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Linkage analysis of the visible mutations *Sel* and *Xan* of *Bombyx mori* (Lepidoptera: Bombycidae) using SSR markers

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Abstract. Wild type silkworm larvae have opaque white skin, whereas the mutants *Sel* (Sepialumazine) and *Xan* (Xanthous) are yellow-skinned. Previous genetic analysis indicated that *Sel* and *Xan* are on established linkage groups 24 (0.0) and 27 (0.0), respectively. However, in constructing a molecular linkage map using simple sequence repeat (SSR) loci, we found that the two mutations were linked. To confirm this finding, we developed a set of SSR markers and used them to score reciprocal backcross populations. Taking advantage of the lack of crossing-over in female silkworms, we found that the progeny of backcrosses between F_1 females and males of the parental strains (BC_1F) of the two visible mutations had the same inheritance patterns linked to the same SSR markers. This indicated that the two visible mutations belonged to the same chromosome. To confirm this finding, we tested for independent assortment by crossing *Sel* and *Xan* marker strains with each other to obtain F_1 and F_2 populations. Absence of the expected wild type class among 5000 F_2 progeny indicated that the two visible mutations were located on the same linkage group. We carried out recombination analysis for each mutation by scoring 190 progeny of backcrosses between F_1 males and parental females (BC_1M) and constructed a linkage map for each strain. The results indicated that the *Sel* gene was 12 cM from SSR marker S2404, and the *Xan* gene was 7.03 cM from SSR marker S2407. To construct a combined SSR map and to avoid having to discriminate the two similar dominant mutations in heterozygotes, we carried out recombination analysis by scoring recessive wild type segregants of F_2 populations for each mutation. The results showed that the *Sel* and *Xan* genes were 13 cM and 13.7 cM from the S2404 marker, respectively, consistent with the possibility that they are alleles of the same locus, which we provisionally assigned to SSR linkage group 24. We also used the F_2 recessive populations to construct two linkage groups for the *Sel* and *Xan* genes.

INTRODUCTION

The silkworm, *Bombyx mori* L., is one of the best characterized insects genetically. At least 400 visible and biochemical mutations have been identified and more than 200 of them have been placed on linkage maps (Doira, 1992; Goldsmith, 1995). These silkworm mutants are invaluable biological resources for basic and applied science. Among visible mutations, yellow-skinned mutants are important for linkage analysis as these genes are the only available markers in a few established linkage groups (ELGs). The five yellow-skinned larval mutants reported so far are: lemon (*lem*: 3–0.0), lemon lethal (*lem^l*: 3–0.0), Yellow molting (*Ym*: 27–?), Sepialumazine (*Sel*: 24–0.0), and Xanthous (*Xan*: 27–0.0) (Fujii et al., 1998). Fujii et al. (1998) described both *Sel* and *Xan* as spontaneous dominant mutations in which 5th instar larvae are pale yellow, with a lighter color in heterozygotes. *Sel* larvae are reported to accumulate sepialumazine (7,8-dihydro -6-lactyllumazine) as a by product of pteridine metabolism, accompanied by high levels of sepiapterin deaminase activity (Mazda, 1980); the *Xan* mutation, possibly introgressed from the silkworm's wild ancestor, *B. mandarina*, has not been characterized biochemically. *Sel* and *Xan* are usually selected as visible markers for linkage groups 24 and 27, respectively (Fujii et al., 1998).

In the current study, we used microsatellite markers as part of a larger project to construct a microsatellite map for the silkworm (Miao et al., 2005). Microsatellites, or simple sequence repeats (SSRs), are tandemly repeated units of one to six nucleotides and are abundant in prokaryotic and eukaryotic genomes (Weber, 1990; Field & Wills, 1996). They are ubiquitously distributed in both protein-coding and non-coding regions (Toth et al., 2000). The advent of the polymerase chain reaction (PCR) and the availability of high-throughput automated sequencers have made them a highly informative and versatile class of genetic markers. The SSR technique is a convenient and reliable tool to generate highly polymorphic molecular markers which greatly facilitate building linkage maps (Litt & Luty, 1989; Tautz, 1989; Weber & May, 1989; Schlötterer, 2004). We established a genetic linkage map of 28 linkage groups for *B. mori* employing 518 SSR markers (Miao et al., 2005). However, when we used *Sel* and *Xan* to identify linkage groups 24 and 27, respectively, we discovered that these markers were not

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independent and appeared to be located on the same chromosome. A similar finding was reported recently (Yasukochi et al., 2005). In the present study, our objectives were to screen additional silkworm SSR markers linked to *Sel* and *Xan* genes in order to confirm linkage of the two visible mutations and to localize the *Sel* and *Xan* markers on the SSR linkage map.

MATERIAL AND METHODS

Silkworm strains and culture

Four silkworm strains, Dazao, C108, K07 (homozygous for *Sel*), and K14 (homozygous for *Xan*), were obtained from the Chinese Sericultural Research Institute (Zhenjiang, China), and four strains, 10–740 (homozygous for *Sel*), 10–700 (homozygous for *Xan*), and 08–101 and 18–050 (wild type), were obtained from The Key Sericultural Laboratory of Agricultural Ministry, Southwest University (Chongqing, China). Mutant stocks of *Sel* (strain e23) and *Xan* (strain l90) were originally obtained from the Institute of Genetic Resources, Kyushu University (Fukuoka, Japan). The larvae were reared on mulberry leaves. Fifth-instar larvae or pupae were used as sources of DNA.

Genetic crosses

Three types of progeny were prepared for linkage and recombination analysis: (i) progeny of backcrosses between F_1 females and males of a parental strain (BC_1F), e.g., (K07 × Dazao) female × Dazao male, (K14 × Dazao) female × Dazao male, or (Dazao × C108) female × C108 male, (ii) progeny of backcrosses between F_1 males and females of a parental strain (BC_1M), e.g., Dazao female × (K07 × Dazao) male or Dazao female × (K14 × Dazao) male, and (iii) F_2 progeny of crosses between F_1 heterozygotes, e.g., (K07 × K14) × (K07 × K14) or (10–700 × 08–101) × (10–700 × 08–101).

Genomic DNA preparation

Genomic DNA was extracted from the posterior silk glands of fifth-instar larvae or pupae by a standard phenol-chloroform procedure as described in Sambrook et al. (1989).

Genomic library construction and SSR locus screening

The genomic DNA from the silkworm strain Dazao was digested with *Sau*3AI or *Tsp*509I (New England Biolabs, Boston, MA, USA) according to the manufacturer's instructions. DNA fragments of 7 kb were transformed into competent *Escherichia coli* DH10B cells using standard procedures (Sambrook et al., 1989). A total of 230,000 recombinant clones were picked from the library by blue-white color selection. Oligonucleotides $(CA)_{15}$ and $(CT)_{15}$ were end-labeled with γ-[^{33}P]-dATP and used as hybridization probes to select positive clones for sequencing (Reddy et al., 1999). The microsatellite primers were designed from the sequences flanking the repeats using the program Primer 5.0 (Premier Biosoft International, Palo Alto, CA).

Linkage and recombination analysis

Genomic DNA from 20 offspring of BC_1F progeny was used for linkage analysis. The 20 individuals were selected as ten yellow-skinned larvae and ten normal larvae. For each of the 28 linkage groups, SSR markers were characterized by PCR. Each heterozygous BC_1F individual showed either a homozygous (normal skin) or heterozygous (yellowish skin) SSR pattern.

To obtain the recombination rate, DNA samples were prepared from 190 progeny of a male informative backcross (BC_1M) between Dazao and *Sel* from the K07 strain or *Xan* from the K14 strain. The skin-colour phenotypes of the segregants were recorded before DNA extraction. To determine the gene order and relative distances with respect to *Sel* and *Xan*, the genotypes of all SSR markers were determined for each of the segregants.

To verify the results, 10–740 (*Sel*) was crossed with 18–050 ($+^{Sel}$) to produce the F_1 and the F_1 individuals were crossed with each other to produce the F_2 generation. Similarly, strain 10–700 (*Xan*) was crossed with 08–101 ($+^{Xan}$) to produce F_1 individuals which were then crossed to obtain the F_2 generation. In each F_2 cross, larval progeny with the visible mutant phenotypes were separated from normal individuals.

The data were analyzed by Mapmaker/Exp ver. 3.0 (Lander et al., 1987; Liu & Meng, 2003). The "GROUP" command was used to segregate all informative markers into linkage groups with a LOD score of 3.0 and a maximum recombination fraction of 0.25.

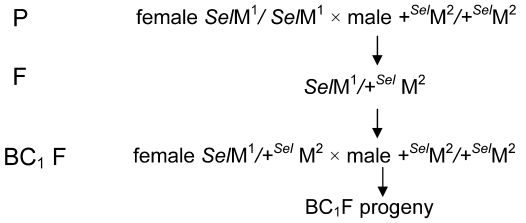
RESULTS

Screening SSR markers

To confirm the inheritance characteristics of the two genes, we constructed two large-insert plasmid libraries to screen for informative SSR markers in the silkworm strains available for mapping, and used hybridization with radioactively labeled $(CA)_{15}$ and $(CT)_{15}$ probes to select positive clones containing $(CA)_n$ or $(CT)_n$ repeats for sequencing. Among 500 clones we found 300 robust SSR loci that could amplify single bands in K07 and K14 which could be used for mapping relative to Dazao, a wild type strain used to construct a first generation genome-wide SSR linkage map (Miao et al., 2005).

Linkage analysis of SSR markers with visible mutations *Sel* and *Xan*

In *B. mori*, linkage analysis can be carried out efficiently because no crossing over occurs in females (Sturtevant, 1915; Goldsmith, 1995). To find which SSR markers were linked to *Sel* or *Xan*, we screened the genotypes of 20 BC_1F individuals using Dazao as a standard wild type to determine which SSR markers (e.g., M) showed a heterozygous (M^1M^2) or homozygous (M^2M^2) pattern among the yellow-skinned ($Sel+^{Sel}$ or $Xan+^{Xan}$) or normal skin ($+^{Sel}+^{Sel}$ or $+^{Xan}+^{Xan}$) individuals, respectively



Male gamete	Female gametes	
	$Sel M^1$	$+^{Sel}M^2$
$+^{Sel}M^2$	$Sel M^1/+^{Sel}M^2$	$+^{Sel}M^2/+^{Sel}M^2$

Fig. 1. Scheme of the cross-breeding analysis showing inheritance of the visible mutation *Sel* and a polymorphic SSR marker from the same linkage group, with complete linkage in the female. P, parental cross; F_1, first filial generation; BC_1F, female F_1 backcross with parental male; M, a microsatellite locus; M^1 and M^2, two different alleles of the M locus.

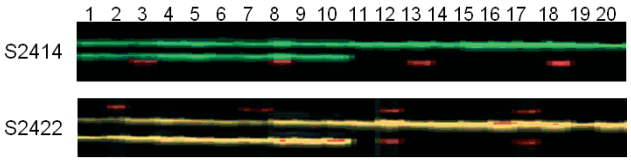


Fig. 2. Electrophoretic patterns of two fluorescent SSR markers (S2414, HEX; S2422, TAMRA). Shown are typical SSR patterns for the same linkage group, on which the *Sel* or *Xan* genes are located, amplified from genomic DNA of 20 BC_1F individuals of female (K07 × Dazao) × Dazao male. Individuals in lanes 1–10 had the yellow-skinned larval phenotype ($Sel M^1/+^{Sel}M^2$); individuals in lanes 11–20 had the normal larval skin phenotype ($+^{Sel}M^2/+^{Sel}M^2$). K07, homozygous for *Sel*.

(Fig. 1) (Kadono-Okuda et al., 2002). In Fig. 2, the first 10 individuals are yellow-skinned and show two bands indicating that they were heterozygotes; the remaining 10 normal skin individuals show a single band indicating that they were homozygotes. These results corresponded to the visible phenotypes of backcross progeny and indicated that these markers were linked with *Sel* or *Xan* genes. In all, a total of 14 SSR markers were found to be linked to *Sel* or *Xan* (Table 1).Using the same strategy with Dazao and C108 as wild type strains we confirmed that all 14 SSR markers belonged to the same linkage group (data not shown).

Phenotypes of the F_2 generation from crosses between *Sel* and *Xan*

Both *Sel* and *Xan* are dominant mutations with similar yellow-skinned larval phenotypes. To test whether these mutations are located on the same chromosome, we crossed K07 (homozygous for *Sel*) with K14 (homozygous for *Xan*) and obtained the F_1 and F_2 segregants. Among 5,000 F_2 progeny all had yellow larval skin, indicating that the two mutations are located on the same chromosome. As indicated in Fig. 3, if the two genes were on different chromosomes, one-sixteenth of the F_2 larvae (approximately 300 individuals) would have been

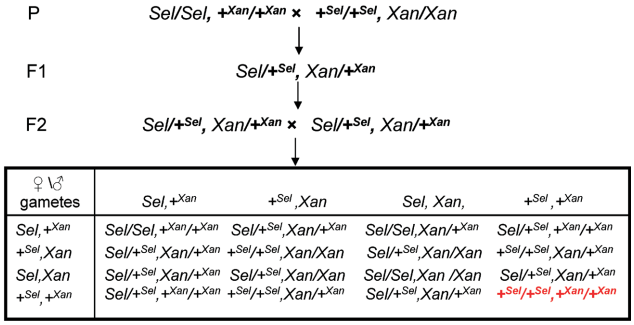


Fig. 3. Predicted inheritance pattern for an F_2 cross between *Sel* and *Xan* homozygotes for mutations on different linkage groups. Because both mutations are dominant and produce the same yellow-skinned phenotype, only 1/16 of the progeny will be homozygous recessive for both mutations and have normal skin. P, parental cross; F_1, first filial generation; F_2, second filial generation.

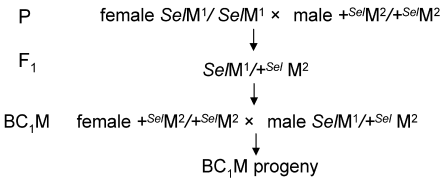
homozygous recessive for both loci and have normal skin color.

Localization of visible mutant markers with SSR markers

Because there is no crossing over in silk moth females, the initial analysis only found the SSR markers that were linked to the two mutations. To determine where the *Sel* and *Xan* genes were located, we employed F_1-male informative backcross mapping (BC_1M) (Fig. 4) with each mutant strain and the wild type strain Dazao, and calculated the recombination frequencies relative to the SSR markers. Seven SSR loci showed polymorphism between strains K07 (*Sel*), and Dazao ($+^{Sel}$), but only one SSR marker showed polymorphism relative to strain K14 (*Xan*). We scored polymorphic SSR markers in 190 progeny from each BC_1M backcross in comparison to the two *Sel* and *Xan* parents and analyzed the data by MAPMAKER/Exp ver. 3.0 (Lander et al., 1987). Fig. 5 shows the linkage map for *Sel*, which was found to be located between SSR markers S2404 and S2414 at dis-

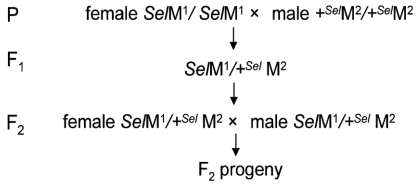
TABLE 1. SSR loci used in this study.

Locus	Repeat motifs	F primer (5'---3')	R primer (5'---3')	Allele size (bp)
S2404	$(CA)_{12}$	ATTAATATTTAGTATACAAAGCGTCCCAT	GAATGTCGGTAGTTTGTACTTTGTTTG	201
S2405	$(CT)_8$	GGCAAGCCCAGAACAACGG	ACACGCTACATTTCACTTTGTTACG	238
S2407	$(CT)_{16}$	CAAATTGCTATTGGTATGCGGTA	ACTGGAAGAAATCGCTTTTAACG	292
S2412	$(CA)_8$	GCTAAAGCAAAAAGTGGGATACATT	GGTGTAGGGATACCGTGAGCAT	234
S2414	$(CT)_{11}$	AAACCAAACCTTGCCATTCCT	CGGTTGCCTGGAAGATATCG	295
S2416	$(CT)_{17}$	CTTATCTTTAGTTGCCCCGTTGA	CGGGGTGCCGATTGCG	210
S2417	$(CT)_{21}$	AACGGGACGCCTACGGAAT	GACCGCCTATTGTACAAATGTATCTG	311
S2418	$(CT)_{10}$	TCGGGTGAGACGAACGGAA	TTAGTTTTAATTGTGGCTTTCCATTAT	189
S2419	$(CA)_{18}$	TCGCGGAAGGAAGATTATGAA	CCCCAGATAACGGGAACAGG	310
S2425	$(CT)_4$	CGGGAATTTAGCCCTTATTGTGAA	TTATCCTCCTGATGGTTGTCTGG	273
S2428	$(CA)_9$	CGCAATTTGCTTTGACCACT	CCGGGTGAAATGCTATTGACG	290
S2429	$(CT)_7$	AGATAACAAACCGATAACGGAGATT	GGTTCTCCTTACCTCTACCCTCCAT	213
S2430	$(CA)_{14}$	GAGCACTTGTGGGCAACTTCA	GCATCCCCACTATCGTCTACTAAACA	239
S2431	$(CA)_8$	TGAGCTCTGAAAACCGACCCT	AGCATGTTAATGTCATTAGCGACTC	284



♀\♂ gametes		Male gametes			
		"parental" type		"recombinant" type	
		*Sel*M^1	+^{Sel}M^2	*Sel*M^2	+^{Sel}M^1
Female gametes	+^{Sel}M^2	+^{Sel}M^2/ *Sel*M^1	+^{Sel}M^2 /+^{Sel}M^2	+^{Sel}M^2 / *Sel*M^2	+^{Sel}M^2 / +^{Sel}M^1

Fig. 4. Scheme of the F_1-male backcross (BC_1M) showing inheritance of the visible mutation *Sel* and a polymorphic SSR marker from the same linkage group, with complete linkage in the female. M, a microsatellite locus; M^1 and M^2, two different alleles of the M locus.



♀\♂ gametes		Male gametes			
		"parental" type		"recombinant" type	
		*Sel*M^1	+^{Sel}M^2	*Sel*M^2	+^{Sel}M^1
Female gametes	*Sel*M^1	*Sel*M^1/ *Sel*M^1	*Sel*M^1 /+^{Sel}M^2	*Sel*M^1 / *Sel*M^2	*Sel*M^1 / +^{Sel}M^1
	+^{Sel}M^2	+^{Sel}M^2 / *Sel*M^1	+^{Sel}M^2 /+^{Sel}M^2	+^{Sel}M^2 / *Sel*M^2	+^{Sel}M^2 / +^{Sel}M^1

Fig. 6. Inheritance pattern of SSR loci in the F_2 generation, with complete linkage in the female. P, parental cross; F_1, first filial generation; F_2, second filial generation. M, a microsatellite locus; M^1 and M^2, two different alleles of the M locus.

tances of 12cM and 22.8cM, respectively. We calculated a recombination distance of 7.03cM between *Xan* and the single polymorphic SSR marker, S2407.

Verifying the localization of *Sel* and *Xan* in F_2 progeny

Fig. 6 shows the inheritance pattern of *Sel* with a polymorphic SSR marker which was produced by mating a homozygous mutant parent (*Sel*M^1/*Sel*M^1) with a wild type parent (+^{Sel}M^2/+^{Sel}M^2). M is a polymorphic SSR marker expressed as M^1 in one parent and M^2 in the other. In the mutant F_2 progeny, the genotype was *SelSel* (M^1M^1) or *Sel*+^{Sel} (M^1M^2, M^2M^2) and their phenotypes were yellow-skinned; however, it was difficult to score homozygous and heterozygous *Sel* phenotypes by SSR markers, and thus it was difficult to identify the parental and recombinant types. But in the wild type F_2 progeny, in which the genotype was homozygous for recessive alleles of the *Sel* gene (+^{Sel}+^{Sel}) and the phenotype was normal larval skin, we could distinguish the parental types (+^{Sel}M^2/+^{Sel}M^2) from the recombinants (+^{Sel}M^2/+^{Sel}M^1) based on the homozygosity (M^2M^2) or heterozygosity (M^1M^2) of SSR bands (Fig. 6). The *Xan* alleles had the same kind of inheritance patterns. The genotypes of all SSR loci were determined in 62 recessive homozygotes for *Sel* (+^{Sel}+^{Sel}) and 95 recessive homozygotes for *Xan* (+^{Xan}+^{Xan}) using segregants from each F_2 generation. The data were analyzed using Mapmaker/Exp ver. 3.0 (Lander et al., 1987). Fig. 7 shows the linkage maps obtained for *Sel* and *Xan*. The *Sel* gene was 13 cM from the S2404 marker, and the *Xan* gene was 13.7 cM from the same marker.

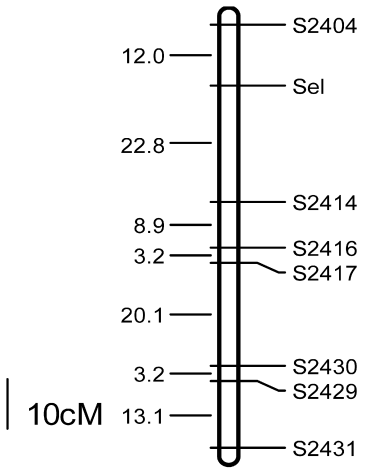


Fig. 5. SSR linkage map of the visible mutation *Sel* determined by analysis of the BC_1M progeny of the cross Dazao female × (K07 × Dazao) male.

DISCUSSION

We found two visible silkworm mutations, *Sel* and *Xan,* originally used to define ELGs 24 and 27, to be linked to SSR linkage group 24 as defined by S2404, a marker in our first generation SSR linkage map (Miao et al., 2005). As shown in Fig. 5, the *Sel* gene was 12 cM from S2404. As there was only one polymorphic SSR marker between the parents of Dazao and *Xan*, only the recombination frequency could be calculated. Therefore, the location of

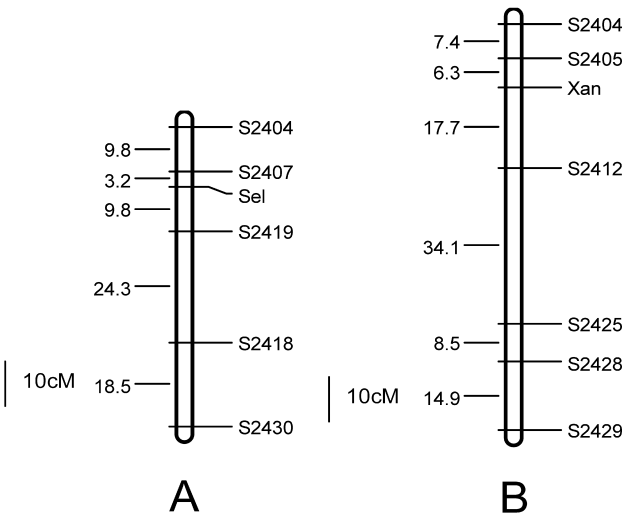


Fig. 7. SSR linkage map of *Sel* (A) and *Xan* (B) defined by analysis of F_2 wild-type progeny. The F_1 populations used to produce the F_2 generations were (18–050 × 10–740) (A) and (10–700 × 08–101) (B). Strain 10–740 is homozygous for *Sel*, 10–700 is homozygous for *Xan*, and 08–101 and 18–050 are wild type.

Xan on the chromosome was difficult to decipher. However, *Xan* was found to be close to marker S2407, at a recombination distance of 7.03cM based on 190 BC_1M individuals. To confirm this, we used F_2 wild-type progeny (homozygous for recessive alleles of both the *Sel* and *Xan* genes) containing common SSR markers to construct a combined linkage map (Fig. 7). The *Sel* gene was 13 cM and the *Xan* gene was 13.7 cM from the common reference marker, S2404. These results were consistent with those from the progeny of BC_1M crosses. Different populations and individuals yielded slight variations. Determining whether the two visible markers are alleles of the same gene will require more common reference markers and larger mapping populations.

The linkage analysis was based on the lack of crossing over in female meiosis (Goldsmith, 1995). This provided a convenient and effective method for linkage analysis but also caused some difficulties. For example, because the available software (i.e., Mapmaker 3.0) does not allow for no crossing over in one sex, we could not use it to construct the linkage map directly from the F_2 generation. Tan et al. (2001) used a backcross population to construct an AFLP genetic linkage map of *B. mori*. In the present study, we also used the BC_1 strategy for SSR linkage analysis and mapping of the dominant mutations *Sel* and *Xan*. As the two dominant phenotypes were difficult to score in heterozygotes, we used the recessive wild type segregants of the F_2 generation to confirm the BC_1 results. The two types of crosses yielded similar recombination values. Using the F_2 segregants homozygous for the recessive wild-type alleles avoided the complication of incomplete dominance of the visible marker, which could have introduced scoring errors.

Our results further confirmed the SSR technique as a reliable tool for constructing linkage maps and for linkage and localization of visible mutations. Our strategy in the linkage analysis was to group BC_1 individuals to screen the potential SSR markers efficiently. This made marker screening much easier even though linkage analysis was not carried out for the entire genome.

Our work also indicated the need for care in selecting the parental combination for linkage analysis. We used two combinations from laboratories in Zhenjiang and Chongqing, China. Polymorphic markers were more plentiful between strains K07 and Dazao than between strains K14 and Dazao; consequently, seven SSR markers showed the same inheritance pattern between K07 and Dazao for the *Sel* marker, but only one polymorphic marker cosegregated with the *Xan* marker in the cross between K14 and Dazao. Evidently, the low polymorphism rate was caused by the close relationship between the two parental strains. For future linkage mapping and recombination analysis, we recommend that the genetic relationships between the prospective parental strains be ascertained first in order to achieve the highest polymorphic rate.

The finding that *Sel* and *Xan* map so close together suggests that they may be alleles of the same gene, especially considering that the small differences in map distance from the reference SSR marker were within expected experimental error. No biochemical analysis has been reported on *Xan*, but the strong similarity in phenotype between the two mutations is consistent with this possibility. Similarly, *Ym*, which is found in *B. mandarina*, the possible source of *Xan*, also appears superficially to involve a step in pteridine metabolism. Early second and third larval instars secrete yellow crystals from Malpighian tubules, and the epidermis is coated with a yellow powder after molting. This locus has been assigned to ELG27, which should be confirmed. Even if *Ym* is not allelic to *Sel* and *Xan*, it would be of interest to find that genes affecting related pathways in pteridine metabolism are on the same linkage group. Finally, ELG24 is now defined by only one additional morphological mutation, *l-li* (lethal larva at 5th instar). The confirmation of linkage group assignments for both of these loci and construction of an associated molecular genetic map can be accomplished efficiently using the strategy employed here.

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