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PCR-multiplexes for a genome-wide framework of simple sequence repeat marker loci in cultivated sunflower

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Abstract Simple sequence repeat (SSR) and other DNA sequence-tagged site markers can be genotyped more rapidly and cost efficiently by simultaneously amplifying multiple loci (multiplex PCR). The development of PCR-multiplexes for a nearly genome-wide framework of 78 SSR marker loci in cultivated sunflower (*Helianthus annuus* L.) is described herein. The most outstanding single-locus SSR markers in the public collection (300 out of 1,089) were identified and screened for polymorphisms among 24 elite inbred lines, preparatory to selecting SSR markers for testing in multiplex PCRs. The selected SSR markers produced robust PCR products, amplified a single locus each, were polymorphic among elite inbred lines (minimum, mean and maximum heterozygosities were 0.08, 0.53 and 0.85, respectively), and supply a dense genome-wide framework of predominantly or completely codominant, single-locus DNA markers for molecular breeding and genomics research in sunflower. Thirteen six-locus multiplex PCRs were developed for 78 SSR marker loci strategically positioned throughout the sunflower genome (three to five per linkage group) by identifying compatible SSR primer combinations and optimizing multiplex PCR protocols. The multiplexed SSR markers, when coupled with 17 complementary SSR marker loci, create a 'standard genotyping' set ideal for first-pass scans of the genome, as are often needed when screening bulked-segregant DNA samples or mapping phenotypic trait loci. The minimum, mean and maximum heterozygosities of the multiplexed SSR markers were 0.38, 0.62 and 0.83, respectively. The PCR-multiplexes increase genotyping throughput, reduce reagent costs, and are ideal for repetitive genotyping applications where common sets of SSR marker loci are required or advantageous.

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Keywords *Helianthus* · Compositae · Simple sequence repeats · Microsatellite DNA · Genome mapping · Multiplex PCR

Introduction

The widespread application of simple sequence repeat (SSR) and other DNA sequence-tagged site (STS) markers in medical, biological and agricultural research has been stimulated, in part, by the development of increasingly high-throughput systems for genotyping DNA fragments (Carrano et al. 1989; Ziegle et al. 1992; Butler et al. 1999; Ehrlich and Matsudaira 1999; Schmalzing et al. 1999; Carrilho 2000; Mueller et al. 2000; Medintz et al. 2001a, b; Emrich et al. 2002; Paegel et al. 2002). SSR and STS markers are commonly genotyped by post-PCR multiplexing (pooling individually produced amplicons for two or more SSR markers) (Reed et al. 1994; Schwengel et al. 1994; Lindqvist et al. 1996; Diwan and Cregan 1997; Bredemeijer et al. 1998; Macaulay et al. 2001; Blair et al. 2002; Coburn et al. 2002). PCR multiplexing (simultaneously amplifying two or more loci in a single PCR) is typically used in forensic, clinical or laboratory settings for repetitively genotyping a small number of SSR or STS marker loci (Chamberlain et al. 1988; Mutirangura et al. 1993; Shuber et al. 1993; Edwards and Gibbs 1994; Henegariu et al. 1994, 1997). The widespread use of multiplex PCR for SSR genotyping in crop plants has been limited by several factors. First, PCR-multiplexes have either not been developed or have only been developed for a limited number of SSR markers in most crop plants (Mitchell et al. 1997; Chavarriaga-Aguirre et al. 1998; Dean et al. 1999; Hopkins et al. 1999; Liu et al. 2000a, b; Narvel et al. 2000a, b; Gethi et al. 2002). Second, the number of

polymorphic SSR marker loci required for specific mapping or molecular breeding applications is often greater than the number incorporated into PCR-multiplexes. Third, some SSR primers and primer combinations are recalcitrant to multiplex PCR. Fourth, the required assemblage of SSR markers needed for a particular mapping problem typically changes out of necessity or practicality.

PCR-multiplexes are ideal for genotyping applications where common sets of SSR marker loci are required or advantageous, e.g., routine, repetitive DNA fingerprinting. Two to 11-locus PCR multiplexes have been developed for sets of four to 74 SSR marker loci in rapeseed (*Brassica napus* L.) (Mitchell et al. 1997), cassava (*Manihot esculenta* Crantz) (Chavarriaga-Aguirre et al. 1998), peanut (*Arachis hypogaea* L.) (Hopkins et al. 1999), sorghum [*Sorghum bicolor* (L.) Moench] (Dean et al. 1999), cotton (*Gossypium hirsutum* L.) (Liu et al. 2000a, b), soybean (*Glycine max* L.) (Narvel et al. 2000a, b) and maize (*Zea mays* L.) (Gethi et al. 2002). Most of the aforementioned PCR multiplexes amplify a limited number of SSR marker loci and were primarily designed for DNA fingerprinting, e.g., identifying genotypes, protecting intellectual property, and testing for seed purity, rather than for genetic mapping. PCR-multiplexes have not been described for amplifying SSR or other STS markers in cultivated sunflower (*Helianthus annuus* L.).

Despite the long history of breeding and genetics research in cultivated sunflower (Fick and Miller 1997; Miller and Fick 1997) and global importance of the crop, very few phenotypic trait loci have been mapped (Knapp et al. 2001), partly because high-throughput DNA markers and genetic linkage maps vital to the process have only recently been developed (Tang et al. 2002; Yu et al. 2002, 2003). The goal of the present study was to assemble PCR-multiplexes for a genome-wide framework of outstanding, single-locus SSR marker loci, principally to create resources for repetitive, high-throughput genotyping applications and for accelerating the process of identifying SSR markers linked to phenotypic-trait loci.

Of the 1,089 public SSR markers described for sunflower thus far, roughly three-fourths have only been screened for polymorphisms among four to six elite inbred lines and roughly half have been mapped (Tang et al. 2002; Yu et al. 2002, 2003; Tang and Knapp 2003). Naturally, the heterozygosities and genotyping characteristics of the mapped and unmapped SSR markers are variable. Some of the SSR markers produce null alleles or amplify multiple loci or non-target bands, while others amplify a single locus, produce no null alleles or non-target bands, and are universally codominant. The present research was undertaken to identify the most-outstanding subset of the latter, preparatory to selecting SSR markers for testing in PCR-multiplexes. While more than 300 dominant or multilocus SSR marker loci have been mapped, and incidentally supply STS loci throughout the sunflower genome, codominant, single-locus SSR markers are superior or essential for most genotyping applications, e.g., identifying genotypes (Heyen et al. 1997; Luikart et al. 1999; Berry et al. 2002), cross-referencing genetic linkage maps, and mapping phenotypic and quantitative trait loci.

Materials and methods

SSR marker selection and germplasm screening

Polymorphism and genetic mapping databases for 1,089 public SSR markers (Tang et al. 2002; Yu et al. 2002, 2003; Tang and Knapp 2003) were searched to identify mapped, codominant, single-locus SSR markers. The genotyping characteristics and qualities of the SSR markers were assessed from the original electropherograms and the ABI377 GeneScan (Foster City, Calif.) outputs. Three-hundred putatively codominant, single-locus SSR markers were selected and screened for polymorphisms among 24 elite inbred lines: two high-oleic sterility maintainer (B) lines (HA341 and HA349) and one high-oleic fertility restorer (R) line (RHA345) (Miller et al. 1987); two Pioneer Hi-Bred International (Johnston, Iowa) inbred lines (PHC and PHD) (Yu et al. 2003); and 19 public B- and R-lines (HA287, HA292, RHA280, RHA282, HA89, HA369, HA370, HA371, HA372, HA383, HA407, HA821, RHA274, RHA373, RHA377, RHA392, RHA409, RHA417 and RHA801) (Tang and Knapp 2003). Genomic DNA was isolated from fresh young leaf tissue from each inbred line. SSR genotyping assays were performed as described by Tang and Knapp (2003) using post-PCR multiplexing of six to eight amplicons (samples were diluted 15- to 20-fold). The number of loci amplified, heterozygosities (Ott 1991), allele numbers, and null allele numbers and frequencies were estimated for each SSR marker.

Developing and testing the PCR-multiplexes

Based on heterozygosities, genotyping performance and map positions of the 300 SSR markers, 96 were selected for testing in PCR-multiplexes. The criteria used to select SSR markers for the PCR-multiplexes were primer compatibility, genotyping performance when amplified by multiplex PCR, allele length range (estimated from the SSR polymorphism screening analysis), map position and heterozygosity. The SSR markers were sorted by allele-length range and combined so as to minimize the comigration of identically labelled non-allelic bands. Our goal was to identify 4 to 5 uniformly spaced multiallelic SSR markers per linkage group with heterozygosities greater than 0.4 among the elite inbred lines tested, while keeping the distances between SSR marker loci below 15 cM.

Before the final PCR-multiplexes were assembled and tested, we identified an optimal protocol for multiplex PCR by assessing the effects of dNTP, *Taq* polymerase, template DNA and PCR buffer concentration on the performance of four six-plexes (Sets A, B, C and D). PCR tests were performed by amplifying SSR alleles from five elite inbred lines (HA89, HA370, HA383, HA372 and RHA373). The SSR marker loci amplified by each six-plex were ORS675, ORS608, ORS1011, ORS774, ORS437 and ORS778 (Set A), ORS668, ORS13, ORS620, ORS750, ORS735 and ORS691 (Set B), ORS815, ORS1271, ORS1161, ORS887, ORS1246 and ORS677 (Set C), and ORS872, ORS1067, ORS326, ORS1179, ORS832 and ORS912 (Set D). We tested the effect of increasing *Taq* polymerase from 1 to 2 units, dNTPs from 0.2 to 0.4 mM, template DNAs from 15 to 30 ng, and PCR buffer from 0.75 to 1.6 × on the amplification of PCR products by multiplex PCR.

The compatibilities of different SSR primer combinations were tested and the genotyping performances of the 13 six-plexes (Sets 1–13) were assessed by screening six elite inbred lines (RHA280, RHA287, HA369, HA370, RHA392 and RHA801). Multiplex PCRs were performed in 20-μl of reaction mixture containing 1.6 × PCR buffer, 2.5 mM of Mg^{++}, 0.4 mM each of dNTPs, 0.1% Tween-20, 4 to 16 pmol of each primer (Table 1), 2 units of *Taq* polymerase (Qiagen, Valencia, Calif., USA) and 30 ng of genomic

DNA. 'Touchdown' PCR (Don et al. 1991) was used to minimize spurious amplification. The initial denaturation step was performed at 95 °C for 2 min, followed by 1 cycle of 94 °C for 30 s, 63 °C for 30 s and 72 °C for 45 s. The annealing temperature was decreased 1 °C per cycle in subsequent cycles until reaching 57 °C. PCR products were subsequently amplified for 33 cycles at 94 °C for 30 s, 57 °C for 30 s, and 72 °C for 45 s with a final extension for 20 min. The PCR products were initially separated and checked on 1.5% agarose gels. The amplicons were diluted eight- to 12-fold before analysis on polyacrylamide gels on an ABI 377 as previously described by Tang et al. (2002). Filter set C was used to genotype multiplex sets 1–3 and 10–13, while filter set D was used to genotype multiplex sets 4–9.

Results

Genotyping characteristics and polymorphisms for a dense genome-wide framework of single-locus SSR marker loci

Three hundred putatively single-locus, predominantly or completely codominant SSR markers were selected from 1,089 public SSR markers for analysis. Of the selected SSR markers, 180 had previously been screened for polymorphisms on four to six elite inbred lines, while 120 had previously been screened for polymorphisms on 12 or more elite inbred lines (Tang et al. 2002; Yu et al. 2002, 2003; Tang and Knapp 2003). The SSR markers were screened for polymorphisms among 24 elite inbred lines to estimate allele-length ranges, null-allele frequencies and heterozygosities, assess genotyping qualities, and identify candidate SSR markers for testing in PCR-multiplexes. The raw data (SSR allele lengths), linkage group assignments and summary statistics (e.g., heterozygosities, number of alleles and number of null alleles) for each SSR marker have been supplied as supplemental data.

The selected SSR markers amplified a single polymorphic locus each among the 24 inbred lines (Table 1). The minimum, mean and maximum number of alleles per locus were 2, 3.6 and 9, respectively, and the minimum, mean and maximum heterozygosities were 0.08, 0.53 and 0.85, respectively (Fig. 1 and Table 1). Three-fourths of the SSR markers amplified three or more alleles, 46 had heterozygosities greater than 0.7, 180 had heterozygosities greater than 0.5 and 56 produced null alleles (Fig. 1 and Table 1). Of the latter, five (ORS352, ORS825, ORS897, ORS1143 and ORS1270) were strictly dominant (produced a null allele and one other allele each), whereas 51 were mixed dominant-codominant (produced a null allele and two to eight other alleles each). The minimum and maximum null allele frequencies for the 56 SSR markers were 0.042 and 0.667, respectively. The mean frequency of null alleles across SSR markers and inbred lines (300 × 24 = 7,200 genotypes) was 0.047. Four-fifths of the SSR markers in the selected set (244 out of 300) were universally codominant (produced no null alleles). The mean number of alleles per locus was lower for universally codominant (3.52) than for null allele-producing SSR markers (3.96).

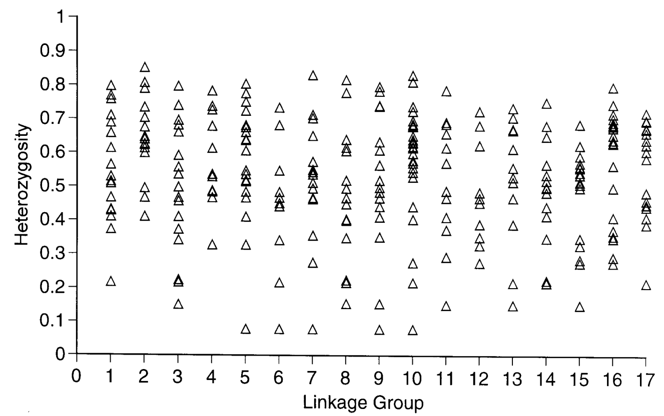


Fig. 1 Heterozygosities by linkage group for 300 simple sequence repeat markers genotyped on 24 inbred lines of sunflower

Table 1 The number of simple sequence repeat (SSR) markers per linkage group, minimum, mean and maximum heterozygosities (H), mean and maximum numbers of alleles (n_A), and the single-most polymorphic SSR marker on each linkage group for 300 single-locus SSR markers screened for polymorphisms among 24 elite inbred lines of cultivated sunflower (*H. annuus* L.)

Linkage group	Number of SSR markers	min[H]	$\bar{H}$	max[H]	n_A	max[n_A]	Single-most polymorphic SSR marker
1	17	0.22	0.56	0.80	3.8	7	ORS662
2	17	0.41	0.65	0.85	4.4	9	ORS1264
3	17	0.15	0.50	0.80	3.3	7	ORS1021
4	11	0.33	0.58	0.79	4.0	6	ORS785
5	20	0.08	0.58	0.81	4.1	7	ORS1159
6	10	0.08	0.45	0.74	3.0	6	ORS1256
7	15	0.08	0.52	0.83	3.7	7	ORS1178
8	16	0.16	0.48	0.82	3.8	8	ORS599
9	18	0.08	0.52	0.80	3.6	7	ORS1265
10	28	0.08	0.55	0.83	3.8	8	ORS541
11	12	0.15	0.52	0.79	3.5	5	ORS1227
12	10	0.28	0.48	0.73	3.0	4	ORS761
13	12	0.15	0.52	0.74	3.4	6	ORS317
14	15	0.22	0.49	0.75	3.3	6	ORS694
15	18	0.15	0.47	0.69	2.9	4	ORS420
16	24	0.28	0.56	0.80	3.8	7	ORS126
17	18	0.22	0.55	0.72	4.1	7	ORS512

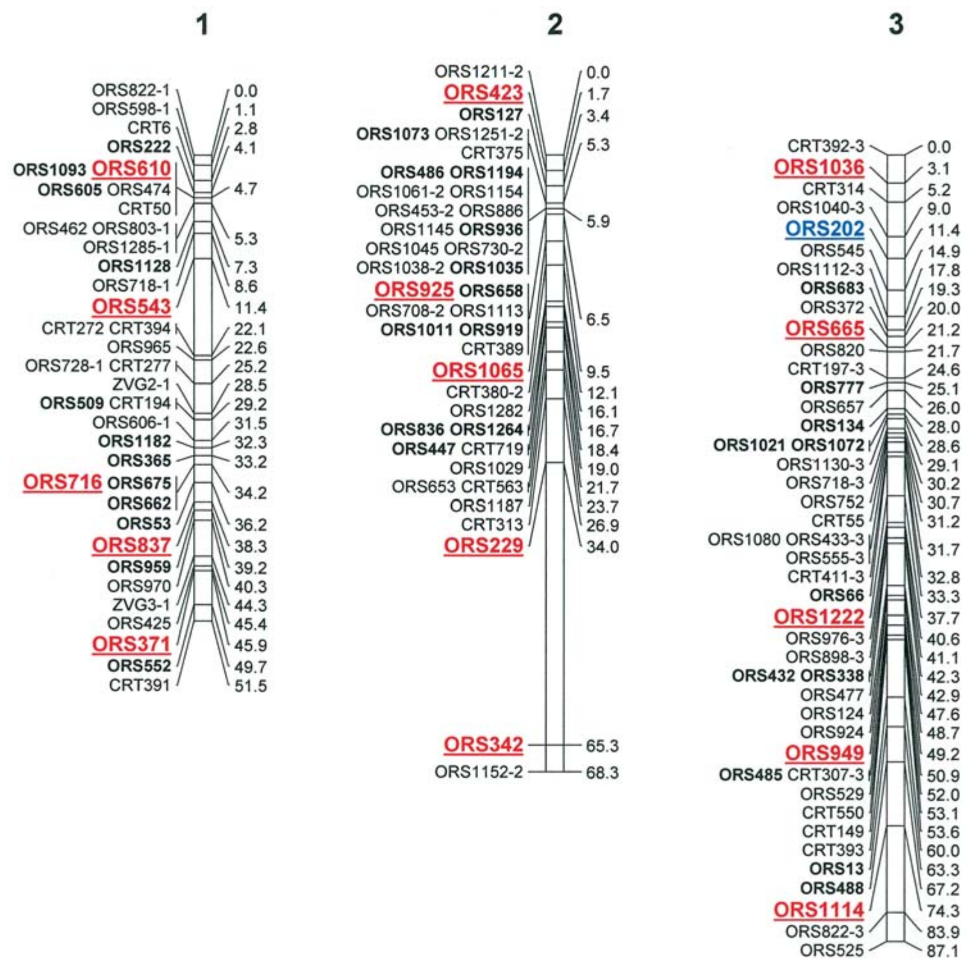


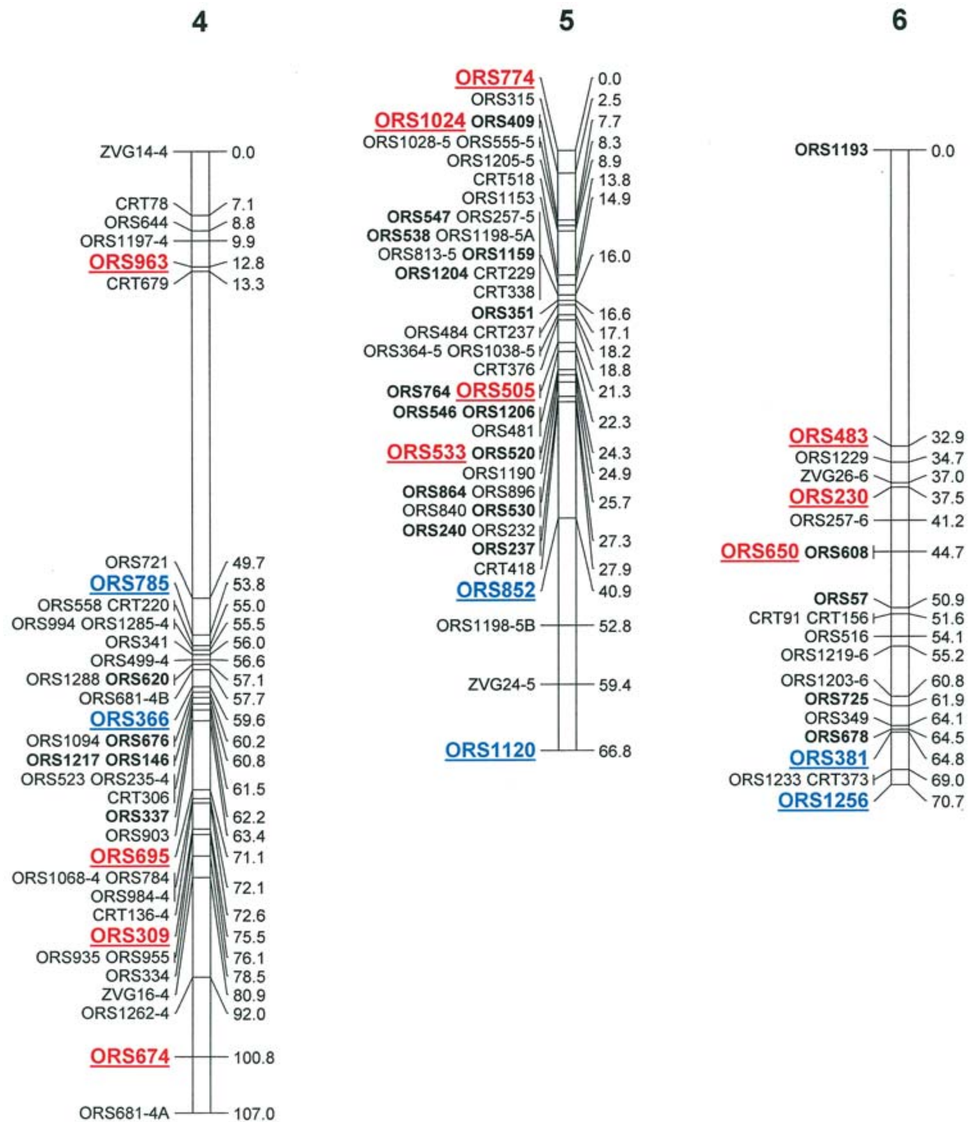
Fig. 2 Composite genetic linkage map of sunflower showing the positions of 78 single-locus simple sequence repeat (SSR) marker loci amplified by 13 six-locus PCR multiplex sets (*boldface red type*), 17 single-locus SSR marker loci (*boldface blue type*) in the 'complementary' set, 183 additional single-locus SSR marker loci (*boldface black type*), and 379 public or proprietary multilocus or dominant SSR markers and proprietary single-locus SSR markers (*plain black type*)

Distribution of the single-locus SSR markers on the genetic linkage map

Of the 300 SSR markers in the selected set, 278 had been mapped and 198 were present on the 577-locus RHA280 × RHA801 recombinant inbred line (RIL) SSR map (Tang et al. 2002; Yu et al. 2003). We constructed a 657-locus 'composite' SSR map by adding 80 SSR marker loci from the HA370 × HA372 F_2, PHA × PHB RIL and HA89 × ANN1238 F_2 maps (Burke et al. 2002; Yu et al. 2003) to the RHA280 × RHA801 RIL SSR map (Fig. 2). The composite SSR map was constructed from previously published individual maps and is presented here purely to illustrate the locations of the single-locus SSR marker loci across the genome, especially the subset of SSR marker loci amplifed by the PCR-multiplexes (see below), and as a guide for selecting SSR markers for new genetic analyses. The original 577 SSR marker loci on the RHA280 × RHA801 RIL map were treated as a fixed framework, while 80 'unique' SSR marker loci from the other maps were inserted by using common flanking SSR marker loci as anchors and interpolating map positions. The 657-locus composite SSR map was 1,423-cM long, had a mean density of 2.2 cM per locus, and had gaps longer than 30 cM on linkage groups 2 (31.3 cM), 4 (36.4 cM), 6 (32.9 cM) and 13 (30.7 cM) (Fig. 2).

The 278 mapped SSR markers in the selected single-locus set spanned 1,295 cM (91%) of the composite map (Fig. 2). The full length of the composite map was not covered because robust, single-locus, codominant SSR markers could not be identified in some regions, e.g., the terminal-most regions of some chromosomes arms. The uncovered regions only spanned approximately 128 cM on the composite map, and the only uncovered regions longer than 10 cM were on linkage groups 3 (12.8 cM), 4 (12.8 cM), 7 (10.5 cM and 19.2 cM) and 17 (17.2 cM) (Fig. 2). Regardless, most loci should be within linkage distance of the single-locus SSR marker loci. While multilocus and dominant SSR markers were not selected for testing in PCR-multiplexes, they are nevertheless essential for mapping phenotypic and quantitative trait loci residing in regions lacking single-locus SSR marker loci and supply a significant number of genomic landmarks for isolating bacterial artificial chromosome clones and developing new DNA markers. Genomic DNA sequences for the SSR markers have been released to Genbank (http://www.ncbi.nlm.nih.gov) and our public website (http:/www.css.orst.edu/knapp-lab). GenBank ac-

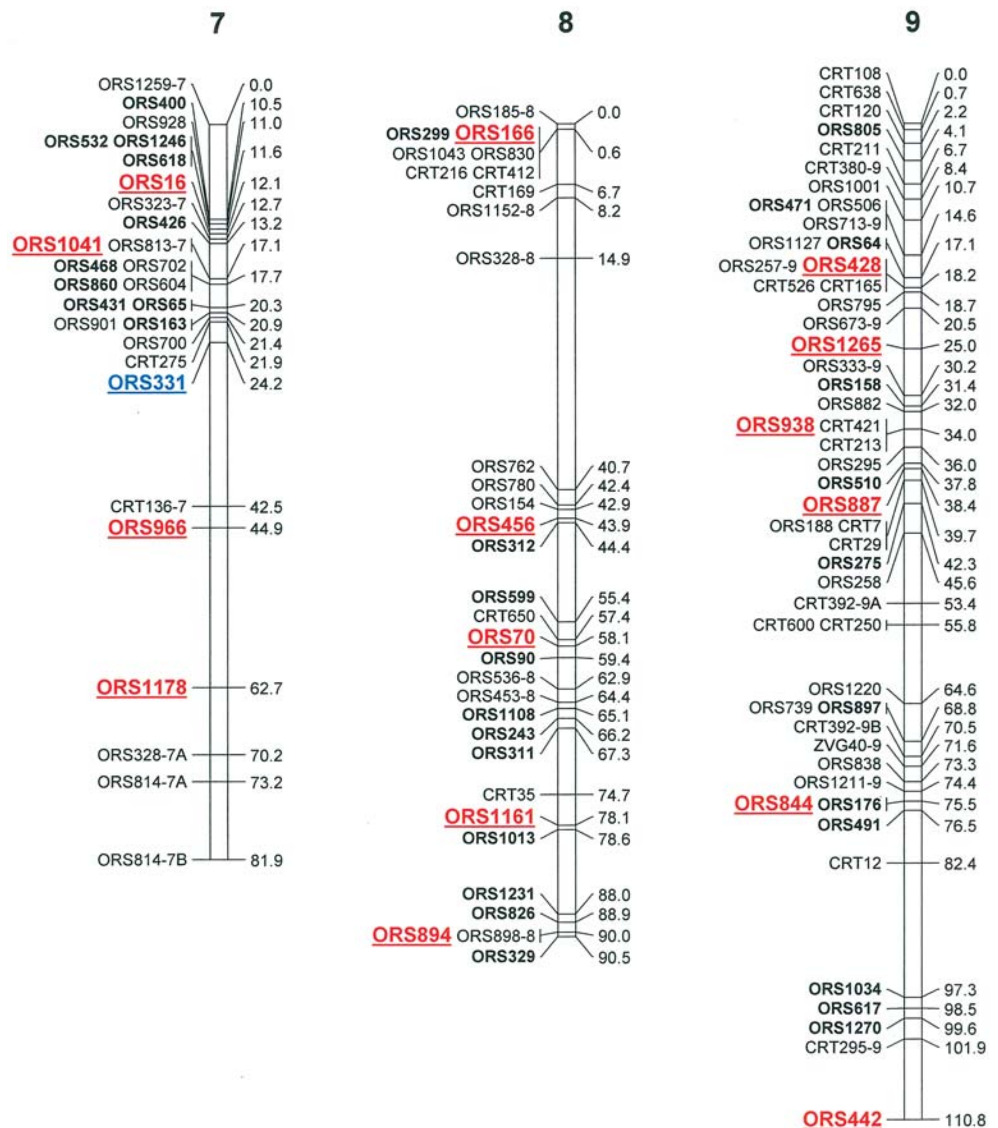
Fig. 2 (continued)



cession numbers for the sequences are BV005815-BV006919 and BV12416-BV012524.

The number of SSR markers per linkage group (LG) in the selected set of 300 ranged from 10 on LG6 and LG12 to 28 on LG10 (Table 1). Based on plots of heterozygosities by map position (data not shown) and linkage group (Fig. 1), SSR polymorphisms were found to be randomly distributed throughout the sunflower genome. Mean heterozygosities ($\bar{H}$) by linkage group ranged from 0.45 (LG6) to 0.65 (LG2), and heterozygosities for the single-most polymorphic SSR marker on each linkage group ranged from 0.69 for ORS420 on LG15 to 0.85 for ORS1264 on LG2 (Table 1). The SSR markers in the single-most polymorphic set and many others in the selected set are powerful for identifying genotypes, e.g., three SSR markers (ORS599, ORS1159 and ORS1264) in the set of 17 (Table 1) distinguished the 24 inbred lines we genotyped (produced unique haplotypes for each inbred line). Several other combinations of SSR markers are equally powerful for identifying and discriminating genotypes. Starting with the single-most polymorphic set, we identified a set of 17 SSR markers (one per linkage group) for post-PCR multiplex genotyping in a single lane or capillary. Three SSR markers in the single-most polymorphic set (Table 1) were replaced with the next most polymorphic SSR markers from the corresponding linkage groups to assemble a combination of 17 SSR marker loci that can be genotyped with minimal or no overlap between identically labeled non-allelic bands (a database listing the 17 SSR markers and potential fluorophore combinations have been supplied as supplemental data). Many other dense post-PCR multiplexes can be assembled.

Fig. 2 (continued)

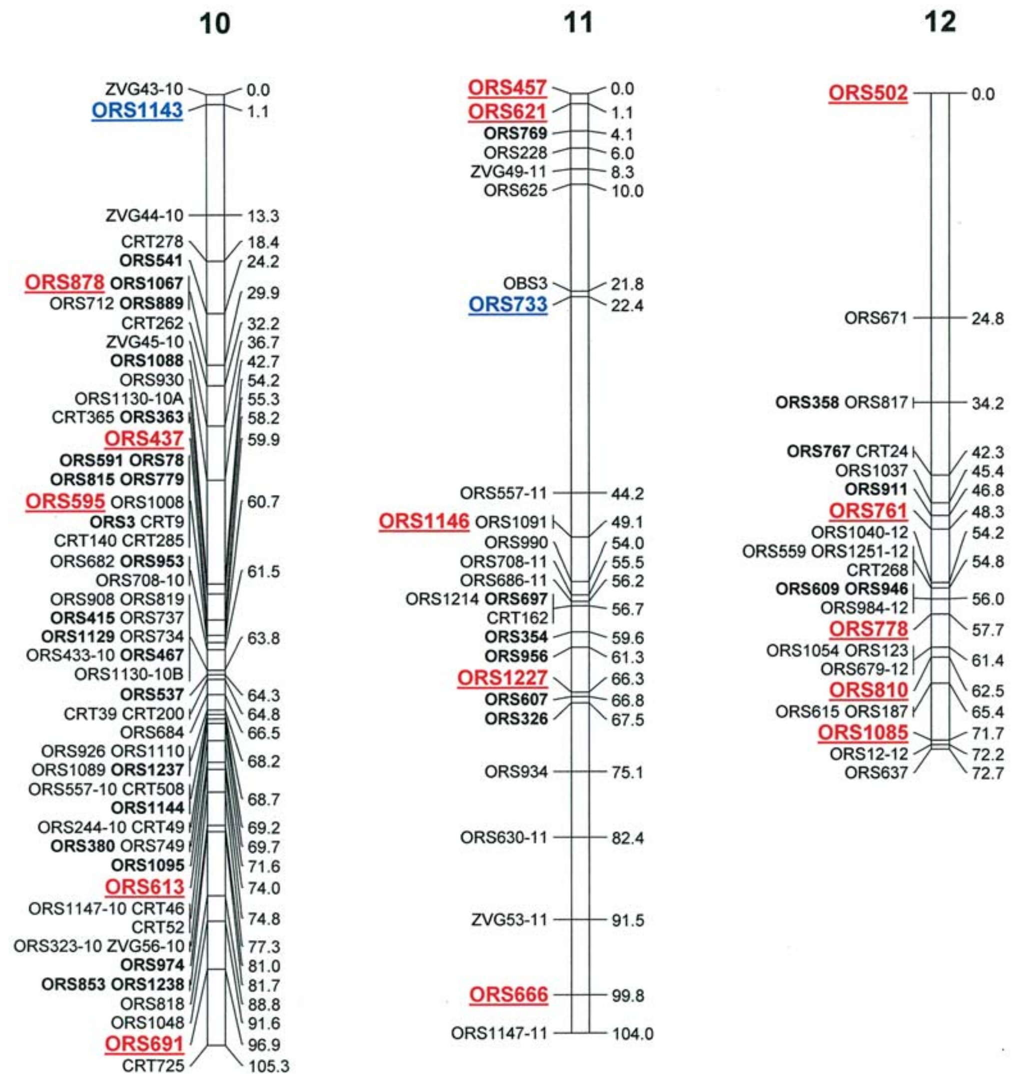


PCR-multiplexes for a nearly genome-wide framework of SSR marker loci

PCR-multiplexes were developed for sunflower by empirically testing SSR primer combinations and using the multiplex PCR principles described by Edwards and Gibbs (1994) and Henegariu et al. (1997). Polymorphisms (heterozygosities), PCR robustness and map position were used to select 96 SSR markers out of the 278 mapped, and single-locus SSR markers for testing in multiplex PCRs. The former produced robust PCR products on agarose and sharp and intense genotypes on polyacrylamide gels when amplified by one-plex PCR, and had heterozygosities greater than 0.4. Map position was a significant constraint and limited the number of candidate SSR marker loci in some genomic regions. We did not check for primer-primer interactions before assembling multiplexes (Henegariu et al. 1997); however, stringent SSR primer design criteria were used to maximize the potential for multiplex PCR, e.g., we used a T_m range of 60 to 63 °C, restricted poly-X to four or less, maintained a G-C content in the 35 to 55% range, checked for 5'-3' complementarity and manually checked automatically selected primers for the presence of repeats, and selected new, repeat-free primers when necessary.

We assembled 78 SSR markers into 13 six-plexes (Sets 1–13) by dropping and adding SSR primers as necessary until optimal combinations were identified (Table 2). Eighteen of the original 96 SSR markers tested could not be incorporated into multiplex PCRs. Several changes to the one-plex PCR protocol were tested to identify an optimal protocol for multiplex PCR. Three changes (increased dNTP, *Taq* polymerase and template DNA concentrations) increased amplicon quantities but did not correct for unevenly amplified loci or pull up unamplified loci (data not shown). dNTP concentrations were increased from 0.2 to 0.4 mM, *Taq* polymerase concentrations were increased from 0.75 to 2.0 U and DNA

Fig. 2 (continued)



template quantities were increased from 15 to 30 ng for multiplex PCR. The most important change and the only change that affected SSR primer compatibility was an increase in buffer concentration from 1.0 to 1.6 × (Fig. 3). The increase generally pulled up faintly amplified and unamplified loci, as illustrated for four six-plexes (Sets A, B, C and D) (Fig. 3). Sets A–D (Fig. 3) are different from the final 13 six-plexes (Sets 1–13) we assembled (Table 2).

Once an optimal multiplex PCR protocol was identified, individual SSR primer pair quantities were modified as necessary to obtain equally intense fluorescent signals for each SSR marker in each six-plex (Fig. 4). The process of calibrating primer pair quantities was done by trial and error by comparing fluorescent signal strengths (Fig. 4). The ratios of SSR primer pairs were more important than the absolute quantities. The multiplex PCR protocols we developed require adding 4 to 16 pmoles per SSR primer pair to a 20-μl reaction (Table 2).

The genotyping performances of the PCR-multiplexes were tested by screening six inbred lines from the original panel of 24 (Fig. 4). Multiplex PCRs produced the same SSR genotypes as were produced by one-plex PCR and post-PCR multiplexing (the latter data are not shown) (Fig. 4). Three of the six-plexes produced anomalous bands (bands that were not produced when the SSR markers were amplified by one-plex PCR). Multiplexed SSR primers occasionally amplify extra bands analogous to random amplified fragment length polymorphisms (RAPDs) (Williams et al. 1992; Mitchell et al. 1997; Ponce et al. 1999; Yue et al. 1999). The anomalous bands in the present study were produced by PCR-multiplex Set 4 (112 bp), 9 (134 bp) and 13 (103, 115 and 129 bp) (Fig. 4). Because they were short, migrated to the lower end of the gel, and did not comigrate with any of the SSR alleles, the extra bands did not interfere with or confound genotyping of the SSR marker loci. Only one of the SSR markers tested in PCR-multiplexes produced stutter bands (ORS1079 in Set 6).

The selection of SSR markers for multiplexing was balanced by map position, polymorphisms (heterozygosities), allele-length ranges and genotyping qualities to

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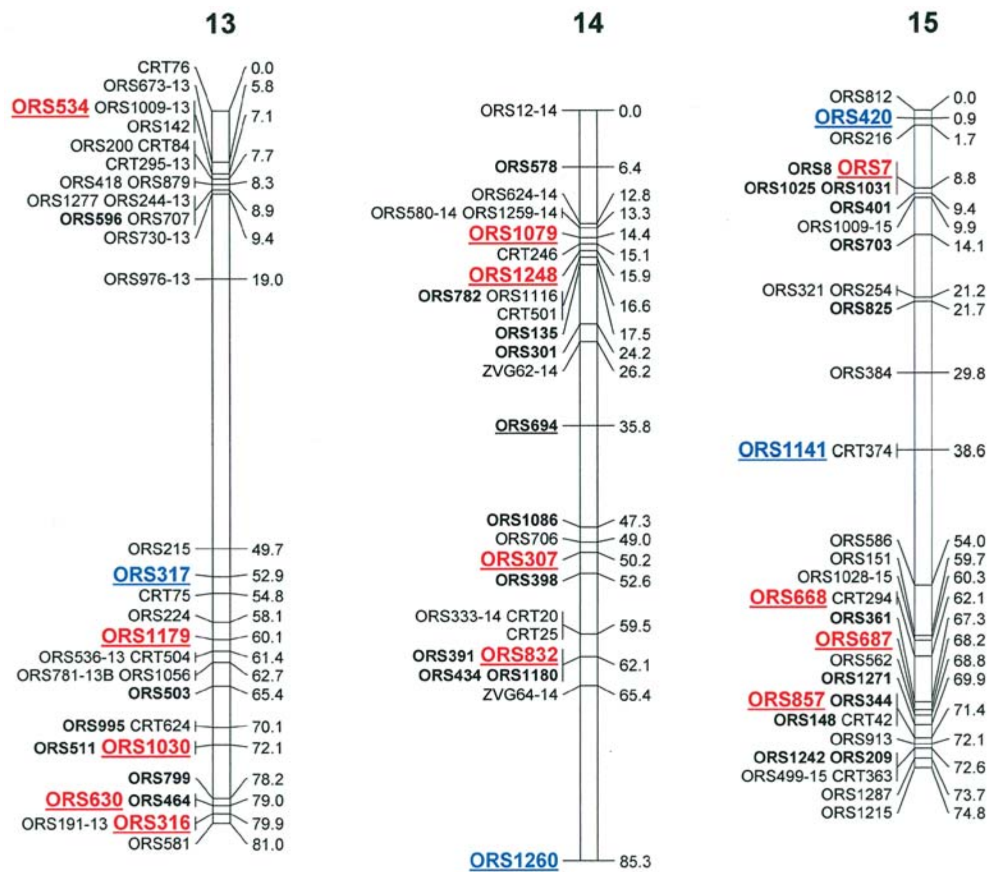


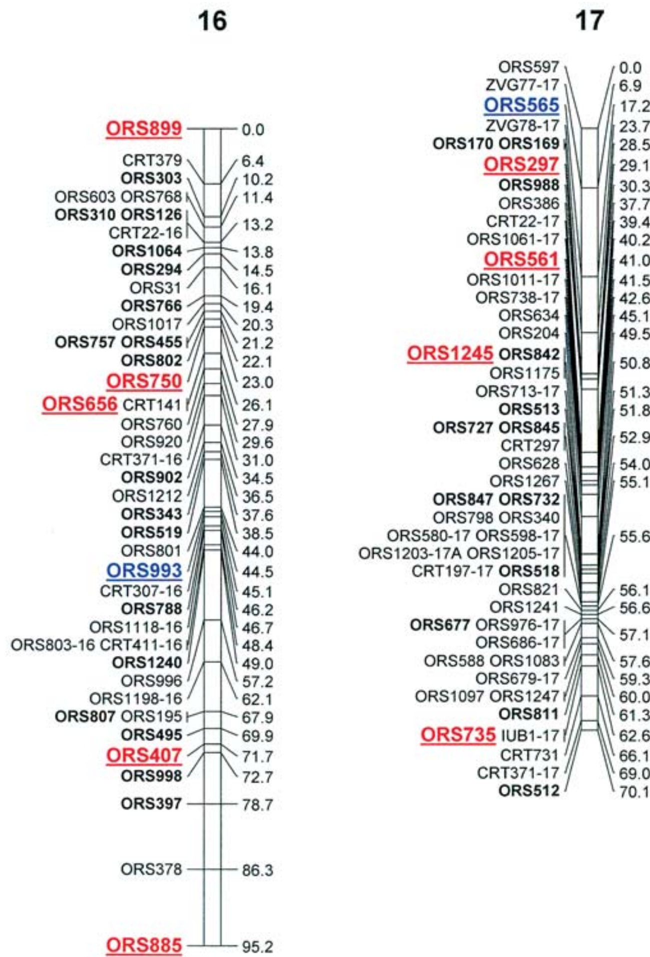
create PCR-multiplexes for amplifying a nearly genome-wide set of SSR marker loci. The 78 SSR markers in the 13 six-plexes spanned 1,067 cM (75% of the length of the composite map) and included three to five SSR markers from each linkage group (shown in underlined red boldface type) (Fig. 2). The only linkage group with fewer than four SSR marker loci was LG6, one of the shortest and least polymorphic linkage groups in sunflower (Yu et al. 2003). The minimum, mean and maximum heterozygosities of the 78 markers among the 24 inbred lines were 0.38, 0.62 and 0.83, respectively (Table 2), and the mean interlocus spacing was 13.7 cM (Fig. 2).

The six-plexes were developed to facilitate genotyping in diverse sunflower germplasm. While denser multiplexing can be performed, the six-plexes were set up to minimize and possibly eliminate the comigration of identically labeled non-allelic bands. This was accomplished by systematically alternating fluorophores and maximizing allele length differences between SSR marker loci as much as possible. The allele lengths of the SSR markers ranged from 95 bp (ORS502) to 466 bp (ORS691) (Table 2) and the distances separating non-allelic bands ranged from 4 to 190 bp. Non-allelic bands within each six-plex were separated by 8 bp or more among the 24 elite inbred lines, aside from a 5-bp gap between alleles amplifed by ORS423 and ORS666 and a 4-bp gap between alleles amplified by ORS666 and ORS371 in multiplex Set 12.

Selected single-locus SSR marker loci for genomic regions missed by the PCR-multiplexes

Several genomic regions were not covered by the SSR marker loci amplified by the PCR multiplexes, e.g., ORS533 in Set 1 was 42.5 cM downstream from the terminal-most SSR marker locus on LG5 (ORS1120), the gap between ORS483 in Set 3 and the terminal-most SSR marker locus on LG6 (ORS1193) was 32.9 cM, and so on (Fig. 2). Key SSR marker loci in the uncovered genomic regions (ORS1193 on LG6, ORS805 and ORS1034 on LG9, ORS358 on LG12 and ORS596 on LG13) either failed to perform in multiplex PCR or were never tested because they failed to meet one or more of the original selection criteria, e.g., ORS1193 (the terminal-most SSR marker locus on LG6) was monomorphic among 23 of the 24 inbred lines tested and only produced a second allele in one confectionery inbred line (RHA280).

We identified 17 outstanding, highly polymorphic, single-locus SSR markers in the uncovered regions to more-thoroughly cover the genome and create a ’standard genotyping set’. The 17 SSR markers in the complementary set (Set C) were not incorporated into multiplex PCRs. Set C SSR marker loci are identified by underlined



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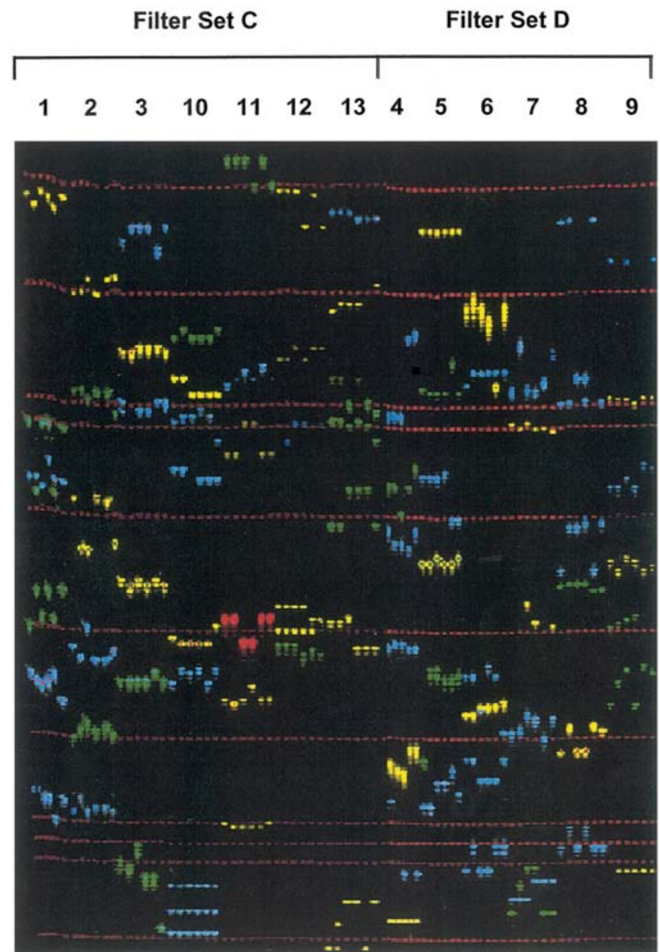

Fig. 4 Simple sequence repeat (SSR) alleles for 78 loci amplified by six-plex PCRs (Sets 1 to 13) from six inbred lines (RHA280, RHA287, HA369, HA370, RHA392, and RHA801) of sunflower shown in order, left to right, for each PCR-multiplex set. SSR alleles amplified by PCR-multiplex sets 1–3 and 10–13 were labelled with 6FAM (*blue*), HEX (*yellow*), TET (*green*), or NED (*red*) and screened using filter set C and the TAMRA500 (*red*) internal lane standard. SSR alleles amplified by PCR-multiplex sets 4–9 were labelled with 6FAM (*blue*), TET (*blue*), HEX (*green*), or NED (*yellow*) and screened using filter set D and the ROX500 (*red*) internal lane standard.

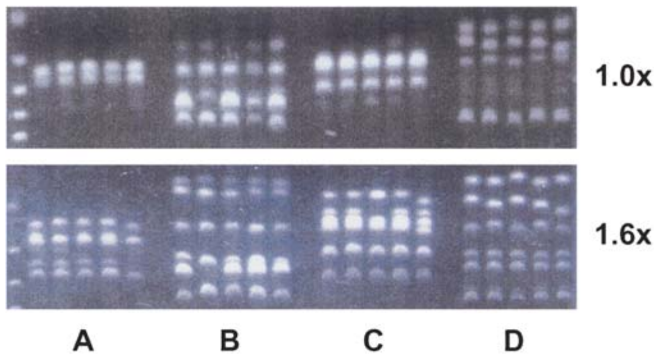

Fig. 3 PCR products amplified by four six-locus multiplex PCRs (sets A, B, C and D) for two buffer concentrations (1.0× and 1.6×) from five inbred lines (HA89, HA370, HA383, HA372 and RHA373) of sunflower shown in order, left to right, for each PCR-multiplex set. Twenty four SSR marker loci were amplified by sets A (ORS675, ORS608, ORS1011, ORS774, ORS437 and ORS778), B (ORS668, ORS13, ORS620, ORS750, ORS735 and ORS691), C (ORS815, ORS1271, ORS1161, ORS887, ORS1246 and ORS677), and D (ORS872, ORS1067, ORS326, ORS1179, ORS832 and ORS912)

blue boldface type on the composite map (Fig. 2). The SSR markers in the complementary set, when coupled with the SSR markers amplified by the PCR-multiplexes, create a genome-wide set for first-pass scans of the sunflower genome, as are often needed in the process of assigning unmapped loci to linkage groups, screening near-isogenic lines (NILs) (Tanksley et al. 1989) or bulked-segregant DNA samples (Michelmore et al. 1991), and constructing new genetic linkage maps by laying down a framework of single-locus linkage-group specific DNA markers. The 95 SSR markers overlay 1,224 cM of the composite map (86% of the length) and supply five to six SSR markers per linkage group with a mean interlocus spacing of 12.9 cM (Fig. 2 and Table 2). The standard genotyping set can be genotyped by post-PCR multiplex-

Table 2 Linkage groups, heterozygosities (H), number of alleles (n_A), minimum and maximum allele lengths (L), null allele frequencies (f_N), fluorescent labels (label), and primer concentrations (pmol) for 78 sunflower simple sequence repeat (SSR) markers assembled into 13 six-plexes for PCR-multiplex genotyping (Sets 1–13) and for a complementary set (Set C) of 17 SSR markers for cultivated sunflower

Set	SSR marker	Linkage group	H	n_A	min[L], max[L]	f_N	Label	pmol
1	ORS533	5	0.639	5	144, 174	0.00	6FAM	14
1	ORS297	17	0.608	5	217, 229	0.00	6FAM	6
1	ORS543	1	0.712	5	244, 268	0.00	TET	8
1	ORS456	8	0.608	3	310, 322	0.00	6FAM	8
1	ORS342	2	0.601	3	308, 341	0.00	TET	8
1	ORS1222	3	0.663	3	436, 442	0.00	HEX	8
2	ORS687	15	0.545	3	165, 171	0.00	6FAM	8
2	ORS656	16	0.694	6	199, 209	0.00	TET	8
2	ORS621	11	0.694	5	234, 251	0.00	6FAM	6
2	ORS844	9	0.741	4	285, 309	0.00	HEX	6
2	ORS674	4	0.740	5	345, 358	0.00	TET	12
2	ORS810	12	0.469	2	399, 406	0.00	HEX	8
3	ORS595	10	0.813	6	105, 148	0.00	TET	8
3	ORS457	11	0.573	4	225, 231	0.00	TET	6
3	ORS483	6	0.486	2	270, 272	0.00	HEX	6
3	ORS630	13	0.708	4	339, 349	0.25	6FAM	16
3	ORS1248	14	0.652	3	372, 376	0.00	HEX	8
3	ORS1178	7	0.833	7	420, 431	0.17	6FAM	12
4	ORS70	8	0.618	6	125, 176	0.13	6FAM	8
4	ORS1245	17	0.628	7	184, 196	0.00	NED	8
4	ORS505	5	0.753	5	235, 249	0.00	6FAM	6
4	ORS665	3	0.684	5	272, 295	0.00	TET	8
4	ORS899	16	0.705	5	301, 322	0.00	HEX	8
4	ORS1146	11	0.618	4	346, 383	0.00	6FAM	8
5	ORS229	2	0.628	5	167, 185	0.00	6FAM	8
5	ORS1265	9	0.799	7	189, 236	0.00	HEX	8
5	ORS1085	12	0.486	2	280, 283	0.00	NED	4
5	ORS716	1	0.771	7	299, 323	0.00	6FAM	8
5	ORS695	4	0.469	3	356, 370	0.00	HEX	12
5	ORS837	1	0.615	3	430, 442	0.00	NED	8
6	ORS16	7	0.552	4	135, 152	0.00	6FAM	8
6	ORS316	13	0.531	3	181, 191	0.00	TET	8
6	ORS857	15	0.517	3	212, 218	0.00	NED	6
6	ORS613	10	0.688	4	204, 234	0.00	TET	6
6	ORS230	6	0.469	2	360, 366	0.00	TET	6
6	ORS1079	14	0.684	6	359, 398	0.00	NED	4
7	ORS309	4	0.486	2	121, 130	0.00	TET	10
7	ORS307	14	0.538	3	114, 136	0.00	HEX	8
7	ORS925	2	0.792	7	201, 215	0.00	6FAM	6
7	ORS894	8	0.642	4	253, 263	0.25	NED	8
7	ORS885	16	0.684	3	339, 342	0.00	HEX	12
7	ORS949	3	0.698	6	359, 398	0.00	6FAM	14
8	ORS610	1	0.517	5	145, 159	0.00	TET	6
8	ORS878	10	0.726	5	194, 214	0.00	NED	6
8	ORS7	15	0.538	3	267, 273	0.00	HEX	6
8	ORS1065	2	0.705	4	275, 297	0.00	6FAM	6
8	ORS761	12	0.726	4	349, 352	0.21	TET	6
8	ORS561	17	0.701	5	362, 434	0.13	6FAM	6
9	ORS1024	5	0.778	6	216, 234	0.00	HEX	8
9	ORS534	13	0.674	5	251, 257	0.50	HEX	8
9	ORS1041	7	0.715	5	276, 284	0.00	NED	6
9	ORS938	9	0.785	5	312, 322	0.00	6FAM	8
9	ORS1227	11	0.788	4	351, 357	0.00	NED	14
9	ORS650	6	0.453	3	415, 416	0.29	6FAM	12
10	ORS1161	8	0.521	5	226, 235	0.00	6FAM	8
10	ORS887	9	0.635	3	245, 253	0.00	HEX	8
10	ORS1179	13	0.517	3	317, 320	0.08	6FAM	10
10	ORS437	10	0.684	4	334, 348	0.00	6FAM	10
10	ORS832	14	0.486	2	355, 362	0.00	HEX	10
10	ORS778	12	0.455	2	380, 384	0.00	TET	10
11	ORS668	15	0.500	2	158, 160	0.00	HEX	10
11	ORS428	9	0.521	4	217, 225	0.00	HEX	8
11	ORS1036	3	0.413	2	245, 255	0.00	NED	6
11	ORS750	16	0.678	3	328, 344	0.00	HEX	10
11	ORS735	17	0.663	5	359, 373	0.00	6FAM	6
11	ORS691	10	0.573	3	451, 466	0.00	TET	8
12	ORS423	2	0.615	4	238, 244	0.00	TET	10
12	ORS666	11	0.375	3	249, 251	0.21	HEX	8
12	ORS371	1	0.434	3	255, 263	0.00	HEX	8

Table 2 (continued)

Set	SSR marker	Linkage group	H	n_A	min[L], max[L]	f_N	Label	pmol
12	ORS963	4	0.531	3	333, 341	0.54	6FAM	10
12	ORS966	7	0.653	3	370, 375	0.38	HEX	16
12	ORS407	16	0.656	4	430, 466	0.00	HEX	8
13	ORS502	12	0.625	4	95, 119	0.00	HEX	8
13	ORS1114	3	0.594	3	242, 256	0.00	HEX	8
13	ORS774	5	0.517	3	296, 312	0.00	TET	12
13	ORS166	8	0.781	5	334, 345	0.00	TET	6
13	ORS442	9	0.521	5	391, 409	0.04	HEX	8
13	ORS1030	13	0.469	2	434, 437	0.00	6FAM	14
C	ORS202	3	0.742	6	292, 423	0.00	6FAM	–
C	ORS785	4	0.785	6	138, 166	0.00	6FAM	–
C	ORS366	4	0.729	6	185, 215	0.00	6FAM	–
C	ORS1120	5	0.521	4	295, 322	0.00	6FAM	–
C	ORS852	5	0.608	4	199, 459	0.08	NED	–
C	ORS1256	6	0.736	6	149, 163	0.00	6FAM	–
C	ORS381	6	0.684	3	212, 218	0.00	TET	–
C	ORS331	7	0.705	4	167, 179	0.00	6FAM	–
C	ORS1143	10	0.444	2	Null, 401	0.42	HEX	–
C	ORS733	11	0.490	4	204, 210	0.00	TET	–
C	ORS317	13	0.736	6	191, 218	0.00	HEX	–
C	ORS1260	14	0.486	3	355, 357	0.17	NED	–
C	ORS694	14	0.753	5	165, 186	0.00	HEX	–
C	ORS420	15	0.688	4	133, 139	0.00	HEX	–
C	ORS1141	15	0.573	3	238, 244	0.00	HEX	–
C	ORS993	16	0.719	5	318, 334	0.17	NED	–
C	ORS565	17	0.642	4	148, 166	0.21	HEX	–

ing using a combination of one-plex PCRs for the 17 complementary SSR markers and six-plex PCRs for the 78 SSR markers. Optimal one-plex and six-plex SSR marker combinations for post-PCR multiplexing were identified and have been supplied as supplemental data.

Discussion

The PCR-multiplexes described herein (Table 2 and Figs. 2 and 4) are the first developed for sunflower and one of the first developed for a nearly genome-wide collection of SSR marker loci for a crop plant. PCR-multiplexes for genome-wide or nearly genome-wide collections of SSR marker loci have only been developed for two other plant species thus far, *Arabidopsis thaliana* L. (Ponce et al. 1999) and maize (*Z. mays* L.) (Gethi et al. 2002). The 21-plex described by Ponce et al. (1999) simultaneously amplifies a genome-wide set of 21 SSR marker loci known to be polymorphic between two widely used ecotypes (*Ler* and Col-0). The 21-plex is a powerful tool for mapping phenotypic loci in Col-0 × *Ler* segregating populations, e.g., Serrano-Cartagena et al. (1999), Quesada et al. (2000) and Robles and Micol (2001) employed the 21-plex to map numerous mutant loci (76 leaf mutant loci alone were mapped). Gethi et al. (2002) assembled a genome-wide collection of 44 SSR marker loci into 17 two- to five-plexes for multiplex PCR genotyping in maize.

Numerous PCR-multiplexes have been described for genotyping SSR marker loci in humans and animals (Chamberlain et al. 1988; Williams et al. 1992; Mutirangura et al. 1993; Shuber et al. 1993; Henegariu et al. 1994, 1997; Fritz et al. 1996; Heyen et al. 1997; Chavarriaga-Aguirre et al. 1998; Luikart et al. 1999; Neff et al. 1999; Yue et al. 1999; Ruitberg et al. 2001; Krenke et al. 2002; Master et al. 2002; Wallin et al. 2002; http://www.cstl.nist.gov/biotech/strbase/multiplx.htm). The development of genome-wide PCR-multiplexes for crop plants other than maize (Gethi et al. 2002) and sunflower (Table 2) are surely forthcoming and only limited by time and resources. Liu et al. (2000a, b) assembled 55 SSR markers into 13 multiplexes (two- to seven-plexes) for multiplex PCR genotyping in cotton (*G. hirsutum* L.). Similarly, Narvel et al. (2000a, b) assembled a nearly genome-wide sample of 74 SSR markers into an unspecified number of two- and three-plexes for multiplex PCR genotyping in soybean (*G. max* L.). PCR-multiplexes have been developed on a more limited scale for other crop plants, e.g., Mitchell et al. (1997) developed an 11-plex for DNA fingerprinting rapeseed (*B. napus* L.) germplasm and Dean et al. (1999) developed three five-plexes for DNA fingerprinting sorghum [*S. bicolor* (L.) Moench] germplasm (Grenier et al. 2000; Smith et al. 2000; Ghebru et al. 2002).

The sunflower PCR-multiplexes amplify twice as many SSR marker loci per PCR than the assortment of PCR-multiplexes described thus far for maize, cotton and soybean (Liu et al. 2000a, b; Narvel et al. 2000a, b; Gethi et al. 2002), increase genotyping throughput, reduce PCR costs by an estimated 50 to 70% compared to multiple one-plex PCRs, and are ideal for genotyping applications where common sets of SSR marker loci are required or advantageous, e.g., genetic diversity analysis (Mitchell et al. 1997; Dean et al. 1999; Grenier et al. 2000; Smith et al. 2000; Ghebru et al. 2002) and protecting intellectual

property (Heyen et al. 1997; Luikart et al. 1999; Berry et al. 2002). The 78 SSR marker loci can be genotyped on two DNA samples, for example, by performing 26 six-plex PCRs, as opposed to performing 156 one-plex PCRs.

While Ponce et al. (1999) demonstrated the technical feasibility of developing extraordinarily dense multiplex PCRs for SSR genotyping, pre- and post-PCR multiplexing densities in crop plants are limited by the requirement of eliminating overlap between identically labelled non-allelic bands across diverse genotypes (Blair et al. 2002). The primary factors behind the selection of individual SSR markers for the sunflower six-plexes, apart from technical factors pertinent to multiplex PCR (Edwards and Gibbs 1994; Henegariu et al. 1997), were map position, heterozygosity and allele-length range. Our goal was to create a set of PCR-multiplexes to speed the process of integrating phenotypic and quantitative trait loci into the molecular genetic linkage map of sunflower, a process that necessitates using multiple segregating populations and diverse parental genotypes. While the *Arabidopsis* 21-plex was built around SSR polymorphisms in two ecotypes (for important scientific and technical reasons), the sunflower six-plexes were assembled for maximum versatility in domesticated and wild sunflowers. The band-width windows of the individual SSR markers in each six-plex should be wide enough to unequivocally call alleles amplified from genetically diverse sunflowers. The versatility requirement set an upper limit of six SSR marker loci per multiplex because of the presence of extraordinary allelic diversity in exotic and wild germplasm, particularly among the most-polymorphic SSR markers (Tang and Knapp 2003), which were preferentially selected for the present study.

SSR allele-length ranges tend to be greater in exotic germplasm than elite oilseed inbred lines in sunflower, and alleles uncharacteristic of stepwise mutational changes (Ohta and Kimura 1973; Schlötterer and Tautz 1992; Shriver et al. 1993; Valdes et al. 1993) are commonly amplified from wild sunflowers and cannot be predicted from allelic variants identified in elite oilseed inbred lines. Non-stepwise SSR allelic series are presumably caused by the presence of insertion-deletion (INDEL) polymorphisms in DNA flanking SSRs, e.g., ORS338 produced two alleles (151 and 189 bp) separated by a 38-bp INDEL, and ORS342 produced two alleles (309 and 341 bp) separated by a 33-bp INDEL. The presence of INDEL polymorphisms in flanking sequences create discontinuous SSR allele distributions. Non-stepwise allelic variants are common in sunflower (Tang and Knapp 2003). Of the 120 SSR markers genotyped by Tang and Knapp (2003), more than half produced allelic variants characteristic of a combination of SSR and INDEL polymorphisms when wild populations, land races and elite inbred lines were genotyped. The SSR genotypes produced by ORS326 and ORS547 are typical. The allele-length ranges for ORS326 were 13 bp for oilseed inbred lines and 70 bp for land races and wild populations, and for ORS547 were 10 bp for oilseed inbred lines and 52 bp for land races and wild populations.

The 78 SSR markers amplified by the PCR-multiplexes were systematically distributed among the six-plexes to facilitate orderly screening of bulked-segregant DNA samples (Michelmore et al. 1991), near-isogenic lines (NILs) (Tanksley et al. 1989) and segregating populations, e.g., one or two SSR markers from each linkage group are present in Sets 1 to 3, another one or two SSR markers from each linkage group are present in Sets 4 to 6, and so on. The six-plexes and the standard genotyping set should be especially powerful for rapidly assigning phenotypic loci to the genetic map using leads produced by analyses of bulked-segregant or NIL DNA samples. Tang et al. (2003) used the PCR-multiplexes to screen bulked-segregant DNA samples, identified SSR marker loci linked to the Or_5 gene for resistance to broomrape (*Orobanche cumana* Wallr.) and mapped the Or_5 locus by screening a minimal number of SSR marker loci. The mean interlocus spacing of 12.9 cM for the 95 SSR markers in the standard genotyping set places three to four SSR marker loci within 30 cM or less of virtually any phenotypic trait locus targeted for mapping. The other nearly 200 mapped single-locus SSR markers (identified by black boldface type on the composite map) are ideal for systematically screening bulked-segregant or NIL DNA samples when initial leads are not produced by SSR markers in the PCR-multiplex or standard genotyping sets, e.g., because of the lack of polymorphisms, and are outstanding candidates for developing additional and perhaps denser multiplexes.

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