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A genetic linkage map for the domesticated silkworm, *Bombyx mori*, based on restriction fragment length polymorphisms

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Summary

We present data for the initial construction of a molecular linkage map for the domesticated silkworm, *Bombyx mori*, based on 52 progeny from an F2 cross from a pair mating of inbred strains p50 and C108, using restriction fragment length polymorphisms (RFLPs). The map contains 15 characterized single copy sequences, 36 anonymous sequences derived from a follicular cDNA library, and 10 loci corresponding to a low copy number retrotransposon, mag. The 15 linkage groups and 8 ungrouped loci account for 23 of the 28 chromosomes and span a total recombination length of 413 cM; 10 linkage groups were correlated with established classic genetic maps. Scoring data from Southern blots were analysed using two Pascal programs written specifically to analyse linkage data in Lepidoptera, where females are the heterogametic sex and have achiasmatic meiosis (no crossing-over). These first examine evidence for linkage by calculating the maximum lod score under the hypothesis that the two loci are linked over the likelihood under the hypothesis that the two loci assort independently, and then determine multilocus linkage maps for groups of putatively syntenic loci by calculating the maximum likelihood estimate of the recombination fractions and the log likelihood using the EM algorithm for a specified order of loci along the chromosome. In addition, the possibility of spurious linkage was exhaustively tested by searching for genotypes forbidden by the absence of crossing-over in one sex.

1. Introduction

The domesticated silkworm, *Bombyx mori*, has been used as a model for formal genetic studies since the rediscovery of Mendalian rules of inheritance at the turn of the century (Coutagne, 1902; Toyama, 1906, cited in Tazima, 1964; Toyama, 1912; Tanaka, 1914, cited in Sturtevant, 1915). Currently its genetic linkage map contains more than 200 markers on 28 linkage groups and covers nearly 1000 centimorgans (cM) (Doira, 1992). Most of these markers are morphological or physiological mutations affecting such fundamental processes as egg formation (e.g., see Kawaguchi & Doira, 1973; Kawaguchi *et al.* 1983, 1987, 1988, 1990, 1993; Fujikawa *et al.* 1993) and choriogenesis (Nadel *et al.* 1980*a*, *b*; Gautreau *et al.* 1993), embryonic patterning and segment identity (e.g., see Tazima, 1964; Ueno *et al.* 1992, 1995), larval epidermal pigmentation (e.g., see Tamura, 1977, 1983; Tamura & Akai, 1990; Sakurai & Tsujita, 1976*a*, *b*; Sawada *et al.* 1990; Shimizu *et al.* 1993), hormone action (Shimizu *et al.* 1980; Nagata *et al.* 1992), and diapause (Yamamoto *et al.* 1978; Sonobe *et al.* 1986) (see also Doira, 1978; Goldsmith, 1995). About 20 loci encode identified gene products such as isozymes, hemolymph, silk, and chorion proteins which were mapped using electrophoretic variants, or more recently, cloned neuropeptides localized via the polymerase chain reaction (Shimada *et al.* 1994; Pinyarat *et al.* 1995). Although a small number of new, spontaneous mutations are added to the map each year (H. Doira, personal communication), little effort is now being directed toward large-scale mutagenesis that would significantly augment the pool of morphological markers available for study.

In addition to these traditional genetic resources, hundreds of so-called geographic races and genetically improved silkworm strains are maintained for practical breeding, largely in Asia (e.g. China, Japan, Korea, and India). Many of these stocks show marked variation in polygenic or quantitative traits of immediate economic interest for silk production, such as cocoon yield and silk fiber length and quality (Udupa

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& Gowda, 1988; Datta & Pershad, 1988; Li, 1992); they also differ in characters affecting important life history traits, such as growth rate, heat tolerance, fecundity, feeding behavior (Tazima, 1989), and disease resistance (Kremsky & Machalska, 1988; Bhaskar *et al.* 1989; Narayanaswany *et al.* 1989). Unravelling the genetic basis underlying such traits is certainly important for strain improvement in sericulture; nevertheless, despite considerable effort success in mapping these kinds of genes has been modest (Eguchi *et al.* 1986; Tazima, 1989; Kanda, Tamura & Inoue, 1988). Moreover, although many species of Lepidoptera are significant agricultural pests, genetic studies targeted at pest control in this order have lagged behind those of Diptera.

Beyond their economic importance as pests and producers of silk, the taxonomic diversity of moths and butterflies (150000 named species) provides a strong rationale for examining the genetic mechanisms underlying their ecology and evolution. Comparative genome analysis represents a particularly powerful approach for revealing the extent of conservation of chromosomal segments and for uncovering features associated with evolutionary change (Heckel, 1993). The well-established genetics of the silkworm, coupled with a large body of information on its development and physiology (Tazima, 1978; Goldsmith & Wilkins, 1995), make *B. mori* the species of choice to serve as a reference for comparative molecular genetic studies within the Lepidoptera.

We present here initial steps in the construction of a molecular linkage map for the silkworm using restriction fragment length polymorphisms (RFLPs). Among available approaches for linkage map construction (for example, see Promboon *et al.* 1995, for a parallel map based on Random Amplified DNA Polymorphisms or RAPDs), we chose RFLP analysis using expressed or coding sequences as probes for several reasons: (1) to obtain well-behaved, genetically informative codominant markers; (2) to develop a set of anchor points for correlation with the established genetic maps and for immediate use with other silkworm strains; (3) to have the potential of uncovering new chromosome relationships among known, functioning genetic elements; and (4) to be able to carry out comparative genome analysis with markers that are conserved among the Lepidoptera.

The RFLP linkage map reported here contains 15 characterized and 36 anonymous, expressed sequences, plus 10 loci identified by a retrotransposon; the 15 linkage groups and eight ungrouped loci account for 23 of the 28 chromosomes and span a total recombination length of 413 cM. Ten of these linkage groups have been correlated with the established classic genetic maps. Upon completion and full integration with the latter, the high density molecular linkage maps now being constructed in collaboration with other laboratories (Promboon *et al.* 1995; H. Doira, Kyushu University and W. Hara, National Institute of Sericultural and Entomological Science, Tsukuba, personal communication) will enable positional cloning of genes encoding known mutations for recognizably critical functions, an important tool in an organism for which we do not yet have a method of gene transfer, interval mapping of quantitative trait loci (QTLS; Paterson *et al.* 1988; Lander & Botstein, 1989), development of marker-based selection for silkworm breeding (Lande & Thompson, 1990; Hospital, Chevalet & Mulsant, 1992), and establishment of a framework for long-term studies of genome organization and evolution among the Lepidoptera.

2. Materials and Methods

(i) *Silkworm strains*

Silkworm strains C108 and p50 were originally obtained from the National Institute of Genetics, Mishima, Japan and the Department of Genetic Resources, Silkworm Division, Kyushu University, respectively, and inbred in our laboratory for more than eight generations. Larvae were reared on fresh mulberry leaves. Single-pair matings were set up in plastic Petri dishes. F_1 sibs from a single pair-mating of a C108 female by a p50 male were used to produce an F_2 population, of which 52 randomly picked larvae were used for the experiments reported here. After selection larvae were sexed and scored for the *p* locus (plain; 2-0·0), for which C108 carries a homozygous recessive allele, *p*, and p50 a homozygous dominant allele, p^3.

(ii) *DNA isolation, restriction enzyme digestion, and genomic Southern blotting*

Genomic DNA was extracted from the posterior silk glands (PSG) of mid-fifth instar larvae. A pair of PSG was disrupted in a Dounce homogenizer in 1·5 ml of warm extraction buffer (50 mM EDTA, 0·5% Sarkosyl, and 0·5 μg/μl proteinase K, pH 8·0) and incubated at 50 °C for 2–3 h with occasional swirling. One extraction was performed with chloroform:isoamyl alcohol (24:1), three with phenol, and one with chloroform:isoamyl alcohol at room temperature using standard procedures (Sambrook *et al.* 1989), after which the DNA was precipitated in 70% ethanol and resuspended in TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8). To improve cutting with certain restriction enzymes, we found it necessary to reprecipitate the DNA with 100 μg/ml spermidine, followed by resuspension in TE buffer.

For digestion, approximately 10 μg DNA was incubated overnight with a five-fold excess of restriction enzyme (*Eco*R I, *Hin*d III, *Pst* I, *Hin*f I, *Xba* I, *Sac* I, or *Nde* I) in buffer supplied by the manufacturer, following which DNA concentrations were measured by fluorimetry. Samples of 10 μg were separated by electrophoresis in 0·7% agarose in TPE buffer (0·09 M

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Tris-phosphate, 0·002 M EDTA, pH 8·0), after which the gels were treated with 0·25 N HCl for 15 min and rinsed briefly in water, followed by denaturation in 1·5 M NaCl, 0·5 N NaOH and neutralization in 1 M Tris (pH 7·4), 1·5 M NaCl by standard procedures (Sambrook *et al.* 1989) before transfer. DNA was transferred to nylon membranes (Zeta Probe GT, Bio-Rad) by capillary action using 10 × SSC (1 × SSC contains 0·15 M NaCl, 0·015 M sodium citrate, pH 7·0) for Southern blotting or 0·4 N NaOH for alkaline blotting according to the manufacturer's instructions. The blots were baked at 80 °C in a conventional oven for 1 h before use.

(iii) *Cloned silkworm genes and cDNAs*

Cloned and characterized silkworm sequences used as probes for RFLP markers were kindly provided by the following individuals (Table 1): egg specific protein (ESP) by O. Yamashita (Nagoya University, Nagoya, Japan); sex-specific storage proteins SP-1 and SP-2, 30K protein (C6), and a pupal cuticle protein (PCP2) by S. Tomino (Tokyo Metropolitan University, Tokyo, Japan); fibroin heavy chain (pBmF6) and ribosomal DNA (rDNA; pBmR161) by H. Maekawa (National Institute of Health, Tokyo, Japan); fibroin light chain (exon 7) by S. Mizuno (Tohoku University, Sendai, Japan); chorion early proteins (5H4 and 6C11) by T. H. Eickbush (University of Rochester, Rochester, NY); Antennapedia homeobox-containing fragment (pBm 3.0) by W. Hara (National Institute of Sericultural and Entomological Science, Tsukuba, Japan); chorion DNA binding proteins (BmCBP1, 3, and 4) by N. Spoerel (University of Connecticut Medical Center, Storrs, CT); sericin 2 (pBmSE2002) and mag retrotransposon by A. Garel (Université Claude Bernard-Lyon I, Lyon, France); actin A3 by N. Mounier (Université Claude Bernard-Lyon I, Lyon, France). N. Spoerel also generously provided us with a library of size-selected (0·8–10 kb) early follicular cDNA cloned into the *Eco*R I site of lambda gt11 as a source of expressed sequences (called here Random cDNA from follicles or 'Rcfs').

(iv) *Preparation of probes*

DNA cloned into plasmids was isolated by alkaline extraction (Birnboim & Doly, 1979) and digested with appropriate restriction enzymes to excise inserts. After electrophoresis on 1 % agarose gels, insert bands were cut out and DNA was purified using a Geneclean kit (Bio 101, La Jolla, CA), and then resuspended in distilled water. Linearized recombinant plasmids were also used directly as probes.

The random follicular cDNA library was plated on X-gal plates by standard techniques (Sambrook *et al.* 1989), and well-isolated, white plaques were excised with the tip of a sterile Pasteur pipette. Agarose plaques were placed into 250 μl of distilled water, freeze-thawed twice to release DNA, and then heated at 94 °C for 5 min to inactivate proteases. Recombinant lambda inserts were amplified via the polymerase chain reaction (PCR; Saiki *et al.* 1988). The reaction mixture (50 μl) consisted of 25 μl of the freeze-thawed recombinant phage lysate, 200 μM each of deoxynucleotide triphosphates (Pharmacia LKB Biotechnology, Piscataway, NJ), 200 ng of the forward (22mer) and reverse (24mer) lambda gt11 PCR primers bordering the insertion site (purchased from Invitrogen or synthesized and generously provided by K. Iatrou and J. Drevet, University of Calgary, Canada), 1·5 mM $MgCl_2$, 50 mM KCl, 10 mM Tris-HCl, pH 8·3, and one unit of *Taq* DNA polymerase (Perkin-Elmer Cetus, Norwalk, CT). Mineral oil (50 μl) was added on top of the reaction mixture and PCR was carried out in an original model Perkin Elmer thermocycler using the following protocol: 1 cycle of 2 min at 94 °C, 1 min at 55 °C and 1 min at 72 °C, followed by 30 cycles of 30 s at 94 °C, 30 s at 55 °C, and 1 min at 72 °C, ending with a final extension step at 72 °C for 7 min. Amplification products were analysed by agarose gel electrophoresis of 5 μl of the reaction mixture, following which amplified DNA was precipitated and washed with 70 % ethanol and then resuspended in distilled water at a final concentration of 10 ng/μl.

After detection of certain repetitious clones early in the study, the follicular cDNA library was prescreened to remove rDNA, early chorion (6C11), and ESP clones that were present in high frequency. The prescreen was carried out either before or after the PCR amplification of inserts by probing dot blots with appropriate clones (see Table 1), using conditions described below.

(v) *Probe labeling, hybridization, and signal detection*

A nonradioactive chemiluminescence method (Durrant *et al.* 1990; Pollard-Knight *et al.* 1990), ECL (Amersham), was used for probe labeling, hybridization, and the detection of hybridization sites, following the manufacturer's instructions with a few modifications. DNA probes in distilled water (10 μg/μl) were denatured by boiling for 5 min and rapidly cooled on ice water for 5 min. An equal volume of labeling reagent (HRP-polyethyleneimine complexes) was added, mixed, and incubated on ice for 5 min. Glutaraldehyde was then added and the preparation incubated at 37 °C for 15 min. The membrane was prehybridized for 30 min to 1 h at 42 °C in ECL hybridization buffer containing 6 M urea, with the addition of 0·3–0·5 M NaCl to control stringency, depending on the probe. A hybridization volume of 0·1 ml buffer/cm^2 membrane was used. Labeled probes were added at a final concentration of 10 ng DNA/ml hybridization buffer. Hybridization was carried out at 42 °C in Seal-a-Meal bags in a shaking water bath for 16 h. For multiple probe hybridizations, selected probes that detected RFLPs

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Table 1. *Identified silkworm sequences used as probes for RFLP analysis. Note that mag-specific markers were scored as dominant and are designated by a letter and Roman numeral indicating the restriction enzyme used to resolve them in genomic digests followed by a hyphen and an Arabic numeral and letter indicating gel band number and dominant parental strain, e.g. RI-1C; other markers listed here were scored as codominant*

Clone	Mapped locus	Established linkage group	Map position (cM)	Identified sequence	Gene product	DNA source*	Probe length (kb)	Reference
5H4	*Ch3*	2	~ 2–7	ErB	Early chorion protein	c	0·6	Lecanidou *et al.* 1986; Goldsmith, 1989
6C11	*Ch3*	2	~ 2–7	ErA	Early chorion protein	c	0·6	Lecanidou *et al.* 1986; Goldsmith, 1989
pBmSP2C2	*Pst*	3	16·7	SP-2 (exon 2)	Larval hemolymph storage protein	c	1·1	Fujii *et al.* 1989
pBm3·0	E^{ca}	6	0	Antennapedia (homeobox plus flanking)	Homeotic	g	3	Hara *et al.* 1991; Ueno *et al.* 1992
pBmSE2002	*Src2*	11	9·2	Sericin 2 (internal repeat)	Silk gum (soluble)	g	4	Michaille *et al.* 1990*a*
FibL7	*Fib-L*	14		Fibroin light chain (exon 7)	Silk thread (fibrous)	g	0·5	Yamaguchi *et al.* 1989; Kikuchi *et al.* 1992
ESP	*Pes*	19	0	Egg specific protein	Yolk protein	g	2·5	Sato & Yamashita, 1991
30K C6	*Lp*	20	6·2	30K	Larval hemolymph storage protein	c	0·9	Sakai *et al.* 1988
pBmSP1C2	*Pfl*	23	8·6	SP-1	Larval hemolymph storage protein	c	2	Sakurai *et al.* 1988
pBmF6	*Fib-H*	25	0	Fibroin heavy chain (crystalline region)	Silk thread (fibrous)	g	2·6	Ishikawa & Suzuki, 1985
PCP2				PCP2	Pupal cuticle protein	c	1·2	Nakato *et al.* 1990
pBmR161				3′ end of repeat unit	Ribosomal DNA	g	6	Maekawa *et al.* 1988
mag				mag ORF1	Retrotransposable element	g	2·5	Michaille *et al.* 1990*b*
BmCPB1					Chorion DNA binding protein	c	5	Kafatos *et al.* 1995
BmCPB3					Chorion DNA binding protein	c	3·7	Kafatos *et al.* 1995
BmCPB4					Chorion DNA binding protein	c	4	Kafatos *et al.* 1995

* c, cDNA; g, genomic DNA.

with the same restriction enzyme on nonoverlapping areas of the blot were labeled separately and then each added to the hybridization buffer at the standard concentration for a single probe.

Membranes were washed twice at 42 °C in primary wash buffer (6 M urea, 0·4% SDS, and 0·1–0·5 × SSC to control stringency) for 15 min each, and then rinsed twice in 2 × SSC at room temperature for 5 min each. Hybridization was detected with ECL detection reagents according to the manufacturer's instructions. Membranes were incubated in detecting reagents for 1 min and excess reagents were then drained off. Blots were wrapped in Saran wrap and placed in a film cassette for exposure to X-ray film (Kodak XAR). The signal was enhanced by using a mirror that served as a reflective surface, similar to using intensifying screens in autoradiography. Exposure time varied from 1 min to 1 h. To remove previous probes, membranes were placed for 5 min on a pad of 3MM filter paper saturated with 0·4 N NaOH and then rinsed in 2 × SSC. Membranes were reused up to 30 times.

(vi) *Linkage analysis*

Two Pascal programs (written by D.G.H.) were used to analyse linkage data in Lepidoptera, where females are the heterogametic sex and have achiasmatic

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meiosis (no crossing-over). Program TwoPtLep first examines dominant or codominant loci for sex-linkage by identifying offspring phenotypes allowable under autosomal inheritance but forbidden under sex-linked inheritance. For the remaining loci that cannot be ruled out as sex-linked in this way, TwoPtLep calculates the logarithm of the ratio of the likelihood under sex-linkage over the likelihood under autosomal inheritance, using analytical formulas. Positive values of this log odds score are evidence for sex-linkage; negative values give evidence for autosomal inheritance. Loci classified as sex-linked by this criterion are then excluded from further calculations. Putative autosomally inherited loci are tested for expected segregation ratios (3:1 for dominance, 1:2:1 for codominance) using G-tests on contingency tables.

Next, for every pair of putatively autosomal loci, TwoPtLep examines evidence for linkage by calculating the maximum lod score Z. Z is defined as the natural logarithm of the ratio of the maximum likelihood of obtaining the data under the hypothesis that the two loci are linked, over the likelihood under the hypothesis that the two loci assort independently. Large values of Z indicate linkage when Z is well defined. Conventionally, Z values greater than 3 are taken as statistically significant evidence for linkage (Ott, 1991), since linkage is greater than $10^3 = 1000$ times more likely than independent assortment when $Z = 3$. Because crossing-over in *B. mori* is restricted to males, certain offspring genotypes are impossible in the F_2 if the two loci are syntenic, i.e. on the same chromosome. Observation of these 'forbidden genotypes' in the progeny implies that the two loci must be independently assorting on different chromosomes; the lod score Z is undefined in this case (converging to minus infinity) because the numerator of the likelihood ratio, and hence the argument of the logarithm, is zero. Whether or not there are observable phenotypes corresponding to these forbidden genotypes depends on the dominance or codominance of the two loci being compared.

When forbidden genotypes are not observed, TwoPtLep calculates the maximum likelihood estimate (MLE) of Z, and the associated male recombination rate, r_m, using the closed-form expression appropriate to the dominance–codominance type of the loci. For two dominant loci in repulsion with no forbidden recombinants, there is no unique MLE of r_m and it was arbitrarily set to 0·49. When forbidden genotypes are observed, TwoPtLep sets $r_m = 0{\cdot}5$ and $Z = -100$ for that pairwise comparison. After TwoPtLep has executed, there are three types of locus pairs: (1) pairs that cannot be syntenic because forbidden recombinants were observed ($Z = -100$); (2) pairs that could be syntenic but for which the evidence for linkage is not impressive ($Z < 3$); and (3) pairs that could be syntenic and moreover have strong evidence for linkage ($Z \geqslant 3$).

The second program, MapALep, calculates multi-locus linkage maps for groups of putatively syntenic loci. For a specified order of loci along the chromosome, it calculates the MLE of the recombination fractions and the log likelihood using the EM algorithm (Dempster *et al.* 1977; Lander & Green, 1987). Given an ordered set of loci, MapALep will examine every possible order and rank the 20 most likely according to the log likelihood value, enabling exhaustive search for the most likely order for five or fewer loci. Two recombinational systems can be specified for MapALep, one in which crossing-over is restricted to males and r_m is estimated, and the other in which crossing-over occurs in males and females and r (the recombination rate common to both sexes) is estimated. Both utilize the same EM algorithm to find the maximum likelihood estimates.

In addition, calculations were checked with the program MAPMAKER (Lander *et al.* 1987, version 2.0 running on a VAX under VMS). Strictly speaking, MAPMAKER is inappropriate for F_2 data from Lepidoptera because it assumes that recombination occurs in both sexes and estimates r. There is no control parameter in the program, or any way of recoding the data, to analyse correctly F_2 data with crossing-over restricted to males and to estimate r_m, under the available versions of MAPMAKER (1.0, 2.0, or 3.0). But because MapALep can analyse F_2 data with crossing-over occurring in males only (estimating r_m) or in both sexes (estimating r), MAPMAKER can be used to verify the implementation of the EM algorithm of MapALep numerically in the latter case. This is an important check because the precise implementation of the EM algorithm in MAPMAKER is not obvious (see Lander & Green, 1979). For this purpose, all calculations described in the Results were repeated using MAPMAKER and using MapALep with crossing-over in both sexes switched on. When MAPMAKER's 'tolerance' parameter governing the convergence criterion was set to 10^{-6}, there was perfect agreement in the output of the two programs (Z scores and estimates of r) in all cases (although of course these differed from the earlier MapALep results with crossing-over in females switched off). This shows that the implementations of the EM algorithm in the two programs will produce the same results under the same conditions and assumptions.

As another check on the implementation of the EM algorithm in MapALep, all two-point comparisons calculated by TwoPtLep using closed-form expressions for r_m were re-calculated by MapALep using the EM algorithm with crossing-over in females switched off. There was perfect agreement of the output of these two programs in all cases.

Finally, MAPMAKER was used to estimate an approximation to the maximum-likelihood map for the linkage group with eight loci. MapALep is too slow to calculate the maps for all 20160 ($= 8!/2$) possible orders in a reasonable time, but

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MAPMAKER only took a few hours to do this on a VAX. However, all male recombination rates, r_m, are under-estimated by the r produced by MAPMAKER, because of the erroneous assumption of crossing-over in both sexes. To convert the r calculated by MAPMAKER to estimates of the actual male recombination rates occurring in *B. mori*, and *ad hoc* procedure was used. For the eight loci on the linkage group, the two-point estimates of r_m under the hypothesis of no crossing-over in females were found to be related to the two-point estimates of r under the hypothesis of crossing-over in both sexes by the linear regression $r_m = 0{\cdot}009 + 1{\cdot}759r$ ($R^2 = 0{\cdot}994$). Multi-locus estimates of r_m were then calculated from MAPMAKER's multilocus estimates of r using this same equation. The resulting map is taken as an estimate of the correct MLE map under the case of crossing-over restricted to males. This is only an estimate, because this method provides no way to check whether the optimal order with recombination switched on in both sexes is also optimal with recombination switched off in females.

Following calculation of recombination fractions as above, they were converted into map distances using the Haldane mapping function (Ott, 1991).

3. Results

(i) *Mapping strategy*

We chose wild-type silkworm strains C108 and p50 as parentals for a standard molecular linkage map because of their phenotypic diversity for such complex characters as size, growth rate, diapause, morphology, nutritional requirements, general vigor, and cocoon properties, suggesting that considerable polymorphism would differentiate them at the DNA level. Other experimental advantages included a high degree of inbreeding, which made it likely that these stocks would be relatively homozygous, and relative ease of rearing. Both strains are of Chinese origin; C108 is an improved bivoltine (2 generations per year), while p50 is multivoltine, with many characteristics closer to tropical than standard Chinese races. 52 F_2 progeny from a single pair mating of F_1 sibs served as the source of DNA to verify our experimental approach and for initial map construction.

It is common to use backcrosses for linkage mapping in *B. mori* because of the restriction of crossing-over to males. These backcrosses have differing advantages, depending on the sex of the informative F_1 parent. If the F_1 is female, rapid detection of linkage is possible because of the complete absence of recombinants, but estimation of map distance is not possible. If the F_1 is male, estimation of map distances is possible because of crossing-over, but detection of linkage may be more difficult. Clearly, one approach to RFLP mapping would be to conduct all eight backcrosses obtainable by all possible combinations of the initial cross (C108 × p50 or p50 × C108), sex of the F_1 parent, and recurrent strain for the backcross (C108 or p50). This would permit mapping of codominant loci or dominant loci originating from either strain and data analysis would be straightforward. However, for maximum efficiency in the initial phases of linkage mapping, we conducted a single F_2 cross instead. This yielded an eightfold savings in labor with only about a twofold decrease in information content. Data analysis for this case is somewhat less straightforward, requiring special methods to extract all of the information present in the cross.

(ii) *Probes and detection of polymorphism in silkworm DNA*

Sixteen of the probes used to construct the map were derived from identified silkworm sequences. Of these, 10 had been localized on the conventional linkage maps using biochemical or morphological variants, and could serve as markers to correlate with the molecular maps (Table 1). Other identified sequences included those encoding chorion DNA binding proteins (BmCBPs; N. Spoerel, personal communication), ribosomal RNA (rDNA), a pupal cuticle protein (PCP2), and mag, a well-characterized retro-transposon (Michaille *et al.* 1990*a*, *b*) which is present in 4–15 copies in silkworm genomes (Tamura *et al.* 1993; Garel *et al.* 1994) and yielded 10 segregating markers scored as dominants (Lawson, Shi & Goldsmith, in preparation).

Many mapping studies in plants have shown moderate to low levels of restriction fragment length polymorphism with both cDNA and genomic clones (e.g., see Figdore *et al.* 1988; Miller & Tanksley, 1990; Devey *et al.* 1994; Kesseli *et al.* 1994); however, in *B. mori* known expressed genes and anonymous cDNA clones revealed sufficient levels of polymorphism for our purposes. In general, we observed the highest levels of polymorphism using restriction enzymes with 6 base pair recognition sequences that were slightly enriched for GC content and produced genomic DNA fragments in the range of 10 kb or less. The majority of identified sequences gave strong signals and showed polymorphism with at least one of seven enzymes tested (see Materials and Methods). In contrast, of five sequences encoding chorion DNA binding proteins, only three showed polymorphism and were mapped here; the other two proved to be monomorphic, as was a cytoplasmic actin gene, A3 (data not shown). This suggests that the DNA flanking certain genes, including potential transcription factors, may be more highly conserved than those encoding relatively abundant differentiation-specific proteins.

To obtain additional markers, we screened 93 random clones (Rcfs) from an early follicle cDNA library on Southern blots of parental and F_1 DNAs for probes encoding expressed, single or low copy sequences. Based in part on preliminary tests and relative cost, we systematically screened the Rcfs

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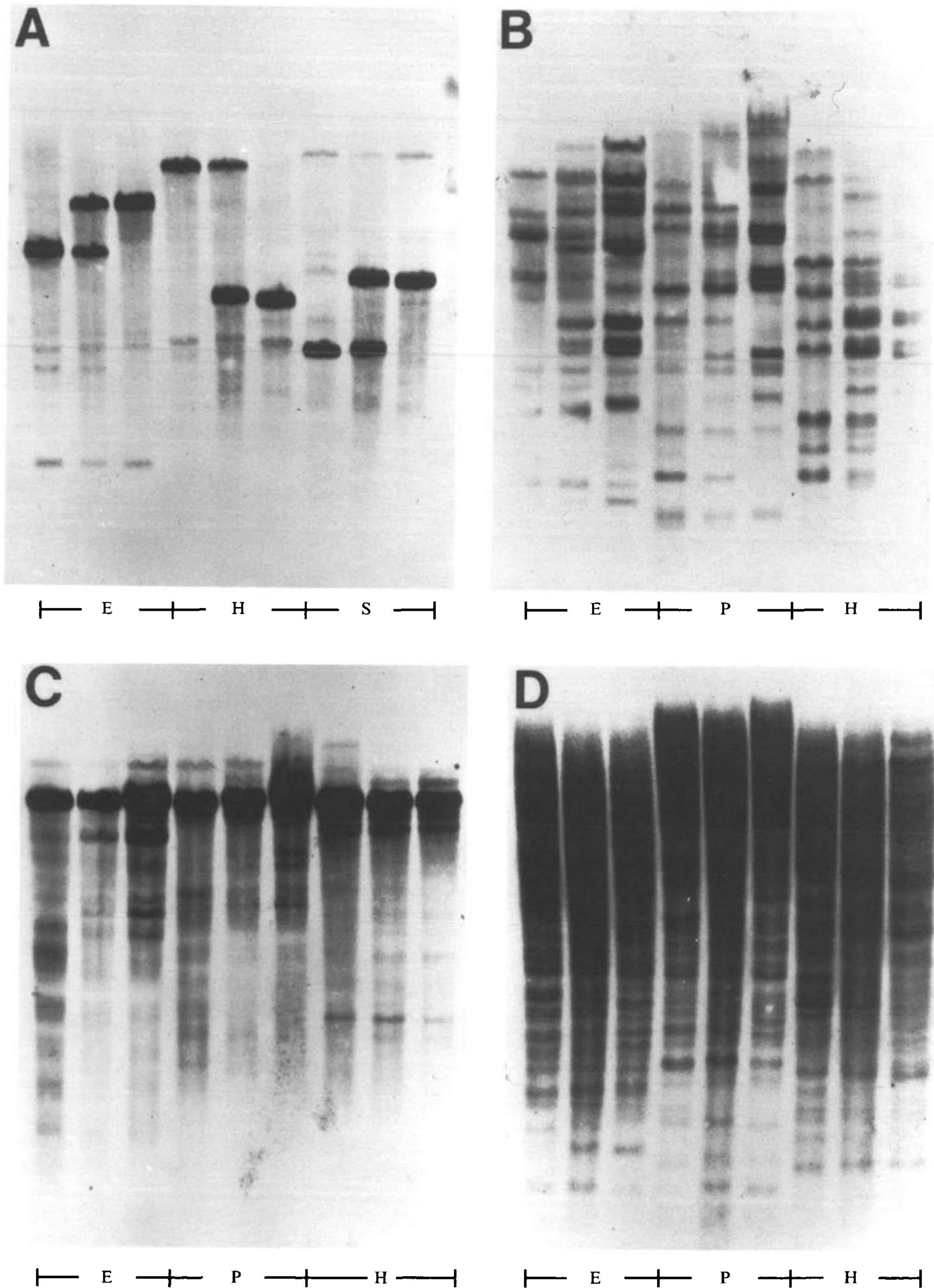

Fig. 1. Typical genomic restriction patterns for Rcfs. Each panel shows a blot containing three sets of parental DNAs in the order p50, F1, C108 (10 μg/lane); each set was digested with one of the following restriction enzymes as indicated: E, *Eco*R I; P, *Pst* I; H, *Hin*d III; S, *Sac* I. Panel A, single copy pattern (Rcf96); panel B, pattern for chorion early multigene gamily, 6C11 (Rcf61); panel C, rDNA pattern (Rcf45); panel D, highly repeated sequence pattern (Rcf253).

using four restriction enzymes which varied somewhat in their usefulness for the two mapping strains as follows: *Sac* I, 67 % of the clones were polymorphic; *Eco*R I, 55 %; *Hin*d III, 51 %; and *Pst* I, 47 %. This confirmed the findings with identified sequences, suggesting that considerable polymorphism exists on

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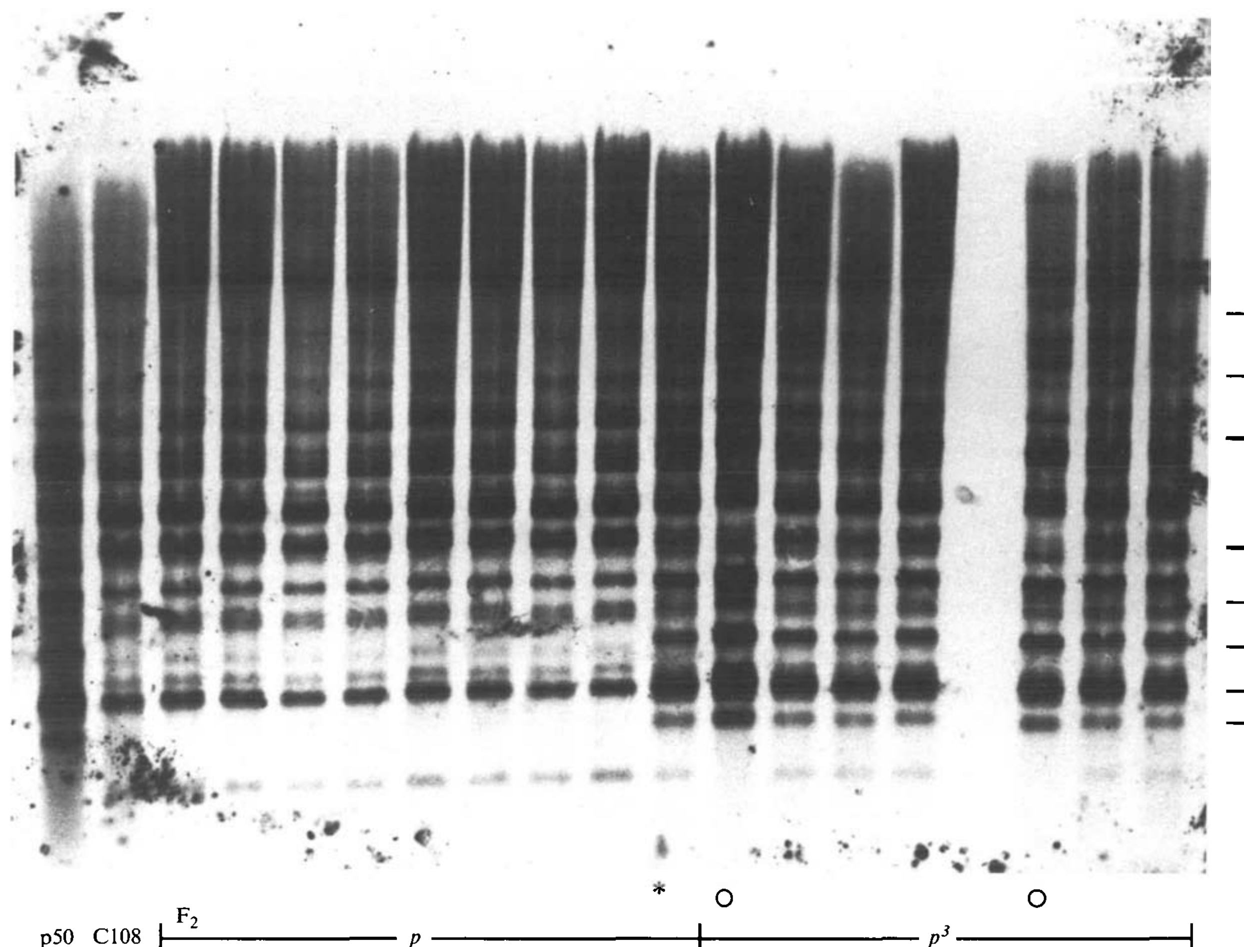

Fig. 2. Hybridization patterns showing cosegregation of early chorion markers with markers representing the *p* locus (2–0·0) in F_2 progeny. A blot of sample DNAs digested with *Pst* I was hybridized to early chorion probe 6C11. Parental p50 and C108 DNAs and F_2 progeny are indicated. p^3 and p indicate the phenotypes of 5th instar larvae at the *p* locus; p^3 (from p50) > p (from C108). All *p* larvae except the sample marked * express the homozygous C108 restriction pattern, consistent with the likelihood that they are homozygous at the proximal end of linkage group 2; similarly, all p^3 larvae except samples marked (o) express the F_1 pattern, as expected. The samples marked (o) show homozygous p50 restriction patterns, consistent with the possibility that they are also homozygous for p^3; the sample marked * shows an F_1 pattern, indicating the likelihood that there was a single crossover between *p* and the chorion locus in this individual. p50 and C108-specific bands are marked with thin and thick lines, respectively.

the DNA level even between strains of related geographic origin. It seems likely that this is due to the variety and high copy number of transposable elements in the silkworm genome (Adams *et al.* 1986; Ogura *et al.* 1994; Eickbush, 1995), which have been detected close to (Eickbush, 1995) and within (Fujiwara *et al.* 1984; Lecanidou *et al.* 1984; Eickbush & Robins, 1985; Ueda *et al.* 1986; Michaille *et al.* 1990 *a*) expressed genes.

Ten (11%) of the random clones produced intense, smeared hybridization patterns characteristic of highly repetitive DNA; 9 (10%) yielded multiple but distinct banding patterns characteristic of mid-repetitious multigene families; 7 (8%) produced very weak signals, perhaps as a result of a complex intron structure or numerous internal restriction sites; and the remaining 67 clones (72%) yielded strong hybridization signals revealing one or a small number of fragments (Fig. 1). Among nearly 100 random sequences examined, we obtained duplicates of only a few of the Rcfs, including members of the chorion early gene family, 6C11, and ESP, both synthesized by follicular cells and therefore likely to have been abundantly expressed in the message population used to construct the library. Ultimately, we introduced a pre-screen for these sequences plus contaminating rDNA by probing unknown Rcfs with them on dot blots.

(iii) *Segregation behavior of representative clones*

As a preliminary test of expected Mendelian behavior for RFLPs in the F_2 progeny, we examined segregation patterns for early chorion genes (5H4, a single copy gene, and 6C11, a multicopy family; Eickbush & Izzo, 1995), which have been localized to a single proximal cluster on chromosome 2 using protein electrophoretic variants (Goldsmith, 1989). As predicted, these genes showed close linkage to morphological markers for the *p* locus on the proximal end of the same

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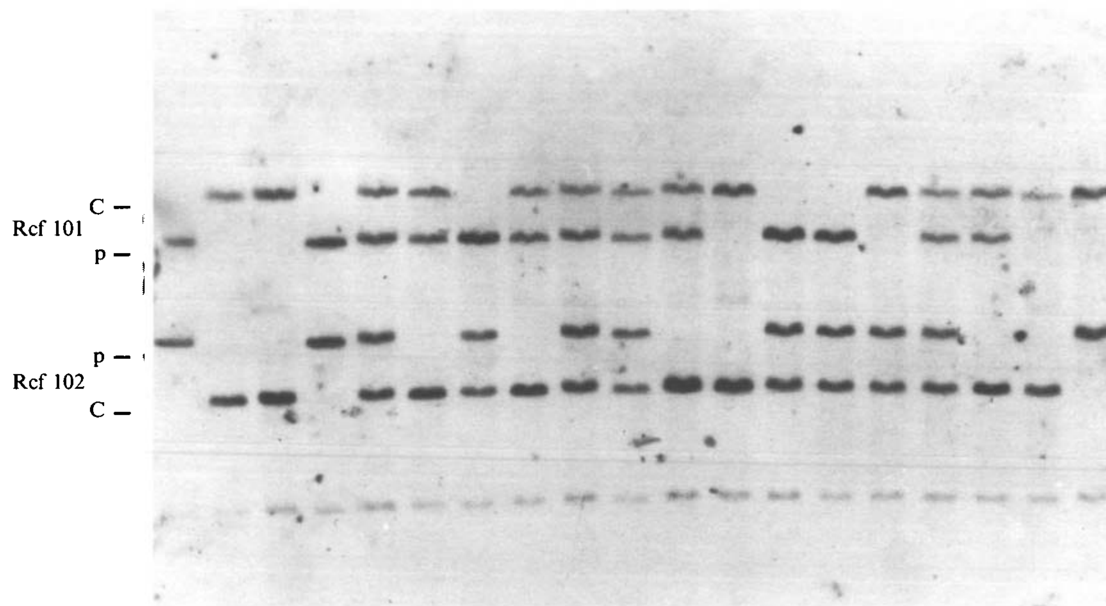

Fig. 3. Multiplex hybridization with well-separated single copy anonymous cDNAs (Rcfs). F_2 progeny DNA was digested with *Eco*R I before electrophoresis, blotting, and hybridization with probes Rcf101 and Rcf102. Left-hand lanes 1 and 2 are homozygous parents p50 and C108, respectively; the remainder are F_2 progeny. p50 (p) and C108 (C) -specific bands are indicated.

chromosome (2-0·0) which were also segregating in the cross (Fig. 2). Each of the other probes associated with the conventional maps were also well behaved, showing independent assortment with respect to the chorion cluster and to each other (see below), and thus provided markers for nine additional established linkage groups. We hybridized the Rcf probes singly or, for those producing well-separated bands, two at a time (Fig. 3), against blots of appropriately digested F_2 progeny DNA. In general, we avoided using Rcfs that produced complex patterns as probes except where it was easy to discriminate strong individual polymorphic bands (Fig. 4). Three pairs of probes, Rcf1 and Rcf42, Rcf 126 and Rcf241, and Rcf40 and PCP2, produced different RFLPs but identical segregation patterns indicating a lack of crossing-over in this experiment, and are treated as single loci Rcf1/42, Rcf126/241, and PCP/Rcf40, respectively, in the following description.

(iv) *Tests for sex-linkage*

We define a dominant locus to be C-dominant if the dominant allele originates from the C108 grandparent, and to be P-dominant if the dominant allele originates from the p50 grandparent. Forty-six of the 59 loci scored were codominant, five were C-dominant, and eight were P-dominant. All codominant loci except one (Rcf96) exhibited genotypes in the F_2 inconsistent with sex-linkage, as did all five C-dominant loci. The log of the likelihood ratio for Rcf96 was 10·84, indicating that sex-linkage is $6{\cdot}92 \times 10^{10}$ times more likely than autosomal inheritance for this locus – strong support for sex-linkage. Because of the nature of the cross (C108 grandmother × p50 grandfather), P-dominant loci cannot produce genotypes that rule out sex-linkage, and the likelihood ratios provide the only evidence. The log-likelihood ratios for seven of the P-dominant loci were negative, ranging from $-2{\cdot}66$ to $-6{\cdot}77$, indicating that sex-linkage was from $4{\cdot}57 \times 10^2$ to $5{\cdot}89 \times 10^6$ times *less* likely than autosomal inheritance – strong evidence for autosomality. The log-likelihood ratio for the eighth P-dominant locus (Rcf208a) was $-1{\cdot}05$, corresponding to an eleven-fold lower likelihood for sex-linkage than autosomality; on this basis Rcf208 was also tentatively classified as autosomal. Thus, only Rcf96 was eliminated from the subsequent linkage calculations that assumed autosomal inheritance.

(v) *Segregation tests*

For the putatively autosomal loci, tests of segregation ratios (13 loci with a dominant allele tested for fit to 1:3, 45 codominant loci tested for fit to 1:2:1) failed to yield any deviations from expectation significant at the 0·05 level adjusted for multiple comparisons, nor did Rcf96 show any significant deviations from predicted ratios under sex-linkage.

(vi) *Preliminary identification of linkage groups*

The 58 autosomal loci were grouped into preliminary linkage groups (PLGs) using the following criteria: two loci are considered to have evidence for linkage if their Z score is greater than 3·0, and this evidence for linkage is sufficient to assign them to the same PLG. According to these definitions, 'evidence for linkage' is not necessarily transitive (A and B can have a Z score greater than 3 and so can B and C, but A and C need not); but the membership in a PLG is transitive

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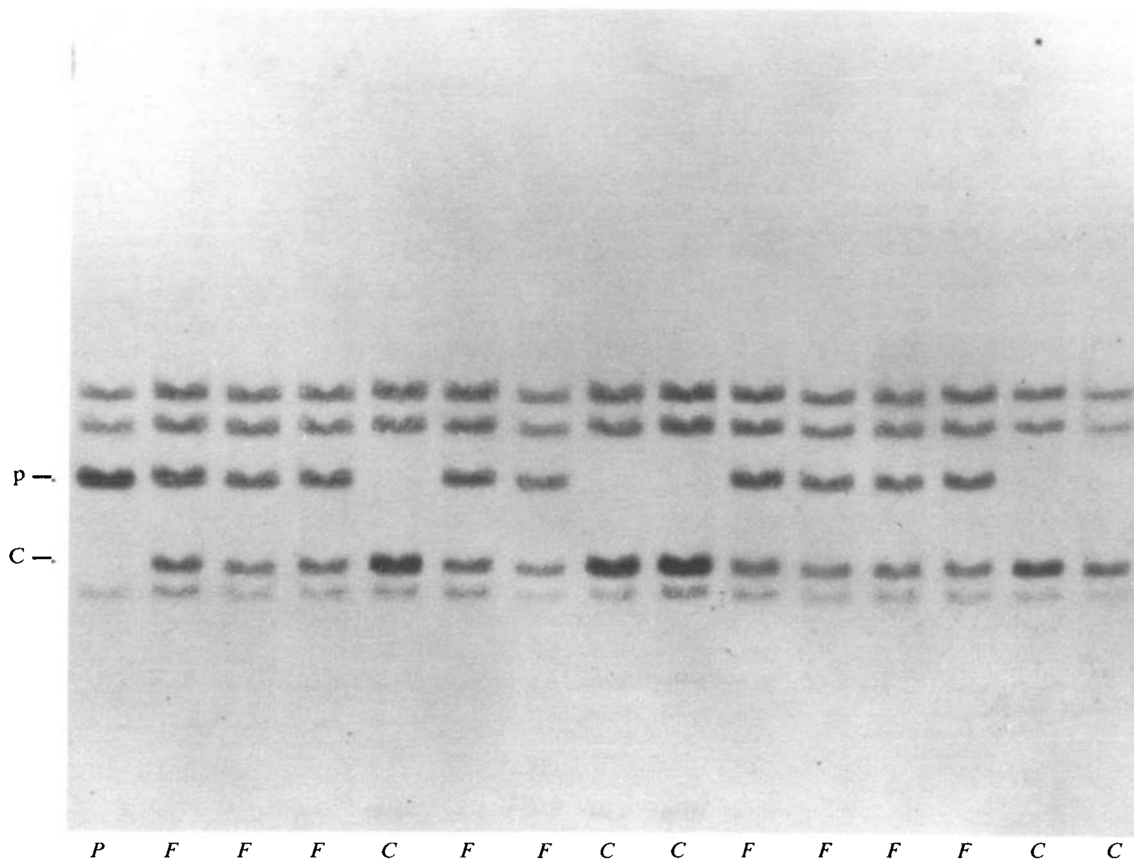

Fig. 4. Hybridization patterns for an anonymous cDNA producing multiple bands. F_2 progeny DNA was digested with *Hin*d III before electrophoresis, blotting, and hybridization with probe Rcf48. Indicated are p50 (p) and C108 (C) -specific bands that were used to score progeny as p50 (*P*) or C108 (*C*) homozygotes, or F_1 (*F*) hybrids.

(if A and B are in the same PLG, and B and C are in the same PLG, then A and C are in the same PLG). Under some circumstances, this transitivity can lead to the creation of large, spurious linkage groups of loci that are not really syntenic but are 'chained' together by chance close linkages between some pairs. With the present data, however, the great majority (75 of 77) of locus pairs that were grouped into the same PLGs also had pairwise Z scores of greater than 3 (range: 3·21 to 22·27). Fifty of the autosomal loci were grouped into a total of 15 PLGs in this way, ranging from two to eight loci each. (Rcf208a was grouped with pBm3·0, and Rcfs 2, 83, and 219 at Z scores between 6·9 and 9·8, strong confirmation of its autosomal inheritance.) The remaining eight loci failing to meet the Z criterion with any other locus were left ungrouped.

(vii) *Tests of synteny within preliminary linkage groups*

Each of the 15 preliminary linkage groups was then re-examined for the possibility of spurious linkage by searching for 'forbidden genotypes'. Fourteen PLGs had pairwise combinations of codominant, P-dominant, and C-dominant loci that would have produced forbidden recombinants had the loci within them been non-syntenic, but all 54 of these pairwise comparisons showed zero forbidden recombinants. This outcome is extremely unlikely unless all of the loci in a PLG are indeed syntenic, providing further support for the integrity of these 14 preliminary linkage groups.

The fifteenth PLG consisted of three C-dominant loci; as dominant loci in coupling, no forbidden genotypes in the F_2 are identifiable by their phenotypes. Thus, qualitative evidence for or against synteny of the fifteenth group cannot be adduced as with the other fourteen. However, quantitative evidence from the linkage map strongly supports the integrity of PLG 15 (see below).

(viii) *Maximum number of chromosomes marked*

If each of the 15 PLGs and each of the eight ungrouped loci occupied a separate chromosome, 23 of the 27 autosomes in *B. mori* would be marked by a polymorphic marker segregating in this family. No more than 23 autosomes could be thus marked (unless the integrity of one of the preliminary linkage groups were disproved; see below for consideration of the fifteenth group), but the number could be less than 23 if pairs of loci with $Z < 3$ were in fact syntenic. To examine this possibility, forbidden recombinants were examined in all pairwise comparisons of loci assigned to *different* linkage groups (or unassigned loci). Two PLGs could be proved to be not syntenic if at least one

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Table 2. *Maximum number of observed forbidden recombinants among and between preliminary linkage groups (PLG) and ungrouped loci. P1 = PLG1, '—' means that there are no phenotypes uniquely identifying forbidden recombinants among loci of PLG15 because they are all dominant in coupling*

	P1	P2	P3	P4	P5	P6	P7	P8	pBmF6	P9	PCP*	Rcf12	P10	P11	P12	P13	P14	Rcf43	Rcf47	Rcf89	Rcf119	RI-3P	P15
P1	0																						
P2	8	0																					
P3	7	7	0																				
P4	7	4	11	0																			
P5	4	4	5	5	0																		
P6	9	7	9	7	8	0																	
P7	4	6	10	7	6	4	0																
P8	6	11	9	7	8	12	4	0															
pBmF6	5	5	5	5	4	6	4	6	0														
P9	3	10	8	9	4	13	6	9	2	0													
PCP*	7	4	7	7	6	8	4	6	3	10	0												
Rcf12	0	3	3	2	2	2	1	4	0	2	1	0											
P10	7	8	9	10	7	9	6	11	3	7	9	0	0										
P11	8	6	7	7	3	11	10	8	6	10	10	3	12	0									
P12	9	6	11	6	8	17	12	8	8	10	8	2	9	6	0								
P13	7	5	12	7	8	12	7	8	3	7	5	3	11	11	8	0							
P14	6	3	6	9	4	8	5	6	3	8	6	0	10	9	9	6	0						
Rcf43	5	5	6	6	2	8	5	6	2	6	6	2	5	4	7	5	6	0					
Rcf47	6	10	7	9	4	10	12	12	1	7	6	2	6	10	11	13	9	6	0				
Rcf89	9	5	8	8	3	6	10	8	3	5	9	3	8	7	9	5	9	3	9	0			
Rcf119	8	4	4	7	4	6	5	7	4	7	9	3	8	5	3	7	9	3	10	4	0		
RI-3P	6	3	4	3	2	4	3	5	1	3	6	1	3	2	5	2	3	1	5	2	0	0	
P15	4	4	3	4	3	7	7	5	4	4	7	3	7	10	5	5	9	3	6	6	4	6	—

* Corresponds to PCP/Rcf40.

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locus in the first exhibited forbidden recombinants with at least one in the second. Similarly, an ungrouped locus with no strong evidence of linkage to a PLG (i.e. $Z < 3$) could be proved non-syntenic with it if forbidden recombinants to any locus on that PLG could be found.

Table 2 summarizes the occurrence of forbidden recombinants between and within all combinations of PLGs and ungrouped loci. The number in the i-th position along the diagonal is the maximum number of forbidden recombinants observed in all pairwise comparisons of loci within that PLG (single ungrouped loci have a zero here by definition). The number in the i-th row and j-th column under the diagonal is the maximum number of forbidden recombinants observed over all pairwise comparisons between loci in group i and loci in the other group j. A nonzero entry in this cell is strong evidence that the two groups must be non-syntenic, occurring on different chromosomes (the only other explanation would be scoring error). A zero entry indicates compatibility with synteny. A dash indicates that forbidden recombinants do not produce identifiable phenotypes in this comparison (dominant loci in coupling), and so such qualitative information on synteny cannot be obtained.

It is evident from Table 2 that the great majority of comparisons are not consistent with synteny. This applies to most ungrouped loci as well as PLGs. In fact, only five of the sub-diagonal cells contain zeroes. Four of these involve the ungrouped locus, Rcf12. The absence of forbidden recombinants to PLGs 1, 10, 14, and ungrouped locus pBmF6 makes synteny of Rcf12 with any one of them feasible; yet these four are mutually non-syntenic so Rcf12 can be syntenic with at most one of them. The Z-score was less than 3·0 for all comparisons between Rcf12 and these four groups, so there is not strong evidence for linkage to any of them. However, examination of the sample sizes suggests another possible reason for this. Rcf12 was only scored for 16 of the 52 offspring in the data set (three other loci were scored for about 30 offspring and all the rest were scored for about 50). Since the sample size sets an upper bound to the Z-score attainable, failure to demonstrate linkage could also be due to low sample size. Since Rcf12 is difficult to place because of small sample sizes, we prefer to leave it unassigned.

Elimination of Rcf12 from consideration yields only one other cell under the diagonal of Table 2 consistent with synteny, corresponding to the zero forbidden recombinants observed between Rcf119 and RI-3P. There was actually an excess of (non-forbidden) recombinants produced among the 43 co-informative offspring (relative to parentals) yielding an MLE of $r_m = 0{\cdot}62$, greater than independent assortment would predict. It is thus not impossible, but still highly unlikely, that Rcf119 and RI-3P could be syntenic.

Table 3. *Summary of Putative Linkage Groups and ungrouped loci. Correspondence is shown to established linkage groups where identified;* (—) *indicates unknown*

PLG or ungrouped locus	Established linkage group
Rcf94	1*
1	2
2	3
3	6
4	11
5	14
6	19
7	20
8	23
pBmF6	25
9	—
10	—
11	—
12	—
13	—
14	—
15	—
PCP/Rcf40	—
Rcf12	—
Rcf43	—
Rcf47	—
Rcf89	—
Rcf119	—
RI-3P	—

* Sex (Z)-linked.

The nonzero cells in the rest of Table 2 show that no other synteny relationship are possible, leading to the conclusion that at least 21 of the 27 autosomes in *B. mori* must be marked by an RFLP scored in this study; and the number is much more likely to be 22. The present data do not permit a unique placement of Rcf12 with any confidence, but 18 possible assignments can be ruled out by the occurrence of forbidden recombinants, even with only 16 informative offspring.

By reference to sequences with known map positions used in this study (Table 1), eight of the PLGs plus Rcf96 could be assigned to linkage groups in the established genetic maps (Table 3); the remaining previously mapped locus for which we scored RFLPs, Fib-H, was ungrouped.

(ix) *Order of loci and map distance within linkage groups*

For the linkage groups with three, four, or five loci, the most likely gene order and the MLEs of the male recombination fractions between adjacent loci (i.e. the most likely map) could be obtained by exhaustive calculation for all possible locus orders. For each linkage group, we will call a map 'well supported' by the data if it is the only possible map, or if it is at least 10 times more likely than the MLE map for any other

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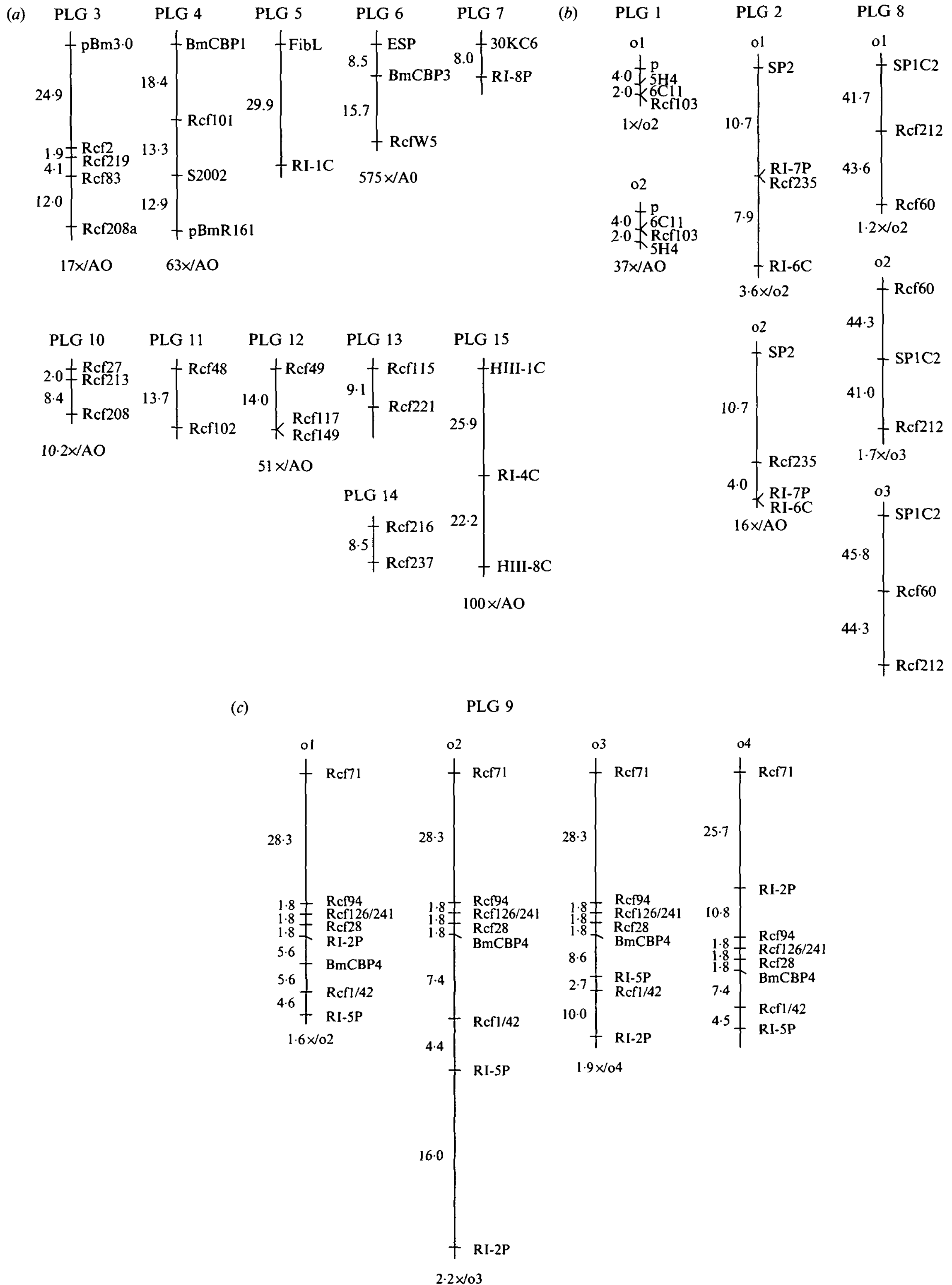

Fig. 5(*a*). Well-supported PLGs. Units are Haldane centimorgans. Notation at bottom, e.g. 17×/AO, means that this order is at least 17 times more likely than any other. (*b*) and (*c*) Less well-supported PLGs. Units are Haldane centimorgans. o1 = order 1 (most likely) 3·6×/o2 means this order is at least 3·6 times more likely than order 2. AO = all other orders. PLG 8 is drawn to 0·4× scale of the other maps.

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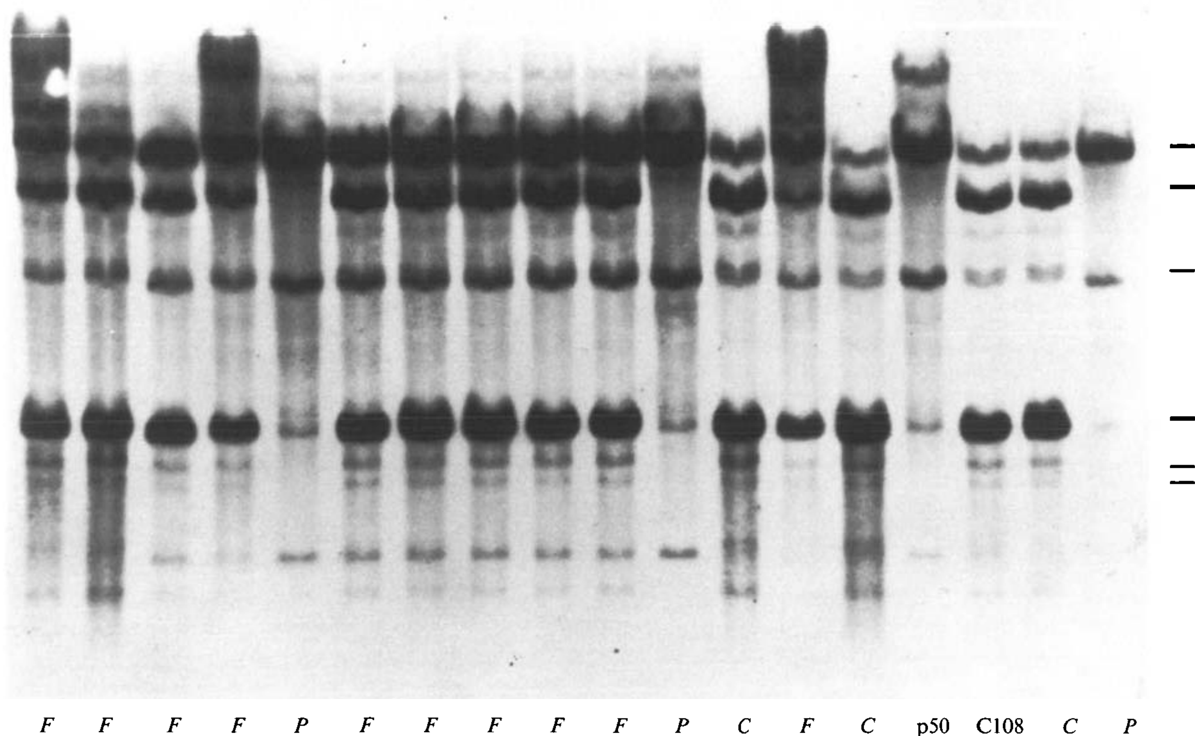

Fig. 6. Hybridization patterns for ribosomal genes. DNA was digested with *Xba* I before electrophoresis, blotting, and hybridization with probe pBmR161. p50 and C108 are parental DNAs; the remainders are F_2 progeny. Thick and thin lines indicate the major and minor bands that were used to score progeny as p50 (*P*) or C108 (*C*) homozygotes, or F_1 (*F*) hybrids.

possible gene order. Most likely maps for the 11 well-supported cases are shown in Fig. 5*a*.

The map for PLG 15 had the second highest level of support of any PLG in Fig. 5*a*; the map shown is at least 100 times more likely than any alternative. Although the pairwise Z values for HIII-1C and HIII-8C was less than 3 for this linkage group, this is probably due to the very large distance (49 cM) and the fact that dominant loci in coupling are less informative for linkage than codominant loci. A separate test for linkage of HIII-1C to the linked pair RI-4C–HIII-8C gave a Z value greater than 4. Thus the integrity of this PLG is strongly supported in spite of the absence of evidence on forbidden recombinants as discussed above.

The three other linkage groups for which exhaustive calculation was possible each have a most likely map that is less well supported by the data, being only 10 times more likely (or less) than one or more alternatives. For these linkage groups, all possible maps within that tenfold range are depicted in Fig. 5*b*. The two maps shown for PLG 1 have nearly identical likelihoods, but both are at least 37 times more likely than all other possible maps. The most likely map for PLG 2 is 3·6 times more likely than the next most likely, which in turn is at least 16 times more likely than all other possibilities. PLG 8 is poorly resolved by these data; the most probable map is only two times more likely than the least probable map.

PLG 9 had to be analysed by special methods with the assistance of MAPMAKER (Lander *et al.* 1987), as described above. The most likely map found by MAPMAKER was not well supported according to our criterion. In fact the four most likely maps had likelihoods within a factor of 7 or less of each other; these are shown in Fig. 5*c*. All other maps found by MAPMAKER were at least 23 times less likely than the most likely map.

(x) *Localization of rDNA*

B. mori carries 240 copies of the ribosomal genes per haploid genome (Gage, 1974). Using interspecific crosses between *B. mori* and *B. mandarina*, Maekawa and colleagues (Maekawa *et al.* 1988) mapped rDNA genes to a single nucleolus organizer region by means of RFLP analysis. Using a probe for the 3′ end of the rDNA repeat unit, we found differences in size and intensity for a major band as well as some minor bands in the restriction patterns of C108 *v.* p50 produced by digestion with *Xba* I (Fig. 6). These markers cosegregated with PLG4, confirming the finding of a single major rDNA locus in *B. mori*.

4. Discussion

The map presented here has only 413 cM, in contrast to 900 cM reported in the established, conventional

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linkage maps, but contains one or more markers on 22+1 of the 27+1 chromosomes. This statement is possible because of the relatively unique test for synteny in Lepidoptera. Although it is difficult to cover all chromosomes in a single cross, we were able to make maximum use of the available genetic information by the felicitous combination of most loci being co-dominant, use of an F_2 cross, and the achiasmatic meiosis of one sex, which allowed a very efficient use of markers. Thus, only one of the 61 molecular markers examined here remains unassigned in the sense that its synteny relationships to the others is ambiguous.

The use of RFLPs proved a straightforward strategy that provided a set of single-copy, polymorphic anchor loci that can be used for map integration across strains and for coordinating independent mapping efforts (Promboon *et al.*, in preparation). We recommend that additional anchor loci be identified so that future crosses can score one anchor locus per chromosome in order to reduce the overall work. Moreover, the yield of anonymous RFLPs from an early oocyte library was relatively high; approximately 2/3 of tested clones provided useful probes, and continuing efforts indicate that the library is not exhausted (D. Mills, personal communication). Other molecular approaches using PCR, such as RAPDs (Promboon *et al.* 1995) and microsatellites (Zheng *et al.* 1993) will be necessary for projected work on complex traits and marker-assisted selection, and are now being investigated (E. Abraham and J. Nagaraju, personal communication).

Multigene families with unknown linkage arrangements present particular difficulties in detection of signals from individual family members, and may require alternative strategies, such as the use of PCR with gene-specific primers. Thus, we were able to confirm the map position of only one member each for the hemolymph protein families SP1, SP2, and 30K, because of the relatively narrow limits within which we could control hybridization stringency with the ECL nonradioactive detection system, originally chosen for its relative simplicity and ease of use with a large variety of sequences. This presents no problems for anonymous probes, which can be selected on strict empirical criteria, but needs to be considered when mapping known genes. Nevertheless, unique advantages may be realized in working with dispersed multigene families. For example, we found the low copy number retrotransposon, mag, to be particularly useful both for multiplexing (eight bands resolved in *Eco*R I digests and two in *Hin*d III digests) and for marking several linkage groups simultaneously (five PLGs confirmed here, with an additional assignment possible for RI-3P). The efficiency of mapping with this kind of probe in an organism with 28 potential linkage groups outweighs the potential disadvantage that sites may not be conserved in different strains (Garel *et al.* 1994). At least one other low copy multigene sequence is known in the silkworm genome, k1·4 (Ueda *et al.* 1986; Tamura *et al.* 1993). Moreover, a Bkm element with similar properties has been reported in the genome of *Ephestia kuehniella* (Traut, 1987), suggesting that this strategy may also be applied to mapping other lepidopteran genomes. The recent development of conditions for *in situ* hybridization of multicopy sequences using silkworm chromosomes (Okazaki *et al.* 1993; Ogura *et al.* 1994) opens up the possibility of obtaining independent confirmation for some of the findings reported here, such as linkage between the rDNA locus and the sericin 2 gene, which has many internal repeats (Garel *et al.* 1994) on PLG 4 (established linkage group 11), and whether there are additional minor rDNA loci in the silkworm genome.

One extension of the RFLP strategy could be to look for linkage or clustering of new, previously undetected functional units expressed in specific tissues or at specific times in development. Although our data are too preliminary to draw strong conclusions, the high yield of closely linked Rcfs on PLG 9 – seven markers, including three closely spaced loci, Rcf94, Rcf 126/241, and Rcf28, and two sets of markers (Rcf1/42 and Rcf126/241) that were not separated by recombination – hints that this region may represent such a possibility. In addition, it will be important for future comparative genome analysis to map more of the available identified sequences, such as those for tissue- and differentiation-specific proteins and transcription factors, as a foundation for investigating the extent of conservation of chromosome units within the Lepidoptera, whose unusual dispersed centromeres (Friedländer & Wahrman, 1970; Murakami & Imai, 1974; Maeki, 1981) may contribute to distinctive evolutionary behavior compared to organisms with more common unitary centromeres.

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