MOLECULAR TOOLS FOR MARKER-ASSISTED BREEDING OF BUFFELGRASS

A Dissertation

by

RUSSELL WILLIAM JESSUP

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

August 2005

Major Subject: Plant Breeding

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ABSTRACT

Molecular Tools for Marker-Assisted Breeding

of Buffelgrass. (August 2005)

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The increasing availability of molecular tools is facilitating marker-assisted selection (MAS) in plant improvement programs. The objectives of this research were to: 1) populate the framework buffelgrass genome map with additional molecular markers, 2) develop polymerase chain reaction (PCR)-based markers from selected, informative restriction fragment length polymorphism (RFLP) markers on the buffelgrass genome map, and 3) increase marker resolution near the locus conferring apomixis (*PApo1*). Buffelgrass [*Pennisetum ciliare* (L.) Link syn. *Cenchrus ciliaris* L.] (2n=4x=36), a highly polymorphic, apomictic, perennial forage grass, is well-suited for genetic linkage analyses. One hundred and seventy one probes from an apomictic, spikelet-specific, complementary deoxyribonucleic acid (cDNA) library and 70 expressed sequence tag simple sequence repeats (EST-SSRs) from apomictic pistil cDNAs were evaluated and added to the framework buffelgrass genome map. The improved linkage map contains 851 markers from 11 grass species and covers approximately 80-85% of the buffelgrass genome. Two RFLPs from the buffelgrass genome map were converted to PCR-based markers for both the identification of hybrids

and quantification of sexual versus apomictic reproduction. A gel-free, high-throughput technique was developed to analyze these markers directly in 96-well plates. Five additional markers were placed onto the buffelgrass linkage group with the *PApo1* apomixis locus through comparative mapping of candidate orthologs from the sorghum genome map and bulked-segregant analysis of amplified-fragment-length-polymorphisms (BSA-AFLP). Increasing the mapping population size did not increase map resolution in the *PApo1* region. Association mapping revealed that the recombination suppression near *PApo1* is moderate and would complicate comparative map-based cloning efforts of the orthologous region in sorghum.

DEDICATION

I dedicate this work to the magic Jeannie who has completed me.

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I would like to express my sincere appreciation to those who gave their support and wisdom to further my academic development at Texas A&M University:

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CHAPTER I

INTRODUCTION

Molecular research in perennial forage grasses has been restricted by a declining number of public breeding programs, limited extramural funding opportunities, and unique biological properties (polyploidy, severe inbreeding depression, minuscule flowers, etc.). In addition, a large portion of comparative genomic information is distinct between grain crops and tropical grasses (Bowers et al., 2003). This indicates that the abundant genomic tools in major grain crops will be of limited use in forage grasses. Molecular research is therefore warranted in additional grass species. Some perennial forage grasses also provide the opportunity to investigate apomixis (asexual reproduction through seed), a valuable trait that is prevalent in tropical forage grasses but absent in major grain crops.

The establishment of a buffelgrass [*Pennisetum ciliare* (L.) Link syn. *Cenchrus ciliaris* L.] genome map (Jessup et al., 2003) has produced genomic information with utility across perennial forage grasses and apomictic species. The buffelgrass genome map contains molecular markers from several grass species and is aligned to a high-density genome map of grain sorghum [*Sorghum bicolor* (L.) Moench.] (Bowers et al., 2003). The locus that exerts major control over apomixis (*PApo1*) has been identified and placed onto the buffelgrass genome map (Jessup et al., 2002). The buffelgrass genome map provides a foundation for marker-assisted breeding in buffelgrass and

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This dissertation follows the style and format of Crop Science.

genomic characterization of other perennial forage grasses. *PApo1* serves as a resource for breeding programs of apomictic forage grasses, molecular investigations of apomixis, and the potential transfer of this valuable trait into major grain crops.

The objectives of this investigation were to: 1) populate the buffelgrass genome map with additional molecular markers; 2) develop PCR-based markers from selected, informative RFLP markers on the buffelgrass genome map; and 3) increase marker resolution near the *PApo1* locus.

CHAPTER II

IMPROVEMENT OF THE BUFFELGRASS GENOME MAP

INTRODUCTION

Approximately 75% of the species grown as forage crops are grasses (Nelson and Moser, 1995), and grassland acreage worldwide is estimated to be twice that of cropland (Jauhar, 1993). About 40 species account for 99% of the pastures in the USA (Moser and Hoveland, 1996), where forages exceed the cash value of any other crop based on livestock feed costs (Barnes and Baylor, 1995).

Despite their importance, genetic mapping of forage grasses lags behind that of major cereal crops. Small flowers and incompatibility mechanisms make many forage grasses difficult to hybridize. Inbreeding depression and polyploidy complicate the development of mapping populations. In addition, limited economic resources restrict molecular research to a minority of forage grasses. Genetic maps have been constructed in a few diploid forage grasses, such as pearl millet [*Pennisetum glaucum* (L.) R. Br.] (Liu et al., 1994), ryegrass (*Lolium perenne* L. x *Lolium multiflorum* Lam.) (Hayward et al., 1998), and bahiagrass (*Paspalum notatum* Flügge) (Ortiz et al., 2001). Most forage grasses are perennial polyploids (Masterson, 1994), and genetic maps have been made for only a limited number. These include tall fescue (*Festuca arundinacea* Schreb.) (Xu et al., 1995), Kentucky bluegrass (*Poa pratensis* L.) (Porceddu et al., 2002), and buffelgrass (Jessup et al., 2003).

Despite being grown as forage on millions of hectares in the arid and semi-arid

tropics (Bogdan, 1977; Bray, 1978; Hussey, 1985), buffelgrass breeding efforts have been limited because apomixis is prevalent in the species (Fisher et al., 1954, Snyder et al., 1955). Most buffelgrass cultivars have resulted from selecting and increasing superior apomictic ecotypes. These natural accessions contain both desirable and undesirable characteristics and are limited by adaptations to the locale in which they were selected (Bashaw and Funk, 1987). Rare sexual genotypes (Bashaw, 1962) have facilitated the production of buffelgrass hybrids (Bashaw and Funk, 1987) and interspecific hybridization between buffelgrass and birdwoodgrass (*Cenchrus setigerus* Vahl) (Read and Bashaw, 1969). This has provided an opportunity to develop improved germplasm. Superior forage production and cold tolerance of the cultivars 'Llano' and 'Nueces' (Bashaw, 1980) demonstrated the benefits of hybrid buffelgrass. Despite the potential of hybrid buffelgrass, additional hybrid cultivars have not been developed.

Buffelgrass has several advantages as a model for genetic mapping studies of polyploid, perennial forage grasses. It is protogynous, which allows hybridizations to be made without hand emasculations of florets. Obligate apomictic buffelgrass genotypes can be maintained long-term as seeds instead of vegetative plants. The high levels of heterozygosity in buffelgrass result in adequate levels of DNA polymorphism for genetic mapping in hybrids between most genotypes. Genetic mapping of hybrid buffelgrass populations was first accomplished by Gustine et al. (1997), and the genome map (Jessup et al., 2003) subsequently placed molecular markers across a majority of the buffelgrass genome, distinguished between genomic regions with disomic and tetrasomic inheritance, and included markers capable of screening genotypes for apomictic reproduction.

In addition to its intraspecific utility, the buffelgrass genome map is a comparative mapping resource for other perennial forage grasses. The buffelgrass map contains more than 500 RFLP markers from 10 grass species that can be used to construct genetic maps throughout forage grasses. For example, more than 140 markers from the buffelgrass genome map were used in the construction of a linkage map of barnyardgrass [*Echinochloa crus-galli* (L.) Beauv.] (Fukao et al., 2004). The buffelgrass map is also aligned to a high-density map of grain sorghum (Bowers et al., 2003) by more than 200 markers. Utilizing the sorghum and buffelgrass genetic maps, comparative mapping can be extended from perennial forage grasses to the established genetic maps of all major cereal crops. In this manner, comparisons of macrocolinearity (cf. Bennetzen et al., 1998) can be used to develop molecular tools for MAS in perennial forage grass breeding programs.

Microcolinearity is required for comparative map-based cloning efforts; however, it is not conserved between distantly related species (cf. Keller and Feuillet, 2000). Extensive genome rearrangements have been found to exist between cereal crops and forage grasses (Bowers et al., 2003), indicating that many forage-specific genome regions are not present in genetic maps of cereal crops. A high-density map of a perennial forage grass would thus be required to establish microscopic synteny between closely related forage grasses. Buffelgrass is in the grass subfamily Panicoideae, along with several other apomictic perennial forage grasses: signalgrass (*Brachiaria decumbens* Stapf), guineagrass (*Panicum maximum* Jacq.), bahiagrass, and eastern gamagrass (*Tripsacum dactyloides* L.). Improvement of the buffelgrass genome map would therefore benefit its utilization as a tool for comparative mapping of

microcolinearity in perennial forage grasses. The overall objective of this study was to saturate the buffelgrass genome map with additional molecular markers.

MATERIALS AND METHODS

Plant Materials

The full-sib buffelgrass population used to construct the buffelgrass genome map (Jessup et al., 2003) was derived from crossing a heterozygous, highly sexual genotype (90C48507) with a heterozygous, highly apomictic genotype (PI 409164). This mapping population consisted of 86 F_1 hybrids. The pedigree for this population is shown in Figure 1.

RFLP Markers

A cDNA library constructed from spikelets of an obligate apomictic buffelgrass plant selected from the full-sib population was utilized. Bacterial clones from the library were obtained by *en masse* phagemid excision, followed by two cycles of selection for recombinant clones on ampicillin plates containing X-gal and IPTG (Sambrook et al., 1989). Inserts were amplified by PCR from bacterial lysate (McCabe, 1990), and an aliquot of the products were electrophoresed in 1% agarose. Clones that gave multiple products were discarded. Sephadex G50 (Sigma) spun mini-columns were used to separate PCR products from excess reaction components (Sambrook et al., 1989). Dot blots with 20 ng of DNA from each probe were hybridized with leaf cDNA to identify and eliminate repetitive elements. The resulting suitable probes were designated "pPAS," for Plasmid-*Pennisetum*-Apomictic-Spikelet.

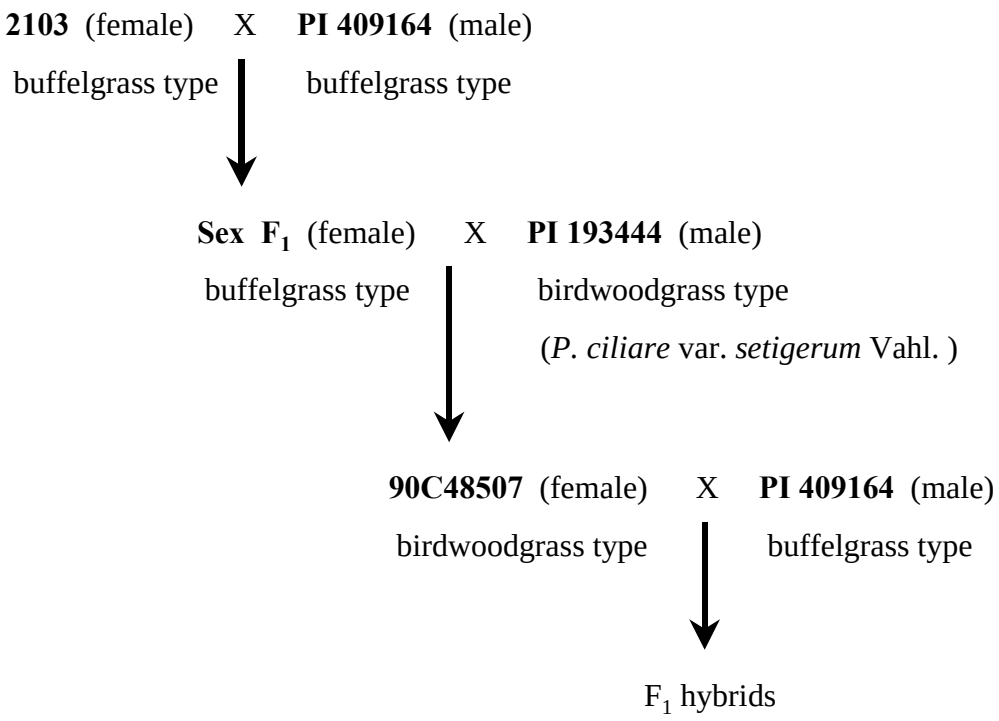

Fig. 1. Pedigree of the buffelgrass mapping population. The parental lines (90C49507 and PI 409164) differed in many traits, including inflorescence type (birdwoodgrass vs. buffelgrass), rhizomes (non-rhizomatous vs. rhizomatous), and method of reproduction (sexual vs. apomictic).

Genomic DNA extraction was adapted from the protocol of Causse et al. (1994). Ten μg of buffelgrass genomic DNA were digested with *Eco*RI, *Hind*III, or *Xba*I, according to the manufacturer's instructions. Southern blotting, radioactive labeling, and autoradiography were performed as described by Chittenden et al. (1994).

EST-SSR Markers

A total of 1,027 partial or full-length cDNA sequences that belong to *P. ciliare* were downloaded from GenBank (National Center for Biotechnological Information; http://www.ncbi.nlm.nih.gov/Genbank/index.html). SSR identification and primer design were performed using the web-based 'SSR Primer Discovery Tool' (Plant

Biotechnology Centre, La Trobe University; http://hornbill.cspp.latrobe.edu.au/ssrdiscovery.html). Selected SSRs contained at least 10 dinucleotide or five tri-, tetra-, or pentanucleotide repeats. Primers design was based on the criteria of 50% GC content, minimum melting temperature of 50° C, absence of secondary structure, length of 20-27 nucleotides, and amplified product range of 100-400 bp. The resulting suitable probes were designated "PCAR," for *Pennisetum-ciliare*-Apomictic-Repeats.

PCR reactions were conducted in a total volume of 20 uL, using 50 ng of buffelgrass DNA, 1X Promega $MgCl_2$-free PCR Buffer, 2.5 mM $MgCl_2$, 0.2 mM dNTPs, 2 mM of each primer, and 1 unit of Promega Taq polymerase. The PCR method included: 1) an initial denaturation at 95° C for 3 min, 2) 10 touchdown decrement cycles at 95° C for 25 sec, 64-55° C for 25 sec, and 70° C for 45 sec, 3) 36 cycles at 95° C for 25 sec, 55° C for 25 sec, and 70° C for 45 sec, 4) an elongation cycle at 70° C for 10 min, and 5) a final hold at 4° C. Electrophoresis was run using a MEGA-GEL High Throughput Vertical Unit (C.B.S. Scientific, Del Mar, CA) as described by Wang et al. (2003). Allele bands were distinguished from minor bands that occur in nondenaturing polyacrylamide gel electrophoresis as described in Rodriguez et al. (2001) in order to prevent band scoring errors.

Linkage Analysis

Each polymorphic band was treated as a locus with dominant gene action. Individual bands present in one parent and absent in the other parent were scored for presence or absence in the progeny. A χ^2 test was used to identify single dose restriction fragments (SDRFs) by their 1:1 segregation ratio at a significance level of 1% (Wu et al.,

1992). A nonsignificant test indicated that a given band was an SDRF and could be considered in the linkage analysis.

SDRFs derived from the pPAS and PCAR markers were combined with the data set from the initial buffelgrass genome map (Jessup et al., 2003). Because an SDRF only reveals segregation in the gametes of one parent, an RFLP map of each parent's respective SDRFs was constructed using MAPMAKER 3.0 (Lander et al., 1987). SDRFs were treated as backcross data. Based on the use of 86 individuals, a log odds difference (LOD) score of 4.0 and recombination fraction of 0.30 were set as the linkage thresholds. The maximum detectable recombination fraction (max_r) in the buffelgrass mapping population was 0.435 at a 98% confidence level for linkage in the coupling- and repulsion-phase of an allotetraploid, as well as in the coupling-phase linkage of an autotetraploid (Wu et al., 1992). However, max_r would be only 0.126 for repulsion-phase linkage of an autotetraploid. Map units, in centiMorgans (cM), were derived using the Kosambi (1944) function. Maximum likelihood orders of markers were verified using the "ripple" function, with those at LOD $\geq$ 4.0 placed on the framework map and all others with LOD $\geq$ 2.0 added at the most likely interval between framework markers. Linkage maps were drawn using MapChart 2.1 (Voorrips, 2002). Repulsion-phase linkages were identified by repeating the Mapmaker analysis with a data set combining the original and inverted SDRF marker scores.

Genome length, G, was estimated from partial linkage data according to the equation of Hulbert et al. (1988):

$$G = MX/K,$$

where M= the number of informative meioses, X= an interval in cM at some minimum

LOD score, and K= the actual number of pairs of markers found to border the interval X or less. A maximum value for M equals the number of pairwise combinations for linked markers (N), or N(N-1)/2. The proportion of genome coverage (C), in terms of the probability (P) of a random point not being covered, was calculated as suggested by Bishop et al. (1983). The equation used was:

$$C = 1-(2r)/(n+1)[(1-x/2t)^{n+1} - (1-x/t)^{n+1}] + (1-rx/t)(1-x/t)^{n},$$

where r= the number of linkage groups, x= an interval in cM, n= the number of intervals, and t= the sum of linkage group lengths in cM.

An evaluation of preferential pairing between chromosomes was accomplished by comparing the ratio of repulsion- versus coupling-phase linkages. To detect repulsion-phase linkages, two-point linkage analyses were repeated with the allele states inverted. Using a χ^2 test, a 1:1 ratio would indicate allotetraploidy (disomic inheritance). Two linkage groups with markers in repulsion along their length would be interpreted as pairing partners (homologous chromosomes). A 0.25:1 ratio would indicate autotetraploidy (tetrasomic inheritance). Any intermediate ratio would suggest partial preferential or multivalent chromosomal pairing and a more complex form of polyploidy (Wu et al., 1992).

RESULTS AND DISCUSSION

pPAS Polymorphism

Four hundred and five uncharacterized repetitive elements were eliminated. A survey of the remaining 171 low-copy pPAS cDNAs yielded 153 polymorphic probes. For the three restriction enzymes that were used, the parents were polymorphic for 89%

of the low-copy pPAS probes: 57% with *Eco*RI, 55% with *Hind*III, and 48% with *Xba*I (Appendix A). The pPAS cDNAs included a slightly lower percentage (26%) of low-copy cDNAs than the pPAP (plasmid-*Pennisetum*-Apomictic-Pistil) cDNAs (31%). This difference could have been caused by gene expression variation between the tissue sources for the two cDNA libraries. The pPAS library was derived from spikelets, which likely included housekeeping genes involved with photosynthesis. The pPAP library, in contrast, was derived from pistils and contained no photosynthetically active tissue. The polymorphism frequency of low-copy pPAS cDNAs (89%) was nearly equivalent to the low-copy pPAP cDNAs (90%), indicating that the pPAS library is also a source of markers that are useful for genetic mapping of buffelgrass.

PCAR Polymorphism

Ninety two out of the 1027 cDNAs analyzed were found to contain at least one microsatellite. Seventy unique sequences remained after 22 duplications were removed from the data set. Sixty-seven of the 70 sequences contained a single SSR, two sequences contained two SSRs, and one sequence contained four SSRs. The 70 PCAR EST-SSRs included 33 di-, 28 tri-, 6 tetra-, and 3 pentanucleotide repeats (Appendix B). Thirty three of the 70 PCAR EST-SSRs were polymorphic between the parents of the buffelgrass mapping population. Three of the polymorphic PCAR EST-SSRs were derived from PPAP cDNAs that had already been placed onto the buffelgrass genome map as RFLPs; therefore, these PCAR probes were not utilized. Polymorphisms occurred with 47% of the PCAR EST-SSRs and 3% of the original sequences that were analyzed. These results are similar to reports in tall fescue (66% and 0.8%, respectively) (Saha et al., 2004), hexaploid wheat (53% and 2%, respectively) (Yu et al., 2004), and

barley (36% and 1.3%, respectively) (Thiel et al., 2003).

Segregation Analysis

The respective 153 and 30 polymorphic pPAS and PCAR probes yielded a total of 181 SDRFs in the maternal parent and 151 SDRFs in the male parent. The pPAS and PCAR SDRFs were combined with the data set from the framework buffelgrass genome map (Jessup et al., 2003) to give a total of 1059 SDRFs (594 on the maternal map and 465 on the paternal map).

Linkage Analysis

Linkage analyses of all SDRFs were conducted using MAPMAKER 3.0. The maternal map included 476 SDRFs in 38 linkage groups, which spanned 4376 cM and had an average interval between markers of 9.2 cM (Appendix C). The paternal map included 375 SDRFs in 34 linkage groups with 3351 cM and an average interval between markers of 8.9 cM (Appendix D). One hundred and eighteen and 90 markers remained unlinked in the maternal and paternal maps, respectively. Analysis of linkage data gave estimates for overall genome length of 5149 cM vs. 4137 cM and coverage of 83% vs. 80% in the maternal and paternal maps, respectively. Final estimates of genome coverage (85% for the maternal map and 81% for the paternal map) were obtained by dividing the map lengths into the estimated genome lengths. The incomplete genome coverage in both parents' maps may be at least partially explained by the slight inbreeding in the pedigree of the buffelgrass mapping population (Fig. 1). The genome coverage estimates of 81-85% were slightly higher than that expected based on the inbreeding coefficient (F=0.25). However, these results suggest that construction of a complete genome map for buffelgrass will require the utilization of more divergent

genotypes.

Placing additional markers onto the genome map allowed several small linkage groups to be consolidated on both parents' maps. Updated linkage group designations are listed in Table 1. As a result, the improved map more closely approximates each of the 36 buffelgrass chromosomes with an individual linkage group than the framework map. The number of linkage groups in the maternal parent's map was reduced from 47 to 38, and the number of linkage groups in the paternal parent's map was reduced from 44 to 34. However, the lack of 36 linkage groups in either parent's map and the presence of numerous unlinked markers provide further evidence that inbreeding in the pedigree has prevented some portions of the buffelgrass genome from being identified.

Table 1. Correspondence of linkage group designations between the framework and improved buffelgrass genome maps.

	Framework Map Linkage Group	Improved Map Linkage Group
Maternal Parent (90C48507)	**15**	**13**
	16	**12b**
	18	**13**
	20	**8a**
	23	**3a**
	24	**12a**
	29	**9b**
	30	**8a**
	31	**25**
	32	**4a**
	33	**12a**
Paternal Parent (PI 409164)	**40**	**2a**
	48	**8b**
	49	**40**
	50	**39**
	51	**33**

Chromosome Associations

Ratios of loci linked in repulsion-phase to loci linked in coupling-phase were calculated for each linkage group to determine the frequency of preferential or random chromosome assortment (Table 2). Repulsion-: coupling-phase SDRF ratios of 1:1 would indicate disomy, while 0.25:1 ratios would indicate tetrasomy (Wu et al., 1992). Disomy was most common in the female parent. The detection of 1:1 repulsion-: coupling-phase associations within 24 linkage groups in the maternal map suggests that 12 bivalents occur during meiosis. This result supports cytological evidence of 10 to 14 bivalents during meiosis in buffelgrass (Fisher et al., 1954; Snyder et al., 1955). In

Table 2. Repulsion- versus coupling-phase linkage ratios across linkage groups in the maternal and paternal maps.

	1 : 1 disomy	0.25 : 1 tetrasomy	0.25-1 : 1 complex
Maternal Parent (90C48507)	**24**	**7**	**2**
Paternal Parent (PI 409164)	**16**	**6**	**4**

contrast, a 1:1 repulsion-: coupling ratio was detected in only 16 linkage groups of the paternal map. This result indicates that the paternal parent is a segmental allopolyploid, but it also suggests chromosome pairing differences between the parental genotypes.

Repulsion was observed for 46% of the SDRFs in the maternal map, compared to 15% of SDRFs in the paternal map. Assuming random chromosome assortment, diploids and allopolyploids would have repulsion at 50% of their loci; whereas, a segmental allopolyploid with 14 bivalents and two quadrivalents would have repulsion at 44% of its loci. This large difference in repulsion-phase associations across loci in the maternal and paternal maps further suggests that chromosome behavior varies between female and male gametes (Fogwill, 1958; Mogensen, 1977; Ross et al., 1996; Havekes et al., 1997). With the general acceptance that chromosome 'alignment' and 'synapsis' are different processes (Loidl, 1990; Kleckner, 1996; Cook, 1997; Moore, 2000), one possible explanation is that similar preferential pairing and dissimilar chiasma formation occurs between the parental genotypes. Alternatively, the paternal parent may have a shorter meiotic cycle (Havekes et al., 1997) or greater chromatin modifications.

CONCLUSIONS

This improved buffelgrass genome map contains 851 markers that can be utilized in breeding programs. The pPAS and PCAR markers have increased saturation and coverage of the buffelgrass genome map. Because many of the pPAS markers have also been placed onto the sorghum genome map, they could facilitate future comparative mapping studies between buffelgrass and sorghum.

CHAPTER III

DEVELOPMENT OF MARKERS TO IDENTIFY HYBRIDS AND QUANTIFY APOMIXIS

INTRODUCTION

There are several markers on the buffelgrass genome map (Jessup et al., 2003) that have potential as MAS tools. However, RFLPs are not convenient for the high-throughput population screens that are desired in plant breeding programs. PCR-based markers developed from these RFLPs would be more efficient and cost effective.

Because buffelgrass hybridizations are not completely reliable, populations used for genetic mapping studies may not always consist of the intended hybrids. This causes major errors in mapping studies and indicates the need for reliable methods to identify these erroneous plants. One approach is to identify paternal-specific markers. A total of 19 paternal-specific markers in the buffelgrass genome map were present in the paternal parent, absent in the maternal parent, and present in all of the F_1 hybrids. Each of these RFLPs represented an allele present in 3 or 4 doses in the paternal parent's genome. These paternal-specific markers could be used to identify and remove self-pollinated maternal plants and undesired outcrosses from the mapping population.

The two markers (pPAP8C08 and pPAP3A07) closely linked to *PApo1* (1.5 cM) could be utilized as a preliminary screen to determine the method of reproduction (sexual versus apomictic) in hybrids. This reduces the number of labor-intensive cytological examinations and field-based progeny tests required to confirm method of

reproduction. The conversion of pPAP8C08 and pPAP3A07 into PCR-based markers is not necessary because three sequence characterized amplified regions (SCARs) (OPC4, QH8, and UGT197) developed in *P. squamulatum* Fresen (Ozias-Akins et al., 1998) are also closely linked to *PApo1* (Jessup et al., 2002). These SCARs could be used as PCR-based selectable markers to identify apomictic reproduction in buffelgrass.

However, these SCARs and other markers developed to distinguish sexual versus apomictic reproduction have an underlying limitation. Most if not all apomictic genotypes are facultative, in that both apomictic and sexual processes occur in the same genotype (Savidan, 2000). The inability to accurately quantify this range of apomictic expression (i.e. facultativeness) across genotypes has resulted in the use of arbitrary phenotypic classes (sexual, apomictic, and facultative apomict). The genetics of apomixis have been further oversimplified in segregation and mapping studies by combining the apomictic and facultative apomict classes into one group. Consequently, difficulties in determining the reproductive behavior of plants have hindered the characterization of apomixis.

Alternative methods are needed to study the quantitative variation that occurs across apomictic phenotypes. One approach would involve using markers that are equivalent to alternate alleles of a gene pair (Aa). Mendelian segregation of these alleles would be 1:1 in a population derived from an obligate sexual plant, 1:0 for the maternal allele in a population derived from an obligate apomict plant, and an intermediate frequency in a population derived from a facultative apomict plant. The transmission genetics of these markers would therefore quantify the degree of 'facultativeness' (ratio of sexual and apomictic reproduction) and distinguish between facultative and obligate

apomictic plants. The maternal parent of the buffelgrass mapping population was a highly sexual plant and could provide markers suitable for this approach. Several maternal-specific markers on the buffelgrass genome map revealed RFLP fragments equivalent to the alternate alleles of a gene pair (Aa). These markers followed simple Mendelian genetics in that they segregated exactly 1:1 across the 86 hybrids in the buffelgrass mapping population and were located in regions of the buffelgrass genome with preferential (disomic) chromosome pairing.

The conversion of RFLPs to PCR-based markers usually involves techniques that identify the unknown DNA sequence between a cDNA or gDNA clone and the neighboring restriction site responsible for an RFLP of interest. Techniques such as inverse PCR (Triglia et al., 1988), capture PCR (Lagerstrom et al., 1991), panhandle PCR (Jones et al., 1992), and vectorette PCR (Arnold et al., 1991) require restriction enzyme digestions, adapter ligations, PCR amplification, product purification, sequencing, and primer design.

In contrast, restriction-site PCR (RS-PCR) is a direct method of amplifying the region between a known sequence and an adjoining restriction site (Sarkar et al., 1993). RS-PCR utilizes one or more sequence-specific primer and a universal primer that recognizes a given restriction enzyme recognition site (Fig. 2). RS-PCR is therefore preferable to the above methods when a selectable marker is desired and the DNA sequence of the unknown region is not required.

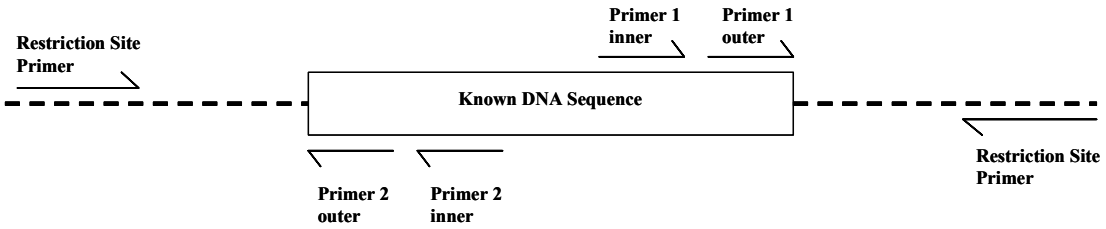

Fig. 2. Schematic representation of RS-PCR.

The objective of this research was to develop RS-PCR markers from RFLPs on the buffelgrass genome map for: 1) paternal-specific hybrid confirmation, and 2) quantitative classification of apomictic reproduction. An additional objective was to develop a gel-free detection protocol for these markers.

MATERIALS AND METHODS

RFLP Selection

RFLPs that were produced using *Hind*III were selected to allow the use of the same universal RS-PCR primer (5'-NNNNNNNNNNAAGCTT-3') for each marker. RFLPs that were smaller in size than 1.5 Kb and their source cDNA were chosen to ensure the expected product could be amplified by RS-PCR. Two informative RFLPs were chosen for each desired marker to compensate for the possibility of RS-PCR failure caused by multiple *Hind*III restriction sites within a cDNA. Because pPAP cDNAs average 1.9 Kb and *Hind*III is a 6-cutter that restricts DNA on an average of every 6 Kb, the occurrence of two *Hind*III recognition sites in a single pPAP cDNA would be rare.

DNA Sequencing

Plasmid DNAs were prepared from selected pPAP cDNAs using a Qiaprep miniprep plasmid DNA kit (Qiagen, Valencia, CA) by following the manufacturer's protocols. Cycle sequencing with ABI (Columbia, MD) Big Dye, followed by analysis on an ABI 3700, was performed for both forward and reverse primers according to the manufacturer's protocols. For any cDNA in which a complete sequence was achieved, the sequence was searched for *Hind*III recognition sites. If multiple *Hind*III recognition sites were found in the cDNA sequence, another marker was substituted in its place.

Primer Design

PCR primers were designed using the internet-based software 'Primer3' (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) based on the criteria of 50% GC content, minimum melting temperature of 50° C, absence of secondary structure, and length of 20-27 nucleotides (Table 3). Primers were commercially synthesized (MWG-Biotech; High Point, NC).

Table 3. RS-PCR primers for selected pPAP markers.

	Outer Primers 5'---------->3'	Inner Primers 5'---------->3'
Hybrid Confirmation		
pPAP3E08	AACTGGTACTCCAGCTAACG ATCTGCGATGCGGTCACTGC	ACCTGAGTTCGACACTCGGC CACTGTGAACGTTCAGTCAC
pPAP7C09	CTACTGTTCAGCGCAGTTCA GACGTCACTAAGCTAACGTG	AGTCGTCATTACGGACTGAT GGCTATACGATCCAGTAGTC
Reproductive Potential		
pPAP1D08	TTCGAAGCTGCTAGATCGAC GACTTCAGCCTACGATACGA	CGCATCGACTAACTGCTACG AGCTCGATTCGATTCGATAG
pPAP10C11	AGTTCCTTTCTTCGGGTACC TATTGACCGAGGGGTTGGTG	GCCAGATATGCTAGGCTCCA TGTCCCGTTGAAGGATATGA

Plant Materials

To determine if all the plants in the mapping population were hybrids, paternal-specific markers were used. The paternal parent (PI 409164) and all 87 plants in the original buffelgrass mapping population (Jessup et al., 2003) were analyzed.

To evaluate the reliability of using molecular markers to predict method of reproduction, the following buffelgrass hybrids were used: one obligate apomict (BWB266), one obligate sexual (BWB178), five facultative apomicts (BWB62, BWB103, BWB131, BWB147, BWB281), and 200 random seedlings from open-pollinated seed collected during the spring, summer, and fall from each of the above mentioned plants.

DNA Extraction

Genomic DNA was extracted using a modified protocol from Ikeda et al. (2001). DNA extraction was performed in 96-well polypropylene plates. About 1 cm of leaf tissue was collected, cut into small pieces, and placed into a plate well. One hundred and fifty uL of TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) were added to each well, and the leaf tissue was pulverized using a disposable pipette tip. The plates were covered and placed into a 96-well hot plate for 20 min at 100° C. The plates were then centrifuged for 10 min at 3500 rpm. The supernatant was recovered and used as template DNA for PCR.

RS-PCR

PCR reactions were conducted in a total volume of 10 uL, using 1 uL of buffelgrass genomic DNA solution, 1X Promega $MgCl_2$-free PCR Buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 2 pmoles of the outer primer from the known sequence, 20

pmoles of the universal *Hind*III primer, and 0.5 units of Promega Taq polymerase. The PCR method included: 1) 30 cycles of 1 min at 94° C, 1 min at 50° C, and 3 min at 72° C, 2) a final elongation cycle at 70° C for 10 min, and 3) a final hold at 4° C. The nested PCR was performed the same manner except that the inner primer from the known sequence was used and 1 uL of the product from the first PCR was used as template.

DNA Detection

Initial RS-PCR products were electrophoresed on 1% agarose gels in TBE for 1 h at 90 V. Gels were stained with ethidium bromide and visualized on a UV transilluminator. RS-PCR markers that produced a single product in the desired parental plant were analyzed in the appropriate populations.

To analyze progeny populations from each hybrid, RS-PCR markers were visualized directly in the 96-well plates in which the PCR was performed. Serial dilutions of 1 mg mL^{-1} ethidium bromide was added to the RS-PCR reaction products and viewed on a UV transilluminator to determine the optimum concentration for discerning positive and negative amplifications.

Phenotypic Classification for Method of Reproduction

The reproductive behavior of the obligate sexual, obligate apomict, and facultative apomict plants was determined by two traditional methods. The first method involved microscopically observing mature megagametophytes in cleared pistils (Young et al., 1979). More than 200 mature megagametophytes from each plant were classified for method of reproduction. Second, a field progeny test was conducted. A clonal ramet and 20 open-pollinated progeny from each hybrid were transplanted into a space-planted nursery on 1 m centers. Once the plants in the nursery were flowering, three observers

independently classified the method of reproduction of each hybrid based on the phenotype of its progeny. Those hybrids with morphologically uniform progeny were scored as apomictic. Those hybrids with progeny exhibiting partial uniformity and a degree of variability were classified as facultative apomicts, and hybrids with completely variable progeny were scored as sexual. Both cytological and progeny testing data were used to classify the method of reproduction of each hybrid.

RESULTS AND DISCUSSION

The first round of RS-PCR produced faint smears upon gel electrophoresis, while the nested cycle RS-PCR produced distinct allele bands (Fig. 3). RS-PCR of pPAP1D08 produced two allele bands, suggesting the presence of two *Hind*III recognition sites in the nearby flanking DNA. In order to avoid gel electrophoresis and optimize the 96-well plate approach, all tests for reproductive potential were therefore carried out using pPAP10C11.

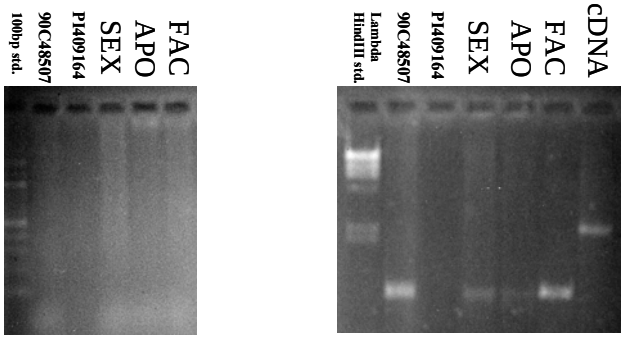

Fig. 3. Agarose gel electrophoresis of pPAP10C11 RS-PCR products: (Left) First PCR. (Right) Second PCR.

Detection of the RS-PCR products in 96-well plates was successful and was improved by filling each well with additional H_20. Optimal results were achieved by

adding 0.1 ug mL^{-1} ethidium bromide in H_20 to each well before visualization on a UV transilluminator. Omission of the nested RS-PCR produced identical results, further simplifying the technique by requiring only a single RS-PCR cycle.

RS-PCR of pPAP3E08 and pPAP7C09 gave identical results and identified the plant in the buffelgrass mapping population that was not of hybrid origin (BWB77) (Fig. 4). These markers can be used in the future to screen any hybrid populations that have PI 409164 as the paternal parent.

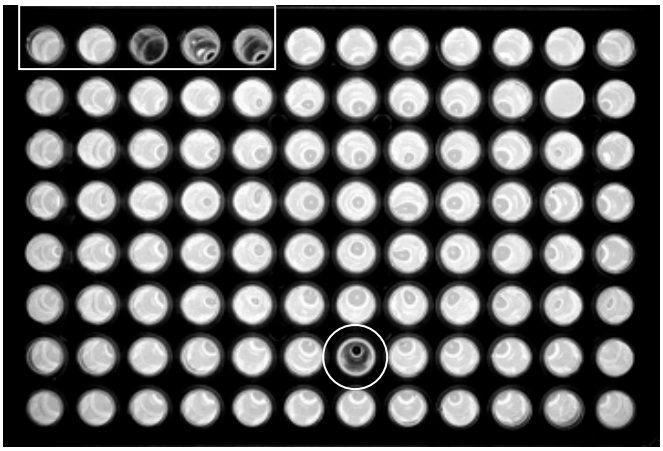

Fig. 4. Ninety six well plate of first cycle RS-PCR products for pPAP3E08. Control samples are denoted by a white box. The non-hybrid plant is denoted by a white circle.

RS-PCR of pPAP10C11 clearly distinguished between sexual, apomictic, and facultative plants (Fig. 5). Transmission of pPAP10C11 was 50% in the obligate sexual plant (BWB178), 100% in the obligate apomict plant (BWB266), and 92.3% in the facultative apomict plant (BWB281). Because sexual reproduction in the facultative apomict would result in an equal number of progeny with and without pPAP10C11, the frequency of sexuality would be twice the number of wells with no RS-PCR product.

Thus, approximately 15.4% of the progeny from BWB281 resulted from sexual reproduction.

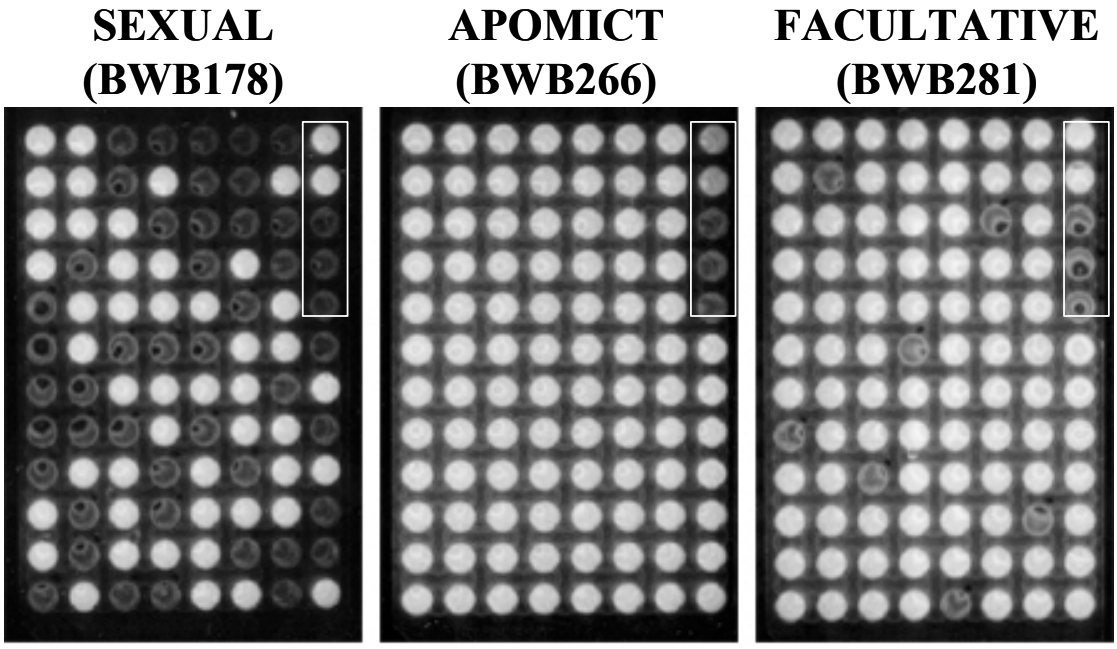

Fig. 5. Ninety six well plates of first cycle RS-PCR products for pPAP10C11. Control samples are denoted by white boxes.

RS-PCR results of pPAP10C11 were also very consistent with the traditional methods for determining mode of reproduction: cytological examinations and progeny tests (Table 4). Statistical analyses were not possible because of the unequal sample sizes of the methods used. In addition, the cytological method could detect individual

Table 4. Comparison of reproductive classifications by RS-PCR, cytological, and progeny test methods.

	RS-PCR n=200	Cytological n>200	Progeny Test n=20
		%	
Obligate Sexual			
BWB178			
Sexuality	**100**	**100**	**100**
Apomixis	**0**	**0**	**0**
Obligate Apomict			
BWB266			
Sexuality	**99**	**100**	**100**
Apomixis	**1**	**0**	**0**
Facultative Apomict			
BWB131			
Sexuality	**2**	**<10**	**0**
Apomixis	**98**	**>90**	**100**
BWB103			
Sexuality	**9**	**<20**	**5**
Apomixis	**91**	**>80**	**95**
BWB147			
Sexuality	**10**	**<20**	**5**
Apomixis	**90**	**>80**	**95**
BWB62			
Sexuality	**12**	**<30**	**10**
Apomixis	**88**	**>70**	**90**
BWB281			
Sexuality	**15**	**<30**	**10**
Apomixis	**85**	**>70**	**90**

ovules that contained both sexual and apomictic megagametophytes. The frequency of this phenomenon varied among facultative genotypes, providing additional phenotypic information about apomixis and suggesting that modifier genes may affect the trait. However, determining which megagametophyte successfully developed into progeny in these cases was not possible. In this study these ovules were divided evenly between the sexual and apomictic classes. This arbitrary placement of 'mixed' type ovules assumes

equal survival of sexual and apomictic megagametophytes. Because this assumption may not be true, statistical tests between the cytological and either RS-PCR or progeny test methods could be confounded by differences in phenotypic measurements. Therefore, only general interpretations were made between the three classification methods.

All three methods gave identical results for the obligate sexual genotype (BWB178). The RS-PCR method revealed a slight amount of facultativeness in the obligate apomictic genotype that was not observed in the cytological and progeny test analyses. This suggests that the RS-PCR method may have a higher precision than traditional methods, as well as suggesting that the obligate apomict genotype (BWB266) is a facultative apomict.

Each of the three classification methods yielded a range of 'facultativeness' values for the facultative apomict genotypes. Progeny tests consistently gave the lowest estimates of sexuality; whereas, the cytological observations consistently gave the highest estimates of sexuality. This trend may be related to the developmental stage at which plants were evaluated for mode of reproduction. The cytological, RS-PCR, and progeny test methods examined gametophytes, seedlings, and mature plants, respectively. In most apomictic species, the amount of sexuality observed decreases as development proceeds due to differential fitness of individuals (Savidan, 2000). Comparisons of the three classification methods support this trend.

The RS-PCR method is advantageous compared to cytological or progeny test approaches because of its efficiency and accuracy. From DNA isolation to RS-PCR product visualization, the method can be completed for a genotype by one person in a

single day. Control experiments using > 500 repeated DNA extractions of a single genotype did not yield false negatives (data not shown), suggesting the method is very accurate. The RS-PCR method would also allow quantitative measures of apomixis to be obtained from a population in a relatively short time period. The RS-PCR method would therefore facilitate QTL studies of apomixis and further reveal the genetics of this complex trait. However, a limitation of this method is that it cannot detect ovules that contain both sexual and apomictic megagametophytes.

CONCLUSIONS

The buffelgrass genome map was shown to be a source of markers that can be used as MAS tools. Informative RFLPs were converted into RS-PCR markers capable of paternal-based hybrid confirmation and quantitative determination of sexual versus apomictic reproduction. The development of a gel-free method increased the high-throughput potential and cost-effectiveness of these markers for use in buffelgrass breeding programs.

CHAPTER IV

SATURATION OF THE *PApo1* REGION

INTRODUCTION

Apomixis, asexual seed production (Nogler, 1984), has the capacity to improve crop yields by producing genetically fixed F_1 hybrids (cf. Bashaw et al., 1970). Apomixis has been reported in more than 300 species within at least 35 plant families (Hanna and Bashaw, 1987), but more than 125 of these species are apomictic forage and turf grasses (Bashaw and Hanna, 1990). This bias towards grasses may be caused by pre-adaptive requirements for the evolution of apomixis that are prevented by ontogeny or reproductive barriers in most plants (Mogie, 1992). The distribution of apomixis is even further biased within the grass family. Of the five subfamilies within the Poaceae, approximately two-thirds of the apomictic genera are in the subfamily Panicoideae (Gould and Shaw, 1983; Carman, 1997). This subfamily includes such important crop species as maize (*Zea mays* L.), sorghum, sugarcane (*Saccharum* spp.), and pearl millet. Through comparative mapping, the buffelgrass map can be used to locate orthologous regions to *PApo1* in the genomes of these important grasses. The *PApo1* linked markers can also be used to screen germplasm collections of these species for genotypes with apomictic potential.

The presence of suppressed recombination near the *PApo1* locus in buffelgrass (Jessup et al., 2002) suggests that the 1.4 cM gap between the locus and flanking markers may be a large physical region (Fig. 6). Similar findings of suppressed

recombination have been reported from cytological (Gustafsson, 1946), genetic mapping (Grimanelli et al., 1998; Ozias-Akins et al., 1998; Noyes and Rieseberg, 2000), and *in-situ* hybridization (Goel et al., 2003) studies of apomixis. The probable cause for limited recombination near the apomixis region is its close proximity to the centromere (Bowers et al., 2003; Goel et al., 2003) on an inverted chromosome segment (Chittenden et al., 1994).

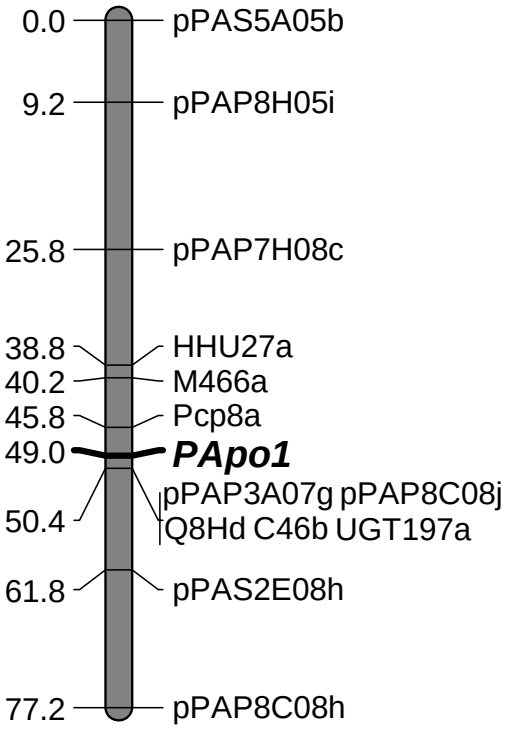

Fig. 6. Linkage group 7b in apomictic buffelgrass.

The buffelgrass linkage group containing *PApo1* (7b) shares synteny with sorghum linkage group D (Jessup et al., 2002). However, improved map resolution near *PApo1* is necessary in order to establish the microcolinearity required for comparative

map-based cloning efforts of the region in sorghum. To do so would require the identification of additional markers and recombination events near *PApo1*. The syntenic region of sorghum linkage group D (Bowers et al., 2003) is one source to obtain candidate markers for the *PApo1* region in buffelgrass. Use of the bulked-segregant-analysis AFLP (BSA-AFLP) methodology that produced markers linked to apomixis in *P. squamulatum* (Ozias-Akins et al., 1998) is also a source of candidate markers for the *PApo1* region in buffelgrass. Increasing the buffelgrass mapping population size could reveal hybrids that are recombinant for markers near *PApo1*. However, this approach may not be sufficient because of the suppressed recombination in the *PApo1* region. Association mapping has the potential to complement linkage mapping efforts in buffelgrass and identify recombination events that have occurred within the *PApo1* region over a long time period.

The objective of this research was fine-mapping of the *PApo1* region of buffelgrass linkage group 7b using: 1) candidate markers from the orthologous region of the sorghum genome, 2) candidate markers from BSA-AFLP analysis of buffelgrass, 3) an increased buffelgrass mapping population, and 4) association mapping.

MATERIALS AND METHODS

Candidate Sorghum Markers

The following 22 markers within the 3.1 cM (56.2-59.3 cM) region surrounding pPAP3A07 (57.7 cM) on the sorghum map (Bowers et al., 2003) were analyzed on the buffelgrass mapping population: CDSC05, CDSR084, CDSR046, CDSR063, CSU034, pSB0314, pSB0747, pSB1343, pSB1847, pSB0161, pSB0866, pSB1450, pSB0520,

PRC1096, PRC0162, PRC0219, PRC0090, PRC0185, PRC0247, PRC1132, PSHR0063, and RZ782. RFLP analyses followed the methods described by Jessup et al. (2003).

Candidate BSA-AFLP Markers

A total of 18 cloned AFLPs previously isolated by bulked-segregant analysis between apomictic and sexual buffelgrass (Yang and Renganayaki, unpublished data) were analyzed. Plasmid DNAs were prepared with a Qiaprep miniprep plasmid DNA kit (Qiagen, Valencia, CA) using the manufacturer's protocols. Cycle sequencing with ABI (Columbia, MD) Big Dye, followed by analysis on an ABI 3700, was performed for both forward and reverse primers according to the manufacturer's protocols. Primers were designed for each clone using the internet-based software 'Primer3' (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) based on the criteria of 50% GC content, minimum melting temperature of 50° C, absence of secondary structure, oligo length of 20-27 nucleotides, and product length of 100-400 bp (Table 5). Primers were commercially synthesized (MWG-Biotech; High Point, NC). The markers were designated 'PCAB,' for *Pennisetum-ciliare*-Apomictic-Bulks.

PCR reactions were conducted in a total volume of 20 uL, using 50 ng of buffelgrass DNA, 1X Promega $MgCl_2$-free PCR Buffer, 2.5 mM $MgCl_2$, 0.2 mM dNTPs, 2 mM of each primer, and 1 unit of Promega Taq polymerase. The PCR method included: 1) an initial denaturation at 95° C for 3 min, 2) 10 touchdown decrement cycles at 95° C for 25 sec, 64-55° C for 25 sec, and 70° C for 45 sec, 3) 36 cycles at 95° C for 25 sec, 55° C for 25 sec, and 70° C for 45 sec, 4) an elongation cycle at 70° C for 10 min, and 5) a final hold at 4° C. Electrophoresis was run using a MEGA-GEL High Throughput Vertical Unit (C.B.S. Scientific, Del Mar, CA) as described by Wang et al.

(2003). Allele bands were distinguished from minor bands that occur in nondenaturing polyacrylamide gel electrophoresis as described in Rodriguez et al. (2001) in order to prevent scoring errors.

Table 5. PCR Primers for PCAB clones.

PCAB	Forward Primer	Reverse Primer	bp
1	TTTGGAACATTGCATCAAGA	CTGCGTACCAATTCACACTGA	241
2	CAGGATAATTTTCCAGTTGACAAG	ACCAATTCACTCGTGCAAAA	151
3	CAGGTGAATGAGGAAATGGAG	TCAGTTGGCTGCTTGAGTGT	330
4	GCTAGCACGTCCGAAAAAGT	GCATCAAAATCGGAAGGAAA	236
5	GGGTCCCCAAGGTATAATCAA	GTACCAATTCAGGGCGTGAC	215
6	CCTGAGTAACTCCTGATGAGTCC	TGTTCGGTTCTTGGCTTTCT	111
7	TCGAAGCTCTCTCGATGATG	CCGCCTTCTAAGTTCATTGAC	250
8	AGAGATCTTGGCCTTGAGCA	AACACATGGCAAGCGGTATT	157
9	GCTAAATTGTTTGCTCTCAAGATG	CCCATCCCACAGGTAGAGTG	227
10	TTCGAAATCGCATAGGTGAG	GAGCCTTTCTTTATTTACCCAGTG	211
11	TTCAGCCTTCCCACAATTTC	CGGTGGAAGAAGAGATGGAG	321
12	GAACAAGGGACTATGTGGTTCA	CTCGATGGCAAGTGTCACAA	211
13	AAGCAAAGACAGGGTGATGC	TGAGTCCTGAGTAACTCCAATGTT	222
14	ACAACGAACTACTAATTGCTTGATT	TCACGCTAAAATGACGAACC	112
15	GAACTCCACCATGGCTTCAC	AAGCAAAGATCTCATGCAAGG	234
16	GGGAGGTAAACTGGACTTCG	TCCTGAGTAACTCCTAGAGCACAA	129
17	AGGCACAAGAGCACAAGAAAG	GATGAGTCCTGAGTAACTCCTTG	130
18	GGCAATGAAAGATAAGTTCATGTC	TTCCGAACTAGCAGTAGATCA	106

Mapping Population Size

An additional 120 F_1 hybrids were added to the buffelgrass mapping population (Appendix E). Phenotype analysis followed the methods in Jessup et al. (2002). RFLP analysis of the increased mapping population was performed using *PApo1* linked markers pPAP3A07, pPAP8C08, Q8H, C46, and UGT197) as described by Jessup et al. (2003).

Association Mapping

A total of 171 apomictic buffelgrass plant introductions (PIs) (Appendix F) for which euploidy (2n=4x=36) had been confirmed with flow cytometry (B.L. Burson, unpublished data) were analyzed. DNA extraction, Southern blotting, and RFLP methods were performed according to Jessup et al. (2003). Nine markers within five cM of *PApo1* (P8C08, P3A07, Pcp8, OPC4, QH8, UGT197, PCAB5, PCAB10, and PCAB13), were analyzed across the population. Five unlinked RFLPs and five unlinked EST-SSRs were randomly selected from the buffelgrass genome map and used to test for population structure as described in Pritchard and Rosenberg (1999). Similarity of marker distribution was calculated by distance analysis between binary variables (simple similarity) using SPSS® software (München, Germany). Marker genotype classes were tested for association with phenotypic scores with the non-parametric Mann-Whitney-U test using SPSS® software (München, Germany). The null hypothesis of no association was rejected at $P < 0.05$.

RESULTS AND DISCUSSION

Two of the 22 candidate markers from sorghum linkage group D mapped to buffelgrass linkage group 7b (Fig. 7). Both markers were distal to the closest flanking markers of *PApo1*, with pSB1817 at 54.3 cM and pSB1312 at 57.9 cM. The comparative mapping approach placed markers onto linkage group 7b more efficiently than randomly surveying molecular markers. However, marker resolution near *PApo1* was not improved. The finding that most sorghum markers did not map to buffelgrass linkage group 7b also suggests that synteny in the *PApo1* region is limited between

sorghum and buffelgrass.

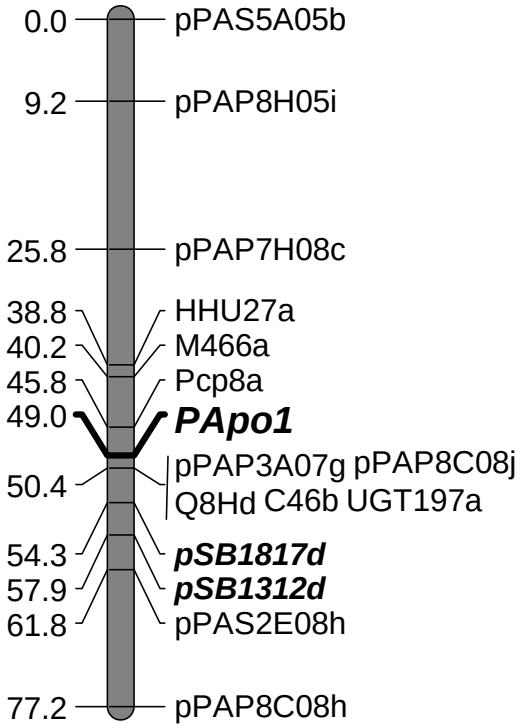

Fig. 7. Syntenic sorghum marker placement on buffelgrass linkage group 7b.

Six of the PCAB markers (PCAB1, PCAB3, PCAB4, PCAB9, PCAB12, and PCAB17) produced no polymorphisms across the parental plants or the mapping population. Five additional copies of these clones were sequenced to investigate the possibility that multiple AFLP fragments were cloned. No additional sequences were found, suggesting that the correct BSA-AFLP fragment was not recovered from the original gels for these markers. Nine of the PCAB markers (PCAB2, PCAB6, PCAB7, PCAB8, PCAB11, PCAB14, PCAB15, PCAB16, and PCAB18) were polymorphic between the sexual and apomictic parents but not across the mapping population. These

markers are probably from hemizygous genome regions that do not occur in the sexual parent's genome. The three remaining PCAB markers (PCAB5, PCAB10, and PCAB13) mapped to buffelgrass linkage group 7b and were closely linked to *PApo1* (Fig. 8). However, these markers cosegregated with five other markers at 50.4 cM and did not improve marker resolution of the *PApo1* region.

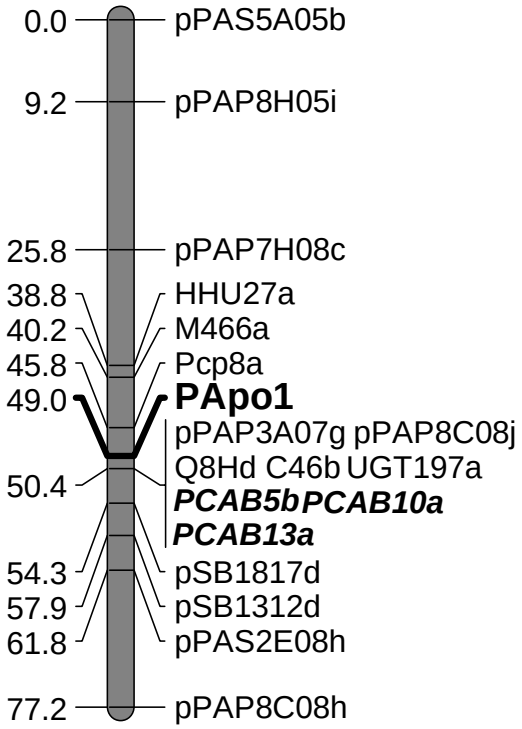

Fig. 8. PCAB marker placement on buffelgrass linkage group 7b.

Marker resolution near *PApo1* was not improved by increasing the size of the buffelgrass mapping population. The markers closely linked to *PApo1* also were not separated from one another. This confirms that recombination is suppressed near *PApo1* and screening additional hybrids would not be effective.

Population stratification was detected across the entire collection of 171 buffelgrass PIs. Removal of the 21 PIs from Morocco (2), Ethiopia (2), Kenya (2), Australia (5), India (5), and Pakistan (5) from the data set was required to prevent spurious associations between *PApo1* and unlinked markers. Population stratification was not detected across the remaining 150 PIs from South Africa (140), Tanzania (5), and Zimbabwe (5). Association tests of Pcp8 and *PApo1* across this population were not significant (Table 6). This indicates that linkage disequilibrium (LD) was not maintained over the 3.2 cM interval between *PApo1* and Pcp8. The 3.2 cM interval may therefore be a large physical distance. Association tests between the other eight *PApo1*-linked markers and *PApo1* were highly significant (Table 6). LD was maintained over the 1.2 cM interval between these markers and *PApo1*. The failure of association mapping to separate these eight cosegregating markers could indicate that they are in close physical proximity to one another as well as to *PApo1*. Alternatively, the results could indicate that recombination is suppressed in this genomic region to an extent that it will complicate comparative map-based cloning efforts between buffelgrass and sorghum.

Table 6 Association tests between marker classes and apomixis.

Marker	P ($\alpha \leq 0.05$)
pPAP3A07	0.001
pPAP8C08	0.003
Q8H	0.003
C46	0.004
UGT197	0.009
PCAB5	0.001
PCAB10	0.002
PCAB13	0.003
Pcp8	0.487

Conclusions

Several new markers linked to *PApo1* were identified. However, new recombination events that would decrease the genetic distances between markers in the region were not detected. The LD found near *PApo1* could be partially explained by apomictic reproduction because meiosis is bypassed. Apomictic reproduction is a barrier to meiosis and recombination events that diminish LD. The founder effect resulting from the evolution of apomixis (Stebbins and Babcock, 1939; Stebbins, 1950) also increases LD throughout a species' genome. The level of LD in buffelgrass is greater than that of arabidopsis (*Arabidopsis thaliana* L.) (Nordberg et al., 2002), maize (Tenaillon et al., 2001) and sorghum (Hamblin et al., 2004), while less than that of sugarcane (Jannoo et al., 1999). This indicates that possible founder and bottleneck effects in the evolution of apomictic buffelgrass are not severe. Association mapping of additional buffelgrass PIs in the future may be successful in delineating the cluster of *PApo1*-linked markers.

CHAPTER V

CONCLUSIONS

This molecular investigation has produced information and valuable tools for studying buffelgrass, perennial forage grasses, and other apomictic species. The buffelgrass genome map contains 851 markers that can be used to characterize the genomics of traits of interest. This map includes information regarding chromosome pairing (disomic versus tetrasomic) that can be used to design appropriate breeding methods for the inheritance of traits of interest. Markers from the map were used to develop high-throughput methods to identify hybrids and quantify apomictic reproduction. Additional markers have been mapped near the *PApo1* locus, and suppressed recombination in this region was moderate yet potentially breakable. Continued development of the buffelgrass genome map will facilitate the production of additional MAS tools for breeding buffelgrass and other perennial forage grasses, as well as achieving the microcolinearity required for map-based cloning of apomixis.

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APPENDIX A

PPAS	Expression	Polymorphism	PPAS	Expression	Polymorphism
1A08	LC	R H X	**3B01**	LC	H X
1A09	LC	R H	**3B05**	LC	R H X
1A11	MC	R H X	**3B07**	MC	R H X
1B01	LC	R H X	**3B08**		NP
1B08	LC	R H X	**3B10**	LC	X
1B11	LC	R H X	**3B11**	LC	R H X
1C03	LC	R H X	**3B12**	LC	R H X
1D02	LC	H	**3C08**	MC	R H X
1D03	MC	R H X	**3C12**	LC	R H X
1D09	MC	R H X	**3D03**	LC	R H X
1E07	LC	R H X	**3D04**	LC	R H X
1E08	LC	R H X	**3D05**	MC	R
1E10	LC	R H X	**3D09**	LC	R H X
1F03	LC	R H X	**3D10**	LC	R H X
1F07	LC	R H X	**3D11**	LC	R H X
1G01	LC	R X	**3E03**	LC	H X
1G04	LC	R H X	**3E07**	LC	R H X
1G08	MC	R H X	**3E10**	LC	R H X
1H01	LC	R H X	**3E12**	LC	R H X
1H08	MC	R H X	**3F02**	LC	R H X
1H10	MC	R H X	**3F04**	LC	R H X
1H11	MC	R H X	**3F10**	MC	R H X
2A03	MC	R H X	**3G01**	LC	R X
2A11	MC	R H X	**4A05**	LC	R H X
2B02	LC	R H X	**4B01**	MC	R H X
2B08	LC	R H X	**4B02**	LC	R H X
2C04	LC	R H	**4B03**	LC	R H X
2C06	MC	R H X	**4B10**	LC	R H X
2C07	LC	R H X	**4C03**	MC	R H X
2C09	LC	R H X	**4D04**		NP
2C10	MC	R X	**4D05**	LC	R H X
2C12		NP	**4D08**	MC	R H X
2D05	MC	R H X	**4D09**	LC	R H X
2D06	LC	R H X	**4E01**	MC	R H X
2D08	LC	R H X	**4E02**	MC	R H X
2E08	MC	R H X	**4E07**	MC	R H X
2E09	LC	R H X	**4E11**	MC	H X
2F02	MC	R H X	**4F03**		NP
2F06	LC	R H X	**4F04**	LC	R H X
2F11	LC	R H X	**4F08**	LC	R H X
2G04	MC	R H X	**4F11**	MC	R H X
2G08	LC	H X	**4F12**	LC	R H X
2G11	LC	R H X	**4G02**	MC	R H X
2H02	LC	R H X	**4G03**	LC	R H
2H04	LC	R H X	**4G04**	LC	R
2H07	LC	R H	**4G06**		NP
2H11	LC	R H	**4G08**	LC	R H X

PPAS	Expression	Polymorphism	PPAS	Expression	Polymorphism
3A04	LC	R H X	**4G10**		NP
3A08	LC	H	**4G11**		NP
3A09	LC	R H X	**4G12**	MC	R H X
3A10	LC	R H X	**4H01**	MC	R H
4H02	LC	R H	**5G12**	MC	R H X
4H05	LC	R H X	**5H05**	MC	R H X
4H10	MC	R H X	**5H06**	MC	R H X
4H11	MC	R H X	**5H10**	MC	R H X
4H12		NP	**6A04**	LC	R H X
5A03	MC	R H X	**6A06**	MC	R H X
5A05	LC	R H X	**6A08**	LC	R H X
5A06	LC	R H X	**6A12**	LC	R H X
5A08		NP	**6B01**		NP
5A10		NP	**6B03**	LC	R H X
5B02	LC	R H X	**6B06**	MC	R H X
5B03		NP	**6B08**	LC	X
5B10	MC	R H X	**6B11**	LC	R H X
5C04	MC	R H X	**6B12**	LC	R H X
5C05	MC	R H X	**6C07**	MC	R H X
5C06		NP	**6C09**	LC	R H X
5C10		NP	**6C11**	MC	R H X
5C11	LC	R H X	**6D08**	LC	R H X
5D07		NP	**6E01**	MC	R H X
5D12	MC	R H X	**6E03**	LC	R H X
5E07	LC	R H	**6E10**	LC	X
5E11	LC	R H X	**6E12**	MC	R H X
5E12	MC	R H X	**6F02**		NP
5F01	MC	R H X	**6F11**	LC	R H X
5F02	LC	X	**6G05**	LC	R H X
5F03	LC	X	**6G08**	LC	R H X
5F04	LC	X	**6G10**	LC	R H X
5F09		NP	**6G11**	LC	R H X
5F10		NP	**6H02**	LC	R H X
5F11	LC	R H X	**6H03**	LC	H
5F12	MC	R H X	**6H06**	LC	H X
5G02	LC	R	**6H07**	LC	R H X
5G03	LC	R H	**6H08**	LC	R H X
5G07	LC	X	**6H12**	LC	R H X
5G08	MC	R H X			

LC = low copy (<5 bands); MC = multiple copy (>4 bands)
NP = no polymorphism
R = EcoRI; H = HindIII; X = Xba1

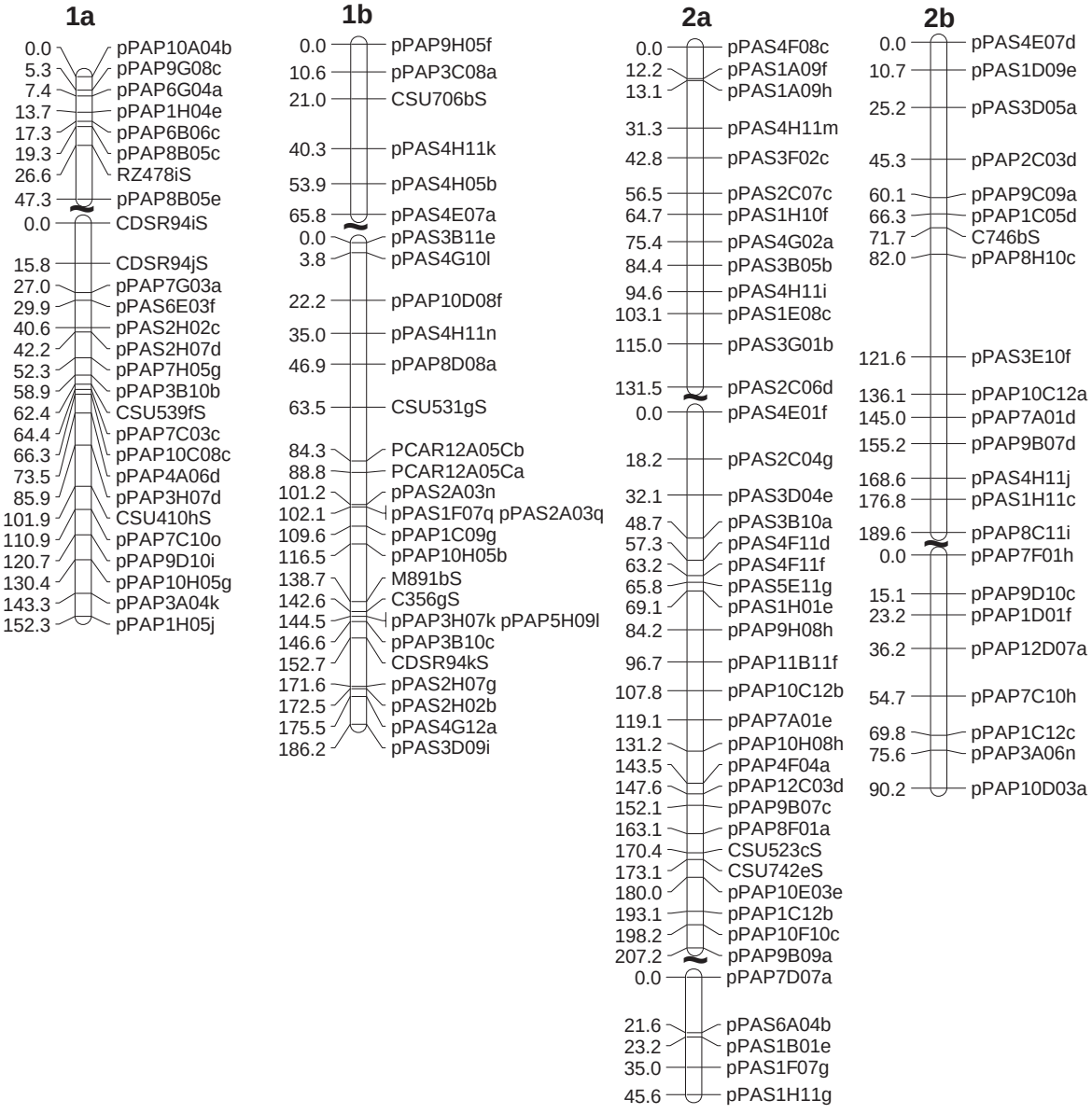
APPENDIX B

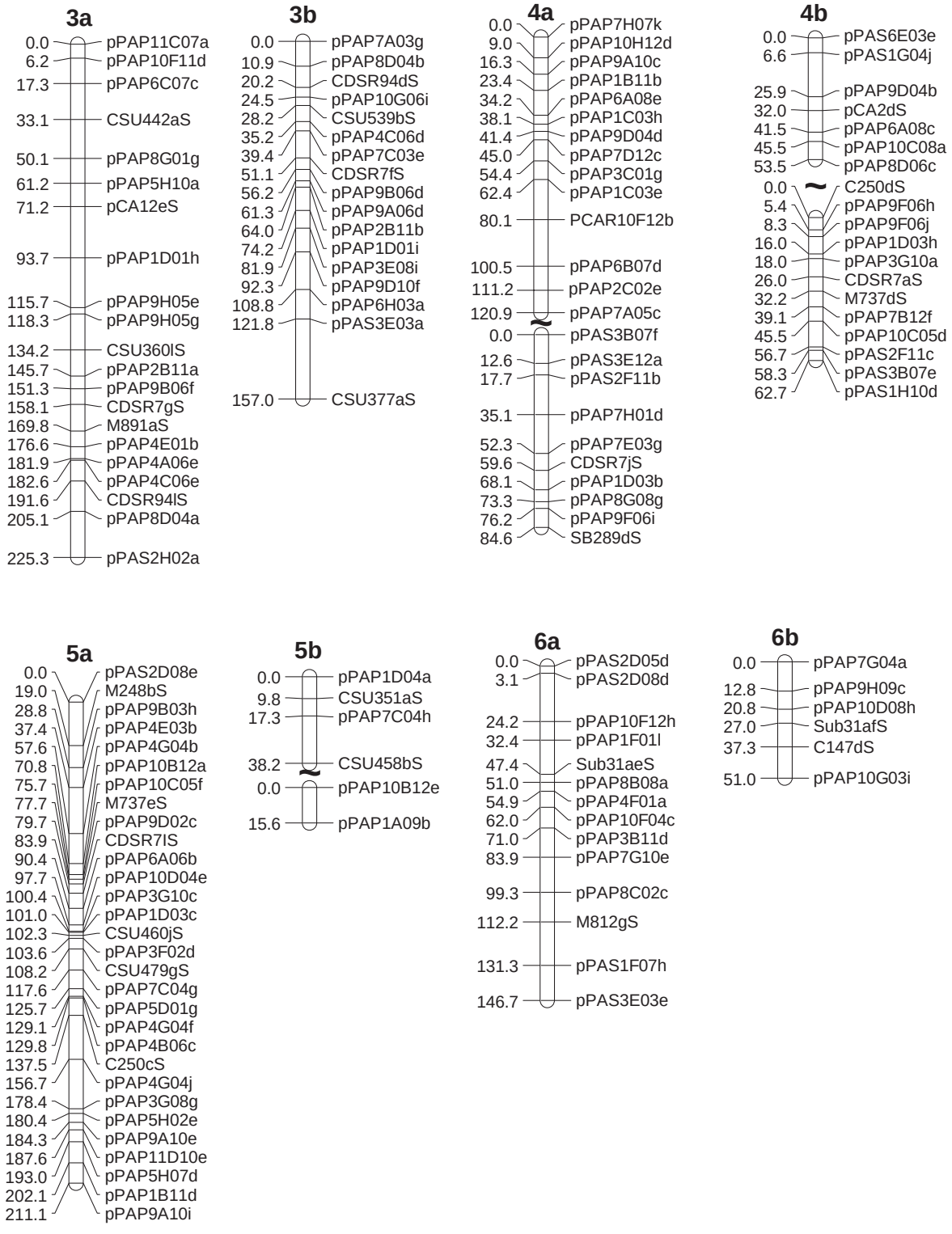
PPAP	Repeat	Left Primer	Right Primer	Size bp
1D08	CG	CGATCGAACGCTAAATCCTC	GACTGGGAGGTGGACGAC	266
1G08	CCG	CTTTCTCCCTCCTCCCATTC	CCAGCACCAGTCTCACTGAA	236
1H07	AT	CAATGTTGCGTAAACAGATGG	CGAGTGAGGGTTTGACGAAT	166
2C10	AC	GAAAGGGAAGAAACAAACAA	ATCCAGAGGTGATGAAGATG	188
2D03	GC	ATCGTCTGTCCGTCCTGGT	TAGCCCTGTCTCCCAACGCC	211
2D03	AT	CAATGGGAGCTCAAATTAGCA	CGGGGAAGAAGTTTGTTCTTT	250
2D09	AAC	CAAATCGGAGCAAATCGG	AGGAAAGCCTCGGGAAAC	358
2D11	GA	CACACACCACACTCGTCA	CAGGCACGGCATCAGGGT	222
2F12	AG	ATCGTGTCGAGGATGAGGAT	AGTGGTCTCATTTCCCCAAA	152
2G07	GT	GTAGCCGTGAACGTTGGAAG	TCGGCGATTCTCCAACAC	226
3A04	AC	TTCAAGTTTTGCCAATGCAG	CAAGAGCGTGGAGAAGAAGG	249
3E04	TA	AATGGCATATTGGCTTCCAG	CAGGATCGTGGAATGCTTTT	181
3E06	GCT	GCAGAACTCCATCTCGCT	CTCTCCTCCACCAATCATC	390
3F01	TCG	CAAATACTCAAATCCATCCAC	CTTCACCGTCGTGCTCTT	274
3H03	CT	ACCCCACAAAACGTGTCACT	CCATTAGGACTATAGACAGTCGTTG	207
4C12	AGCC	CATCCCTCATTTCACAACTAC	CACCAACAACTACGCCTT	118
4D10	CGG	TAGCCGACCAGACCCTCTC	ACGCCGACACAGGAAGCAC	311
4E06	TGG	GGTGCTCACAGAAACCACAA	GACCACCACCACGGTAGAAG	169
4H08	GCTCT	TTTATTCCCATAGCATCTTTAC	CCCTTCTCGTTCTTCTTCTT	274
5A01	CCA	AGCCAGATACGAGCGAAG	GTCTAATCAAGGTGGTGGTG	394
5A09	TCG	TAATCAATCAGCAGGAGCA	GGAACGAAGCAGAATCAGT	388
5B01	TGC	CATAAACACCGAATGAGAGAA	CAGAGGAAGAGGATTGAGTAG	284
5B06	GCG	TTATTTGTCTCCACCCTCC	GGAAGAATGACCAGAGCC	356
5D11	CA	TCTCTACCACAGCTACATGCAA	GTGGAACTCGTTCGGTTGAT	172
5E03	CAG	ATAGAACTGAAGGCACACAAA	GAAGTCAAGAACGCACCTC	343
5F02	CT	CCCGAGCACAATCCATTATC	AACCCGATATCAAGCGTCTG	231
6B03	GCCC	CACCCGCCAATCCGATAA	AGCTCCGAGAGGGTGAGAG	245
6B10	TG	ATAGTGAAATGAGTTGTCGGG	GGAAGAGGTGTAGGGTAAGAG	335
6C03	CTTC	CGAAACAAAGATGGACAGA	GAACTCAACATAGACGCACC	235
6E03	GGT	TGTAATCCAACCAACCAAA	ATCCCGCACCCGCAGTTC	371
6E10	GCC	ACTCCACTGCTGCCTCCT	CTTCCACCACCATACCCT	389
6H06	TACAC	TTACAGGGAGAGTCCAGAAAG	CGTGTGTGTTTGCCAGTT	355
7A02	CG	CGGTGTAGAGGAGGAAGTCG	AGCTGAGGAAAGTGGAGCTG	238
7A04	AGCA	GGGTAGAGGGAGAGAGGTT	AGATTTAGGAGGTCAGGAATG	175
7B11	GA	CTCCATTCGCCTCCCTAC	GTTTCGTCTCTCCCATCAG	391
7E09	CGG	GGAGGTAGATGTTGATGTTGA	CCCTTTGTCCCGCCATAC	360
7H12	CT	TCTTATTCCTCCGAGCCGTA	GGAAAATTGGGACCCTTTGT	182
8E03	CG	GTGGTGGAGACGTCCATCTT	CAGCACCTTGTTGAGCTCCT	240
8F05	AT	ACCTGTCCATCTGTTTACCTT	CAGCCATCTCCTTATTTCAGT	345
8G06	GCA	CCTCCTGGTGATGATGAA	AGTGGCTGATTATGGTGCT	281
9D08	AC	GGTCTCCGCATTACTTATTT	GCCAACAGCAACTACCAG	130
9F06	TGT	GTGAACAAGACGAAAGGAGA	ATCTACGCAAGAAGGAGACC	392
9G12	CGC	CGTCCCTCAAGGTGAACAAT	CGGCGAAGAGGTACAGGTC	194
9H01	TA	CGAAGGAGTTGGACTTGG	GTGGACTGAACACAAACGA	215
10E12	GT	CTCTTGAACCCCGAGGCTAT	ATCTCGCGTCATCGTTTAGG	196
10F12	TC	ATGACCCAAACGGGGTAAAT	TTTTCTCCTCCACTCGCTGT	250

PPAP	Repeat	Left Primer	Right Primer	Size bp
10G10	TGAC	AAGAAGAAGAAGAAGAAGAAGGA	GGAAGAGGAGACCAACAAA	193
10G10	AAG	GTGTTGTCTGGCTGGCTT	GCCGAGTAGGTGTCAGTC	213
10H10	CGA	CGACTCAGACCACCTCTC	GGCTCCCAGTTCTTCATC	307
11A08	CT	CGCAGACGCATTTCTCTC	CTCCTCCGCCCTCTCCAGT	268
11A12	GAG	GTGCCCATCAAGTCCAAG	TTCTTCCTCCTCGCTCTC	294
11E04	TA	TACTCCATCAGCACCTCC	CAAGTCTCCTCGGTTCCA	264
11E09	GGT	ATCTGACTTGCTTGGCGGT	AATCTCCGAATCCTCCTG	158
12A05	GA	GGGAGAGAGAGGAGGAGGTG	CGCTCTCCCTTTTCTGCTG	160
12A05	AG	AGAGCAGCAGAAAAGGGAGA	CGCCTCTGTCTATCCTCGTC	216
12A05	GC	CTCCAATCCATCCATCCATC	CTCCTCAGGTCGTTCTCAGC	197
12A05	AG	AGAGAAGTCCGAGCAGCAAG	TCTCGCTCTCTCCTCTACGC	238
12E02	GGC	CACTCCTCATCCATTCCTC	GCTCCTCTTCCTCGGGTC	311
12E10	CCG	TCTGACCTCTCCTCTCCTTC	AAGCCCTCCTTTCTTCAC	244
12F11	GCG	GGAACCCTAACTGGGAAG	CAAGGTGTGAAAGCAGAAG	173
12H03	CT	ATCACCGACAGCAACAAA	GGATGGATTGGGAGAGAC	194
12H04	CGC	AGTGGAAGAACTGAAGCC	GTAGTAGAGGTGACGGATTGT	225
13A04	TTCTA	GACCGATTCCAATTCCGTTA	TGAAAATTAGGTCCGCTTGC	234
13A07	AC	ATTGTTGTTCCATACCGCC	CGCACGCAGAAGATAAAG	322
13A12	ATCC	CAGAGAACAAGTAGAGGGTGA	CAGGTTTCCAATCTTACGG	228
13B03	GAC	CTGACTCTGACACAACACACA	ACTCCGATGGTGGGAACT	188
13B10	GCC	TAGGACGACACACAGGAATAA	GGGATGAACGGCAAGGAG	359
13C01	AC	CCAAAGAATAATAGGAATCAACT	TCATCAGTAGCACAGCAATAA	169
13C10	GCT	TAGTTACACCCTTTCTTTGGT	CATTTCCTCCGCCACCTC	333
13C11	CA	TGCAATATACATTTATTTTTGCTGAA	CCGCATATCGAAACGGTACT	156

APPENDIX C

Linkage Map of the Maternal Parent: 90C48507

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93.7 pPAP1D01h
115.7 pPAP9H05e
118.3 pPAP9H05g
134.2 CSU360lS
145.7 pPAP2B11a
151.3 pPAP9B06f
158.1 CDSR7gS
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191.6 CDSR94lS
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225.3 pPAS2H02a
3b
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20.2 CDSR94dS
24.5 pPAP10G06i
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35.2 pPAP4C06d
39.4 pPAP7C03e
51.1 CDSR7fS
56.2 pPAP9B06d
61.3 pPAP9A06d
64.0 pPAP2B11b
74.2 pPAP1D01i
81.9 pPAP3E08i
92.3 pPAP9D10f
108.8 pPAP6H03a
121.8 pPAS3E03a
157.0 CSU377aS
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9.0 pPAP10H12d
16.3 pPAP9A10c
23.4 pPAP1B11b
34.2 pPAP6A08e
38.1 pPAP1C03h
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45.0 pPAP7D12c
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62.4 pPAP1C03e
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17.7 pPAS2F11b
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52.3 pPAP7E03g
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8.3 pPAP9F06j
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125.7 pPAP5D01g
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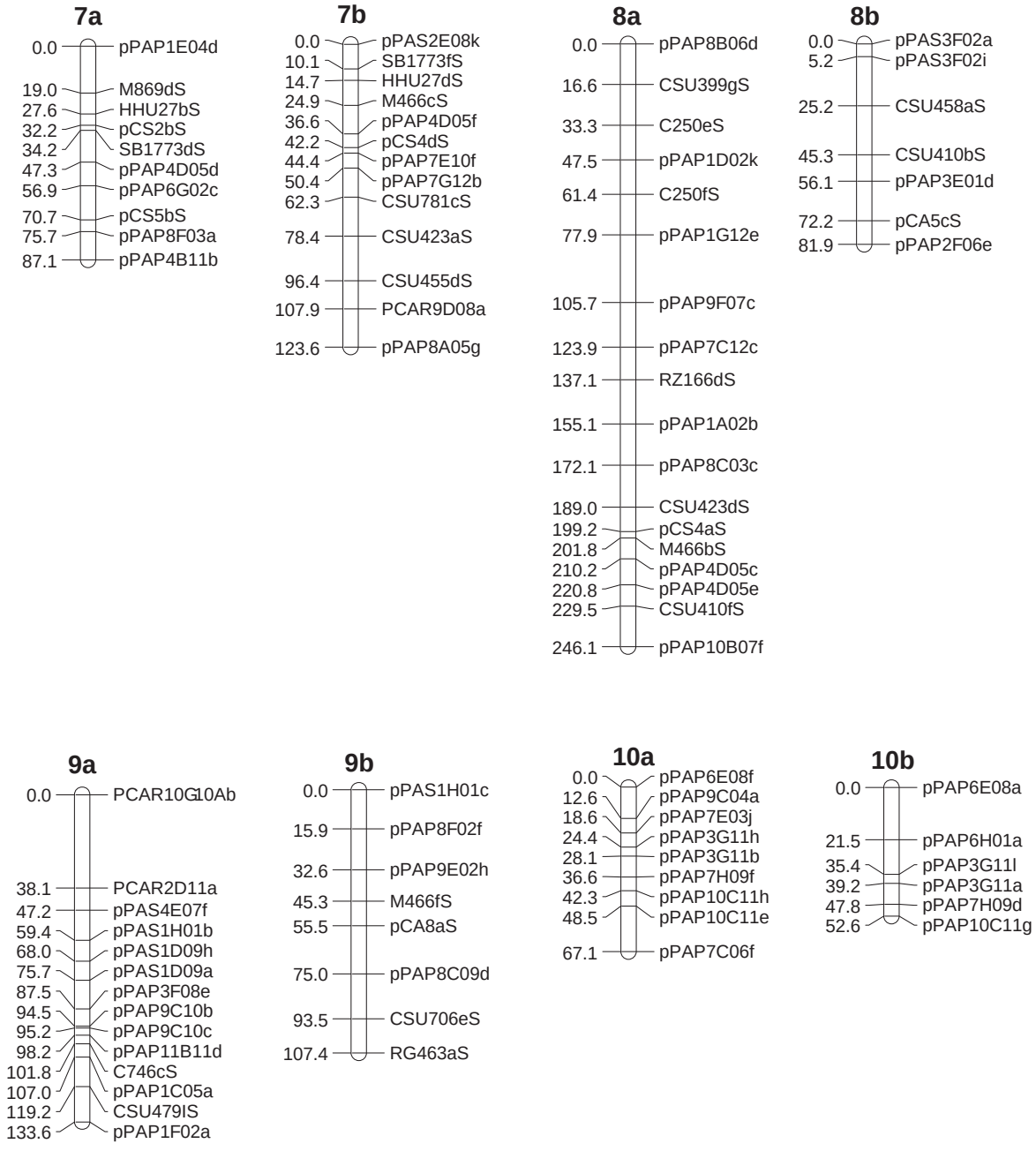
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8a
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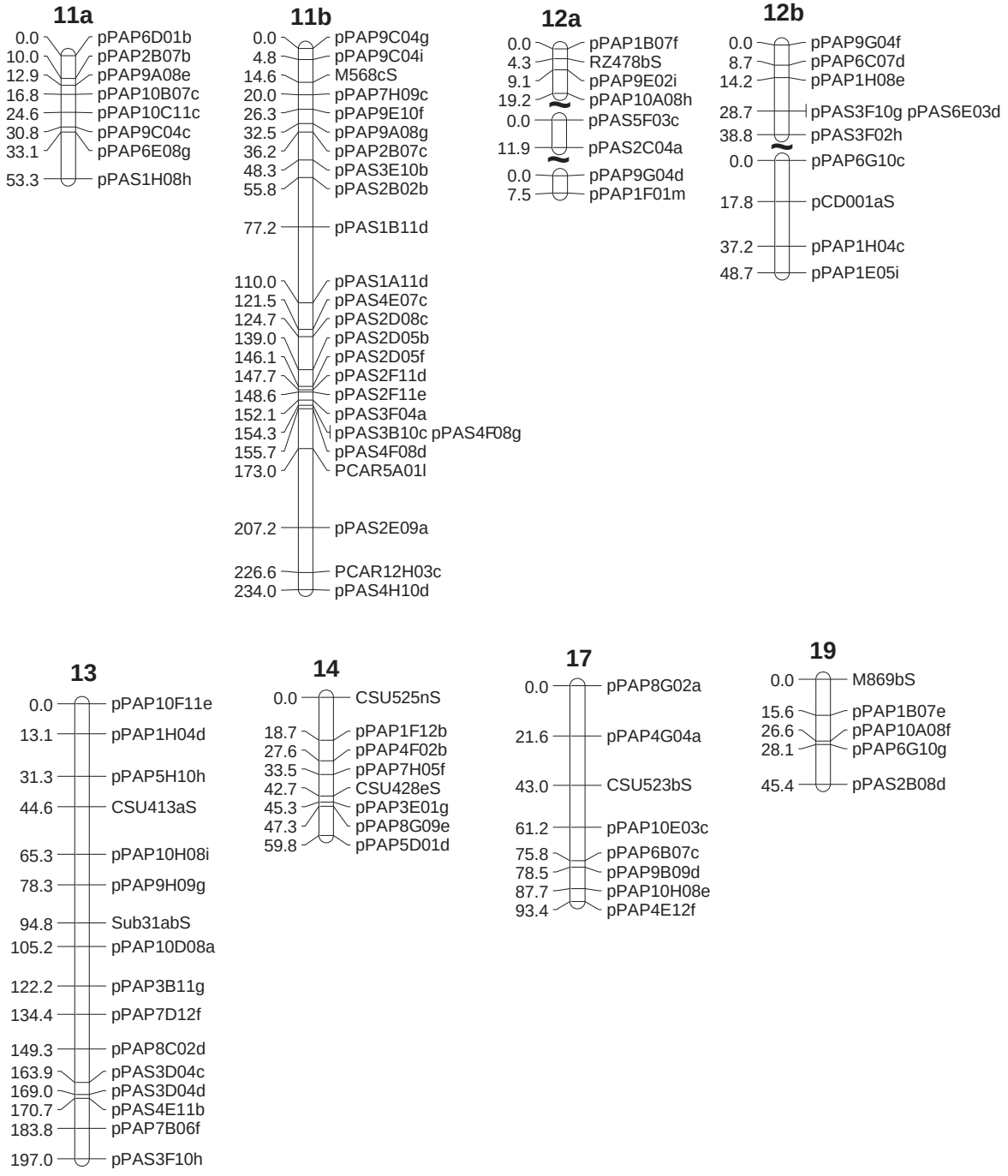
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11b
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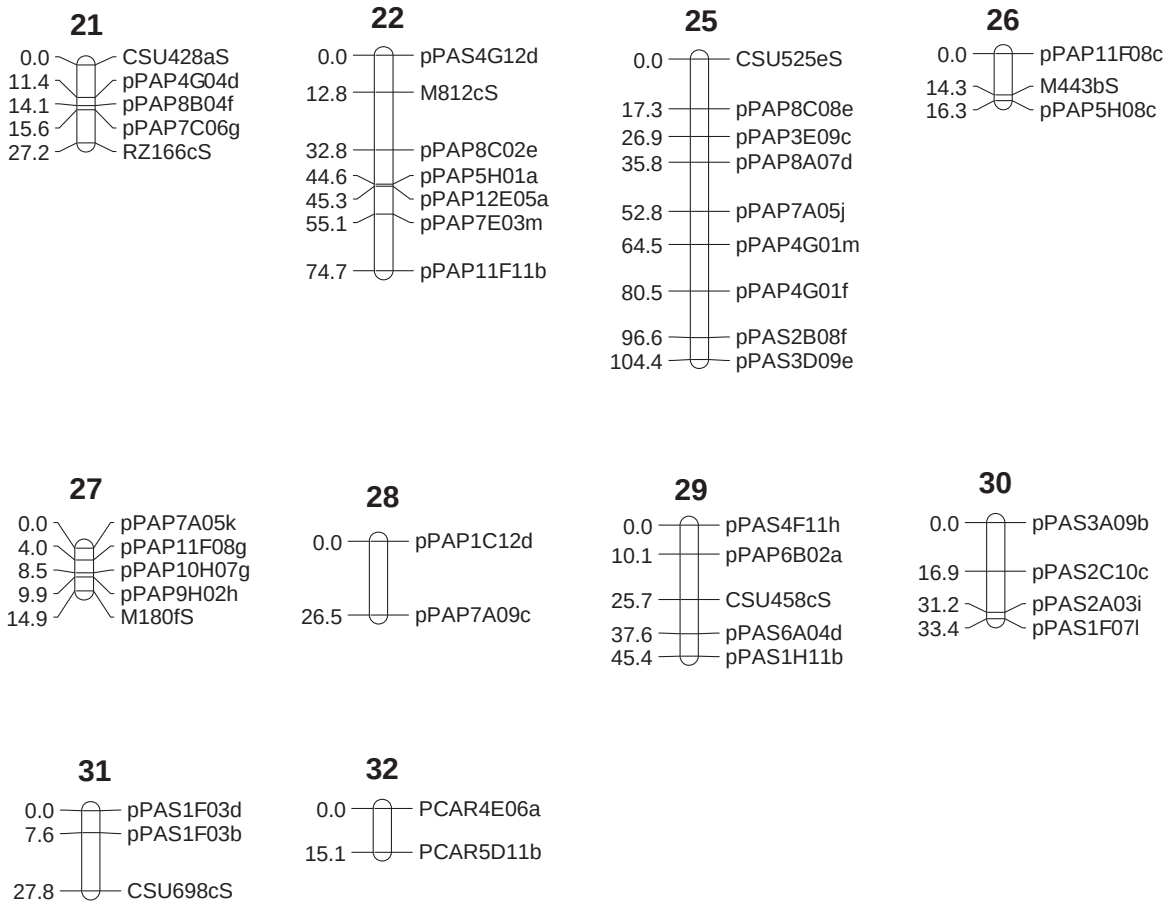

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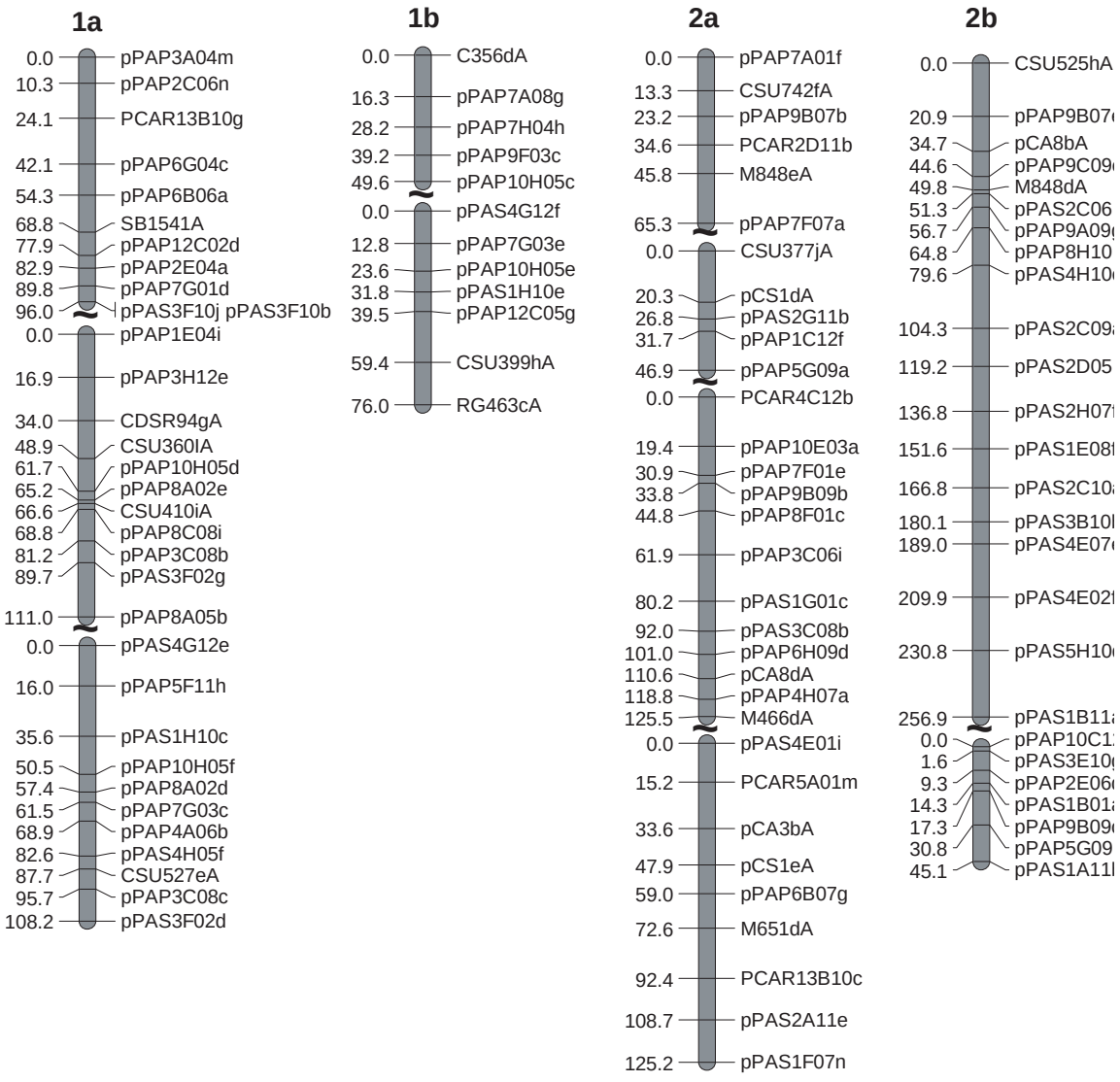
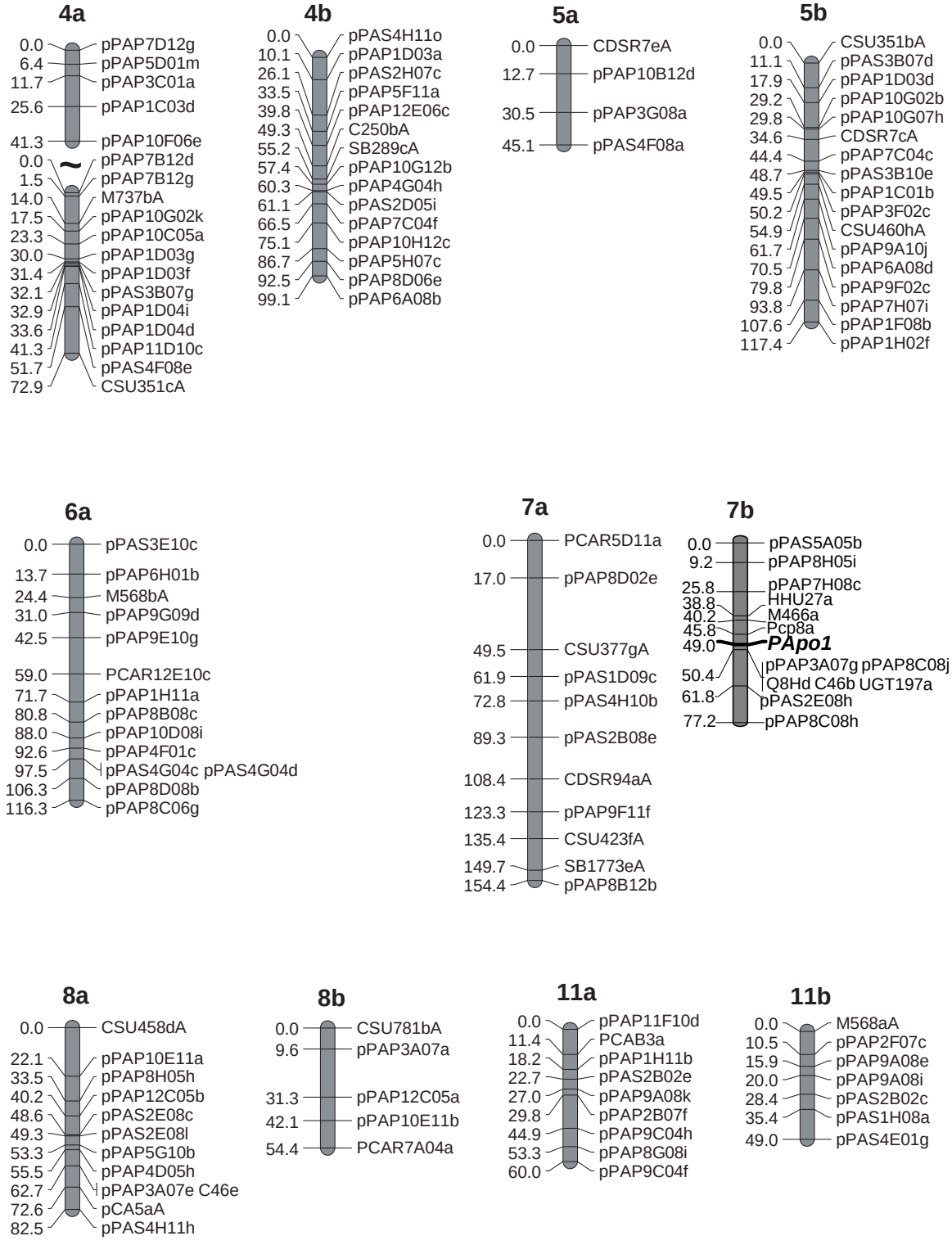
APPENDIX D

Linkage Map of the Paternal Parent: PI 409164

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17.5 pPAP10G02k
23.3 pPAP10C05a
30.0 pPAP1D03g
31.4 pPAP1D03f
32.1 pPAS3B07g
32.9 pPAP1D04i
33.6 pPAP1D04d
41.3 pPAP11D10c
51.7 pPAS4F08e
72.9 CSU351cA
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26.1 pPAS2H07c
33.5 pPAP5F11a
39.8 pPAP12E06c
49.3 C250bA
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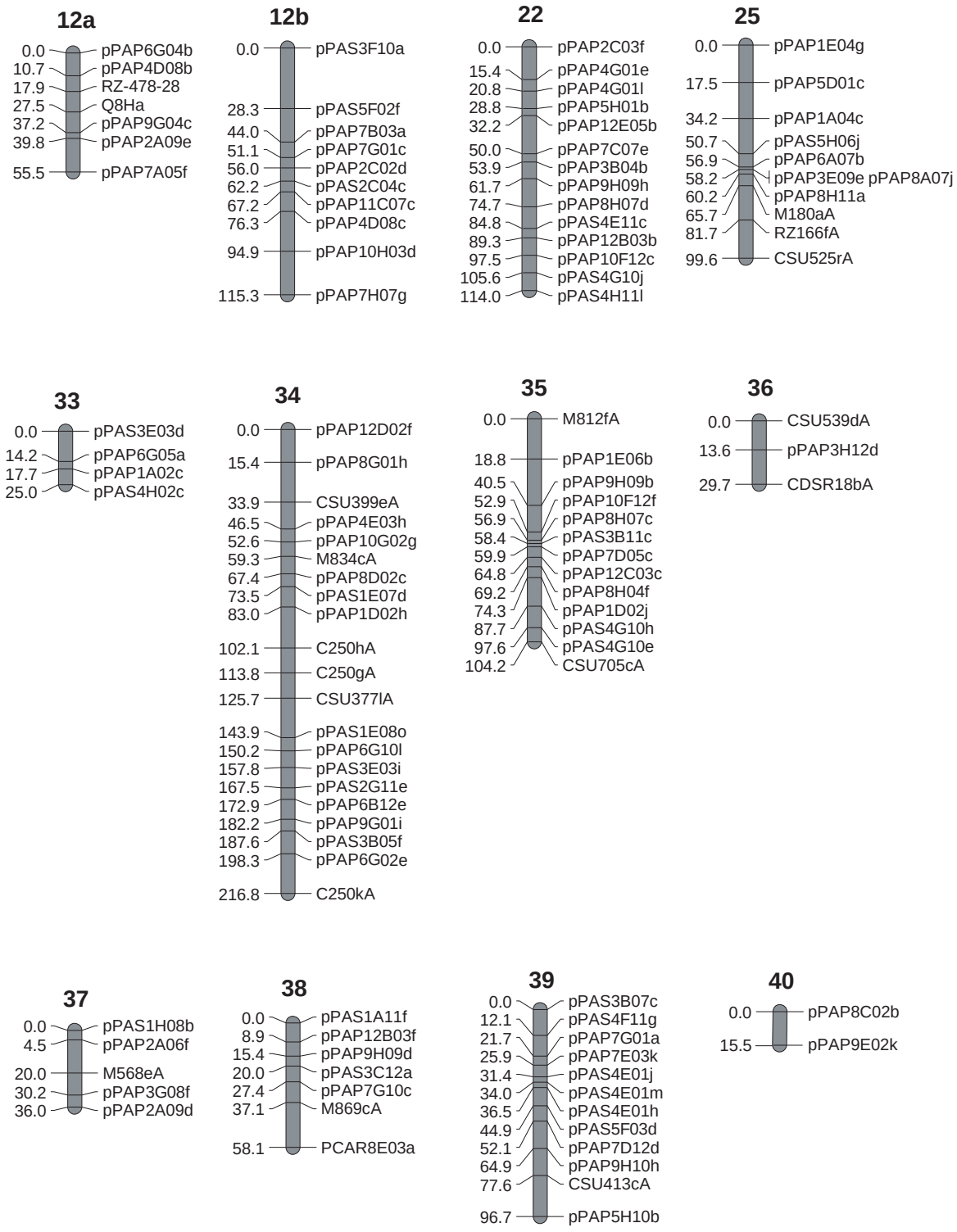
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59.3 M834cA
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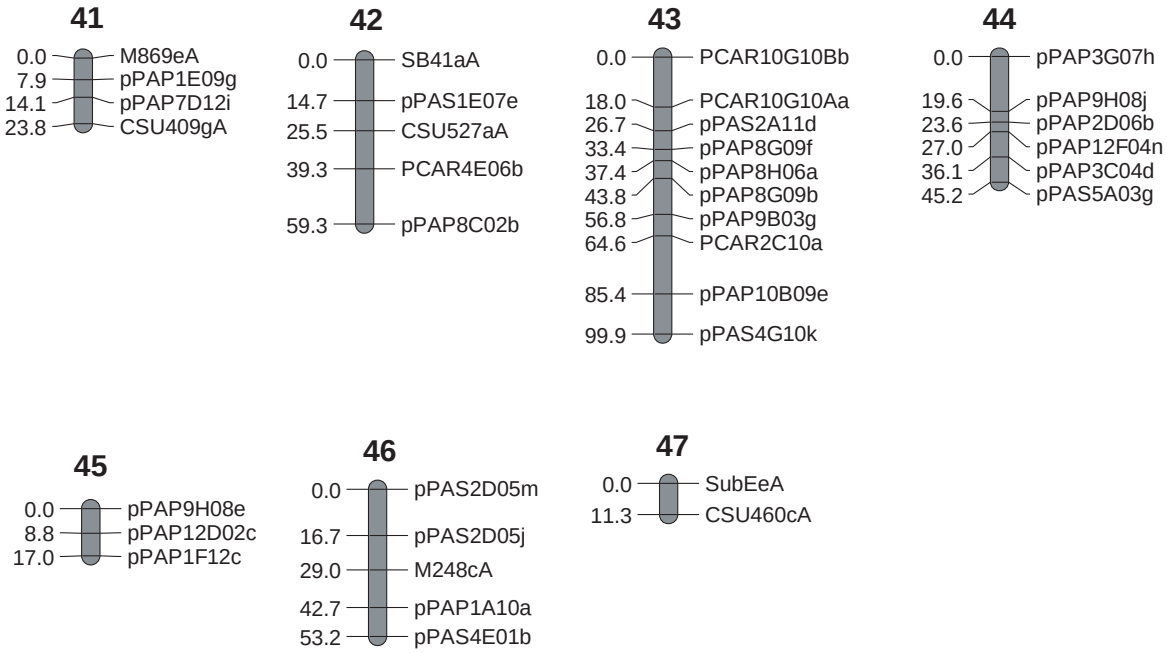
73.5 pPAS1E07d
83.0 pPAP1D02h
102.1 C250hA
113.8 C250gA
125.7 CSU377lA
143.9 pPAS1E08o
150.2 pPAP6G10l
157.8 pPAS3E03i
167.5 pPAS2G11e
172.9 pPAP6B12e
182.2 pPAP9G01i
187.6 pPAS3B05f
198.3 pPAP6G02e
216.8 C250kA
35
0.0 M812fA
18.8 pPAP1E06b
40.5 pPAP9H09b
52.9 pPAP10F12f
56.9 pPAP8H07c
58.4 pPAS3B11c
59.9 pPAP7D05c
64.8 pPAP12C03c
69.2 pPAP8H04f
74.3 pPAP1D02j
87.7 pPAS4G10h
97.6 pPAS4G10e
104.2 CSU705cA
36
0.0 CSU539dA
13.6 pPAP3H12d
29.7 CDSR18bA
37
0.0 pPAS1H08b
4.5 pPAP2A06f
20.0 M568eA
30.2 pPAP3G08f
36.0 pPAP2A09d
38
0.0 pPAS1A11f
8.9 pPAP12B03f
15.4 pPAP9H09d
20.0 pPAS3C12a
27.4 pPAP7G10c
37.1 M869cA
58.1 PCAR8E03a
39
0.0 pPAS3B07c
12.1 pPAS4F11g
21.7 pPAP7G01a
25.9 pPAP7E03k
31.4 pPAS4E01j
34.0 pPAS4E01m
36.5 pPAS4E01h
44.9 pPAS5F03d
52.1 pPAP7D12d
64.9 pPAP9H10h
77.6 CSU413cA
96.7 pPAP5H10b
40
0.0 pPAP8C02b
15.5 pPAP9E02k



UNLINKED SDRFs

CDSR94fA CSU360kA CSU409dA CSU525cA CSU525dA CSU525kA CSU527gA CSU531cA CSU539cA CSU698aA CSU698dA M812aA M869aA M869gA pCA12cA PCAB4b PCAR10E12a PCAR10E12b PCAR10F12a PCAR10G10Bd PCAR11E04a PCAR12E10a PCAR13C11a PCAR2F12a PCAR3E04a PCAR3E06a PCAR5B06a PCAR5E03a PCAR6E03a PCAR7A02a PCAR7B11a PCAR7H12a PCAR9H01b pCS1cA pPAP10A03f pPAP10B09c pPAP10D03a pPAP10H08f pPAP12C02a pPAP1C09h pPAP2C06c pPAP2C06j pPAP3C01b pPAP3D12a pPAP3G08e pPAP6C01d pPAP6C01f pPAP8B05c pPAP9E01a pPAP9E02k pPAS1B08b pPAS1E08a pPAS1E08e pPAS1E08l pPAS1E08n pPAS2A03a pPAS2A03g pPAS2B08b pPAS2C04b pPAS2C06e pPAS2D08a pPAS2E09b pPAS2G11c pPAS2H07e pPAS3A09c pPAS3C12c pPAS3D05e pPAS3D09h pPAS3E03b pPAS3F02b pPAS3F02f pPAS4E02c pPAS4E02d pPAS4E07b pPAS4E11a pPAS4E11e pPAS4F08b pPAS4G02d pPAS4G10i pPAS4H02a pPAS4H02b pPAS5E07c pPAS5F02e pPAS5H05b pPAS5H05f RG463dA RZ166bA SB1365cA Sub31aaA Sub31acA

APPENDIX E

92BWB Hybrid Number	Method of Reproduction	92BWB Hybrid Number	Method of Reproduction
1	Apomictic	160	Sexual
4	Sexual	161	Sexual
15	Apomictic	163	Apomictic
16	Apomictic	168	Apomictic
18	Apomictic	169	Sexual
23	Sexual	170	Apomictic
25	Facultative	172	Sexual
27	Facultative	174	Apomictic
30	Sexual	175	Sexual
38	Apomictic	179	Apomictic
46	Apomictic	182	Facultative
50	Facultative	189	Facultative
51	Sexual	192	Apomictic
55	Sexual	193	Sexual
56	Apomictic	202	Facultative
61	Apomictic	204	Apomictic
63	Apomictic	211	Sexual
71	Facultative	214	Sexual
72	Sexual	215	Facultative
74	Sexual	217	Apomictic
78	Apomictic	219	Apomictic
79	Sexual	224	Sexual
80	Apomictic	225	Apomictic
81	Apomictic	228	Apomictic
83	Sexual	229	Sexual
92	Sexual	231	Apomictic
97	Sexual	243	Sexual
98	Sexual	245	Apomictic
100	Sexual	251	Sexual
101	Facultative	253	Facultative
102	Apomictic	256	Apomictic
106	Facultative	257	Apomictic
107	Sexual	259	Facultative
112	Apomictic	260	Apomictic
113	Facultative	261	Sexual
114	Sexual	262	Sexual
117	Sexual	267	Facultative
119	Sexual	270	Sexual
120	Sexual	274	Sexual
125	Facultative	277	Sexual
126	Sexual	279	Facultative
127	Sexual	280	Sexual
128	Sexual	285	Sexual
138	Sexual	289	Sexual
139	Sexual	290	Sexual
148	Sexual	293	Sexual
151	Facultative	295	Sexual
156	Sexual	301	Apomictic

APPENDIX F

PI240170	Morocco	PI161633	S. Africa	PI409283	S. Africa	PI409607	S. Africa
PI240171	Morocco	PI161634	S. Africa	PI409286	S. Africa	PI409608	S. Africa
		PI161637	S. Africa	PI409287	S. Africa	PI409613	S. Africa
PI196008	Ethiopia	PI171944	S. Africa	PI409290	S. Africa	PI409614	S. Africa
PI199975	Ethiopia	PI208141	S. Africa	PI409306	S. Africa	PI409616	S. Africa
		PI209204	S. Africa	PI409311	S. Africa	PI409618	S. Africa
PI226090	Kenya	PI225585	S. Africa	PI409327	S. Africa	PI409621	S. Africa
PI299542	Kenya	PI253267	S. Africa	PI409329	S. Africa	PI409627	S. Africa
		PI253271	S. Africa	PI409331	S. Africa	PI409642	S. Africa
PI365656	Tanzania	PI273256	S. Africa	PI409334	S. Africa	PI414458	S. Africa
PI365671	Tanzania	PI274182	S. Africa	PI409338	S. Africa	PI414461	S. Africa
PI365680	Tanzania	PI284831	S. Africa	PI409345	S. Africa	PI414463	S. Africa
PI365702	Tanzania	PI299506	S. Africa	PI409346	S. Africa	PI414465	S. Africa
PI365744	Tanzania	PI299510	S. Africa	PI409348	S. Africa	PI414466	S. Africa
		PI299512	S. Africa	PI409351	S. Africa	PI414467	S. Africa
PI210695	Zimbabwe	PI299514	S. Africa	PI409352	S. Africa	PI414468	S. Africa
PI284829	Zimbabwe	PI299521	S. Africa	PI409354	S. Africa	PI414469	S. Africa
PI295655	Zimbabwe	PI299523	S. Africa	PI409361	S. Africa	PI414470	S. Africa
PI295657	Zimbabwe	PI299534	S. Africa	PI409365	S. Africa	PI414471	S. Africa
PI295660	Zimbabwe	PI299538	S. Africa	PI409367	S. Africa	PI414472	S. Africa
		PI299543	S. Africa	PI409381	S. Africa	PI414473	S. Africa
PI162399	Australia	PI364428	S. Africa	PI409391	S. Africa	PI414474	S. Africa
PI193445	Australia	PI364429	S. Africa	PI409397	S. Africa	PI414475	S. Africa
PI209101	Australia	PI364431	S. Africa	PI409398	S. Africa	PI414476	S. Africa
PI253726	Australia	PI364436	S. Africa	PI409399	S. Africa	PI414477	S. Africa
PI284838	Australia	PI364440	S. Africa	PI409403	S. Africa	PI414478	S. Africa
		PI409142	S. Africa	PI409405	S. Africa	PI414479	S. Africa
PI164414	India	PI409143	S. Africa	PI409408	S. Africa	PI414481	S. Africa
PI215599	India	PI409147	S. Africa	PI409409	S. Africa	PI414482	S. Africa
PI245374	India	PI409150	S. Africa	PI409413	S. Africa	PI414483	S. Africa
PI271212	India	PI409152	S. Africa	PI409420	S. Africa	PI414486	S. Africa
PI349651	India	PI409153	S. Africa	PI409426	S. Africa	PI414489	S. Africa
		PI409154	S. Africa	PI409427	S. Africa	PI414492	S. Africa
PI217951	Pakistan	PI409155	S. Africa	PI409429	S. Africa	PI414496	S. Africa
PI218095	Pakistan	PI409156	S. Africa	PI409432	S. Africa	PI414497	S. Africa
PI284836	Pakistan	PI409158	S. Africa	PI409444	S. Africa	PI414505	S. Africa
PI323444	Pakistan	PI409165	S. Africa	PI409445	S. Africa	PI414510	S. Africa
PI323445	Pakistan	PI409168	S. Africa	PI409447	S. Africa	PI414517	S. Africa
		PI409170	S. Africa	PI409467	S. Africa	PI414518	S. Africa
		PI409173	S. Africa	PI409502	S. Africa	PI414521	S. Africa
		PI409186	S. Africa	PI409506	S. Africa	PI414523	S. Africa
		PI409190	S. Africa	PI409515	S. Africa	PI414525	S. Africa
		PI409192	S. Africa	PI409522	S. Africa	PI414526	S. Africa
		PI409193	S. Africa	PI409526	S. Africa	PI414534	S. Africa
		PI409195	S. Africa	PI409528	S. Africa		
		PI409240	S. Africa	PI409535	S. Africa		
		PI409247	S. Africa	PI409585	S. Africa		
		PI409280	S. Africa	PI409590	S. Africa		

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