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Marker-Assisted Backcrossing for Simultaneous Introgression of Two Genes

Matthias Frisch and Albrecht E. Melchinger*

ABSTRACT

Marker-assisted selection is used to accelerate recovery of the recurrent parent genome (RPG) in backcross programs. Our objectives were to compare various selection strategies and breeding plans for the simultaneous introgression of two genes with respect to the RPG recovered and the number of marker data points (MDP) required. Computer simulations were performed with a published genetic map in maize (*Zea mays* L.) consisting of 80 markers and assuming selection for dominant target genes on the basis of phenotypic evaluation. For unlinked as well as for linked target genes, a shortening of the backcross program from six to three generations resulted from applying marker-assisted background selection. In breeding programs with three backcross generations, the least MDP were required when (i) applying selection strategies consisting of three or four selection steps on the basis of presence of the target genes and selection indices calculated from the marker genotype, (ii) increasing the population size from early to advanced generations, and (iii) merging the target genes in an early generation. These principles can be used for optimizing the design of marker-assisted backcross programs for the simultaneous introgression of two genes.

Marker-assisted backcrossing has become an established tool in plant breeding, and its importance is increasing with the availability of transgenes. The simultaneous introgression of two genes from different sources into the genetic background of one recipient genotype by recurrent backcrossing is a common task, for example, in the development of inbred lines for hybrid varieties or line cultivars in autogamous species. Besides the transfer of the target gene(s), the main goal in such a breeding program is to recover the RPG as rapidly and completely as possible. Analysis of DNA markers with a good coverage of the entire genome can be used to select for individuals with a high proportion of the RPG and, thus, reduce the number of backcross generations required.

Institute of Plant Breeding, Seed Science, and Population Genetics, University of Hohenheim, 70593 Stuttgart, Germany. Received 1 Sept. 2000. *Corresponding author (melchinger@uni-hohenheim.de).

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Monitoring the parental origin of alleles at markers throughout the genome in backcrossing was originally proposed by Tanksley et al. (1989) and later termed *background selection* (Hospital and Charcosset, 1997). Background selection for introgression of a single gene with known map position was investigated by various authors (Hospital et al., 1992; Openshaw et al., 1995; Frisch et al., 1999a,b; Frisch and Melchinger, 2001a,b). Hospital and Charcosset (1997) provided theoretical and simulation results on background selection for the introgression of favorable alleles at quantitative trait loci. To our knowledge, no conceptual frame-work exists for background selection for introgression of two genes with known map positions.

In this study, we extend results on the efficiency of background selection for the introgression of one target gene (Frisch et al., 1999b) to the simultaneous introgression of two genes. We consider phenotypic selection for presence of two dominant target genes combined with background selection for a high RPG at markers covering the whole genome. Our research objectives were to (i) examine alternative breeding plans, (ii) compare different selection strategies, and (iii) examine the effects of varying the population size from early to late generations with respect to the level of RPG attained and the number of MDP required.

METHODS

Genetic Map

Our simulations were based on a published linkage map of maize (Schön et al., 1994) previously used to investigate introgression of one target gene (Frisch et al., 1999b). The map was constructed using Haldane's (1919) mapping function, with marker data from a population of 380 F_2 individuals derived from the cross of two flint inbred lines. The total map length was 1612 cM. Our of the 89 polymorphic RFLP markers used by Schön et al. (1994), 80 were chosen for the simula-

Abbreviations: BC_t, *t*-th backcross generation; cM, centimorgan; MDP, marker data points; RPG recurrent parent genome.

tions. Markers *umc128*, *umc5*, *umc175*, *bnl6.06*, *umc54*, *umc51*, *umc110*, *bnl7.61*, and *bnl9.44* were tightly linked to other markers, and, therefore, excluded from our study. The map has two large gaps, a 90-cM marker interval on Chromosome 3, and a 89-cM marker interval on Chromosome 9.

We investigated two scenarios: (i) the two target loci are unlinked, one being located on Chromosome 5, 30 cM from the most distal marker, the second being located on Chromosome 7, 40 cM from the most distal marker; and (ii) the two target loci are linked and located on Chromosome 5 with map distances 30 and 70 cM from the most distal marker.

Breeding Plans

We compared a breeding scheme (Breeding Plan 1) for a backcross program with selection only for presence of the target genes with five alternative breeding schemes (Breeding Plans 2–6), which employ selection for presence of the target genes as well as background selection (Fig. 1). In Breeding Plan 1, the target genes A and B are introgressed in separate branches of the breeding program from the donor genotypes D^A and D^B into the genetic background of the recipient genotype R. After an initial cross, six to eight generations of backcrossing with selection for presence of the respective target genes are carried out. The BC_6^A to BC_8^A individuals are crossed with the respective BC_6^B to BC_8^B individuals and a carrier of both target genes (denoted by BC_6–BC_8) is selfed to generate homozygous carriers of the target genes (denoted by BC_6-S_1–BC_8-S_1).

Breeding Plans 2 to 6 start with (i) one initial cross of the donors with the recipient, followed by (ii) three backcross generations, (iii) one cross to merge the two target genes into one genotype, and (iv) one selfing generation to generate homozygous carriers of the target genes. The various plans differ with respect to the generation in which the two target genes are merged into one individual. This is performed in one of the following generations: P (Plan 2), F_1 (Plan 3), BC_1 (Plan 4), BC_2 (Plan 5), or BC_3 (Plan 6). In the generations before merging the target genes, a separate branch of the backcross program is carried out for each target gene. In all generations except the F_1, selection for presence of the target genes and marker-assisted background selection are carried out according to one of the later described selection strategies.

Population Size

For conducting a successful breeding program, it is necessary that both target genes are recovered in each cross and backcross generation, and that a homozygous carrier of both target genes is present in the progeny of the selfing generation. These conditions can be used to determine the minimum population size for each generation in Breeding Plans 1 to 6.

Let p denote the probability that an individual has the desired genotype in a crossing, backcrossing, or selfing generation. We first consider generations before merging the target genes, in which the desired genotype is heterozygous for one target gene: $p = 1$ if the parent carrying the target gene is homozygous and $p = 1/2$ if it is heterozygous. In the generation of merging the target genes, the desired genotype is heterozygous for both target genes, and each parent carries one target gene. If the parents are homozygous, $p = 1$; if both are heterozygous, $p = 1/4$. In generations after merging the target genes, the desired genotype carries both target genes, which are originating from one of its parents. The probability p depends on (i) the linkage between the two target genes and (ii) whether both target genes originate from the same ancestor of the crossing parent (coupling phase) or each target gene originates from a different ancestor of the crossing parent (repulsion phase). Repulsion phase occurs in the first generation after merging the two target genes into one genotype; coupling phase occurs in all subsequent generations. For these cases the probabilities p are calculated with the equations given in Table 1 in terms of the recombination frequency r, which is obtained from the map distance d under the assumption of no interference (Stam, 1979) with the inverse of Haldane's (1919) mapping function

$$r = [1 - \exp(-2d)]. \quad [1]$$

The population sizes in the two branches of a breeding program in a certain generation t before merging the target genes are denoted with n_{ta} and n_{tb} such that the total number of individuals employed in generation t is $n_t = n_{ta} + n_{tb}$. The probability q_t that both target genes are recovered in generation t can be derived from the probability function of the binomial distribution as

$$q_t = 1 - 2^{-n_{ta}} - 2^{-n_{tb}} \qquad \text{for } n_{ta} \neq n_{tb}, \quad [2]$$

which reduces to

$$q_t = 1 - 2^{1-n_t/2} \qquad \text{for } n_{ta} = n_{tb} = n_t/2. \quad [3]$$

In generations after merging the target genes

$$q_t = 1 - (1 - p)^{n_t}. \quad [4]$$

The (a priori) probability that at least one selfing progeny is homozygous for both target genes is obtained by multiplying the values for the s generations of a breeding program:

$$q = \prod_{t=1}^{s} q_t \quad [5]$$

When $q_1 = 1$ and $q_t = q^{1/(s-1)}$ for all $t \in \{2, \ldots, s\}$, Eq. [2] can be solved to find the minimum population size for generations before merging the target genes in one individual as

$$n_{ta} = n_{tb} \geq 1 - \log_2(1 - q^{1/(s-1)}). \quad [6]$$

For generations after merging the target genes, solving Eq. [4] yields

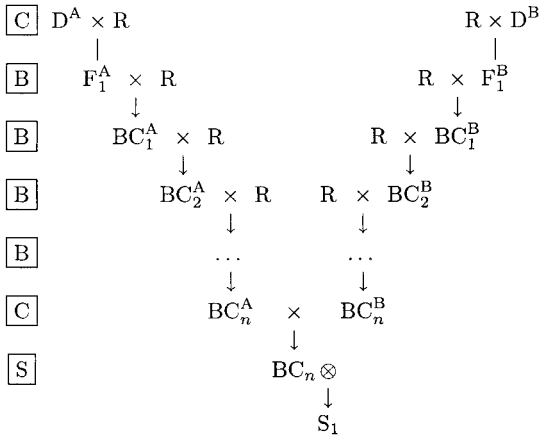
$$n_t \geq \ln(1 - q^{1/(s-1)})/\ln(1 - p) \qquad \text{for } p \neq 1. \quad [7]$$

Applying Eq. [6] and [7] to Breeding Plans 2 to 6 yields, for two unlinked target loci and $q = 0.99$, a minimum populations size of 22 individuals ($p = 1/2$) in crossing and backcrossing generations and 97 individuals in selfing generations ($p = 1/4$). The minimum population sizes required for linked target loci ($d = 0.4$, $r = 0.275$) are shown in Table 2.

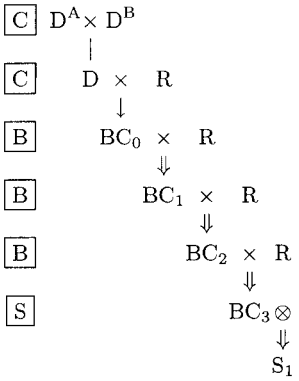
Previous investigations (Frisch et al., 1999b) showed that background selection reduced the number of generations required for introgression of one target gene from six to three when employing 60 individuals (per target gene) in each generation. In order to verify whether this result also applies to introgression of two target genes, we employed a fixed number of 80, 120, and 160 individuals per generation with Breeding Plan 6, and then extended the scheme to generations BC_4, BC_5, BC_4-S_1, and BC_5-S_1.

For Breeding Plans 2 to 6, we employed altogether $3 \times 2 \times 60 = 360$ individuals for the three backcross generations. They were allocated to the generations in three variants: (i) constant population size—in each backcross generation 120 individuals were generated; (ii) increasing population size—in generations BC_1 to BC_3 a total of 60, 120, and 180 individuals, respectively, were generated; (iii) decreasing population size—in generations BC_1 to BC_3 a total of 180, 120, and 60 individuals, respectively, were generated. In generations before merging the tar-

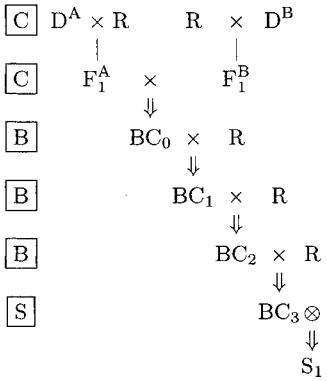
Breeding Plan 1: Reference scheme



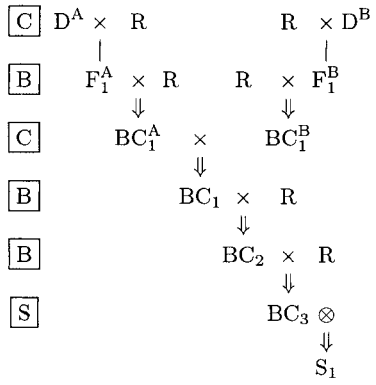
Breeding Plan 2: Merging of target genes in generation P



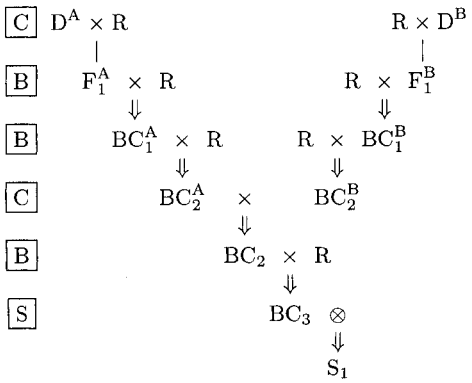
Breeding Plan 3: Merging of target genes in generation F_1



Breeding Plan 4: Merging of target genes in generation BC_1



Breeding Plan 5: Merging of target genes in generation BC_2



Breeding Plan 6: Merging of target genes in generation BC_3

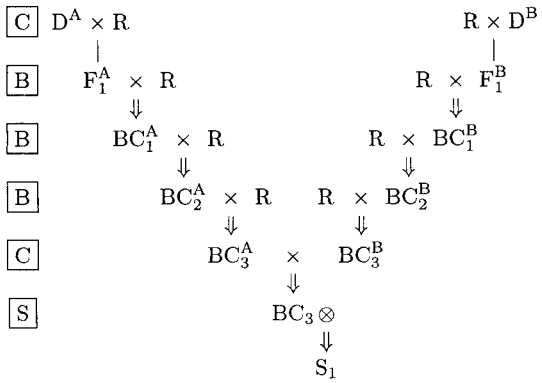


Fig. 1. Five breeding schemes (Breeding Plan 2 to 6) for marker-assisted introgression of two genes and a reference scheme (Breeding Plan 1) for introgression of two genes without background selection. D^A and D^B are the donor lines of the target genes, R is the recipient line. The symbols denote: | no selection, ↓ selection only for presence of the target gene(s), ⇓ selection for presence of the target gene(s) and marker-assisted background selection, × crossing, and ⊗ selfing, C, B, and S indicate generations with crossing, backcrossing, and selfing, respectively.

get genes, the total number of individuals per generation was split equally among the two branches of the breeding program. None of these population sizes is smaller than the respective minimum population sizes determined above.

We employed 120 individuals in the crossing generation to merge the target genes and in the selfing generation. This resulted in a constant total number of individuals applied in the breeding program. However, when two linked target genes

Table 1. Probability *p* that an individual derived by selfing or crossing/backcrossing, is homozygous (selfing) or heterozygous (crossing/backcrossing) for both target genes, provided one of its parents was heterozygous for both target genes. The values depend on the recombination frequency *r* between the two target loci and the gametic phase of the target genes in the parent.

Gametic phase	Selfing	Crossing/backcrossing
Unlinked	$p = \left(\frac{1}{4}\right)^2$	$p = \frac{1}{4}$
Coupling	$p = \left(\frac{1-r}{2}\right)^2$	$p = \frac{1-r}{2}$
Repulsion	$p = \left(\frac{r}{2}\right)^2$	$p = \frac{r}{2}$

are merged in generation BC_3, the minimum population size according to Table 2 is not reached and, hence, in an applied breeding program, a larger population size should be used.

Selection Strategy

We applied the two-, three-, and four-stage selection strategies, originally developed for studying the introgression of one target gene (Frisch et al., 1999b) to the above breeding plans. We considered c chromosomes of length l_i ($i = 1 \ldots c$), those carrying at least one target locus are referred to as "carrier chromosomes", the remaining are referred to as "non-carrier chromosomes". Positions on the chromosomes are represented by a scale in Morgan units ranging from 0 to l_i. We considered 2 target loci at positions $x_{i,j}$ ($j = 1, 2$) on the respective carrier chromosomes and two flanking markers at positions $y_{i,j,n1}$ and $y_{i,j,n2}$, and m additional markers on the carrier chromosomes are located at positions $z_{i,k}$ ($k = 1 \ldots m$). In total e markers at positions $u_{i,f}$ ($f = 1 \ldots e$) are located on the non-carrier chromosomes. Let $X_{i,j}$, $Y_{i,j,n1}$, $Y_{i,j,n2}$, $Z_{i,k}$, and $U_{i,f}$ be indicator variables, which take the value 1, if the corresponding locus is homozygous for the recurrent parent allele and 0 otherwise. From these random variables we obtain the count variables $X = \Sigma_{i,j} X_{i,j}$, $Y = (\Sigma_i Y_{i,j,n1} + Y_{i,j,n2})$, and $U = Y + \Sigma_{i,k} Z_{i,k} + \Sigma_{i,f} U_{i,f}$. Furthermore, the indicator variable Z is 1, if all m additional markers on the carrier chromosomes are homozygous for the recurrent parent allele and 0 otherwise.

By using the random variables X, Y, Z, and U as selection indices, three sequential selection strategies were applied. The first step always involved selection of individuals carrying the target allele ($X = 1$). Subsequently followed one, two, or three steps with background selection. In each selection step, only those individuals selected in the previous step are subjected to marker analyses. In the individual selected for producing the next backcross generation, all markers not fixed in the previous generation(s) are analyzed to determine which of them are homozygous and, hence, need not be examined in the subsequent generation(s).

In two-stage selection, selection in the second step is based on the index U, which takes into account all marker loci irrespective of their position in the genome. In three-stage selection, the second selection step rests on the flanking markers (index Y), while the final step is again based on all markers (index U) irrespective of their genomic location. Four-stage selection is similar to three-stage selection, but adds after the second step one additional selection step exclusively based on the markers located on the carrier chromosome (index Z).

We further investigated a modification of two-, three-, and four-stage selection, which we call reduced two-, three-, and four-stage selection. In generations before merging the target genes, in each branch of the breeding program, selection is performed for presence of one target gene. In such generations, the markers located on the carrier chromosome of the second target gene were not analyzed.

Table 2. Minimum population size in each generation of Breeding Plans 2 to 6 required for recovering two linked ($d = 0.4$ M) target genes with probability $q = 1$ in generation 1 and $q = 0.99^{1/5}$ in Generations 2 to 6. The symbols denote: C, crossing generation; B, backcross generation; S, selfing generation; /R, repulsion phase; and /C, coupling phase.

Breeding Plan	Generation 1	2	3	4	5	6
2	C: 1	C/R: 42	B/C: 14	B/C: 14	B/C: 14	S/C: 45
3	C: 2 × 1	C: 22	B/R: 42	B/C: 14	B/C: 14	S/C: 45
4	C: 2 × 1	B: 2 × 10	C: 22	B/R: 42	B/C: 14	S/C: 45
5	C: 2 × 1	B: 2 × 10	B: 2 × 10	C: 22	B/R: 42	S/C: 45
6	C: 2 × 1	B: 2 × 10	B: 2 × 10	B: 2 × 10	C: 22	S/R: 325

Simulations

Each backcross program was simulated 10 000 times with PLABSIM (Frisch et al., 2000). In the simulations, the entire map was additionally covered with equally spaced (1 cM) background loci to monitor the parental origin of the entire genome. For each simulation run, the percentage of the RPG was assessed in the selected individuals of each generation by dividing the number of loci (marker and background loci) homozygous for the recurrent parent allele by the total number of loci monitored. The values obtained from the 10 000 repetitions can be regarded as realizations of random variables that describe the proportion of RPG attained in a backcross program with the parameter settings considered. The 10% percentile of the empirical distribution of the RPG in the selected individual (Q10) was used as an estimator for the proportion of RPG reached after selection in the final generation with probability 0.90. For example, a Q10 value of 98% means that "with probability 0.90 an RPG proportion greater than 98% is attained" under the parameter combination being considered.

The number of MDP required in each generation was counted and summed over the entire backcross program. The

Table 3. Simulation results for the mean and 10% percentile (Q10) of the distribution of the recurrent parent genome in generations BC_1 to BC_6 and BC_6-S_1 to BC_8-S_1 with random selection (Breeding Plan 1) of individuals carrying the target genes at both unlinked or linked ($d = 0.4$ M) loci and expected values for the mean without selection.

Generation	No selection	Selection for presence of both target genes			
		Unlinked target genes		Linked target genes	
	Mean	Mean	Q10	Mean	Q10
	%				
BC_1^A/BC_1^B†	75.0	74.0/74.1	67.4/67.5	73.9/73.8	67.3/67.3
BC_2^A/BC_2^B	87.5	86.1/86.2	80.6/80.7	86.1/85.9	80.6/80.5
BC_3^A/BC_3^B	93.8	92.3/92.4	88.3/88.4	92.4/92.2	88.3/88.1
BC_4^A/BC_4^B	96.9	95.6/95.6	92.7/92.8	95.6/95.4	92.7/92.6
BC_5^A/BC_5^B	98.4	97.3/97.3	95.2/95.3	97.3/97.2	95.2/95.1
BC_6^A/BC_6^B	99.2	98.2/98.2	96.7/96.8	98.3/98.1	96.7/96.6
BC_7^A/BC_7^B	99.6	98.7/98.7	97.6/97.6	98.7/98.6	97.6/97.4
BC_8^A/BC_8^B	99.8	99.0/99.0	98.1/98.1	99.9/98.9	98.1/98.0
BC_6	99.2	97.4	95.8	97.3	95.7
BC_7	99.6	98.0	96.7	97.9	96.6
BC_8	99.8	98.3	97.3	98.3	97.2
BC_6-S_1	99.2	96.0	93.8	97.2	95.2
BC_7-S_1	99.6	96.7	94.8	97.8	96.2
BC_8-S_1	99.8	97.2	95.5	98.2	96.8

† The superscripts A and B refer to the branches of the breeding program to introgress target genes A and B, respectively.

Table 4. Simulation results for the 10% percentile (Q10) of the distribution of the recurrent parent genome in the selected F_1 to BC_5-S_1 individuals and total number of marker data points (MDP) required in a backcross program using Breeding Plan 3 to introgress two unlinked or two linked (d = 0.4 M) target genes with constant, increasing or decreasing population size from early to advanced backcross generations. Values for MDP are rounded to multiples of ten.

Population size	Generation	Unlinked target genes			Linked target genes		
		Two-stage selection	Three-stage selection	Four-stage selection	Two-stage selection	Three-stage selection	Four-stage selection
		Q10[%]/MDP					
		Constant population size					
80	F_1	54.8/1600	44.9/250	44.8/270	54.6/1600	42.9/220	42.7/240
	BC_1	79.7/2610	72.6/520	72.6/570	79.5/2150	71.7/460	71.6/500
	BC_2	91.3/3100	86.5/730	85.9/740	91.8/2860	86.0/740	85.9/760
	BC_3	95.8/3260	94.3/910	93.5/880	96.0/3070	94.4/1010	94.1/990
	BC_4	97.6/3310	97.5/970	97.4/950	97.5/3150	97.7/1090	97.6/1080
	BC_5	98.4/3320	98.4/980	98.4/960	98.3/3180	98.4/1100	98.4/1080
	BC_3-S_1	94.6/3270	94.1/930	93.7/900	94.6/3100	94.8/1040	94.5/1030
	BC_4-S_1	96.5/3310	96.7/980	96.7/950	96.3/3160	96.9/1090	96.9/1080
	BC_5-S_1	97.4/3320	97.6/980	97.5/960	97.3/3180	97.5/1100	97.5/1080
120	F_1	56.2/2390	45.2/200	45.4/330	55.9/2400	42.8/260	43.1/270
	BC_1	80.9/3870	73.1/640	73.1/680	80.6/3200	71.9/570	72.0/610
	BC_2	92.0/4550	87.3/930	86.6/900	92.5/4190	86.8/960	86.5/920
	BC_3	96.2/4760	95.2/1200	94.4/1110	96.3/4460	95.3/1380	95.1/1291
	BC_4	97.8/4820	97.8/1270	97.8/1190	97.7/4560	97.8/1460	97.8/1377
	BC_5	98.5/4840	98.5/1270	98.4/1190	98.4/4590	98.4/1460	98.4/1379
	BC_3-S_1	95.1/4780	95.1/1220	94.7/1130	95.0/4500	95.5/1400	95.4/1323
	BC_4-S_1	96.8/4830	97.0/1270	97.0/1190	96.7/4570	97.0/1460	97.0/1378
	BC_5-S_1	97.6/4840	97.6/1270	97.6/1190	97.5/4590	97.6/1460	97.6/1379
160	F_1	57.1/3200	45.8/350	45.7/380	56.8/3190	43.3/300	43.4/320
	BC_1	81.4/5130	73.6/750	73.4/800	81.3/4240	72.0/640	72.2/690
	BC_2	92.5/5990	87.9/1140	87.0/1050	92.9/5490	87.6/1200	86.8/1060
	BC_3	96.4/6240	95.8/1510	95.1/1360	96.5/5820	95.9/1750	95.6/1590
	BC_4	97.9/6310	97.9/1570	97.8/1440	97.8/5930	97.9/1830	97.9/1680
	BC_5	98.5/6320	98.5/1570	98.4/1440	98.5/5950	98.5/1830	98.4/1680
	BC_3-S_1	95.4/6260	95.5/1520	95.3/1380	95.4/5860	95.8/1780	95.8/1620
	BC_4-S_1	97.0/6310	97.0/1570	97.0/1440	96.9/5940	97.0/1830	97.0/1680
	BC_5-S_1	97.7/6320	97.7/1570	97.7/1440	97.7/5960	97.6/1830	97.6/1680
		Increasing population size					
120	F_1	56.2/2400	45.3/300	45.3/330	55.7/2400	42.8/260	43.1/270
60	BC_1	80.1/3140	72.8/530	72.8/580	79.7/2800	71.5/470	71.5/500
120	BC_2	91.7/3860	86.8/800	86.3/780	92.2/3850	86.5/810	86.3/790
180	BC_3	96.2/4190	95.1/1180	94.5/1070	96.2/4290	95.2/1370	94.9/1280
120	BC_3-S_1	95.2/4210	95.0/1190	94.7/1090	95.1/4320	95.4/1400	95.4/1320
		Decreasing population size					
120	F_1	56.2/2400	45.3/300	45.2/330	55.7/2400	42.9/260	43.0/280
180	BC_1	81.2/4620	73.2/720	73.2/770	81.0/3600	72.1/630	72.0/680
120	BC_2	92.2/5280	87.5/1030	86.6/990	92.6/4560	87.2/1060	86.5/1000
60	BC_3	96.0/5380	95.0/1180	94.2/1120	96.1/4690	95.2/1280	94.8/1210
120	BC_3-S_1	94.9/5390	94.9/1200	94.5/1140	94.8/4730	95.4/1310	95.3/1250

mean value over the 10 000 repetitions was used to characterize the required number of MDP for any one breeding plan.

RESULTS

In backcrossing, when selection is performed only for presence of the target genes, the mean of the RPG was about 1% below the theoretical values expected without selection (Table 3). After six generations of backcrossing, a Q10 value of 95.8 and 95.7% was reached for two unlinked or linked loci, respectively. In subsequent generations, the increase in Q10 values was 1.5% for both scenarios. Selfing BC_6 to BC_8 individuals resulted in a reduction of the Q10 values of about 2% for unlinked target loci, but only about 0.5% for linked target loci. The BC_6-S_1 individuals reached Q10 values of 93.8 and 95.2% for unlinked or linked target loci, respectively.

In the F_1 generation, two-stage selection resulted in up to 10% greater Q10 values for the RPG than three- or four-stage selection (Table 4). The differences in RPG among the three selection strategies decreased in size in advanced generations and were only marginal in generation BC_5. The RPG values reached with 160 individuals per generation were up to 2% greater than those reached with only 80 individuals. For linked target genes the trends were the same as for unlinked target genes with the exception that in the selfing generations three- and four-stage selection yielded up to 0.5% greater Q10 values than two-stage selection. With two-stage selection about one-half of the total number of MDP required in total were consumed in the first generation of background selection. Three- and four-stage selection resulted in a reduction of the number of MDP required in the F_1 generation of up to 80% compared with two-stage selection.

For two unlinked target genes, the Q10 values for the RPG of selected BC_3-S_1 individuals were greater in all breeding plans with background selection (Table 5) than those of BC_6-S_1 individuals without background selection (Table 3). The numbers of MDP required for this

Table 5. Simulation results for the 10% percentile (Q10) of the distribution of the recurrent parent genome in the selected BC_3-S_1 individual and total number of marker data points (MDP) required in a backcross program to introgress two unlinked target genes. Values for MDP are rounded to multiples of ten.

Merging of target genes in generation	Population size in generation			Selection strategy		
	BC_1	BC_2	BC_3	Two-stage selection	Three-stage selection	Four-stage selection
				Q10[%]/MDP		
P	60	120	180	94.9/2560	94.2/780	93.9/750
	120	120	120	94.9/3500	94.3/820	93.9/800
	180	120	60	94.7/4540	94.2/810	93.8/820
F_1	60	120	180	95.2/4200	95.0/1200	94.7/1090
	120	120	120	95.1/4780	95.1/1220	94.7/1140
	180	120	60	94.9/5390	94.9/1200	94.5/1140
BC_1	2 × 30	120	180	95.4/4590	95.5/1590	95.4/1380
	2 × 60	120	120	95.5/6730	95.8/1780	95.5/1480
	2 × 90	120	60	95.4/8970	95.6/2010	95.4/1550
BC_2	2 × 30	2 × 60	180	95.8/4670	96.0/1910	95.8/1530
	2 × 60	2 × 60	120	95.9/6810	96.1/2240	95.9/1690
	2 × 90	2 × 60	60	95.8/9050	96.2/2590	95.9/1860
BC_3	2 × 30	2 × 60	2 × 90	96.2/4780	96.3/2280	96.2/1960
	2 × 60	2 × 60	2 × 60	96.2/6770	96.4/2340	96.3/1910
	2 × 90	2 × 60	2 × 30	96.1/8900	96.3/2470	96.2/1870
				Reduced selection strategies		
BC_1	2 × 30	120	180	95.4/4380	95.5/1550	95.3/1380
	2 × 60	120	120	95.4/6280	95.7/1720	95.4/1480
	2 × 90	120	60	95.3/8270	95.6/1920	95.4/1550
BC_2	2 × 30	2 × 60	180	95.8/4290	96.0/1780	95.8/1490
	2 × 60	2 × 60	120	95.8/6190	96.1/2080	95.9/1650
	2 × 90	2 × 60	60	95.7/8190	96.1/2370	95.9/1780
BC_3	2 × 30	2 × 60	2 × 90	96.2/4310	96.3/2080	96.2/1850
	2 × 60	2 × 60	2 × 60	96.2/6100	96.3/2140	96.3/1820
	2 × 90	2 × 60	2 × 30	96.1/8030	96.3/2280	96.2/1790

saving of three backcross generations ranged between about 800 for merging the targets in generation P when applying three- or four-stage selection, up to about 9000, for merging the target genes in generations BC_1 to BC_3 when applying two-stage selection with increasing population size.

Variation of the population size for BC_1 to BC_3 had only marginal influence on the Q10 values of the RPG in selected BC_3-S_1 individuals (Table 5). By contrast, in comparison with a constant population size across the backcross generations (ratio 1:1:1 in generations BC_1:BC_2:BC_3), the required number of MDP was reduced up to 25% for a ratio 1:2:3 and increased up to 33% for a ratio 3:2:1.

The selection strategy had a considerable effect on the RPG content only when merging the two target genes in generations P or F_1 (Table 5). In this case, the Q10 values with two-stage selection were up to one percent greater than with four-stage selection. No differences in the Q10 values were observed when the target genes were merged in later generations. However, two-stage selection required two times (merging target genes in generation BC_3, increasing population size) to five times (merging target genes in generation P, decreasing population size) more MDP than did three-stage selection. Four-stage selection further reduced the number of required MDP by about 10 to 20% compared with three-stage selection.

The reduced selection strategies reached practically the same Q10 values as the ordinary selection strategies (Table 5). While the ordinary and reduced four-stage selection required the same number of MDP when the target genes were merged in generation BC_1, applying the reduced two-stage selection saved about 10% of the MDP required for ordinary two-stage selection when targets were merged in generation BC_3.

Merging the target genes in advanced generations resulted in a greater RPG content than in early generations (Table 5). The difference was about 2% in four-stage selection and 1% in two-stage selection. The greater Q10 values resulting from merging the target genes in advanced compared with early generations required a greater number of MDP. For example, merging the target genes in generation BC_3 required two to three times more MDP than merging them in generation P.

For two linked target genes (d = 0.4 M), the Q10 values for the RPG of selected BC_3-S_1 individuals (Table 6) were greater than those of BC_6-S_1 individuals without background selection (Table 3) only when (i) applying three- or four-stage selection and merging the target genes in generation F_1 (Breeding Plan 3), or (ii) merging the target genes in generations BC_1 to BC_3 (Breeding Plans 4–6). In contrast, when (i) merging the target genes in generation P (Breeding Plan 2), or (ii) merging the target genes in generation F_1 (Breeding Plan 3) and applying two-stage selection, the Q10 values for the RPG of selected BC_3-S_1 individuals were smaller than those reached without background selection in generation BC_6-S_1.

For linked target genes, the effects of (i) increasing and decreasing population size, and (ii) the generation of merging the target genes on the RPG and the number of MDP required followed the same trends (Table 6) as for unlinked target genes (Table 5). However, when compared with two-stage selection, three- and four-

Table 6. Simulation results for the 10% percentile (Q10) of the distribution of the recurrent parent genome in the selected BC_3-S_1 individual and total number of marker data points (MDP) required in a backcross program to introgress two linked (d = 0.4 M) target genes. Values for MDP are rounded to multiples of ten.

Merging of target genes in generation	Population size in generation			Selection strategy		
	BC_1	BC_2	BC_3	Two-stage selection	Three-stage selection	Four-stage selection
				Q10[%]/MDP		
P	60	120	180	95.1/3480	94.5/950	94.3/950
	120	120	120	94.9/4920	94.8/1020	94.6/1020
	180	120	60	94.8/6450	94.6/1020	94.5/1050
F_1	60	120	180	95.1/4320	95.4/1400	95.4/1320
	120	120	120	95.0/4500	95.5/1400	95.4/1320
	180	120	60	94.8/4730	95.4/1310	95.3/1250
BC_1	2 × 30	120	180	95.4/4440	95.8/1680	95.7/1640
	2 × 60	120	120	95.4/6560	95.9/1780	95.9/1780
	2 × 90	120	60	95.3/8780	95.9/1960	95.8/1940
BC_2	2 × 30	2 × 60	180	95.8/4580	96.1/1820	96.0/1720
	2 × 60	2 × 60	120	95.9/6750	96.2/2160	96.2/2000
	2 × 90	2 × 60	60	95.8/9020	96.2/2530	96.1/2330
BC_3	2 × 30	2 × 60	2 × 90	96.2/4790	96.4/2290	96.3/2130
	2 × 60	2 × 60	2 × 60	96.2/6790	96.5/2320	96.4/2140
	2 × 90	2 × 60	2 × 30	96.1/8920	96.5/2440	96.4/2240

stage selection attained up to 0.5% greater RPG values with fewer MDP.

DISCUSSION

Genetic Model

Following earlier studies on marker-assisted selection (Hospital et al., 1992; Visscher et al., 1996; Hospital and Charcosset, 1997), we used Haldane's (1919) mapping function for modeling crossover formation during meiosis. It is well know that this is a simplified model because of the assumption of no interference (Stam, 1979). Since Haldane's pioneering paper, numerous researchers (e.g., Kosambi, 1944; Karlin and Liberman, 1978; Zhao and Speed, 1996; Browning, 2000) proposed alternative mathematical models, which include interference. Most of the resulting map functions can be modeled by a stationary renewal process, the inter-event distribution of which can be approximated by gamma distributions (Zhao and Speed, 1996). McPeek and Speed (1995) compared the fit of various crossover formation models and concluded that gamma inter-event distribution fit best the *Drosophila* dataset of Morgan et al. (1935).

We used Haldane's (1919) mapping function due to its mathematical simplicity and the stochastic independence of crossover formations in adjacent chromosome regions, which allowed us to derive closed analytical formulas for the problems addressed in this study. Applying gamma inter-event distributions would in most instances yield unwieldy formulas, which could only be numerically approximated. Moreover, as pointed out by Stam and Zeven (1981), dropping the assumption of no interference would reduce the generality of the presented approach because it would be necessary to know the type and degree of interference for the chromosome region of each target gene.

With positive crossover interference, multiple crossover events in a given chromosome region occur less frequently than with no interference (Stam, 1979). Assuming positive interference, (i) the reduction in the number of MDP required (unlinked and linked target genes) and (ii) the increase of RPG values (linked target genes), which are observed in three- and four-stage selection compared with two-stage selection, are expected to be lower in magnitude. The reverse holds true under the assumption of negative interference. In conclusion, the reader should be aware that the model presented (as with most mathematical models of biological systems) is not capable of capturing every detail of the underlying biological process, and our results should be interpreted accordingly.

Saving Backcross Generations

Introgression of one gene is usually accomplished by six generations of backcrossing (Allard, 1960). Hence, a straightforward method for introgression of two genes is to transfer each of them into the recipient genotype and then cross these converted lines, leading to the creation of one homozygous individual. We used this concept as motivation for Breeding Plan 1, which was used as a reference to determine the RPG proportion reached in a classical gene introgression program without background selection (Table 3).

With background selection, the number of backcross generations required for the introgression of one target gene was reduced from six to three when using 60 individuals per generation (Frisch et al., 1999b). Consequently, when applying background selection, using 60 individuals per generation in each branch of the breeding program and merging the target genes in generation BC_3 (Breeding Plan 6), RPG values comparable with those reached after six generations of backcrossing without background selection (Breeding Plan 1) is attainable. This expectation proved true for both unlinked and linked target genes (Tables 5 and 6), indicating that saving of three backcross generations due to background selection is a realistic goal for simultaneous introgression of two genes.

Another trend observed for the introgression of one target gene (Frisch et al., 1999b) was confirmed for two genes (Tables 3 and 4): Even with limited resources (population size $n_t = 80$ and 500 to 1 000 MDP), background selection can save one or more generations in a backcross program. However, a direct comparison of the RPG values across both studies was not possible, because in the present study background selection was employed not only in backcross generations but also in crossing and selfing generations. This contrasts with the introgression of one target gene (Frisch et al., 1999b), where background selection was employed only in backcross generations.

Increasing, Constant, or Decreasing Population Size

Variation in the population size n_t for constant total number of individuals Σn_t only marginally affected the RPG proportion in the last generation of the breeding program (Tables 5 and 6). This result can be attributed to two effects that compensated for each other (Frisch et al., 1999b): First, selection from large populations in early backcross generations takes advantage of the large variance in RPG. Second, by contrast, the selection response due to large populations in late backcross generation has a greater carry-over rate with respect to the RPG content in the final breeding product. The latter effect was explained by Hospital et al. (1992) with a mathematical derivation for the special case of a backcross program with one generation of background selection. These effects suggest that in breeding programs with at least three backcross generations, increasing population sizes can be used to minimize the number of MDP required. In contrast, in breeding programs with the goal of maximizing the RPG after two backcross generations, a constant population size in generations BC_1 and BC_2 ($n_1 = n_2$) is preferable (Table 4).

Selection Strategy

For unlinked target genes, two-stage selection resulted in greater RPG values in early generations than three- and four-stage selection. However, in advanced backcross and selfing generations these differences were marginal (Table 4). A high selection pressure was placed on carrier chromosomes in early generations with three- and four-stage selection, and a low selection pressure was placed on noncarrier chromosomes (Frisch et al., 1999b). This results in low overall RPG values for these selection strategies in early generations, because the noncarrier chromosomes form the major part of the genome. Consequently, when the goal of a breeding program is to generate high RPG values after two backcross generations, two-stage selection is preferable. However, with three backcross generations, three- or four-stage selection provides an option to minimize the number of MDP required.

For two linked target genes, three- and four-stage selection yielded greater RPG values in the final generation than two-stage selection (Table 6), indicating that the chromosome segment directly attached to linked target genes is hardly reduced when treating all markers as equal. Individuals showing recombination between the target genes and the flanking markers are preferably selected in three- and four-stage selection. Hence, the probability that the finally selected individual carries a chromosome segment from the recipient between the linked target genes is considerably greater than for two-stage selection. This results in greater Q10 values for the RPG reached for three- and four-stage selection compared with two-stage selection. This result suggests that for linked target genes, three- and four-stage selection increases the proportion of the RPG that can be recovered and decreases the number of MDP required.

The reduced selection strategies for unlinked target genes resulted in the same RPG values observed for the corresponding regular strategies (Table 5). In the generation of merging the target genes, the carrier chromosomes of the selected individuals are inherited from the parent, which is originating from the branch of the breeding program in which background selection on the carrier chromosome was applied. Therefore, omitting background selection on the respective chromosome in the other branch of the breeding program has practically no effect on the RPG values after the merging of the target genes. Consequently, for unlinked target genes with merging of target genes in generation BC_1 to BC_3, the reduced selection strategies are preferable. They allow a reduction in the total number of required MDP, without any appreciable effect on the final recovery of the RPG.

Generation of Merging the Target Genes

Breeding Plans 2 to 6 differ with regard to the number of generations in which background selection is applied, and the expected number of carriers of the target genes per generation. The latter determines the number of individuals that are subjected to background selection.

When merging the target genes in generation P (Breeding Plan 2), only four generations of background selection can be carried out, whereas in other breeding plans five generations are possible (Fig. 1). This explains (i) the lower RPG values reached with Breeding Plan 2 compared with Breeding Plans 3 to 6, and also (ii) the greater RPG values reached with two-stage selection compared with three- and four-stage selection, because two-stage selection yields greater RPG values in early generations.

In a backcross generation before merging the target genes, about one-half of the backcross individuals are expected to carry one target gene and, hence, to be marker assayed. In contrast, in a backcross generation after merging two unlinked target genes, only 1/4 of the individuals are expected to carry both target genes. For linked target genes the expected portion of carriers of both target genes depends on the degree of linkage and the gametic phase of the target genes. For loose linkage ($r \rightarrow 1/2$), it converges to 1/4, whereas for tight linkage ($r \rightarrow 0$) it converges to 1/2, when the target genes are in coupling, or to 0, when they are in repulsion phase.

The greater portion of individuals expected to be marker-assayed in generations before merging the target genes than in generations after merging the target genes results in a higher selection intensity. This explains the greater RPG values attained when the target genes are merged in advanced generations.

Consequently, when the primary objective is reaching a maximum RPG value with a given number of individuals, merging the target genes in generation BC_2 or BC_3 is advantageous. However, when the target genes are merged in generation BC_3, they are in repulsion phase in the individual to be selfed. Depending on the recombination frequency r between the target genes, the population size of the selfing progeny must be fairly large in order to recover with sufficient probability of success q at least one carrier of both target genes (Eq. [7]).

In contrast, when the primary objective is to save three backcross generations with minimum expenditures, merging the target genes in generation F_1 combined with three- or four-stage selection is advantageous. This scheme combines five generations of background selection, resulting in a saving of three backcross generations, with a low consumption of MDP for both unlinked and linked target genes.

Number of Marker Data Points Required

By applying two-stage selection and a constant population size of $n_t = 60$ individuals in generations BC_1 to BC_3 introgression of one target gene required a total of 3340 MDP (Frisch et al., 1999b, Table 3). Introgression of two unlinked target genes with Breeding Plan 2 and three-stage selection required 3500 MDP, when using 120 individuals per generation (Table 5).

In introgression of one target gene, the expected portion of carriers of the target gene in a backcross population, which are undergoing marker analyses, is 1/2, whereas for introgression of two unlinked genes the expected portion is 1/4 according to Breeding Plan 2. Therefore, in a backcross program for two genes, in which twice the number of individuals are employed than in a backcross program for one gene, the number of required MDP is expected to be the same as in the backcross program for one gene. The additional marker analyses required in the selfing generation account for a slight increase (3500 vs. 3340) in the total number of MDP required with two genes. Thus, with Breeding Plan 2 introgression of two unlinked target genes can be accomplished with only a few more MDP than required for the transfer of one target gene, but at the expense of doubling the population size.

The smaller number of MDP required with three- and four-stage selection in comparison with two-stage selection (Tables 3 and 5) is due to a reduction in the number of individuals, which are analyzed for the entire set of markers in the first generation of background selection (Frisch et al., 1999b). The reduction of the number of individuals for which the complete set of markers is analyzed in generation BC_1 explains also that the number of required MDP is reduced when (i) reducing the population size in early generations and increasing it in advanced generations in two-stage selection, or (ii) merging target genes in generation BC_1.

Omitting the marker analyses for one chromosome in the reduced strategies results in fewer MDP required by these selection strategies than by the respective ordinary selection strategies.

Transferability to Other Situations in Breeding

Our simulation results depend on the population sizes employed and the chosen genetic map. In addition to the presented results, we investigated two alternative methods to determine the population size in backcross and selfing generations: First, the population size in the crossing generation was varied in accordance with the varying population size for the backcross generations (e.g., for Breeding Plan 3 for the scenario with increasing population size we used 2×1, 48, 96, 144, 192, 120 individuals in generations C_1, C_2, BC_1, BC_2, BC_3, BC_3-S_1, respectively). Second, the population size in the crossing generation was chosen according to minimum population size calculated with Eq. [7] (e.g., 2×1, 22, 60, 120, 180, 120 individuals in generations C_1, C_2, BC_1, BC_2, BC_3, BC_3-S_1, respectively). While the absolute Q10 values for the RPG under these scenarios differed slightly from the results presented here, the effects of selection strategy, breeding plan, and generation of merging the target genes were essentially the same (data not shown). For both of the above alternatives, the number of MDP required for Breeding Plan 3 were about 10% below the values of the presented results, whereas the differences were only marginal for the other breeding plans.

Important characteristics of the genetic map used in this study are that (i) it consists of 10 chromosomes with a total map length of 16 M, (ii) map positions of markers and target loci are known, and (iii) the average marker density is 20 cM. The ratio between carrier and noncarrier chromosomes determines the differences between the selection strategies with respect to RPG values attained and number of MDP required. For instance, in crops with less than 10 chromosomes, the differences are expected to be smaller, whereas with more than 10 chromosomes, the proportion of genome on the noncarrier chromosomes increases, and, consequently, the differences between the selection strategies are expected to be greater.

When the map position of markers or target genes is unknown, two-stage selection is the only option. In this situation applying increasing population sizes from early to advanced generations and merging the target genes in early generations can reduce the number of MDP required.

Previous simulations for one target gene (Frisch et al., 1999b) have shown that when considering marker densities higher than 20 cM, increases the RPG values are difficult to attain and require substantially more MDP. This can be explained by the fact that given a high marker density, the low frequency of recombination events is the limiting factor for background selection but not the marker coverage to detect the recombination events. However, using evenly spaced markers

resulted in greater RPG values without requiring more MDP (Frisch et al., 1999b).

For linked target genes we assumed a recombination frequency $r = 0.275$ (map distance $d = 0.4$ M, no interference). If the target genes are more tightly linked, then it is expected that the observed effects of the breeding plan and selection strategy on the proportion of RPG reached and the number of required MDP converge to the values observed for one target gene. In contrast, with increasing map distance between the target genes it is expected that these trends converge to those observed for unlinked target genes. In particular, the increase in RPG values reached with three- and four-stage selection compared with two-stage selection is expected to be smaller with extremely tight but also loose linkage between the target genes.

CONCLUSIONS

The presented results suggest the following guidelines for the design of a marker-assisted backcross program for simultaneous introgression of two dominant target genes:

A reduction in the number of backcross generations from six to three can be attained with 1000 to 1500 MDP in maize.

Applying three- or four-stage selection for linked target genes and reduced three- or four-stage selection for unlinked target genes requires fewer MDP than two-stage selection. For unlinked target genes, the saving in MDP is realized without reduction of the RPG content, unless the target genes are merged in generation P. For linked target genes, the saving in MDP is accompanied with an increase in the RPG content.

Small population sizes in early generations and large population sizes in advanced generations require less MDP than constant or decreasing population sizes while attaining the same RPG content.

When merging the target genes in generation P, only four generations of marker-assisted background selection are possible compared with five generations when merging the target genes in generations F_1 to BC_3. Merging the two target genes in an advanced generation results in greater RPG values than merging them in an early generation but requires more MDP.

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