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Hybridization Diversity of the Chorion Multigene Families of *Bombyx mandarina* with Reference to Several Genetic Stocks of *Bombyx mori*

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Genomic Southern blotting analyses of *Bombyx mori* and *B. mandarina* were performed with the 5'-exons or 5'-flanking sequences of two members of genes A/B.L11 and A/B.L12. They represent multigene families encoding chorion proteins that are expressed in the middle period of choriogenesis. Many bands cross-hybridized with the probes in *B. mandarina* and in different strains and races of *B. mori*, supporting the idea that the middle chorion multigene families have remained essentially the same in the two species. Both the exon and the 5'-flanking probes exhibited similar patterns within the European stocks of *B. mori*, indicating that the examined DNA regions have been conserved. Patterns revealed in a Japanese and a Chinese strain showed distinct differences in band number and fragment size, indicating that the chorion gene families are not fixed within the domesticated silkworm. A greater number of fragments hybridized with the exon probes in *B. mandarina* than in *B. mori*; this may have relevance to the previous finding that *B. mandarina* has a large number of proteins with high cysteine content. The banding patterns for both the exon and 5'-flanking probes were markedly different between *B. mori* and *B. mandarina*, indicating that major evolutionary differences exist between the two species.

Key words: *Bombyx mandarina, Bombyx mori*, genomic Southern blots, *Gr* mutations, chorion multigene families

INTRODUCTION

The insect eggshell or chorion has a highly ordered structure (Mazur *et al.*, 1989; Gautreau *et al.*, 1993) made of large numbers of proteins (Nadel and Kafatos, 1980; Bock *et al.*, 1982). In the domesticated silkworm, *Bombyx mori*, several allelic or pseudo-allelic chorion mutants known as grey or opaque eggs (Sado and Chikushi, 1958) are controlled by the *Gr* complex loci, mapped to position 6.9 centimorgans of the second chromosome (Tazima, 1964; cf. Fujii *et al.*, 1998). Variation in chorion architecture has been observed by scanning and transmission electron microscopy, revealing that the *Gr* mutations most extensively affect the middle layer among the tripartite structure of the chorion (Sakaguchi *et al.*, 1973). The mutation named Gr^B, which lacks large regions of chorion, has been analyzed at molecular level and corresponds to a deletion encompassing many chorion structural genes (Iatrou *et al.*, 1980; Nadel *et al.*, 1980; Durnin-Goodman and Iatrou, 1989).

Genetic studies through the systematic analyses of chorion proteins using the wild type strain C108 indicate that the chorion locus is composed of two major clusters named *Ch 1-2* and *Ch 3* (Goldsmith and Basehoar, 1978; Goldsmith and Clermont-Rattner, 1979, 1980). The 2 clusters are separated from each other by a map distance of about 4.0 centimorgans, which may correspond to more than 2,400 kb of the *B. mori* genome (Eickbush and Izzo, 1995). Also a chromosomal walk (Eickbush and Kafatos, 1982), together with extensive analyses of nucleotide sequences of contiguous segments (Goldsmith and Kafatos, 1984; Lecanidou *et al.*, 1983, 1986; Burke and Eickbush, 1986; Spoerel *et al.*, 1986, 1989; Tsitilou and Kafatos, 1989), primarily using the wild type strain 703 (see below), has revealed that the chorion region of the chromosome encompasses about 200 structural genes comprising several large multigene families (reviews: Regier and Kafatos, 1985; Eickbush and Izzo, 1995). On the basis of their periods of expression during choriogenesis as well as sequence similarity, the chorion gene families have been classified into the early (ErA and ErB or CA and CB), middle (A and B) and late (HcA and HcB; Hc comes from the high cysteine content of proteins) members. Two-thirds of the middle genes and all of the late genes appear to reside in the *Ch 1-2* cluster, whereas one-third of the middle genes and all of the early genes seem to be contained in the *Ch 3* cluster (review: Eickbush and Izzo, 1995).

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The sequence analyses cited above have also indicated that the chorion genes are organized in coordinately transcribed pairs separated by a short promoter region of less than 300 nucleotides (Spoerel *et al.*, 1986, 1989). The early genes comprise at least 10 ErA/ErB pairs, the middle genes comprise at least 14 A/B pairs (numbered L1 to L14, where the letter L indicates left relative to the late genes) and the late genes comprise at least 15 HcA/HcB pairs. The middle genes include additional A/B pairs numbered R1 to R3 (right relative to the late genes). These complicated arrangements of the chorion genes are proposed to have occurred as a result of sequence transfers due to events like tandem duplication and gene conversion (Xiong *et al.*, 1988; Spoerel *et al.*, 1989; Regier *et al.*, 1994; Kravariti *et al.*, 1995).

Bombyx mandarina is a wild silkworm closely related to *B. mori* and indistinguishable from the latter in many aspects including the ease of interbreeding (Yoshitake, 1988; Kawarabata, 1998). Nevertheless, the chorion architecture of *B. mandarina* was found to be unique compared to that of *B. mori*. Scanning electron microscopy of vertical sections revealed an outermost layer of chorion composed of prominent lamellate peaks covered by a thin surface coating enclosing a large air space (Sakaguchi *et al.*, 1990; Kawaguchi *et al.*, 1996). Such features have not been observed in the domesticated silkworm and are considered to be an adaptation to the outdoor habitat. Our previous results using two-dimensional gel electrophoresis (Sakaguchi *et al.*, 1998) indicated that the distinctive outer chorion of *B. mandarina* is rich in high cysteine proteins, which would contribute to its strength and rigidity via disulfide bridges.

Despite the dramatic differences in the external morphology between the two species, the bulk of the chorion encompassing the middle region appears similar at the ultrastructural level (Sakaguchi *et al.*, 1990; Kawaguchi *et al.*, 1996). This portion of the chorion is largely encoded by the middle A and B genes, which are responsible for lamellar expansion, an indispensable process of choriogenesis in silkmoths (Regier and Kafatos, 1985; Mazur *et al.*, 1989; Gautreau *et al.*, 1993). In the present study, we tried to detect any differences in middle chorion genes in *B. mandarina* in comparison with several stocks of *B. mori* by using Southern blots. Subclones of two representative gene pairs, A/B.L11 and A/B.L12, were used as probes, since these sequences have been well characterized (Spoerel *et al.*, 1986, 1989) and comprise the majority of middle gene families.

MATERIALS AND METHODS

Animals

The *B. mori* stocks N9 (Japanese), OE16 (old European 16, No.556), 706 (European) and 703 (European) were kind gifts of the National Institute of Sericultural and Entomological Science (NISES), Kobuchizawa, Japan. N9 originated from the race Bunrihaku in 1937 at the Kumamoto branch of NISES. OE16 was derived from the Giallo Ascoli strain imported from Italy in 1926 and was established in the Kumamoto branch of NISES in 1932. Strain 706 carries the Gr^B mutation ("birdeye" egg; 2-6.9) and has been kept by the cross Gr^B p/ + p^3 females × Gr^B p/ Gr^B p males to obtain Gr^B/Gr^B *and* Gr^B/ + eggs. The strain 703, a relative of 706, carries Gr^B linked to p^1 as well as unlinked markers *Ge* (1-14.0) and *oc* (5-14.8). The strain d03 is a stock of the Institute of Genetic Resources (IGR), Faculty of Agriculture, Kyushu University Graduate School, which was supplied from a strain established by Takasaki and Aratake in the Kumamoto branch of NISES in 1969, and carries the Gr^B mutation ("birdeye" egg, 2-6.9). The strain 73 was established by extracting the complete Gr^B chromosome derived from d03 and maintaining it as a heterozygote with a wild type second chromosome from Japanese strain p22 which is marked with p^3 (Iatrou *et al.*, 1980), similar to maintenance of strain 706. C108 (Chinese) was the kind gift of Dr. Y. Tazima, National Institute of Genetics, Mishima, and has been maintained in one of our laboratories (M.R.G.) by small batch matings since 1972. It was used as a standard race for practical breeding around 1940 in NISES. The *B. mandarina* strain 010 was the 15th and 16th generations of a population collected and maintained by Dr. O. Ninagi in NISES. Individuals from 2 batches (nos. 1 and 4) were used.

Preparation of uniformly labeled single-stranded M13 probes

Central parts of the 5'-exons of some of the middle A/B genes, or parts of their 5'-flanking regions, were subcloned into the *Sma*I site of M13 mp19 and uniformly labeled with ^{32}P under the conditions previously reported (Spoerel *et al.*, 1986). The following 4 probes were used in the present hybridization experiments. Probe 1: A.L12 5'-exon probe, containing a central part of the 5'-exon of *A.L12*, from + 326 (*Nae*I) to + 468 (*Ban*I), with a size of 143 nt. Probe 2: B.L12 5'-exon probe, containing a central part of the 5'-exon *B.L12*, from + 262 (*Fok*I) to + 480 (*Hinf*I), with a size of 219 nt. Probe 3: A/B.L11 5'-flanking probe, containing a part of the 5'-flanking sequence of the A/B.L11 pair,

from −34 of A.L11 (*Fnu*4HI) to −15 of B.L11 (*Fnu*4HI), with a size of 230 nt. Probe 4: A/B.L12 5'-flanking probe, containing a part of the 5'-flanking sequence of the A/B.L12 pair, from −25 of A.L12 (DNase I digestion) to −6 of B.L12 (*Taq*I), with a size of 247 nt. The sequences of these probes have been published (probes 1 and 2 in Spoerel *et al.*, 1986; probes 3 and 4 in Spoerel *et al.*, 1989).

DNA isolation and Southern blot hybridization

High molecular weight genomic DNA was isolated from the posterior silk glands of *B. mori* and *B. mandarina* on day 3 or 4 of the fifth instar by means of standard procedures (Maniatis *et al.*, 1982). Silk glands from 5-6 individuals of each inbred line were pooled. *B. mandarina* samples were treated individually or en masse as indicated. The DNA was subjected to Southern blot hybridization as detailed previously (Spoerel *et al.*, 1986). In brief, restriction digests, adjusted to 10 μg per lane as far as possible, were fractionated on 0.7% agarose gels, blotted to Biodyne A nylon membranes (Pall Ultrafine Filtration) in 10 × SSC (SSC = 0.15 M NaCl, 0.015 M sodium citrate) and cross-linked with ultraviolet light. The membranes were hybridized at 50°C in 7% sodium dodecyl sulfate, 0.3 M sodium pyrophosphate, pH 7, 0.5 mg/ml single-stranded herring sperm DNA and about 2×10^6 cpm of uniformly labeled single-stranded probe. After hybridization filters were washed for 10 min in 5% sodium dodecyl sulfate at 50°C, then in 0.3 M sodium pyrophosphate at 50°C, and finally in the latter solution at t_m −1°C for the exon probes (1 and 2) or at t_m −30°C for the flanking probes (3 and 4). The t_m values for probes 1, 2, 3 and 4 were previously determined to be 92, 87, 80 and 82°C, respectively (Spoerel *et al.*, 1986).

RESULTS

Principles of the present hybridization experiments

We used DNA from pooled samples of *B. mori* representing 3 geographic groups (European, Japanese and Chinese) and of *B. mandarina* using single individuals from 2 batches of strain 010 as well as pooled samples from the two batches. To confirm our hybridization conditions and facilitate identification of the middle A/B gene pairs we also included DNA samples from Gr^B homozygotes (2 strains), which carry a large deletion of the chorion locus lacking all of the Hc gene pairs and many A/B pairs, therefore presenting a distinctive and simplified chorion pattern (Iatrou *et al.*, 1980; Durnin-Goodman and Iatrou, 1989).

Genomic DNA samples were digested with *Eco*RI and *Hin*dIII, electrophoresed, blotted and hybridized to probes 1 to 4 representing exons or 5'-flanking regions of middle gene families A/B.L11 and A/B.L12. Different membranes prepared under similar conditions were used for each of the respective probes. Because of their similar genetic backgrounds and the use of stringent hybridization conditions, most of the bands for strains 703 (European, wild type), 706 (European, Gr^B heterozygote) and the Gr^B homozygotes could be assigned to members of the chorion gene families by referring to the previously published physical maps and sequence data (Eickbush and Kafatos, 1982; Spoerel *et al.*, 1986, 1989). A1, A2 etc. in the figures presented below denote the ErA/ErB gene pairs, L1, L2 etc. denote the A/B.L gene pairs and R1, R2 etc. denote the A/B.R gene pairs. Band density is a reflection of the length of hybridizing sequence present on a given DNA fragment and the relative degree of homology between the probe and target. It is not possible to distinguish these two conditions from Southern blots alone. Therefore, because of our high hybridization criteria, we used the simplifying assumption that all hybridizing sequences represented members of these gene families, and used bands to estimate relative gene copy numbers.

Genomic Southern blots hybridized to probe 1 containing the A.L12 5'-exon

Fig. 1 illustrates the results using a central part of the 5'-exon of A.L12 as a probe to reveal the majority of L12-related genes. In *B. mori*, the Gr^B reference strains, 73 (Lane 6) and d03 (Lane 9), were similar to each other, giving signals for a small number of bands (10 total; Table 1), including A1-A3, and L10-L12. These results are in agreement with previous analyses of the homozygous mutants which show a deletion extending from L1 to L8, including middle gene pairs R1-R3 (Durnin-Goodman and Iatrou, 1989; Durnin, 1988). The wild type eggs segregated from the European strain 703 (Lane 5) gave the expected bands for L1-L6, L8, L13 and L14, and A1-A3, for a total of 15 hybridizing bands (Table 1). The European strain 706 (Lane 7), with the genotype of $Gr^B/+$, showed the same set of A/B.L genes, as well as the middle genes A/B.R2 and R3, and A1-A3. This strain had a few more bands than strain 703 (20 bands total; Table 1), which could be explained by the presence of polymorphic bands on the Gr^B chromosome (e.g., compare Lane 6 and Lane 7 in regions near A2, A3 and L2) and the higher loading of the 706 sample, making it easier to detect weakly hybridizing bands (cf. region near L1 and between L3 and A2). In OE16 (Lane 8), a European strain normal with respect to the *Gr* locus, the banding pattern was basically similar to those of 703 and 706; bands corre-

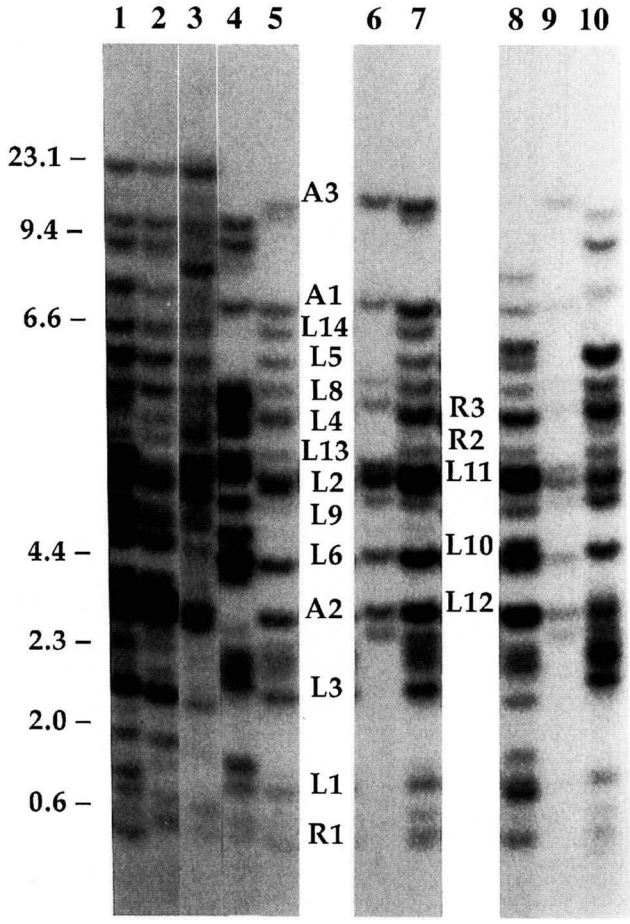

Fig. 1. Genomic Southern blots of DNAs from *B. mori* and *B. mandarina* hybridized with probe 1, a central part of the 5'-exon of the A.L12 gene. DNA was extracted using 5 individuals per stock of *B. mori*. The stock of *B. mori* with the corresponding lane numbers in parentheses are as follows: C108 (4), 703+/+ (5), 73 Gr^B/Gr^B (6), 706 $Gr^B/+$ (7), OE16 (8), d03 Gr^B/Gr^B (9), and N9 (10). The *B. mandarina* strain 010 was analyzed as follows: lanes 1 and 2, DNA from individuals no. 3 and no. 1 of batches nos. 1 and 4 (thus 1-3, 4-1); lane 3, a mixture of DNAs from batches nos. 1 and 4 (5 individuals each). A1, L1, R1 etc. denote the chorion genes ErA1, A/B.L1, A/B.R1 etc., respectively, identified according to previous physical maps and sequencing data (see text).

sponding to those in 703 and 706 were present in the region from about 0.6 to 6.5 kb (e.g., in the size range of bands L1 to L14, A1, A2, R2 and R3), as well as some polymorphisms (e.g., bands L14 and A3 were absent, and relatively stronger bands were present in the region of L1 and L10), for a total of 21 bands (Table 1). Japanese strain N9 (Lane 10) showed a relatively similar pattern to the European strains, with most bands between 2.2 and 6.5 kb and a total number of 18 bands (Table 1). Relative intensities of many bands differed from the European strains, however. For example, the signals in the region of L1, L10, L11, L14, A3 and R3 were relatively weak, whereas bands near L5 and L8 were relatively strong. The normal Chinese strain C108 (Lane 4) gave a markedly different pattern from all of the other strains, with a narrower size range for the mostly strongly hybridizing bands (approximately 4-5 kb), but the same approximate number (20; Table 1). Few if any of the C108 bands comigrated with those of the European and N9 strains.

In *B. mandarina*, the hybridization bands were more widely distributed in size (from 0.5 to 23 kb) than those of *B. mori*. The 2 individuals from different *B. mandarina* batches (nos. 1 and 4) did not coincide completely in banding pattern (Lanes 1 and 2, respectively), in particular in the regions around 5-6 kb and below 2 kb. The bands produced by the mixed DNA samples from both batches (Lane 3) showed relatively high density in the size range from about 2.5 to 23 kb and tended to smear, probably because there were many closely migrating or overlapping bands; this was not seen in DNA isolated from individuals. Total numbers of discernible bands in the individual *B. mandarina* samples (28; Table 1) were significantly higher than those of normal *B. mori* strains.

Genomic Southern blots hybridized to probe 2 containing the B.L12 5'-exon

When a membrane with the same DNA samples as those shown in Fig. 1 was hybridized with a central part of the 5'-exon of B.L12 as a probe (Fig. 2), the rough classification of patterns on the basis of geographical and genetic origins of *B. mori* was virtually reproducible compared to Fig. 1. The European members 703, 706 and OE16, all normal or heterozygous for Gr^B, gave substantially similar patterns, with 703 and 706 most closely matched (Lanes 5, 7 and 8). The estimated band number revealed by this probe was slightly less than for the A.L12 family (13-18 bands; Table 1). In the strains with the Gr^B/Gr^B genotype, a reduced number of bands occurred, as expected (4 bands each; Lanes 6 and 9; Table 1). These corresponded to the B.L10, B.L12 and B.L13 genes plus an additional band greater than 10 kb. Japanese strain N9 again showed several bands that comigrated with those of the European strains, but there were more marked differences in the overall pattern with this probe (Lane 10). These included enhanced banding intensities in the regions near L4 and L2, and fewer bands hybridizing overall (14 bands; Table 1). Chinese strain C108 (Lane 4) also showed fewer bands (14 total; Table 1) with little correspondence to those of the European strains and a narrower size range for the most prominent bands (4-5 kb).

The hybridizations for *B. mandarina* with the B.L12 5'-exon probe gave widely distributed bands from 0.5 to 23 kb, similar to the results with the A.L12 5'-exon

Tabel 1. Numbers of bands observable on the gels shown in Figs. 1 to 4

Species	Strain or race (phenotype)	Lane on gel	Probe			
			A. L12 5'-exon (Fig. 1)	B. L12 5'-exon (Fig. 2)	A/B. L11 5'-flanking (Fig. 3)	A/B. L12 5'-flanking (Fig. 4)
B. mori	C108	4	19 (1)[a]	11 (3)	9	4
	703	5	10 (5)	6 (7)	12	7
	73 (Gr^B/Gr^B)	6	3 (7)	3 (1)	2	4
	706 ($Gr^B/+$)	7	19 (1)	12 (6)	12	8
	OE16	8	15 (6)	8 (8)	13	10
	d03 (Gr^B/Gr^B)	9	3 (7)	3 (1)	2	4
	N9	10	14 (4)	11 (3)	10	5
B. mandarina	1-3[b]	1	26 (2)	17 (3)	7 (5)	11 (1)
	4-2[b]	2	25 (3)	21 (1)	8 (1)	8 (1)
	Mixed[b]	3	20 (*)	15 (*)	12 (*)	10 (5)

[a]Numerals in parentheses indicate approximate numbers of faint bands, which were considered less accurate, sometimes uncountable because of smearing (*).
[b]See the legend to Fig. 1.

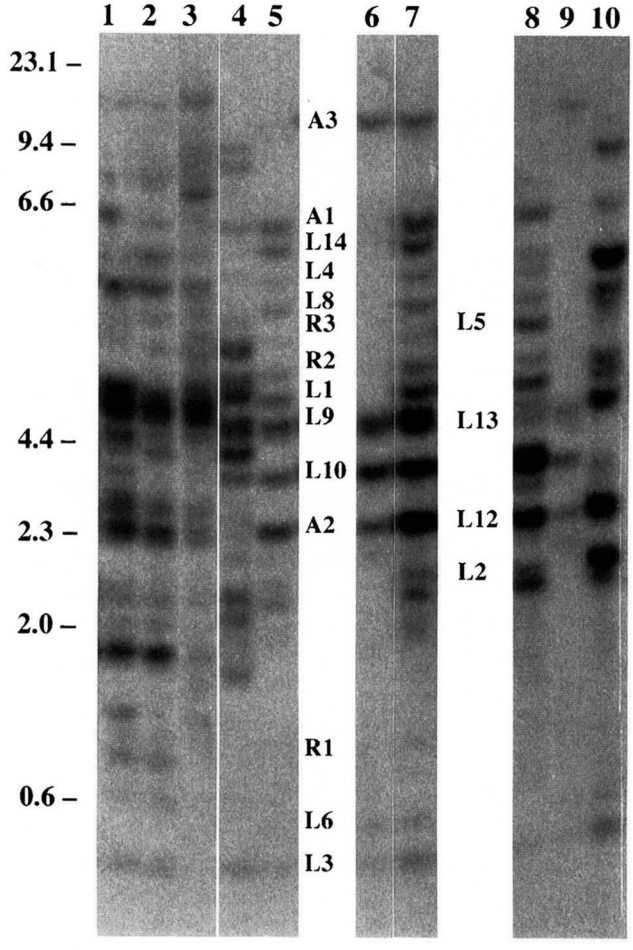

Fig. 2. Genomic Southern blots of DNAs from *B. mori* and *B. mandarina* hybridized with probe 2, a central part of the 5'-exon of the B.L12 gene. For other details, see Fig. 1.

probe, with the densest bands around 1.8 kb, and between 2.3 and 5 kb. Again the 2 individuals from different batches were not completely similar to each other (Lanes 1 and 2), and the mixed DNA samples produced a less discrete banding pattern with evidence for a greater number of hybridizing bands than found in individual samples (Lane 3). For this family *B. mandarina* samples revealed a slightly larger number of hybridizing bands compared to European *B. mori* strains (20-22 bands; Lanes 1 and 2; Table 1).

Genomic Southern blots hybridized to probe 3 containing the A/B.L11 5'-flanking region

Hybridization of a portion of the 5'-flanking region of the A/B.L11 gene pair to the membrane produced the results shown in Fig. 3. The European stocks analyzed here, 703 (Lane 5), 706 (Lane 7) and OE16 (Lane 8), were almost identical to each other, and bands corresponding to the L1, L2, L4, L11, L13, A1-A3, R2 and R3 genes were recognized. In OE16, two additional bands were present while A3 was absent, yielding an estimated count of 13 *vs.* 12 bands (Table 1). The Chinese strain C108 (Lane 4) and Japanese race N9 (Lane 10) had several comigrating bands relative to the European stocks, with total bands numbering 9 and 10, respectively (Table 1). The Gr^B homozygotes showed only 2 bands each, L11 and L13 (Lanes 6 and 9, respectively; Table 1).

In *B. mandarina*, the overall patterns were again different from those of *B. mori* (Fig. 3). Although several bands seemed to comigrate with ones derived from the European strains (e.g., A2, L1, L2, L4, L13 and R3), their intensity differed, often markedly, and there were generally fewer bands than in the typical *B. mori* patterns observed here (12 and 9 bands in batches 1-3 and 4-2, respectively; Table 1). For example, in individual *B. mandarina* samples (Lanes 1 and 2) a band around the size of L2 consisted of a doublet

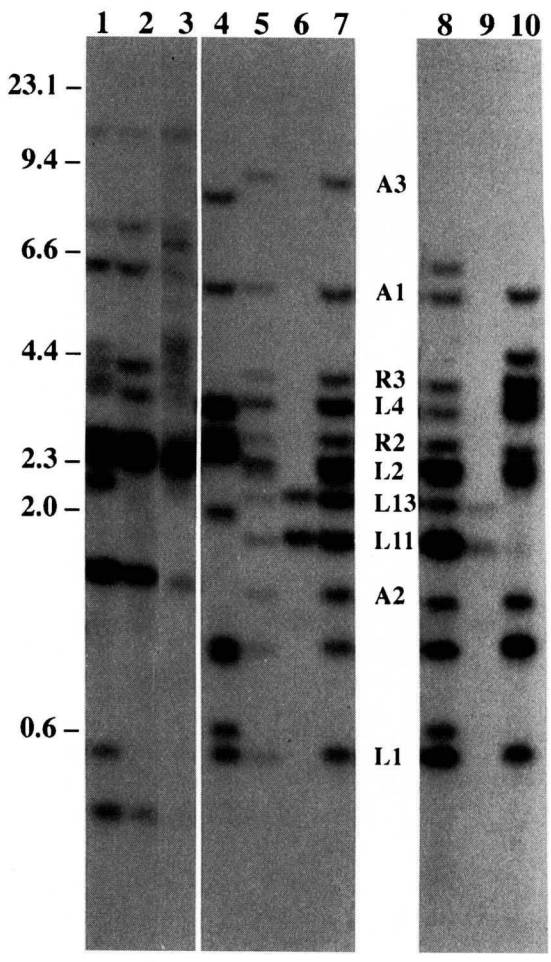

Fig. 3. Genomic Southern blots of DNAs from *B. mori* and *B. mandarina* hybridized with probe 3, a part of the 5'-flanking region of the A/B.L11 gene. For other details, see Fig. 1.

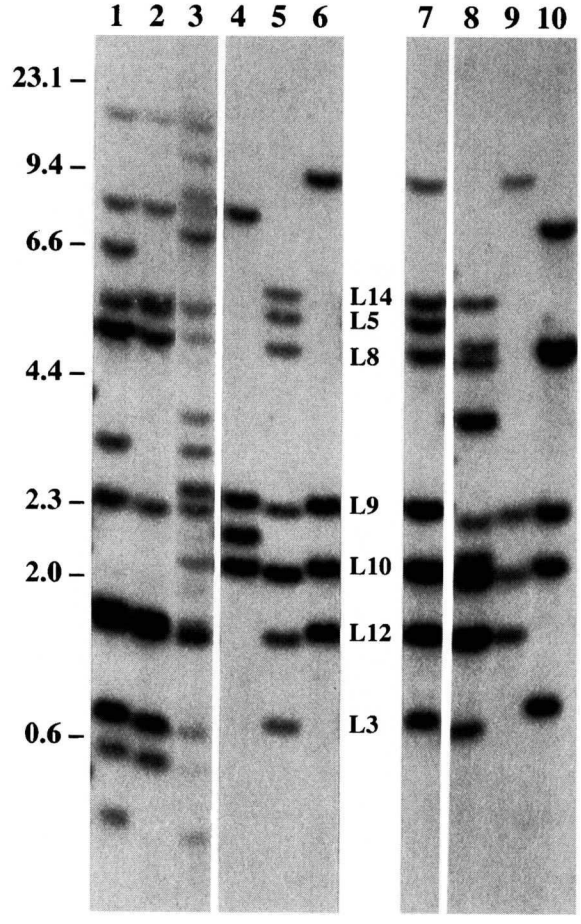

Fig. 4. Genomic Southern blots of DNAs from *B. mori* and *B. mandarina* hybridized with probe 4, a part of the 5'-flanking region of the A/B.L12 gene. For other details, see Fig. 1.

whose intensity appeared to be at least twice that of the L2 band seen in 703, 706 and OE16. Similarly, a *B. mandarina* band migrating near A2 had at least 3 times the intensity of the one derived from *B. mori*, and differed in density by around 2-fold in the individuals from the two batches. These results suggested that the *B. mandarina* chromosomal fragments were probably derived from different regions of the chorion locus than those of *B. mori* and contained variable numbers of L.11-related gene pairs.

Genomic Southern blots hybridized to probe 4 containing the A/B.L12 5'-flanking region

When the 5'-flanking region of the A/B.L12 pair was used as a probe (Fig. 4), the wild type European strain 703 (Lane 5) gave signals for the L3, L5, L8, L9, L10, L12 and L14 genes, which were also found in 706 (Gr^B/ + ; Lane 7). The Gr^B homozygotes (Lanes 6 and 9) yielded discrete bands which included L9, L10 and L12, and an additional band also observed in the 706 heterozygote (Lane 7) which was probably derived from the Gr^B chromosome. Interestingly, the variation in the overall banding patterns among the strains and races was greater with the L.12 flanking probe than with the L.11 flanking probe (cf. Figs. 3 and 4). OE16 (Lane 8) displayed more bands with this probe than the other European strains (10 *vs.* 7 or 8; Table 1); less than half of these (corresponding to L8, L10, L12 and L14) comigrated with the 703 and 706 bands, but the overall density was roughly equivalent. This probe revealed only 5 bands with Japanese strain N9 (Lane 10), of which 3 comigrated with normal European bands (L8, L9 and L10). Chinese strain C108 (Lane 4) had only 4 bands when hybridized with the 5'-flanking region (Table 1), of which 2 (L9 and L10), also apparently present in N9, comigrated with European strains, and 1 additional band comigrated with a band around 8 kb in N9. Based on these patterns, C108 appeared to have the smallest number of gene pairs in this L12 middle A/B family (4), whereas the European strains had the greatest number (7-10).

In *B. mandarina*, the DNA mixture (Lane 3) exhibited not strong but clear bands in this case, with several polymorphisms relative to the samples containing individual DNAs, resulting in a larger number of bands overall. Differences due to the batch of animals were seen in the individual samples (Lanes 1 and 2), consistent with the evidence for polymorphism in the mixture. The total number of bands (9-12; Table 1) and their relative intensities suggested that the size of this family is similar to that found in the European strains, and probably greater than in the Chinese or Japanese strains examined here.

Polymorphism in *B. mandarina* strains

The larger number of bands found in the pooled *B. mandarina* samples probed with the 5'-exon sequences suggested that the batches of animals were polymorphic at the chorion structural gene loci that we examined. As a further test of this possibility, we made a Southern blot containing additional individual DNAs sampled from the two batches and probed them with the A/B.L11 5'-flanking region (Fig. 5). Hybridization patterns revealed that even individuals (4-1 and 4-2) from the same batch (no. 4) were not completely identical (Lanes 5 and 6). However, batch no. 1 showed an invariable pattern from individual to individual (1-1 to 1-4).

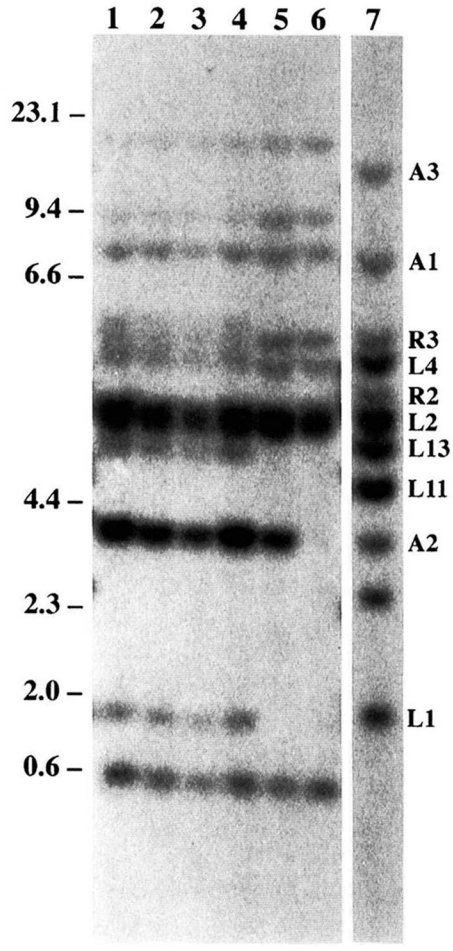

Fig. 5. Genomic Southern blots of DNAs from *B. mandarina* hybridized with probe 3, a part of the 5'-flanking region of the A/B.L11 gene. Lanes 1 to 4, individuals nos. 1 to 4, respectively, from batch no. 1 (1-1, 1-2, 1-3, 1-4). Lanes 5 and 6, individuals nos. 1 and 2, respectively, from batch no. 4 (4-1, 4-2). Lane 7, DNA from strain 706 as a reference (see Fig. 1 for details).

DISCUSSION

The present genomic Southern blotting analyses, using the A.L12 and B.L12 exons or A/B.L11 and A/B.L12 5'-flanking sequences as probes, produced discrete bands in most lanes, and the band numbers could be counted (Table 1). The band numbers that we obtained here seemed to be reasonable, since *B. mori* has 14 pairs of A/B.L genes arranged to the left of the Hc gene clusters and 3 pairs of A/B.R genes to the right of these on the second chromosome (Spoerel *et al.*, 1989); further, many of the bands could be correlated with those of the published reference strains, especially those of the closely related European stocks. Detection of some additional bands was not unexpected despite the stringent hybridization conditions, especially for the exon probes, since there is ample evidence for sequence exchange between chorion genes, creating a mosaic of related sequences within the exon-containing regions which could cross-hybridize with one another (Eickbush and Burke, 1985). Thus, the A.L12 central exon probe revealed a larger number of bands than the B.L12 central exon probe, even though these two genes are considered to be paired and so would be expected to reside on the same restriction fragment, given their close proximity. This suggests that the AL.12 exon probe may have cross-hybridized with some members of other closely related A or B gene families.

By contrast, the short 5'-flanking regions between chorion gene pairs in *B. mori* contain distinctive sequences that correspond to limited temporal classes of chorion genes whose signals can be readily separated from one another by well-chosen hybridization criteria (Spoerel *et al.*, 1986, 1989). Thus, for the 5'-flanking probes the washing step was made at a low stringency of t_m–30°C, but there were fewer bands compared to the results with the exon probes washed at a high stringency of t_m–1°C. We believe that the patterns obtained from the exon probes present a more complete picture of the total number of chorion gene pairs belonging to the middle A/B gene families, whereas the patterns produced by the 5'-flanking probes more accurately reflect the number of gene pairs

belonging to these two temporal subsets of the middle chorion genes.

We found that the different geographical strains or races of *B. mori* with normal or heterozygous Gr^B genotype, i.e., European (703, 706 or OE 16), Japanese (N9) and Chinese (C108) stocks, did not give a common pattern, although some bands seemed to comigrate. Some variation was consistently seen even within the 3 European stocks, which gave closely similar patterns. In general, the European and Japanese strains had a greater number of bands than the Chinese one, suggesting that the former may carry more chorion structural genes. These results are comparable to published chorion protein patterns for several *B. mori* strains, which show many comigrating A and B proteins on two-dimensional SDS gels, but also differences, notably a larger number of middle proteins expressed in 703 and other European strains compared to C108 (Goldsmith and Clermont-Rattner, 1980; Goldsmith, 1989). A smaller number of chorion structural genes may also be reflected in the smaller number of hybridizing bands seen for the 5'-flanking probes in C108 compared to the other strains examined here.

Variation in restriction fragment size among strains of different geographic origin is not surprising, given the variation in *Eco*R1 sites in the middle-late region of the chorion locus in strain 703 *vs.* C108 (T. Eickbush, personal communication); this is attributed largely to differences in the location of mobile elements which are widely dispersed in the silkworm genome (Eickbush, 1995). Thus, the *B. mori* specimens, in particular Chinese and Japanese ones, exhibited many bands whose identification must be considered provisional without additional restriction mapping or sequence data.

The strains with the Gr^B/Gr^B genotype (73 and d03) resulted in only a few hybridizing bands in all of the blots. The bands identified were as expected, since the chromosome containing the Gr^B deletion lacks 225 kb of the 270 kb *Ch 1-2* segment, including the majority of A/B pairs and all of the HcA/HcB pairs (Iatrou *et al.*, 1980; Durnin, 1988; Durnin-Goodman and Iatrou, 1989). Detection of all predicted chorion bands in the mutants provided an internal check on hybridization conditions used for the 4 probes we tested.

The 5'-flanking probes (probes 3 and 4) produced patterns that resembled one another from specimen to specimen within the European stocks, indicating that this region, with important roles for gene expression, was well conserved. The banding patterns between the L.12 and L.11 flanking probes were different from one another, indicating that the promoter regions are divergent in sequence according to the gene pair. Again, agreement with previous results provided a useful control for digestion and hybridization conditions (cf. Spoerel *et al.*, 1989).

The high degree of cross-hybridization observed between the *B. mori* probes and *B. mandarina* exon and 5'-flanking sequences confirmed that many genes have common sequences in the domesticated and wild silkmoth. Based on the number and size distribution of hybridizing bands and their relative intensities, we found that the total number of chorion-containing fragments, including faint ones, was greater in *B. mandarina* compared to the normal strains of *B. mori* (Table 1). This observation was reminiscent of our previous chorion protein analysis (Sakaguchi *et al.*, 1998), wherein high cysteine proteins were more abundant in the wild silkworm than in the domesticated one, although no major difference was seen for other families of proteins. A more complete survey will determine whether the particular strains used represent the full range of chorion polymorphism in terms of restriction sites and gene copy number present in the two species.

The present observations probably in part mirror higher levels of polymorphism in restriction enzyme sites for the A/B families in the *B. mandarina* strain used here, which has not been as extensively inbred after collection from the wild (15-16 generations) as the long-established *B. mori* geographic strains. Consistent with this idea is the greater number of bands found for all probes in the sample containing a mixture of DNAs from individuals of different batches. Indeed, the banding patterns slightly differed in the 2 batches examined (nos. 4 and 1), and a Southern blot containing additional individual DNAs sampled from the two batches and probed with the A/B.L11 5'-flanking region (Fig. 5) revealed that even individuals (4-1 and 4-2) from the same batch (no. 4) were not completely identical (Lanes 2 and 3). However, batch no. 1 showed an invariable pattern from individual to individual (1-1 to 1-4, Lanes 4 to 7, respectively), indicating that it is a promising source for an established line.

We cannot rule out the possibility that some of the putative A/B bands were detected as a result of cross-hybridization to HcA/HcB gene family members, especially with the A and B exon-containing probes. The plausibility of this explanation is reinforced by the proposed model for evolution of the HcA and HcB families by sequence transfers from the A and B families, respectively (Iatrou *et al.*, 1984). Direct genomic hybridization analysis of *B. mandarina* using the HcA/HcB exon and 5'-flanking sequences as probes will be useful to address this question.

As a whole, the present Southern blotting experi-

ments with A/B.L11 and 12 gene sequences as probes revealed a distinctness of *B. mandarina* in comparison with *B. mori* strains. Nevertheless, it appears that the middle A/B chorion gene families did not undergo extensive changes during domestication, consistent with observations that the ultrastructure of the middle chorion region is similar in the two species (Kawaguchi *et al.*, 1996; Sakaguchi *et al.*, 1990, 1998). The animals analyzed here may serve as convenient tools for further analysis of the molecular changes that have occurred in the chorion and the complex and highly integrated genetic system that encodes it, and may help illuminate some of the differing selection pressures imposed during domestication of the mulberry silkmoth. Studies along these lines are in progress in our laboratories.

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