Genet. Res., Camb. (1995), **66**, *pp.* 1–7 *With 5 text-figures*

Linkage map of random amplified polymorphic DNAs (RAPDs) in the silkworm, *Bombyx mori*

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(*Received 13 December 1994 and in revised form 21 February 1995*)

Summary

We have constructed a linkage map of random amplified polymorphic DNAs (RAPDs) in *Bombyx mori*. We screened 320 10-mer primers, and found 243 clear polymorphic bands between C108 and p50 strains. In the F_2 generation, segregation ratios of 168 bands were nearly 3:1 in a chi square test, showing Mendelian inheritance. The MAPMAKER program sorted 168 bands into 29 linkage groups and 10 unlinked loci at minimum LOD score 3·0, and determined orders of loci in each group, which contained 2–11 markers. It also detected typing errors in our data. We calculated map distances between pairs of neighbouring loci using recombination values in males and the Kosambi mapping function. Our RAPD map consists of 169 loci including the *p* locus, and the sum of map distances is approximately 900 cM. Linkage groups 1 and 2 of our map correspond to chromosomes 1 and 2 on the conventional linkage map because of linkage to sex and *p*, respectively.

1. Introduction

Polymorphic DNA markers have been used in agriculturally important species for genetic mapping, evolutionary studies, population genetics and quantitative trait analysis (Watkins, 1988; Lander & Botstein, 1989; Tanksley *et al.* 1989; Dietrich *et al.* 1992; Williams *et al.* 1993). Mainly two types of molecular markers have been used to develop genetic linkage maps. The majority of studies have used restriction fragment length polymorphisms (RFLP) (Botstein *et al.* 1980; O'Brien, 1993). More recently random amplified polymorphic DNA (RAPD) markers have been shown to be useful as genetic markers for a variety of organisms, including humans, fungi, bacteria, higher plants (Welsh & McClelland, 1990; Williams *et al.* 1990; Newberry & Ford-Lloyd, 1993), animals (Levin *et al.* 1993) and insects (Hunt & Page, 1992). RAPDs are detected by gel electrophoresis after carrying out the polymerase chain reaction (PCR) using an arbitrary oligonucleotide primer. Polymorphisms are caused by base changes in the primer annealing sites or by chromosome rearrangements within the amplified sequence. Genetic analysis with RAPD markers is more rapid and simpler than RFLP analysis and requires smaller amounts of DNA. Moreover it does not require cloning of DNA or Southern hybridization. In contrast to most RFLPs, RAPD markers are usually scored as dominant alleles since a RAPD is present in only one of the parents and amplified in the heterozygote.

In recent years, RAPD markers have enabled a significant advance in the ability to generate linkage maps quickly. Linkage maps of RAPD markers have been reported for arabidopsis (Reiter *et al.* 1992), bananas (Faure *et al.* 1994), lettuce (Kesseli *et al.* 1994), eucalyptus (Grattapaglia & Sederoff, 1994), chicken (Levin *et al.* 1993) and so on.

In *Bombyx mori*, 400 or more visible mutations have been found, about 220 of which have been mapped on the conventional linkage maps (Doira *et al.* 1992; Goldsmith, 1994). These mutants involve various abnormalities in morphological, developmental, physiological and behavioral characteristics. Therefore, *B. mori* is a good model for solving a broad range of fundamental biological problems (Goldsmith, 1995). Moreover, more efficient breeding of the silkworm using genetic markers such as marker-assisted selection is expected to increase the silk production. Molecular analysis of the silkworm's

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genome is, however, at an elementary stage. Goldsmith and her collaborators have started the mapping of RFLPs in *B. mori* (Goldsmith & Shi, 1994; Goldsmith *et al.* in preparation). We think that a dense molecular linkage map is essential for applying the conventional phenotype map to molecular biological studies, for example, for positional cloning (Berger *et al.* 1992), and chromosome structure studies (Tanksley *et al.* 1992; Cox *et al.* 1993). It is also useful for classical genetic studies, for example, for the mapping of quantitative trait loci (QTLs) and newly found mutants. In this paper we present our first results of looking for and mapping RAPDs in *B. mori.*

2. Materials and methods

(i) *Insects*

Strain p50 was donated by Dr Hiroshi Doira, Institute of Genetic Resources, Faculty of Agriculture, Kyushu University, Fukuoka, Japan, and maintained by single pair matings as long as the parental generation was itself quite homozygous before hybridization. This strain originated as Chinese strain, Daizo. The strain C108 was supplied by the National Institute of Genetics as strain No. 785, and maintained by single pair matings the same as p50. C108 was originally an improved race bred from Chinese bivoltine strains. To construct a RAPD map, we used 101 individuals in the F_2 hybrid generation between a p50 mother and a C108 father. For a comparative study, ten more strains of *B. mori* were used: 912 *pe-re ch*, 604 Mysore, 502 Ascoli, 312 Kansen, 322 Sekko, 319 Oha, 751 E^{Ns}, 928 *K U* (donated by the Laboratory of Genetic Resources, Department of Insect Genetics and Breeding, National Institute of Sericultural and Entomological Sciences, Kobuchizawa, Yamanashi 408, Japan), UT16 N4 (Shimada), w30 p^M *Ze L q bp* (Dr Doira). Two strains of *B. mandarina*, which originated from Hangzhou, Zhejiang Province, China, and Sakado, Saitama Prefecture, Japan, and were maintained for several years in the University of Tokyo, were also used. *B. mori* and *B. mandarina* were fed on fresh mulberry leaves. *Antheraea yamamai* and *A. pernyi* were supplied from the Tokyo Metropolitan Agricultural Experiment Station and the Sericultural Experiment Station of Nagano Prefecture, respectively, and the insect larvae were raised with fresh leaves of an oak, *Quercus variabilis.*

(ii) *RAPD analysis*

Genomic DNA of individual silkworms was isolated from the posterior silkglands of mid-fifth instar larvae by the method of Bender *et al.* (1983). It was further purified by extraction with phenol/chloroform, precipitated by ethanol, and resuspended in TE (10 mM Tris-HCl, pH 8·0, 1 mM EDTA) buffer. Amplification reactions (15 μl) contained 75 ng of genomic DNA, 0·2 mM each of dATP, dCTP, dGTP and dTTP, 1 μM of 10-mer primer (Operon Tech., Alameda, California, USA) and 0·34 unit of *Taq* polymerase (Wako Pure Chem., Osaka, Japan). Each reaction was overlaid with 100 μl of light mineral oil to prevent evaporation. A thermal cycler 'Thermo Processor TR-100' (Taitec, Tokyo, Japan) was used. Temperature cycling was the same as Williams *et al.* (1990). Amplification products were analysed by electrophoresis on 2% agarose gels and stained with ethidium bromide. The presence or absence of each polymorphic DNA band was scored for every individual of the F_2 generation.

(iii) *Linkage analysis and map construction*

First we compared the RAPDs of the F_1 between a C108 female × p50 male and a p50 female × C108 male, and put aside the data of sex-linked (Z-linked) RAPDs. We calculated a chi square value for each RAPD to determine whether the segregation ratio of presence/absence in F_2 was 3:1, and eliminated the RAPD data showing an unexpected segregation ratio at the 5% level of significance. A data matrix was constructed from the presence/absence of all polymorphic bands for all 101 F_2 individuals. This data matrix was input into the program package MAPMAKER/Exp (version 3.0b) (Lander *et al.* 1987). MAPMAKER sorted the RAPDs into linkage groups, determined the orders of loci, and detected typing errors at a minimum logarithm of the odds ratio (LOD) score of 3·0. After correcting typing errors, we manually calculated map distances between pairs of neighbouring loci from recombination values in the meiotic division in the F_1 male according to the Kosambi mapping function (Kosambi, 1944).

3. Results

(i) *Difference of RAPD patterns among species and strains*

The band patterns of RAPDs were different among species and strains of the silkworm (Fig. 1). Bands were in part shared by strains of *B. mori*; however, many intraspecific polymorphic bands were available as potential markers for the genome analysis. Although any two strains in *B. mori* showed different RAPD patterns and could be used for mapping, we adopted the strains C108 and p50 as standard strains for our mapping studies since RFLP studies had already started using the same strains (Goldsmith & Shi, 1994).

(ii) *Search for RAPDs*

To find well-behaved RAPDs for genetic analysis first we compared the banding patterns among C108, p50 and their F_1 hybrid, and picked up strain-specific bands (Fig. 2). We used 320 10-mer primers, 130 of which showed polymorphism between the two lines. Some of them produced multiple polymorphic bands, and the sum of polymorphic bands was 243. We

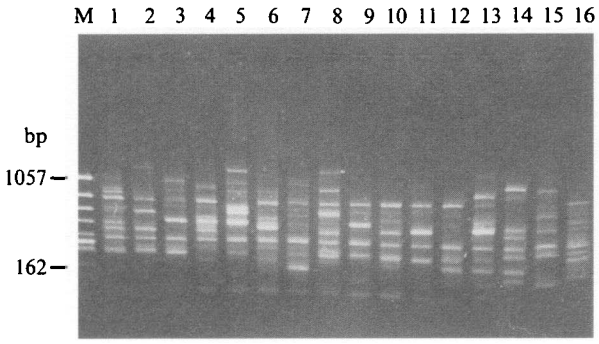

Fig. 1. Band patterns of RAPD-PCR silkmoth among species and strains. Primer is 5′-TGGGAGGGG-3′. M, Size marker; lanes 1–12; *B. mori* strains: 1, Mysore; 2, Ascoli; 3, Kansen; 4, NISES912; 5, w30; 6, Sekko; 7, Oha; 8, NISES751; 9, N4; 10, NISES928; 11, C108; 12, p50 Daizo; 13, *B. mandarina* Japan; 14, *B. mandarina* China; 15, *Antheraea yamamai*; 16, *A. pernyi*.

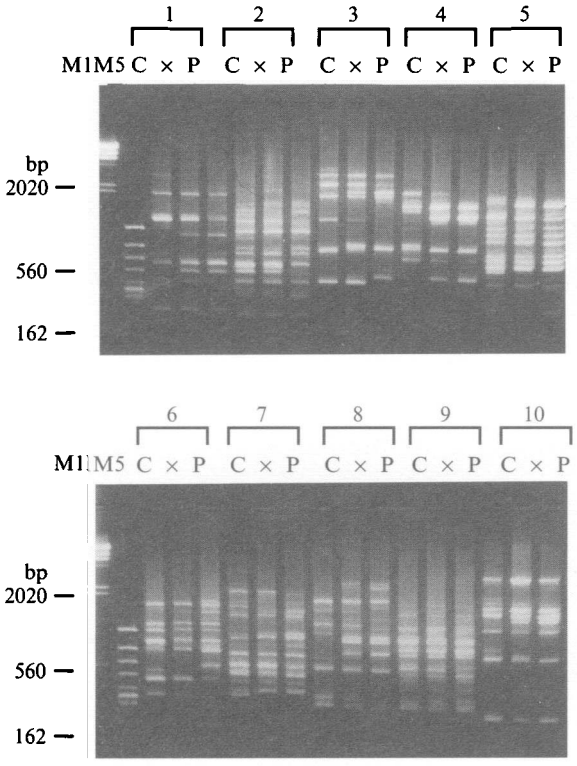

Fig. 2. Comparison of RAPD bands between C108 and p50 strains. Strain-specific RAPD between C108 and p50 were found. 1–10, OPY primers; M, size marker; C, C108 female; P, p50 female; X, F_1 female of p50 female × C108 male.

looked for sex-limited (on the W chromosome) and sex-linked (on the Z chromosome) RAPDs, and found 0 sex-limited and 3 sex-linked ones. After putting them aside, we estimated the segregation ratios of RAPDs in the F_2 population according to a chi square test. At the 5% level, the ratios of 75 RAPDs fell outside of 3:1, and their data were eliminated. Finally we obtained 168 RAPDs which showed Mendelian inheritance. These RAPDs originated from 110 primers giving a ratio of available primer of

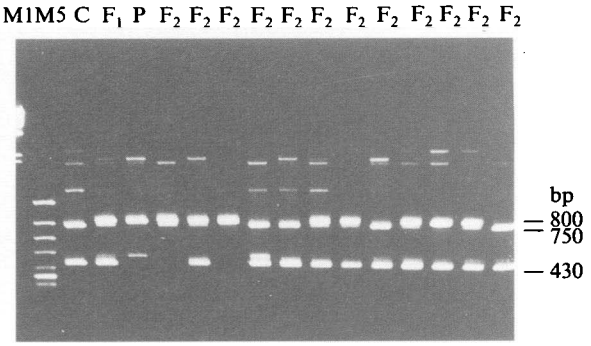

Fig. 3. RAPDs that segregated in a Mendelian fashion in the F_2 generation. Primer: OPY 3. C, C108 strain; P, p50 strain; F_1, (P × C)F_1; F_2, (P × C)F_2; M, size marker.

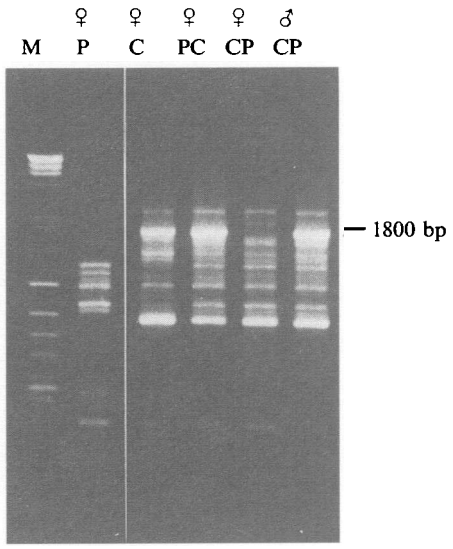

Fig. 4. Sex-dependent RAPDs. Primer: OTP15. M, size marker; P, p50 strain; C, C108 strain; PC, (p50 female × C108 male)F_1; CP, (C108 female × p50 male)F_1.

110/320 = 34·4%. An example of the segregation of RAPDs in the F_2 generation is presented in Fig. 3.

Our data consisted of 168 RAPDs and the *p* alleles. In the homozygotes for *p*, larval skin markings such as lunar markings and star spots, were not melanized. The $p/+^p$ and $+^p/+^p$ individuals expressed visible black markings with melanin on the larval skin. The data of 169 polymorphisms in 101 F_2 intercross individuals were divided into 3 data matrices as follows: the first data matrix contained 86 C108-specific RAPDs, and the second one contained 79 p50-specific RAPDs and *p*. The last one contained 3 sex-linked RAPDs. Sex-linked RAPDs were present in F_1 females from either of two reciprocal crossings, and absent in those from another cross (Fig. 4). Two sex-linked RAPDs were C108-specific bands, and one sex-linked RAPD was p50-specific. To construct the data matrices, we used locus names such as M1.80, which means a 800 bp band produced by the primer OPM1.

(iii) *RAPD linkage map*

To construct the linkage map, we sorted RAPDs into

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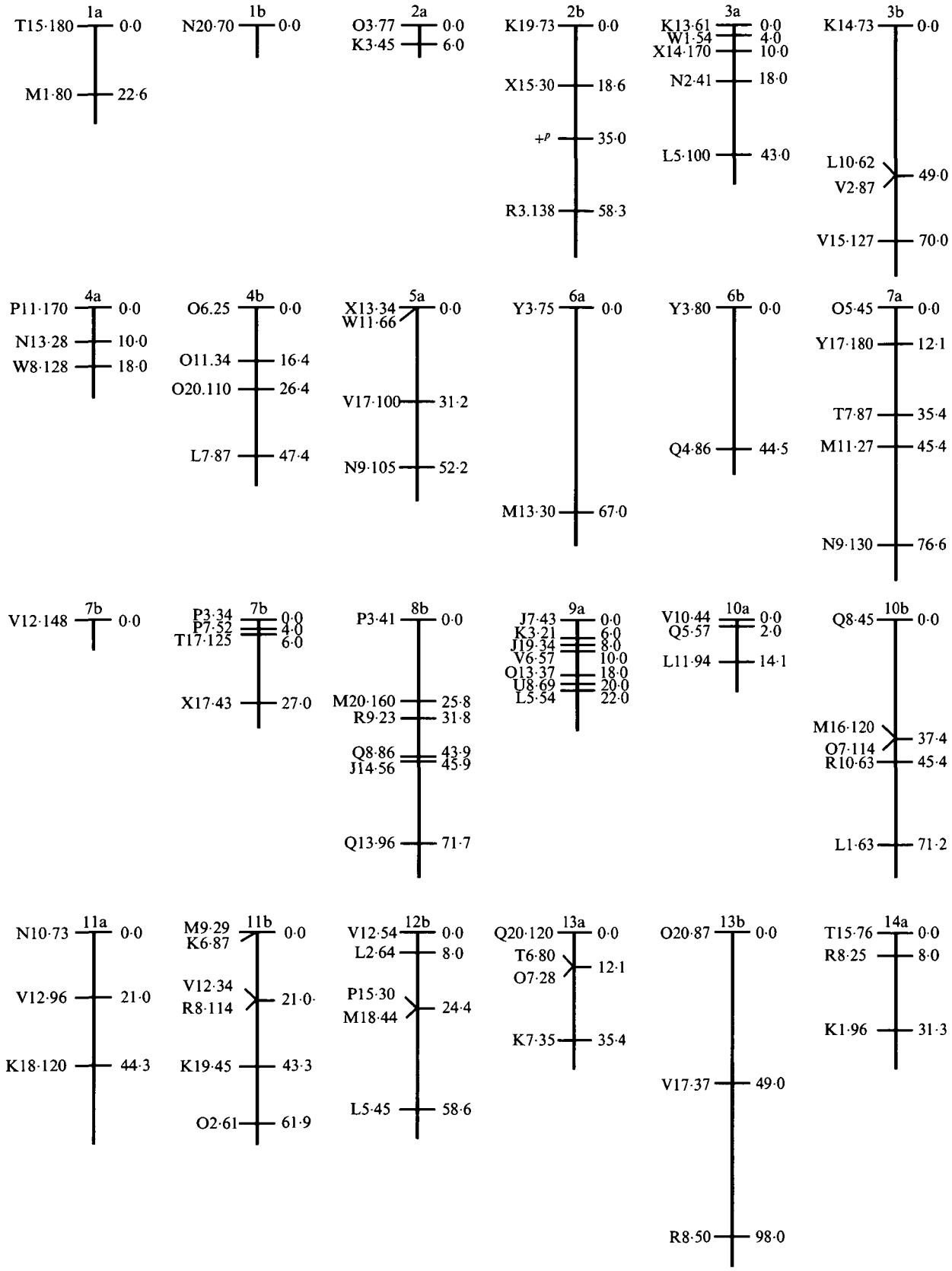

Fig. 5. Linkage map of RAPDs in *B. mori*. Linkage groups 1a and 1b correspond to conventional linkage group 1 (Z chromosome), and linkage groups 2a and 2b to the conventional group 2. The other linkage groups have not yet been correlated with the conventional linkage groups. Linkage groups named 'a' contain C108 dominant alleles, while those named 'b' contains p50 dominant alleles. Weak linkage is found between: linkage groups 6 and 25, linkage groups 8, 26 and 27, linkage groups 13 and 28, and linkage groups 19 and 29.

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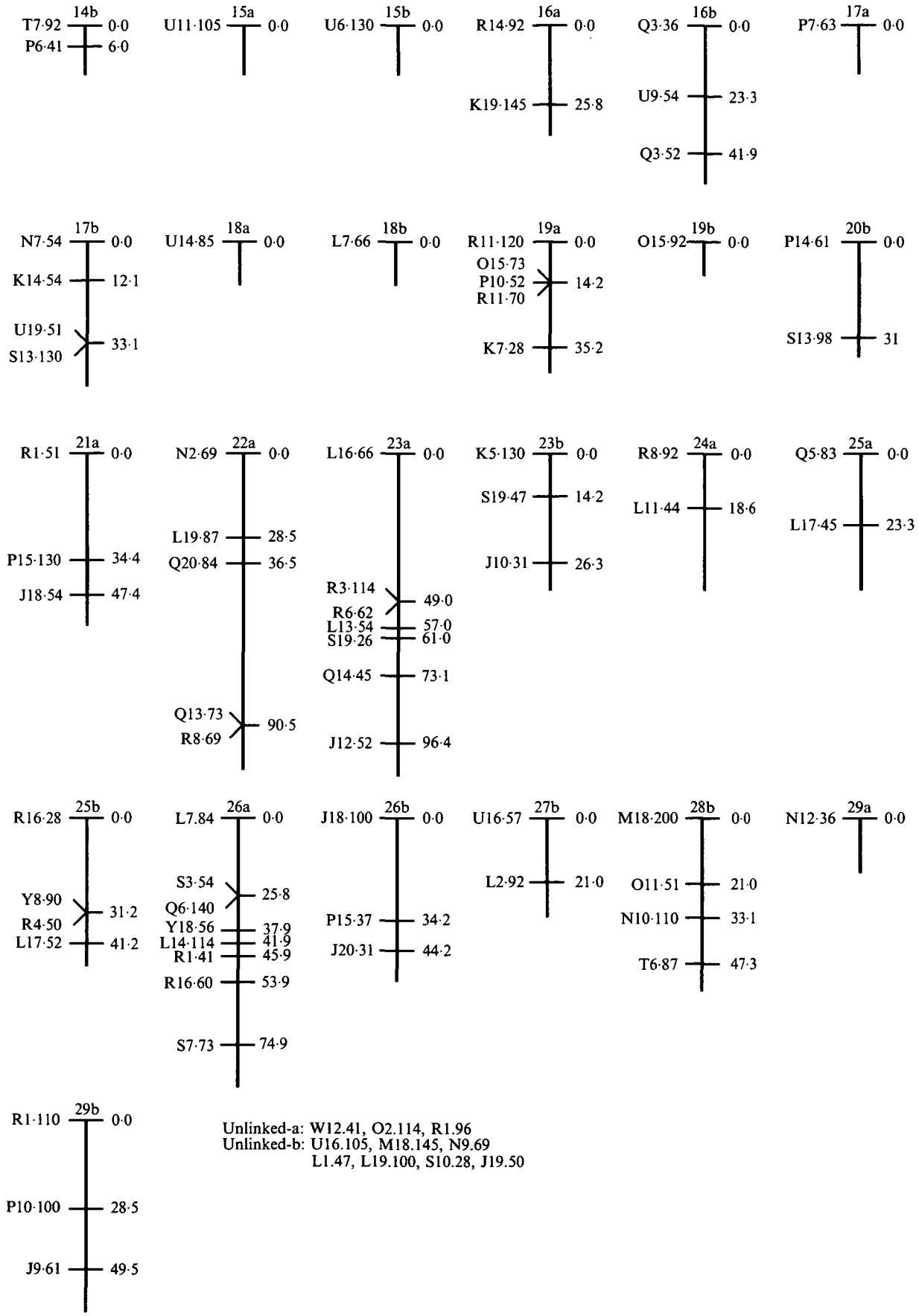

Fig. 5 (continued).

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linkage groups, determined the order of loci, corrected typing errors, and calculated the map distances. The MAPMAKER program sorted 169 loci into 29 linkage groups, and 10 unlinked loci (Fig. 5) at LOD score 3·0. Of 29 linkage groups, there were four weak linkages between groups (linkage groups 6 and 25; linkage groups 8, 26 and 27; linkage groups 13 and 28; and linkage groups 19 and 29). We estimated a probability of typing errors as 2% from unexpected segregation in a sex-linked RAPD, M1.80.

Although genetic recombination occurs in meiosis of both males and females in vertebrates, it occurs only in males in *B. mori* and other lepidopteran insects. This Lepidoptera-specific situation leads to a problem as follows. We cannot determine the order among genetically linked loci that have a 'repulsion' relationship. The repulsion means two loci whose relationship is *X-y/x-Y*, where *X* and *Y* are linked to each other, and *x* and *y* are recessive alleles of *X* and *Y*, respectively. Because the *x* and *y* do not coexist in the same individual in the F_2 intercross of lepidopteran insects, we cannot detect any recombinants. Since we cannot determine the locus order of RAPDs in repulsion, it is impossible to calculate recombination values between the two RAPDs in repulsion. To compensate for this problem, we divided each linkage group into two subgroups, C108-specific RAPDs ('a' group) and p50-specific RAPDs ('b' group). Each linkage group was composed of 2–11 markers. However, the 'a' and 'b' subgroups could not be integrated as a single linkage group. MAPMAKER could not be adjusted to calculate the recombination values or map distances under the condition of no crossing-over in one sex. Therefore, after correcting typing errors, map distances between pairs of neighbouring loci were calculated from recombination values in males using the Kosambi mapping function.

Linkage groups ranged in length from 6·0 to 96·4 and 6·0 to 98·0 cM for the a and b groups, respectively. The sums of lengths for all linkage groups were 871·6 cM for the a group and 923·2 cM for the b group. The distance between adjacent markers ranged from 2·0 to 67·0 and 2·0 to 49·0 cM with an average distance of 9·9 and 11·4 cM for C108-specific and p50-specific markers, respectively. Linkage groups 1 and 2 correspond to chromosomes 1 and 2 of the classical linkage map because of linkage to sex and the *p* allelomorphs, respectively.

4. Discussion

We screened 110 primers, and found 243 polymorphic bands, 169 of which could be used to construct the linkage map. The MAPMAKER program sorted them into 29 linkage groups and 10 unlinked loci. Mapping of RAPDs was very efficient, since we took only 15 months for the PCR work. Silkworm geneticists have taken 90 years or more to map about 200 visible mutants. A great many people have been involved in the mapping. On the contrary, the RAPD mapping required only a few people.

All RAPDs are genetically dominant markers. The lack of allelism is one of the disadvantages in RAPDs (Devos & Gale, 1992). Moreover RAPDs do not correspond only to single copy sequences but also to repetitive DNAs (Williams *et al.* 1990; Echt *et al.* 1992). In *B. mori*, genetic recombination occurs only in males, i.e. meiosis of spermatocytes. Therefore, we cannot obtain double negative F_2 individuals for both C108-specific and p50-specific RAPDs, in what is called 'repulsion'. This meant that we could not integrate the C108-specific linkage group with the corresponding p50-specific group. Instead we are now analysing both RFLPs and RAPDs of the same F_2 DNAs to integrate our RAPD map with an independently constructed RFLP map (Goldsmith & Shi, 1994; Goldsmith *et al.* in preparation).

B. mori has 28 chromosomes with a total map distance of 900·2 cM in the conventional phenotype linkage maps (Doira *et al.* 1992), whereas our RAPD linkage map has 29 linkage groups with a total average map distance of 897·4 cM between C108-specific and p50-specific RAPDs. Linkage groups have lengths of 17·6–53·7 cM in the conventional map (Doira *et al.* 1992), whereas that of our map has its length of 6·0–97·2 cM. The sums of genetic distances were very similar between the RAPD map and the conventional map, suggesting that RAPDs were well interspersed on the genome and unbiased on linkage groups. Although there seem to be too many linkage groups in our RAPD map, some linkage groups may be connected. Indeed, we found some weak linkages between groups. To determine the relationship between our RAPD map and the conventional map, we have already started some crossing experiments to integrate them.

The haploid genome size of *B. mori* is 530 million base pairs (Mbp) (0·5 pg DNA) (Gage, 1974; Rasch, 1974); this is approximately three and a half times the size of the *Drosophila melanogaster* genome (140 Mbp) (Rasch *et al.* 1971) and one-sixth the size of the human genome (3000 Mbp). We can estimate the relationship between the genetic distance and the physical distance. Given the total length of the genome as approximately 1000 cM and 500 Mbp, 1 cM roughly corresponds to 500 kbp. If we plan to use the genetic map for positional cloning, we need to construct a 1 cM-density (500 kbp-density) map. This would allow cloning into yeast artificial chromosomes (YACs), as vectors used for large-scale genomic libraries, which can contain up to 1 Mbp insertions. Our RAPD map has a density of 5·3 cM on average, which corresponds to 2·7 Mbp. Although our map itself cannot be used for positional cloning, it is a milestone for further genome analysis in the silkworm which can also be used for analysis of QTLs, more efficient mapping of newly found mutants and so on.

We wish to thank Dr Marian R. Goldsmith, University of Rhode Island, for her helpful advice, encouragement throughout this study and reviewing this manuscript. We are grateful to Drs Hiroshi Doira and Hiroshi Fujii, Kyushu University for supplying mutant strains and helpful suggestions, Dr Masataka Ozaki, University of Tokyo, and members of the Institute of Sericultural and Entomological Sciences also generously supplied genetic stocks. This work was supported by Grants-in-Aid for Scientific Research from the Ministry of Education, Science and Culture, Japan (Nos. 03304016, 04660058, 06660063).

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