Chromosoma (Berl) (1988) 96:263–269

CHROMOSOMA

Nucleolus organizers in the wild silkworm *Bombyx mandarina* and the domesticated silkworm *B. mori*

Hideaki Maekawa [1], Naoko Takada [1], Kenichi Mikitani [2], Teru Ogura [1,*], Naoko Miyajima [1], Haruhiko Fujiwara [1], Masahiko Kobayashi [2], and Osamu Ninaki [3]

[1] Department of Technology, National Institute of Health, Tokyo 141, Japan
[2] Department of Sericulture, Faculty of Agriculture, University of Tokyo, Tokyo 113, Japan
[3] Department of Silkworm Breeding, The Sericultural Experiment Station, Ibaraki 305, Japan

Abstract. Two types (R^{a1} and R^{a2}) of nucleolus organizers were identified in the genome of *Bombyx mandarina* (Japan) which occurs in Japan. Genetical analysis of a hybrid with *B. mori* suggested that the loci of both nucleolus organizers are allelic and correspond to the R^{o} locus of *B. mori*. These nucleolus organizers segregated and were inherited by the progeny in a Mendelian fashion. The majority of the R^{a1} rDNA units were 10.6 kb in length and had an additional EcoRI site in the transcribed spacer region when compared with the same size unit of R^{o}. On the other hand, the KpnI site present in the non-transcribed spacer region of the R^{o} rDNA was not detected in the R^{a1} unit. The 15.1 kb unit observed in the R^{a2} locus was the same as the unit with the type II intron of R^{o}. The four major components of R^{a2} rDNA, with lengths of 10.6, 15.1, 15.7 and 20.8 kb, were also found in the R^{o} locus, and thus the R^{a2} and the R^{o} loci were considered to be closely related. Usually the functional unit of rDNA in the nucleolus organizers of homologous or non-homologous chromosomes cannot be easily distinguished. However, in the case of *B. mandarina* (Japan), distinct functional 10.6 kb units were observed in the allelic R^{a1} and R^{a2} loci. Therefore the existence of the two distinct units suggest the possibility of introducing the chromosomes of the interspecies in the genus *Bombyx*.

Introduction

rDNA is one of the multigene families which has been investigated from an evolutionary and genetic standpoint (for a review see Bush and Rothblum 1982). Since rDNA is organized in a tandem array in the genome with units usually numbering several hundreds, and although some fractions exist singly, rDNA has been presented as an example in studies on "concerted evolution" in some species (Federoff 1979; Petes 1980; Szostak and Wu 1980; Arnheim et al. 1980; Krystal et al. 1981; Dover 1982; Gerbi et al. 1982; Ranzani et al. 1984). Studies on rDNA have been carried out primarily in *Drosophila* species leading to a large amount of genetic information (Coen et al. 1982a, b; Coen and Dover 1983).

Bombyx mori which probably originated in China from *B. mandarina* (China) [we tentatively designate *B. mandarina* living in China as *B. mandarina* (China) to distinguish it from *B. mandarina* living in Japan] by breeding about 4600 years ago, was introduced into Japan about 2000 years ago for sericulture (Yoshitake 1968). The chromosome number of *B. mori* is $n=28$ (Toyama 1894; Kawaguchi 1928), the same as that of *B. mandarina* (China) (N. Yoshitake, personal communication) whereas that of *B. mandarina* (Japan) is $n=27$ (Kawaguchi 1928). The difference is probably due to fusion of two corresponding chromosomes contained in *B. mori* (Murakami and Imai 1974). The wild silkworm carrying 27 chromosomes is also found in Korea (N. Yoshitake, personal communication). It has been speculated that *B. mandarina* (Japan) segregated from *B. mandarina* (China) long before the islands of Japan were completely separated from the Asian continent about 20000 years ago (Minato 1966). Mating between both species, *B. mori* and *B. mandarina*, is possible and their progeny are fertile (Sasaki 1898; Kawaguchi 1928; Ohmura 1950). The structure of the fibroin (Kusuda et al. 1986) and chorion genes (B. Sakaguchi, personal communication) of *B. mandarina* (Japan) is also different from that of *B. mori* (Tsujimoto and Suzuki 1979; Eickbush and Kafatos 1982). Thus a comparison of genes between the closely related species *B. mori* and *B. mandarina* (Yoshitake 1966) is important in accounting for evolutionary events.

We initiated studies to examine the classes of rDNA genes in the *B. mandarina* genome. The rDNA of *B. mori* which consists of 240 repeating units (Gage 1974) corresponds to one nucleolus organizer (NO) per haploid genome (Toyama 1894). Three length classes, 10.6, 15.1 and 15.7 kb units in EcoRI digests, have been identified in *B. mori* rDNA (Fujiwara et al. 1984). An additional rDNA class with a size of 20.8 kb which contains the tandem duplicated type I intron has been found and these distinct units occur in a random combination in the rDNA cluster (T. Ogura et al., in preparation) and form the R^{o} locus. In the present study we will describe another type of rDNA repeating unit in the *B. mandarina* (Japan) genome that forms the R^{a1} locus. Furthermore, in *B. mandarina* (Japan) we observed that both R^{a1} and the *B. mori* type (R^{a2}) are present in an allelic form in the genome. The origin of *B. mandarina* (Japan) and of the NOs is discussed.

Materials and methods

The standard P22 strain of *B. mori* was a gift from Dr. H. Doira, Kyushu University. The wild silkworm, *B. man-*

* *Present address:* Department of Molecular Genetics, Institute for Medical Genetics, Kumamoto University Medical School, Kumamoto 862, Japan

Offprint requests to: H. Maekawa

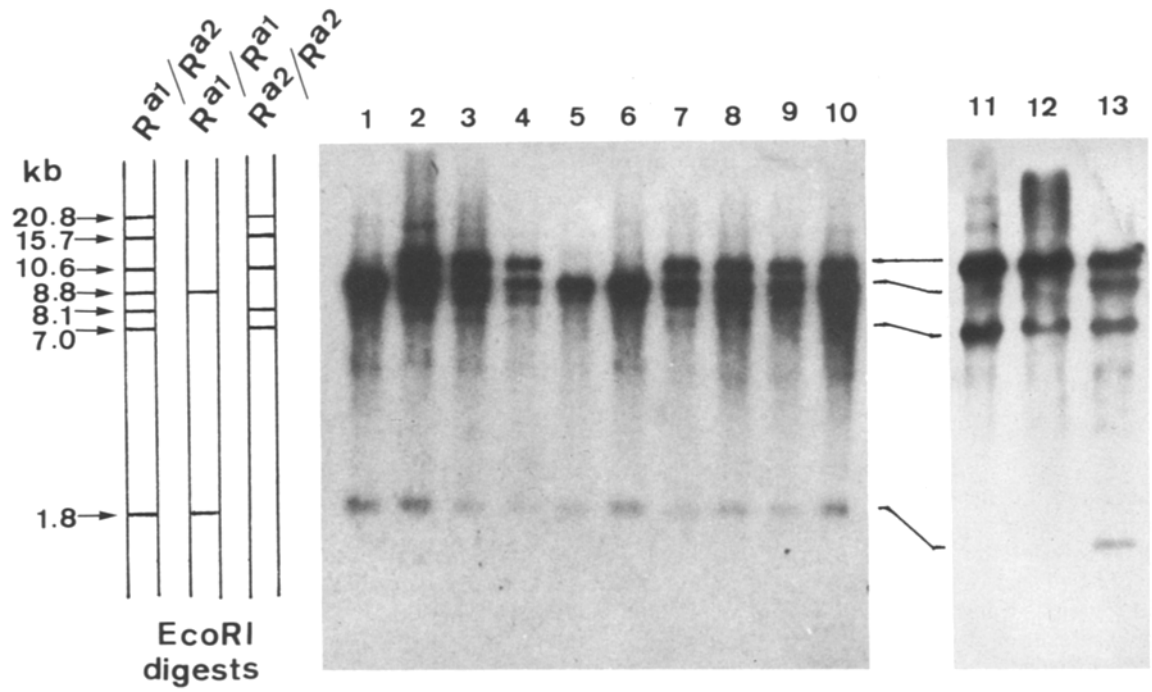


Fig. 1. EcoRi digests of *Bombyx mandarina* (Japan) DNAs hybridized with an rDNA probe. DNA extracted from the posterior silk gland (p.s.g.) of an individual larva was digested with EcoRI, electrophoresed in a 0.7% agarose gel, transferred to a nitrocellulose filter, and the autoradiogram was obtained after hybridization of DNA with an rDNA probe (pBmR145 and 161). Schematic autoradiogram patterns are shown on the *left-hand side*. A homotype (R^{a1}/R^{a1}) is indicated by two bands (8.8 and 1.8 kb); *lanes 1, 5* and *6*). Another homotype (R^{a2}/R^{a2}) is shown in *lanes 11* and *12* although the 8.1 kb fragment is not detectable because of the choice of a probe (pBmR145) carrying the 5′ side of the rDNA unit (see Fig. 4c). The heterotype is also indicated in *lanes 2–4, 7–10* and *13*. In *lane 13*, the pBmR145 probe was used

darina (Japan) was collected in fields at Ibaraki and reared in the Sericultural Experiment Station for three generations after selection of one mating pair among the progeny of the first population. DNA of each individual was extracted from the posterior silk gland (p.s.g.) of the fifth instar larva (Suzuki et al. 1972; Takada et al. 1985) and digested with EcoRI. The digests were electrophoresed on a 0.7% agarose gel, blotted onto a nitrocellulose filter (Southern 1975), BA85 (Schleicher and Schuell, New Hampshire) and hybridized with nick-translated rDNA probes (λBmR11, pBmR145 and pBmR161, derived from clones isolated from *B. mori*) at 65° C for 16 h. The hybridization buffer contained 10× Denhardt's solution (Denhardt 1966), 0.6 M NaCl, 120 mM Tris-HCl, pH 8.0, 8 mM EDTA and 0.2% SDS. The hybridized filter was washed with 3×SSC (1×SSC contains 0.15 M NaCl, 0.015 M sodium citrate), 2×SSC, 1×SSC and 0.1×SSC at 65° C and autoradiographed using Kodak XAR films.

Upon mating between *B. mori* P22(R^o/R^o) and *B. mandarina* (R^{a1}/R^{a2}), the genotype of the F_1 progeny was determined as described below. One of a pair of p.s.g. was dissected out from a larva immobilized on ice. The stage that immediately precedes spinning is suitable for this operation because the larva contains a small amount of hemolymph and can be easily operated on with less damage. The operated larva was kept on ice for 1–2 h and then at 25° C without feeding on mulberry leaves. After spinning, the larva molted and metamorphosed to an adult moth via a pupa. The dissected out p.s.g. was homogenized, DNA was extracted, and the DNA was assayed with rDNA probes as described above. Thus the genotype of 10 adult moths grown from 18 larvae could be determined individually. A male and female were selected, mated and used for the F_2 assay.

Blot mapping of the genomic rDNA unit was performed according to the procedure described above. R^{a1}/R^{a1} DNA was digested with several restriction enzymes (Takara Shuzo Co. Ltd., Kyoto) and hybridized with an rDNA probe (pBmR145 and 161) after the genotype of the larval DNA had been determined. The restriction digests of R^{a1}/R^{a2} DNA were analyzed with a probe specific to the type II intron (pBmR315) of the rDNA.

Results

Alleles at the NO locus

rDNA units in the EcoRI digests of *B. mandarina* (Japan) genomic DNA were analyzed by blot hybridization with an rDNA probe (pBmR145 and 161; Fig. 4c). Autoradiographic patterns of the rDNAs of each individual are shown in Figure 1. These patterns were classified into three groups: R^{a1} which has 8.8 and 1.8 kb fragments, R^{a2} which has 20.8, 15.7, 10.6, 8.1 and 7.0 kb fragments, and R^{a1}/R^{a2} which has a mixture of both the R^{a1} and R^{a2} fragments. The 20.8 and 15.7, and 10.6 kb fragments correspond to the *B. mori* unit with and without the type I intron, respectively. The probe pBmR145 which carries the 5′ side of the 10.6 kb rDNA unit of *B. mori* DNA, hybridized to the 1.8, 7.0 and 8.8 kb fragments, while pBmR161 which

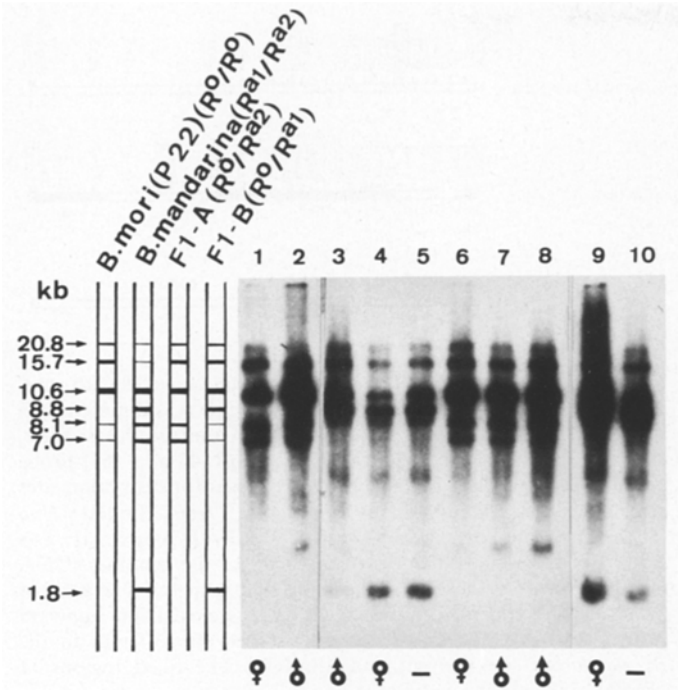


Fig. 2. Determination of the genotype of the F_1 progeny of P22 × *Bombyx mandarina* (Japan). DNA of mature larva from the F_1 progeny of $R^o/R^o \times R^{a1}/R^{a2}$ was digested with EcoRI, blotted, and hybridized to an rDNA probe (pBmR145 and 161). The rDNA restriction pattern of the parental DNA [P22 and *B. mandarina* (Japan)] and that predicted in the F_1 progeny, are schematically illustrated on the *left-hand side* of the figure. The two genotypes in the F_1 progeny were R^o/R^{a1} (*lanes 3–5, 9* and *10*) and R^o/R^{a2} (*lanes 1, 2,* and *6–8*). The sex of the individuals is indicated under each lane

carries the 3′ side of the unit hybridized to the 8.1 and 8.8 kb fragments (data not shown). Therefore, the 1.8 and 8.8 kb, and 7.0 and 8.1 kb fragments seem to be tandem arrays and correspond to the *B. mori* unit without and with the type II intron, respectively, as described below. The 20.8, 15.7 and 10.6 kb units were always associated with the presence of the 15.1 kb unit, which consists of a pair of 7.0 and 8.1 kb fragments, but not with the 10.6 kb unit, which consists of a pair of 1.8 and 8.8 kb fragments. Thus we designated the two large classes of 20.8, 15.7, 15.1 and 10.6 kb fragments, and the 10.6 kb fragment with the EcoRI site, which form the rDNA repeating units of *B. mandarina*, as the R^{a1} and R^{a2} loci, respectively, these represent the NOs.

Genotype determination in hybrids between B. mandarina (R^{a1}/R^{a2}) and B. mori (R^o/R^o)

To determine whether the R^{a1} and R^{a2} loci are allelic, we crossed *B. mandarina* with *B. mori* and analyzed the genomic rDNA patterns of their progeny (F_1 and F_2). The P22 strain of *B. mori* with the R^o locus was mated with *B. mandarina* having the R^{a1}/R^{a2} genotype. The DNAs of the F_2 progeny were digested with EcoRI, and after blot hybridization the autoradiogram was examined.

As shown in Figure 2, five out of ten larvae were found to contain the 20.8, 15.7, 10.6, 8.8 and 1.8 kb fragments which corresponded to the pattern of R^o plus R^{a1}. In the other five larvae, the 15.7, 10.6, 8.1 and 7.0 kb fragments were detected as predicted for the R^o plus R^{a2} pattern. As the 20.8, 15.7 and 10.6 kb fragments are common to R^o and R^{a2}, the content of these fragments in the R^o/R^{a2} class seems to be more abundant than in the R^o/R^{a1} class. The segregation ratio of R^o/R^{a1} and R^o/R^{a2} among the F_1 proge-

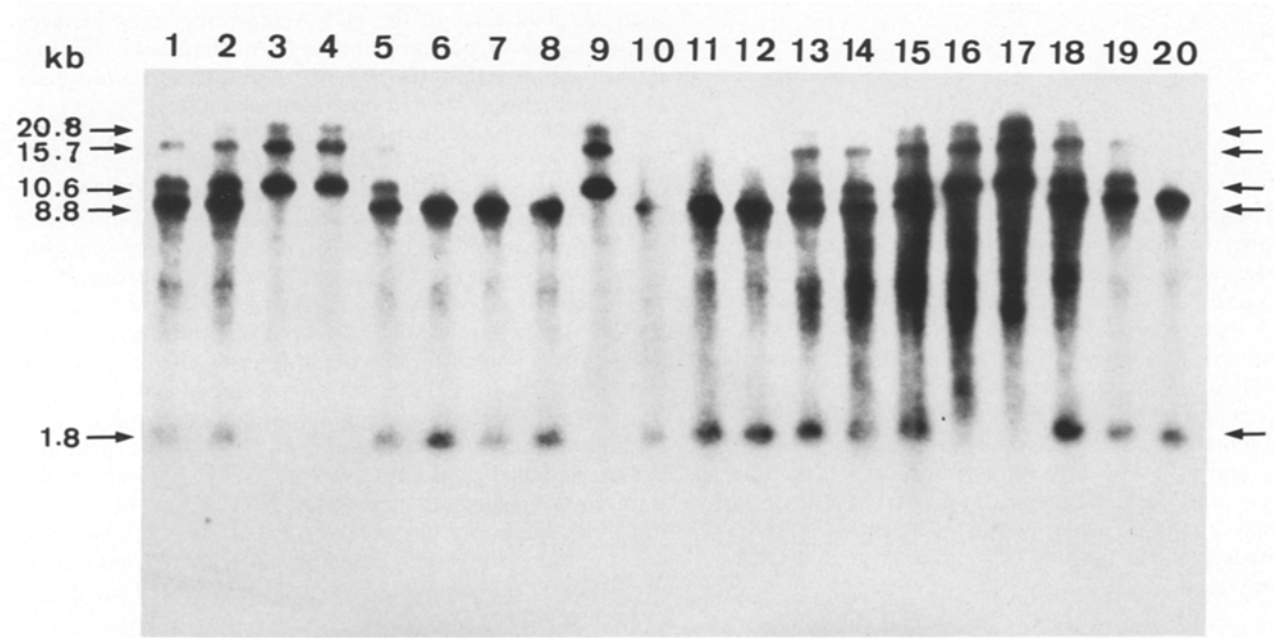
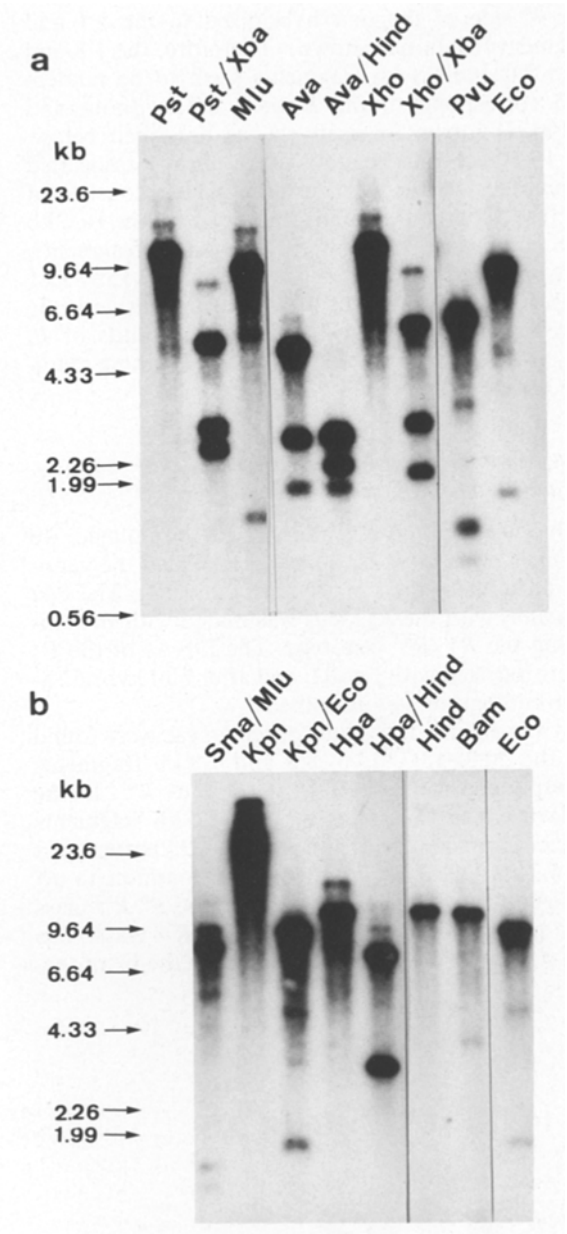


Fig. 3. Determination of the genotype of the F_2 progeny of $R^o/R^{a1} \times R^o/R^{a1}$. The parents whose genotype is R^o/R^{a1}, are shown in *lanes 3* and *4* of Figure 2. Individual DNAs from the F_2 progeny were assayed by a similar procedure to that described in Figures 1 and 2 except for the use of the λ BmR11 rDNA probe. Homotype R^o/R^o is indicated in *lanes 3, 4, 9, 16* and *17*, heterotype R^o/R^{a1} in *lanes 1, 2, 5, 13–15, 18* and *19*, and the other homotype R^{a1}/R^{a1} in *lanes 6–8, 10–12* and *20*



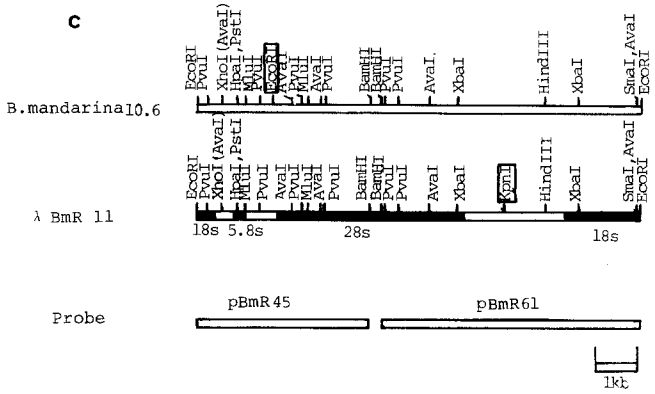


Fig. 4a–c. Blot mapping of the *Bombyx mandarina* 10.6 kb unit. The DNA of larvae with the R^{a1}/R^{a1} genotype was digested with several restriction enzymes. After blotting to a nitrocellulose filter, the filter was hybridized to an rDNA (pBmR145 and 161) probe and autoradiographed (**a** and **b**). *Abbreviations* for restriction sites are: *Ava* AvaI; *Bam* BamHI; *Eco* EcoRI; *Hind* HindIII; *Hpa* HpaI; *Kpn* KpnI; *Mlu* MluI; *Pst* PstI; *Pvu* PvuI; *Sma* SmaI; *Xba* XbaI and *Xho* XhoI. The restriction map of the genomic rDNA unit is shown for the *B. mandarina* 10.6 kb unit (**c**). The map of λBmR11 with the 10.6 kb unit in *B. mori* is cited from Fujiwara et al. (1984) (**c**). The *boxed* restriction sites are different in the *B. mandarina* 10.6 kb unit and λBmR11. The filled regions of λBmR11 indicate the coding regions

ny was roughly estimated as 1:1. The sex of the larvae when grown to moths was discriminated by observation of the external sexual organs and is indicated under each lane in Figure 2. Based on these data it was concluded that the rDNA locus is not linked to the sex chromosomes.

To estimate the segregation ratio of the F_2 progeny, R^o/R^{a1} and R^o/R^{a2} males were mated with R^o/R^{a1} and R^o/R^{a2} females respectively. For a cross between an R^o/R^{a2} male and female, the pairs of moths indicated in lanes 1(♀) and 2(♂), and 6(♀) and 7(♂) were mated with each other, respectively, and the progeny of the pair from lanes 1 and 2 were used for the F_2 assay. Moths of lanes 3(♂) and 4(♀) whose genotypes are both R^o/R^{a1}, were also mated and the progeny used.

Genotype determination in F_2 hybrids ($R^o/R^{a1} \times R^o/R^{a1}$)

The p.s.gs. of F_2 larvae were dissected and the genomic DNAs extracted. The DNA of each larva was digested with EcoRI, blotted, and hybridized with an rDNA probe (Fig. 3). A combination of 15.7 and 10.6 kb fragments was predicted for the R^o/R^o genotype (Fig. 2), 15.7, 10.6, 8.8 and 1.8 kb fragments for the R^o/R^{a1} genotype (Fig. 2), and 8.8 and 1.8 kb fragments for the R^{a1}/R^{a1} genotype (Fig. 1), respectively.

The segregation ratio of the R^o/R^o, R^o/R^{a1} and R^{a1}/R^{a1} genotypes was 5:8:7, respectively (Fig. 3). This pattern of segregation was consistent with that predicted. No combinations of fragments except for the patterns predicted for the R^o/R^o, R^{a1}/R^{a1} and R^o/R^{a1} genotypes were observed in the F_2 progeny examined. Therefore, it is considered that the gene class of the rDNA repeating units behaves like one gene locus according to Mendelian laws. That is, it is concluded that the R^o, R^{a1} and R^{a2} loci are allelic to each other and located on an autosome (see Fig. 2).

A clear separation pattern from the assay of the F_2 progeny in the crosses between R^o/R^{a2} and R^o/R^{a2} could not be obtained, as R^o and R^{a2} contained an rDNA unit with the same fragments. Although we observed a difference in the quantity of each rDNA unit, it was not possible to determine whether the pattern was derived from R^o or R^{a2} (data not shown).

We selected an organ (p.s.g.) but not the whole animal as the DNA source for the determination of the genotype as the dissection of the gland and extraction of the DNA can be easily performed. The amplification of the *B. mori* genome, the uniform replication of all its genes including rDNA, reached a ploidy level of 4×10^5 per cell in the p.s.g. by the last instar stage (Gage 1974). Thus the genome set of the p.s.g. should be the same as that of the germ line and infact the results based on the genotype determination revealed that the NO of the p.s.g. is inherited according to Mendelian laws.

Restriction map of the rDNA unit specific to B. mandarina

A restriction map of the genomic rDNA unit was obtained by blot hybridization of the rDNA probes pBmR145 and

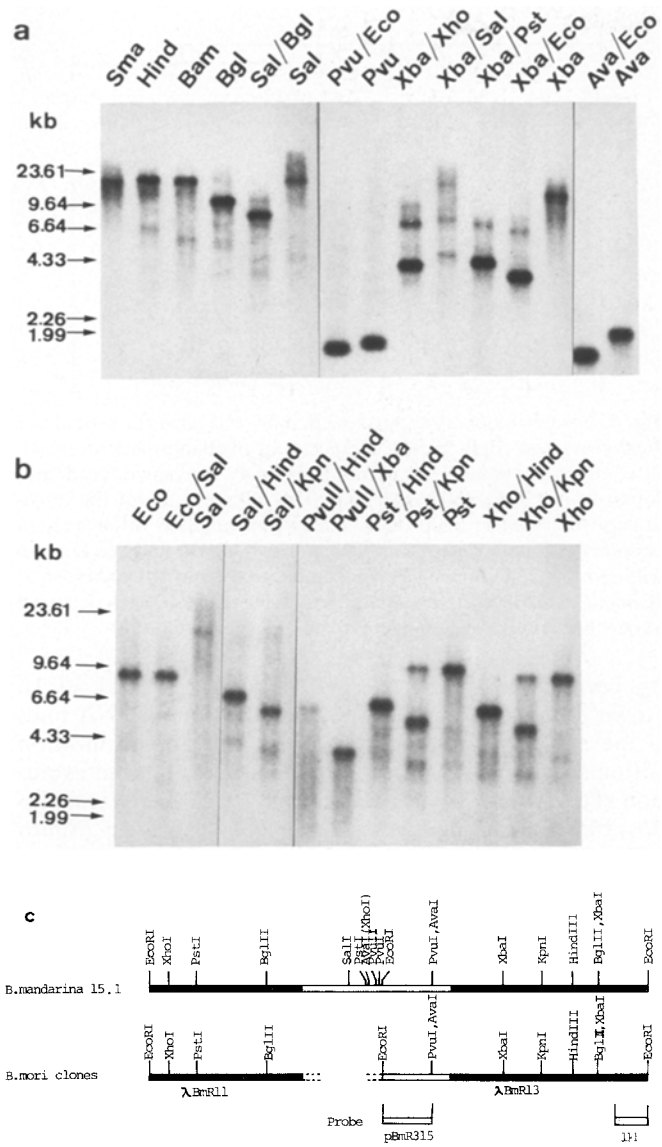


Fig. 5a–c. Blot mapping of the *Bombyx mandarina* 15.1 kb unit. DNA extracted from posterior silk glands (p.s.g.) of larvae with the R^{a1}/R^{a2} genotype was digested with several restriction enzymes and blotted as shown in Figure 4. Hybridization to the type II intron probe (pBmR315) shown in (**c**) was performed. *Abbreviations* for the restriction sites are the same as those in Figure 4a, b except for *Bgl* BglII; *Sal* SalI. The restriction map determined is illustrated in (**c**) and that of the corresponding rDNA of *B. mori* is also indicated under the *B. mandarina* 15.1 kb unit

161 to the genomic DNA of the homozygous larva (R^{a1}/R^{a1}). The restriction map of the R^{a1} major unit was constructed as illustrated in Figure 4c. Most of the restriction sites were consistent with those of λBmR11, a clone of a *B. mori* rDNA unit without an intron. However, an additional EcoRI site was found in the 10.6 kb unit of R^{a1} whereas the KpnI site observed in λBmR11 was not detected. Therefore two differences in the cleavage sites were detected in the transcribed spacer region (EcoRI) and the non-transcribed one (KpnI). Although fragments of less than 0.5 kb produced by AvaI or PvuI digestion could also be detected and identified, they did not appear in the autoradiogram due to the faintness of the bands.

Restriction map of B. mandarina 15.1 kb unit at the R^{a2} locus

The rDNA of *B. mori* includes either a type I or a type II intron within the 28S rDNA coding region (Lecanidou et al. 1984; Fujiwara et al. 1984; Eickbush and Robins 1985). As the 15.1 kb unit found in *B. mandarina* (R^{a2}) probably corresponds to the unit containing the type II intron, we examined the blot mapping of this unit by using a probe specific for the type II intron region (pBmR315) (Fig. 5c). In *B. mandarina* (R^{a2}), this 15.1 kb unit is one of the major units in the genomic rDNA and can be easily mapped by the intron probe (Fig. 5a, b). Although the DNA derived from an R^{a1}/R^{a2} larva was used as the source, this probe (pBmR315) did not hybridize to the R^{a1} DNA (data not shown) and therefore, the autoradiogram obtained is based on the R^{a2} DNA. However, the use of this probe was associated with limitations in mapping as described below. With this probe only limited fragments including the intron region were detected, and even if additional sites for restriction enzymes were present outside of the limited region, these sites could not be identified. The limited restriction map of the 15.1 kb unit is illustrated in Figure 5c and compared with those of λBmR11 and λBmR13. The 5′ and 3′ sides of this unit correspond to a part of the 5′ side of λBmR11 and the right side from the inner EcoRI site of λBmR13 (Fig. 5c). The 15.1 kb unit of *B. mandarina* contained the type II intron in the 28S coding region and corresponded to the cloned rDNA B131 (Eickbush and Robins 1985) regardless of the site limitation.

Discussion

Differentiation of species is an important event in evolution. It is generally considered that the differentiation of a new species from an old one is caused by geographic isolation. When a species which is closely related to another species living in a continent occurs in a neighboring island, the time scale since the species separation can be roughly estimated from the time when the island became separated from the continent. Japan probably separated from the Asian continent about 20000 years ago (Minato 1966). In the genus *Bombyx*, the two species *B. mori* and *B. mandarina* are well-known in both Japan and China. *B. mori* was originally derived from China (Yoshitake 1968) and its chromosome number is different from that of *B. mandarina* in Japan (Kawaguchi 1928) but the phenotypic characteristics of *B. mandarina* (Japan) are similar to those of *B. mandarina* (China) (N. Yoshitake, personal communication). We speculate that *B. mandarina* (Japan) is probably derived from the ancestor of *B. mandarina* (China). The time required for the separation of the two strains (races) is very short if the time when Japan became an island is considered. However, the presence of the Korean wild silkworm suggests that the differentiation of *B. mandarina* (Japan) was associated with the environmental and geological conditions of the period before Japan became an island. The period when the geological separation occurred corresponds to the middle of the Pleistocene (about two million years ago) when the Korean Peninsula was formed by the subsidence of Hwang Hai (Yellow Sea) (Tokuda 1969). This period would probably be sufficient for the fixation of a mutation in a multigene family. For example, in *Drosophila melanogaster*, the fixation time for one rDNA unit mutated

in the cluster corresponds to the order of 10^7 generations (Coen et al. 1982b; Dover 1982; Ohta 1983). Since the repeating number of rDNA units in *B. mori* is approximately the same as that in *D. melanogaster* and since *Bombyx* has three generations per year, the fixation time for the rDNA of *B. mori* corresponds to about three million years.

We observed an rDNA unit peculiar to *B. mandarina* (Japan) which had not been detected in the eight strains of *B. mori* so far examined. As shown in Figure 4c, there are two distinct sites for restriction enzymes in this functional 10.6 kb rDNA unit compared with the *B. mori* 10.6 kb unit. Both sites are localized in the spacer regions and not in the coding region. The R^{a1} locus mainly includes these units and can be distinguished from the R^{a2} locus. The genomic blotting experiments showed that these distinctive sites did not vary (Fig. 4). The heterogeneity in the length of the spacer region has been observed in the human (Krystal and Arnheim 1978), and in Dipteran (for a review see Beckingham 1982; Boncinelli et al. 1983; Israelewski and Schmidt 1982) and other species (Yakura et al. 1984) but in *B. mori* the length of the region is constant in spite of the presence of repeating sequences (Fujiwara et al. 1987). Therefore, the R^{a1} and R^{a2} loci are probably derived from different sources as described later. These rDNAs are suitable candidates for studies on concerted evolution. To obtain further evidence, sequence analyses of the rDNA clones are needed. We have partly determined the sequence of the rDNA clone from the *B. mandarina* gene library (Fujiwara et al. 1987). If these sequences are homologous to that of *B. mori*, however, these data must be considered carefully because the gene library may contain rDNA units of the *B. mori* type (R^{a2}) except from that of the R^{a1} homotype. The same consideration applies to other genes, such as the fibroin gene (Kusuda et al. 1986).

We observed an additional large unit in the R^{a2} locus. This *B. mandarina* 15.1 kb unit was primarily detected in *B. mandarina* and the strains of *B. mori* (T. Ogura et al., in preparation), and has also been cloned from *B. mori* (Eickbush and Robins 1985). This unit coexists with other rDNA units, i.e., the 10.6 kb unit which is the major unit without an intron, the 15.7 kb unit containing the type I intron (Fujiwara et al. 1984; H. Fujiwara, unpublished data) and the 20.8 kb unit with the additional tandem insertion of the 5.1 kb intron to the 15.7 kb unit (T. Ogura, in preparation) (see Figs. 1, 2 and 3). Furthermore preliminary results indicate that the 15.1 kb unit is randomly inserted among other units (H. Maekawa, unpublished data). The R^{o} locus also consists of the same members as in the R^{a2} locus of *B. mandarina*. Thus R^{o} and R^{a2} fundamentally have the same components except for the altered contents of each unit. Therefore, in the F_2 progeny with the R^{o}/R^{a2} genotype, the autoradiograms of the three types of combinations predicted on the basis of Mendel's laws were not clearly distinguished (data not shown). The components of R^{a1} in the R^{a2} locus and in contrast, those of the R^{a2} locus in the R^{a1} locus are usually not detected and as indicated by the Mendelian pattern of the genetical analysis the three R^{o}, R^{a1} and R^{a2} loci are allelic on the autosome. Based on these observations, it appears that the components of the R^{a1} locus can be distinguished from those of the R^{o} and R^{a2} loci.

In *D. melanogaster*, very few units with the type I intron are located in the NO of the *Y* chromosome but the intron is present in 50% of the rDNA units of the NO of the *X* chromosome (Wellauer et al. 1978). A similar tendency has been observed in *D. hydei* (Renkawitz-Pohl et al. 1981). However, the structure of the basic functional rDNA unit, is the same in both chromosomes. The homogenization within species has also been explained by concerted evolution (Dover and Coen 1981; Dover 1982) and revealed a possible mechanism based on unequal exchange (Smith 1974; Ohta 1980). Considering this evidence, the clear separation of the two types of NOs observed in *B. mandarina* cannot be explained. In phylogenically closely related species such as *D. mauritiana* and *D. simulans* (Strachan et al. 1982b), exchanged restriction enzyme sites are found in the spacer region (Coen et al. 1982b), even though two different species, e.g., *Calliphora erythrocephala* and *D. melanogaster*, have a similar restriction map (Beckingham and White 1980). The two types of NOs found in *B. mandarina* may differ in their origin, i.e., the R^{a1} and the R^{a2} units were probably derived from the ancestor of *B. mandarina* (China) and *B. mori*, respectively. It was possible to obtain nine larvae of *B. mandarina* (China) reared at the University of Tokyo. Although the rDNA pattern of each larva was examined, the R^{a1} type unit in the genomic DNA could not be detected. However the R^{a2} type units (data not shown) were observed although the larvae may not clearly represent *B. mandarina* (China) because they were inbred. Therefore it is suggested that the R^{a1} type unit was probably derived from the ancestor (*R*) of the R^{o} type about three million years ago, and became fixed in *B. mandarina* (Japan) and that it is presumably identical with that of the Korean strain through geographical separation. Then it is considered that the chromosome containing the R^{a2} locus was more recently introduced into the population with the different type of NO (R^{a1}) from *B. mori* introduced into Japan and was fixed in it. This speculative history of the genus *Bombyx* is summarized in Figure 6. Similar results were obtained with the fibroin gene. The band specific to *B. mandarina* (Japan) was not detected in *B. mori* and *B. mandarina* (China) when hybridization of the genomic DNAs of the three sources (J. Kusuda, unpublished data) to the fibroin gene probe was carried out.

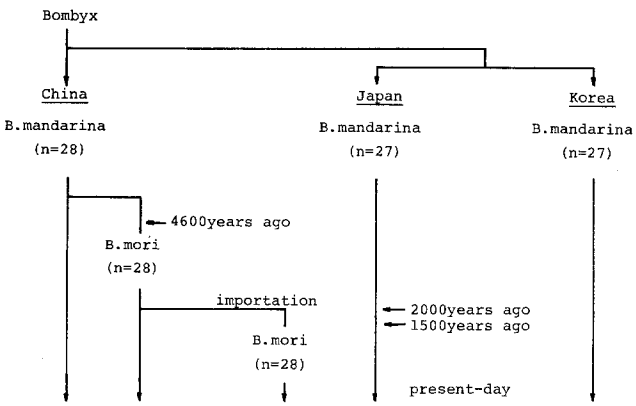


Fig. 6. History of *Bombyx* species. *B. mori* was used for sericulture 4600 years ago. Before that, *B. mori* had probably been domesticated from *B. mandarina* (China). *B. mori* was introduced into Japan from China 2000 years ago. The reference about the introduction of *B. mori* 1500 years ago is also cited in other ancient documents. The ancestor of *B. mandarina* (Japan) and the Korean wild silkworm has probably been separated from the ancestor of *B. mandarina* (China) through the geological events which occurred about three million years ago

Acknowledgements. We are indebted to Drs. Y. Suzuki, J. Kusuda, B. Sakaguchi, H. Doira and A. Watanabe for their useful com-

ments and encouragement and are also grateful to Drs. Y. Suzuki and T. Mukai for their critical reading of the manuscript and to Mr. Kiyota and Miss Sudo for their assistance. This study was partly supported by a grant from the Ministry of Education, Science and Culture of Japan and the Foundation for Promotion of Cancer Research supported by the Japan Ship Building Industry Foundation.

References

Arnheim N, Krystal M, Schmickel R, Wilson G, Ryder O, Zimmer E (1980) Molecular evidence for genetic exchanges among ribosomal genes on nonhomologous chromosomes in man and apes. Proc Natl Acad Sci USA 77:7323–7327

Beckingham K (1982) Insect rDNA. In: Bush H, Rothblum L (eds) The cell nucleus, vol X, rDNA, Part A. Academic Press, New York, pp 205–269

Beckingham K, White R (1980) The ribosomal DNA of Calliphora erythrocephala; an analysis of hybrid plasmids containing ribosomal DNA. J Mol Biol 137:349–373

Boncinelli E, Borghese A, Graziani F, Mantia GL, Manzi A, Mariani C, Simeone A (1983) Inheritance of the rDNA spacer in D. melanogaster. Mol Gen Genet 189:370–374

Bush H, Rothblum L (eds) (1982) The cell nucleus, vol X, rDNA part A. Academic Press, New York

Coen ES, Dover GA (1983) Unequal exchanges and the coevolution of X and Y rDNA arrays in Drosophila melanogaster. Cell 33:849–855

Coen ES, Thoday JM, Dover G (1982a) Rate of turnover of structural variants in the rDNA gene family of Drosophila melanogaster. Nature 295:564–568

Coen ES, Strachan T, Dover G (1982b) Dynamics of concerted evolution of ribosomal DNA and histone gene families in the melanogaster species subgroup of Drosophila. J Mol Biol 158:17–35

Denhardt DT (1966) A membrane-filter technique for the detection of complementary DNA. Biochem Biophys Res Commun 23:641–646

Dover G, Coen E (1981) Springcleaning ribosomal DNA: a model for multigene evolution? Nature 290:731–732

Dover G (1982) Molecular drive: a cohesive mode of species evolution. Nature 299:111–117

Eickbush TH, Kafatos FC (1982) A walk in the chorion locus of Bombyx mori. Cell 29:633–643

Eickbush TH, Robins B (1985) B. mori 28S ribosomal genes contain insertion elements similar to the type I and II elements of Drosophila melanogaster. EMBO J 4:2281–2285

Federoff NV (1979) On spacers. Cell 16:697–710

Fujiwara H, Ogura T, Takada N, Miyajima N, Ishikawa H, Maekawa H (1984) Introns and their flanking sequences of B. mori rDNA. Nucleic Acids Res 12:6861–6869

Fujiwara H, Maekawa H, Ninaki O, Takada N, Miyajima N, Ishikawa H (1987) Comparative studies on the rDNA of the silkworm, Bombyx mori and its presumed ancestor. Comp Biochem Physiol 88B:761–766

Gage LP (1974) Polyploidization of the silk gland of B. mori. J Mol Biol 86:97–108

Gerbi SA, Gourse RL, Clark CG (1982) Conserved regions within ribosomal DNA: locations and some possible functions. In: Bush H, Rothblum L (eds) The cell nucleus, vol X, rDNA, part A. Academic Press, New York, pp 351–386

Israelewski N, Schmidt ER (1982) Spacer size heterogeneity in ribosomal DNA of Chironomus thummi is due to a 120 bp repeat homologous to a predominantly centromeric repeated sequence. Nucleic Acids Res 23:7689–7699

Kawaguchi E (1928) Zytologische Untersuchungen am Seidenspinner und seinen Verwandten. I. Gametogenese von Bombyx mori L. und B. mandarina M. und ihrer Bastarde. Z Zellforsch Mikrosk Anat 7:519–552

Krystal M, Arnheim N (1978) Length heterogeneity in a region of the human ribosomal gene spacer is not accompanied by extensive population polymorphism. J Mol Biol 126:91–104

Krystal M, Eustachio PD, Ruddle FH, Arnheim N (1981) Human nucleolus organizers on nonhomologous chromosomes can share the same ribosomal gene variations. Proc Natl Acad Sci USA 78:5744–5748

Kusuda J, Tazima Y, Onimaru K, Ninaki O, Suzuki Y (1986) A sequence around the 5′-end of fibroin gene from wild silkworm, Bombyx mandarina and its comparison with that of domesticated species, B. mori. Mol Gen Genet 203:359–364

Lecanidou R, Eickbush TH, Kafatos FC (1984) Ribosomal DNA genes of B. mori: a minor fraction of the repeating units contain insertions. Nucleic Acids Res 12:4703–4713

Minato M (1966) The last viaduct of Japan Islands. Earth Sci 85/86:2–11 (in Japanese with English summary)

Murakami A, Imai H (1974) Cytological evidence for holocentric chromosomes of the silkworms, Bombyx mori and B. mandarina (Bombycidae, Lepidoptera). Chromosoma 47:167–178

Ohmura S (1950) Researches on the behavior and ecological characteristics of the wild silkworm Bombyx mandarina. Bull Sericul Exp 13:79–130 (in Japanese with English summary)

Ohta T (1980) Evolution and variation of multigene families, lecture notes in biomathematics, no 37. Springer, Berlin New York

Ohta T (1983) Time until fixation of a mutant belonging to a multigene family. Genet Res 41:47–55

Petes TD (1980) Unequal meiotic recombination within tandem arrays of yeast ribosomal DNA genes. Cell 19:765–774

Ranzani GN, Bernini LF, Crippa M (1984) Inheritance of rDNA spacer length variants in man. Mol Gen Genet 196:141–145

Renkawitz-Pohl R, Glatzer KH, Kunz W (1981) Ribosomal RNA genes with an intervening sequence are clustered within the X chromosomal ribosomal DNA of Drosophila hydei. J Mol Biol 148:95–101

Sasaki C (1898) On the affinity of our wild and domestic silkworms. Ann Zool Jpn 2:33–41

Smith GP (1974) Unequal crossover and the evolution of multigene families. Cold Spring Harb Symp Quant Biol 38:507–513

Southern EM (1975) Detection of specific sequences among DNA fragments separated by gel electrophoresis. J Mol Biol 98:502–517

Strachan T, Coen E, Webb D, Dover G (1982) Modes and rates of change of complex DNA families of Drosophila. J Mol Biol 158:37–54

Suzuki Y, Gage LP, Brown DD (1972) The genes for silk fibroin in Bombyx mori. J Mol Biol 70:637–656

Szostak JW, Wu R (1980) Unequal crossing over in the ribosomal DNA of Saccharomyces cerevisiae. Nature 284:426–430

Takada N, Miyajima N, Ogura T, Maekawa H (1985) The high molecular weight DNA prepared from the posterior silk gland of Bombyx mori. Jpn J Appl Ent Zool 29:83–85 (in Japanese with English summary)

Tokuda M (1969) Biogeography. Tsukiji Shokan, Tokyo, p 137 (in Japanese)

Toyama K (1894) On the spermatogenesis of the silkworm. The Bulletin of the College of Agriculture, vol II. Tokyo Imperial University, Japan, pp 125–157

Tsujimoto Y, Suzuki Y (1979) Structural analysis of the fibroin gene at the 5′ end and its surrounding regions. Cell 16:425–436

Wellauer PK, Dawid IB, Tartof KD (1978) X and Y chromosomal ribosomal DNA of Drosophila: comparison of spacers and insertions. Cell 14:269–278

Yakura K, Kato A, Tanifuji S (1984) Length heterogeneity of the large spacer of Vicia faba rDNA is due to the differing number of a 325 bp repetitive sequence elements. Mol Gen Genet 193:400–405

Yoshitake N (1966) Difference in the multiple forms of several enzymes between wild and domesticated silkworms. Jpn J Genet 41:259–267 (in Japanese with English summary)

Yoshitake N (1968) Phylogenetic aspects on the origin of Japanese race of the silkworm, Bombyx mori L.. J Sericult Sei Jpn 37:83–87 (in Japanese)

Received March 25, 1987 / in revised form December 14, 1987
Accepted by W. Hennig