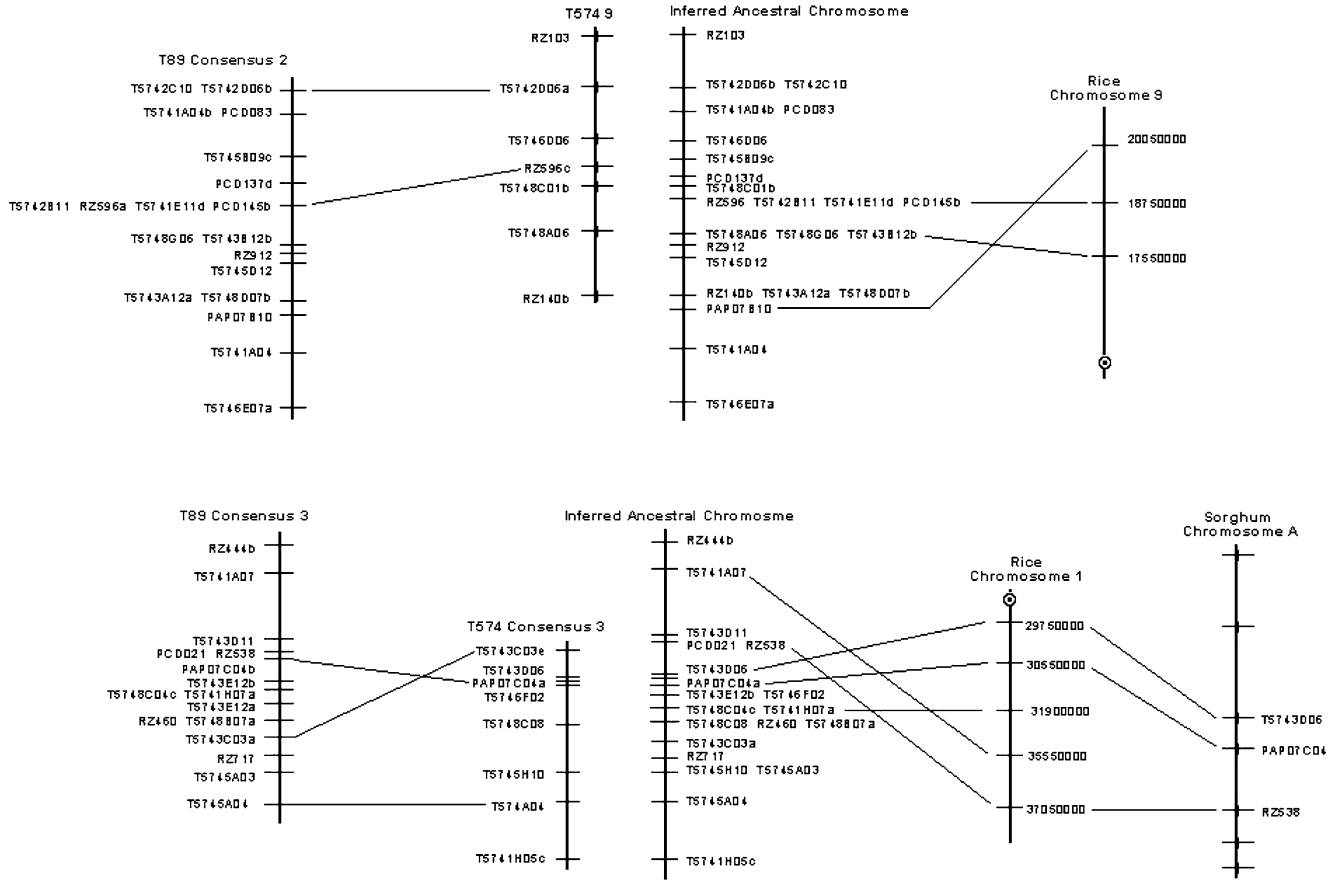
Fig. 3 Construction of inferred bermudagrass ancestral chromosomes between the two parents T89 and T574, with comparisons to rice and *Sorghum*. *Circles* show the relative location of centromeres on rice chromosomes. Construction of inferred bermudagrass ancestral chromosomes between the two parents T89 and T574, with comparisons to rice and *Sorghum*

could resolve only 22 repulsion linkages, involving a total of 17 linkage groups. In contrast, a total of 102 coupling linkages were detected on the same linkage groups. Seven pairs of linkage groups showed two or more repulsion linkages—in virtually all cases, the repulsion linkages suggested similar alignments of homologs as did data from coupling linkages among multilocus probes, supporting the veracity of the repulsion linkages. One incongruity, regarding consensus group 4, was dependent on the map order reported for a pair of markers (T5746B12a, RZ740) that are only 2 cM apart, suggesting that an alternative order for these markers may be revealed by additional data.

Unexpectedly, repulsion-phase linkages were also infrequent in the diploid T574 map, with only 13 found at a rate of 1–4 per consensus group, versus 52 coupling linkages on these groups. This unexpected finding is further addressed in the discussion.

Discussion

We report an early investigation of chromosomal transmission in bermudagrass, and present the first genetic maps of the *C. dactylon* and *C. transvaalensis* genomes. With approximately 61 and 62% estimated genome coverage, and 15.3 and 16.5 cM average marker spacing, these maps permit early investigation involving the two genomes, and represent the foundation for construction of more saturated maps. Moreover, in the case of T574, the genetic map includes both members for seven of the nine expected homologous groups plus three



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unassigned linkage groups, suggesting that we have partial coverage of most *C. transvaalensis* chromosomes. At the same time, although three linkage groups make up T574 homologous group 7, it is possible that linkage groups 7a-1 and 7a-2 represent two regions of the same chromosome that are linked by a segment for which we lack DNA markers. This is based on the observation that 7a-1 and 7a-2 align to separate ends of linkage group 7b (Fig. 2). Therefore, increasing marker density in this region may cause the two linkage groups to join, and T574 homologous group 7 may be reduced to two linkage groups, as expected. In the case of T89, the genetic map includes 35 of the 36 expected linkage groups. However, since some markers remained unlinked to the map, we cannot yet assume that we have partial coverage of all except one of the *C. dactylon* chromosomes. Also, in constructing the map of T89, we detected repulsion linkages that involved single unlinked markers. Presumably, as marker density increases, these markers will adjoin to our linkage groups and allow us to complete the homology sets.

We have demonstrated the usefulness of bermudagrass genetic maps for comparative analysis with other grasses. Notably, comparisons conducted here using our framework map produce significant hits to regions of all 12 rice chromosomes, and common probes with regions of all 10 *Sorghum* linkage groups (Supplementary Table 1). The results from our comparative analyses were able to validate predictions of homology between linkage groups. Fifteen cases exist where separate linkage groups in the same homology set share significant hits to the same rice chromosome, and six cases where they share common probes with the same *Sorghum* linkage group (Supplementary Table 1). For instance, T574-3a and T574-3b each share three significant hits to rice chromosome 1 and *Sorghum* linkage group A. Further, the results of the independent comparisons of bermudagrass to rice and *Sorghum*, when taken together, are consistent with Paterson et al. (2004) where *Sorghum* and rice were compared directly. Our findings here underscore the importance and potential of bermudagrass linkage maps in contributing to evolutionary studies of the grass family.

Finally, for genomes of uncertain constitution, such as bermudagrass, the ratio of repulsion versus coupling linkages found in SDRF mapping can be used to determine auto- versus allopolyploidy. In tetraploid T89, the preponderance of coupling linkages is consistent with autopolyploidy, as expected based on the observation of Forbes and Burton (1963), of largely multivalent chromosomes during meiosis. Our observed ratio of repulsion to coupling linkages (22:102) is not

significantly different from a ratio of 0.25:1 that would indicate polysomic inheritance of an autotetraploid (Wu et al. 1992).

Unexpectedly, in diploid T574, the ratio of coupling to repulsion linkages is also 0.25:1 (13:52). There is no tetraploidy known in the *C. transvaalensis* species, the progeny of this cross are sterile and ostensibly triploid (although each one has not been checked cytologically), and we find only two homologous T574 linkage groups per set (save for one anomalous triplet that appears likely to actually represent only two linkage groups, as detailed above). Further, the number of polymorphisms that fell on T574 chromosomes is almost exactly half that of T89. This will be a topic of further investigation.

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