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Citation	Genome, 50(11), 1061-1065 https://doi.org/10.1139/G07-082
Issue Date	2007-11-01
Doc URL	https://hdl.handle.net/2115/33043
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Type	journal article
File Information	RPViewDoc.pdf



NOTE / NOTE

Conserved synteny of genes between chromosome 15 of *Bombyx mori* and a chromosome of *Manduca sexta* shown by five-color BAC-FISH

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Abstract: The successful assignment of the existing genetic linkage groups (LGs) to individual chromosomes and the second-generation linkage map obtained by mapping a large number of bacterial artificial chromosome (BAC) contigs in the silkworm, *Bombyx mori*, together with public nucleotide sequence databases, offer a powerful tool for the study of synteny between karyotypes of *B. mori* and other lepidopteran species. Conserved synteny of genes between particular chromosomes can be identified by comparatively mapping orthologous genes of the corresponding linkage groups with the help of BAC-FISH (fluorescent in situ hybridization). This technique was established in *B. mori* for 2 differently labeled BAC probes simultaneously hybridized to pachytene bivalents. To achieve higher-throughput comparative mapping using BAC-FISH in Lepidoptera, we developed a protocol for five-color BAC-FISH, which allowed us to map simultaneously 6 different BAC probes to chromosome 15 in *B. mori*. We identified orthologs of 6 *B. mori* LG15 genes (*RpP0*, *RpS8*, *eIF3*, *RpL7A*, *RpS23*, and *Hsc70*) for the tobacco hornworm, *Manduca sexta*, and selected the ortholog-containing BAC clones from an *M. sexta* BAC library. All 6 *M. sexta* BAC clones hybridized to a single *M. sexta* bivalent in pachytene spermatocytes. Thus, we have confirmed the conserved synteny between the *B. mori* chromosome 15 and the corresponding *M. sexta* chromosome (hence provisionally termed chromosome 15).

Key words: BAC-FISH, *Bombyx mori*, chromosome, conserved synteny, Lepidoptera, linkage map, *Manduca sexta*.

Résumé : Combinées aux banques publiques de séquences nucléotidiques, la détermination de la correspondance entre les groupes de liaison (« LG ») existants et les chromosomes individuels de même qu'une carte génétique de seconde génération établie suite à l'analyse d'un grand nombre de contigs de clones BAC (chromosomes bactériens artificiels) chez le ver à soie, *Bombyx mori*, constituent un outil puissant pour l'étude de la syntenie entre les caryotypes du *B. mori* et d'autres espèces de lépidoptères. Il est possible de révéler la syntenie génique entre des chromosomes particuliers en effectuant une cartographie physique comparée de gènes orthologues au moyen d'analyses BAC-FISH (hybridation in situ en fluorescence) sur les groupes de liaison correspondants. Cette technique a été mise au point chez le *B. mori* pour analyser simultanément deux sondes marquées différemment sur des bivalents en pachytène. Afin de permettre une cartographie comparée BAC-FISH plus rapide chez les lépidoptères, les auteurs ont mis au point un protocole BAC-FISH en cinq couleurs, permettant ainsi de faire la cartographie simultanée de six sondes BAC sur le chromosome 15 du *B. mori*. Des clo-

Received 16 May 2007. Accepted 25 August 2007. Published on the NRC Research Press Web site at genome.nrc.ca on 1 November 2007.

Corresponding Editor: W. Traut.

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nes BAC contenant les orthologues de six gènes du *B. mori* (*RpP0*, *RpS8*, *eIF3*, *RpL7A*, *RpS23* et *Hsc70*) ont été obtenus chez le sphinx du tabac, *Manduca sexta*, au sein d'une banque de BAC. Les six clones BAC du *M. sexta* hybridaient à un seul bivalent chez des spermatocytes en pachytène du *M. sexta*. Ainsi, les auteurs ont confirmé la synténie entre le chromosome 15 du *B. mori* et le chromosome correspondant chez le *M. sexta* (provisoirement nommé chromosome 15).

Mots-clés : BAC-FISH, *Bombyx mori*, chromosome, synténie, carte génétique, *Manduca sexta*.

[Traduit par la Rédaction]

Introduction

Moths and butterflies (Lepidoptera) have small and numerous holokinetic chromosomes that are refractory to differential cytogenetic techniques. For a long time, these characteristics prevented identification of individual chromosomes even in the model species, the silkworm, *Bombyx mori* (L.) (Bombycidae). The problems with chromosome identification in *B. mori* remained until the introduction of fluorescence in situ hybridization (FISH) with probes prepared from bacterial artificial chromosome (BAC) clones (Sahara et al. 2003; Yoshido et al. 2005). The so-called BAC-FISH technique provides anchors on chromosomes and is also convenient for direct physical mapping of genes under study (Yasukochi et al. 2004; Niimi et al. 2006). Thus, BAC-FISH appears to be a promising method for karyotyping and genome analysis in other lepidopteran species.

Comparative genomic studies including establishment of synteny between orthologous chromosomes would be possible in silico if genome sequence information were available for the species of interest. However, genome sequencing in many species is unrealistic at present because of high costs. In Lepidoptera, recent advances in *B. mori* genomics make comparative genomic studies feasible. The draft sequence information of this species has been deposited in public databases (Mita et al. 2004; Xia et al. 2004) and all genetic linkage groups have been successfully assigned to individual chromosomes (Yoshido et al. 2005). In addition, a second-generation linkage map has been constructed by mapping a large number of BAC contigs (Yasukochi et al. 2006). In the latter study, it was shown that PCR screening of orthologous genes in BAC libraries and BAC-FISH mapping using selected BACs carrying the orthologs are powerful tools for identification of conserved synteny between *B. mori* chromosomes and the chromosomes of other lepidopteran species. However, two-color probe BAC-FISH, established in *B. mori* (Yoshido et al. 2005), is labour-intensive and requires multiple experiments to localize large numbers of genes from a particular linkage group and determine their order (Yasukochi et al. 2006).

In this study, we present an improved *B. mori* BAC-FISH technique for the study of synteny between orthologous chromosomes in Lepidoptera by combining additional fluorescent dyes and newly constructed filter sets. The improved BAC-FISH has been used to identify successfully the synteny between chromosome 15 of *B. mori* and the corresponding chromosome of the tobacco hornworm, *Manduca sexta* (L.) (Sphingidae).

Materials and methods

Bombyx mori specimens were from strain p50 maintained

in the laboratory of K. Sahara. *Manduca sexta* cultures were provided by Sandra Barns and Stuart Reynolds (Bath University, UK) and reared in the laboratory of F. Marec, essentially as described by Reynolds et al. (1985). Briefly, larvae were maintained individually at 25 ± 1 °C under a photoperiod of 16 h light : 8 h dark, without humidity control, and were fed the artificial diet of Bell and Joachim (1976) slightly modified for local conditions.

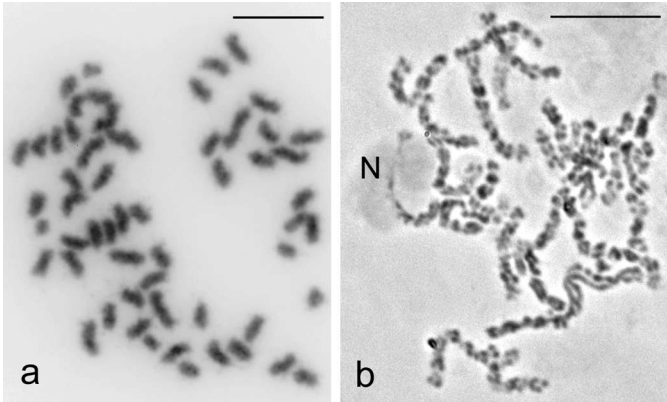
We used 6 *B. mori* BAC clones (18B2A, 10C5D, 15I9C, 18D1A, 20A1F, and 10A11H) that map to chromosome 15. Their isolation and characteristics were described previously (Yasukochi et al. 2006). For isolation of BAC clones in *M. sexta* we used a *Manduca* *EcoRI* library obtained from the GENefinder Genomic Resources (Texas A&M University, College Station, Texas) with a mean insert size of about 165 kb (C. Wu, D. Proestou, D. Carter, E. Nicholson, F.A. Santos, H.-B. Zhang, and M.R. Goldsmith, unpublished data). To select *M. sexta* orthologs of genes mapped to linkage group (LG) 15 of *B. mori*, we performed TBLASTN searches against the whole-genome shotgun sequences of *B. mori* (Mita et al. 2004; Xia et al. 2004) using Kaikoblast (<http://kaikoblast.dna.affrc.go.jp/>). Deduced amino acid sequences of *M. sexta* genes were used as queries; these were obtained from public databases or ButterflyBase (<http://www.butterflybase.org/>; Papanicolaou et al. 2005). We selected *M. sexta* genes showing significant similarity to genome sequences previously localized to LG15 of *B. mori* (Yasukochi et al. 2006) for the design of sequence-tagged site (STS) primer sets (Table 1). Six clones were isolated from the *M. sexta* BAC library with a two-step PCR screening method (Yasukochi 2002) using the STS primers. The PCR amplification was carried out with a 3 min denaturation at 94 °C followed by 45 cycles of 1 min denaturation at 94 °C, 2 min annealing at 55 °C, and 3 min elongation at 72 °C, ending with a 5 min final extension at 72 °C.

Pachytene nuclei were prepared from ovaries (*B. mori*) or testes (*M. sexta*) of last-instar larvae using a modified spreading procedure of Traut (1976) as described in Sahara et al. (1999). BAC clones were labeled by a nick translation system (Invitrogen, Tokyo, Japan) according to the method described in Yoshido et al. (2005) using the following fluorescent dyes: diethylaminocoumarin-5-dUTP (PerkinElmer Inc., Boston, Massachusetts); SpectrumGreen-dUTP, SpectrumOrange-dUTP, and SpectrumRed-dUTP (Abbott Molecular Inc., Des Plaines, Illinois); and double incorporation of Cy5-dCTP plus Cy5-dUTP (Amersham, Tokyo, Japan). BAC-FISH and related procedures including extraction of BAC DNA, DAPI counterstaining of chromosomes, and digital image processing were carried out according to Yoshido et al. (2005). Preparations were observed under a DM6000B fluorescence microscope equipped with a

Table 1. Primers used to search for orthologous genes in *Manduca sexta* showing significant similarity to genome sequences previously localized to LG15 of *Bombyx mori* (Yasukochi et al. 2006).

Gene	Putative gene function	Accession No.	ButterflyBase code	Forward sequence	Reverse sequence	Size (bp)	BAC No.
<i>RpP0</i>	Ribosomal protein P0	AI187668	MSP00336_1	AGGAGGACAAAGGCTACT	CCACGGGTGAAGACAAAAG	ca. 800	02I22
<i>RpS8</i>	Ribosomal protein S8	AI172658	MSP00115_1	GCAAGAAGAGGAAGTATGA	CTGAACCCACGAGAAAT	ca. 280	02B17
<i>eIF3</i>	Translation initiation factor 3 subunit 2	BF707436	MSP00835_1	CGTCGTGTGGTGCTTAGA	TGGCTTTGTCAGTAGTAT	ca. 600	19P12
<i>RpL7A</i>	Ribosomal protein L7A	CA798803	MSP00412_1	GAAAGAAGTCGGGAAGAA	TGAACTGGTTGATTGGTG	ca. 850	30J18
<i>RpS23</i>	Ribosomal protein S23	BE015595	MSP00549_1	GAAAGAGACGACGAAATG	CAAGGTAGGAGGGACAC	ca. 420	09A17
<i>Hsc70</i>	Heat shock 70 kDa protein cognate	BE015303	MSP00378_1	GTATCATCGCTGTGCTCTCA	GCCCTTCTTCTTCTTGTA	263	30P01

Fig. 1. Chromosome complements of the tobacco hornworm, *Manduca sexta*. Fluorescent image (black and white inverted) of a DAPI-stained mitotic spermatogonium (a) showing $2n = 56$ metaphase chromosomes. Phase microscopy image of an orcein-stained oocyte (b) showing pachytene bivalents with a characteristic chromosome/interchromomere pattern; note a conspicuous nucleolus (N) carried by a putative NOR-bivalent. Scale bars are 10 μ m.



DFC350FX CCD camera (both Leica Microsystems Inc., Tokyo, Japan) and the recommended filter sets, A4 (UV), L5 (blue), N3 (green), and Y5 (infrared), which were used for DAPI, SpectrumGreen, SpectrumOrange, and Cy5 fluorescence, respectively. To detect hybridization signals of probes labeled with the remaining fluorescent dyes, 2 additional custom-made filter sets were used, one composed of XF1009, XF2006, and XF3005 filters for diethylaminocoumarin and the other composed of XF1023, XF2020, and XF3023 filters for SpectrumRed (Omega Optical Inc., Brattleboro, Vermont).

Results and discussion

To our knowledge the only published information on *M. sexta* cytogenetics is a conference report (Marec 1995) describing the number of pachytene bivalents in males ($n = 28$). Our preparations from larval testes contained not only pachytene spermatocytes but also some mitotic spermatogonia that showed 56 rod-shaped chromosomes (Fig. 1a), thus confirming the previous report. This suggests that *M. sexta* has the same diploid chromosome number, $2n = 56$, as *B. mori* (Kawamura 1979). In a few preparations of ovaries made from young pupae without hypotonic treatment and stained with lactic acetic orcein, clumps of pachytene bivalents showed a conspicuous nucleolus associated with a bivalent, most probably carrying the nucleolar organizing region (NOR). No other bivalents, including the anticipated sex-chromosome bivalent WZ, were identified (Fig. 1b). In pachytene oocytes of *B. mori*, 6 BAC probes hybridized to the bivalent corresponding to *B. mori* LG15 (chromosome 15) (Fig. 2a). Each probe was labeled with 1 of 5 different fluorescent dyes, except for clone 18D1A, which was double labeled (SpectrumGreen and SpectrumOrange). The order of the BACs containing genes *RpP0*, *RpP1*, *RpS8*, *opsin*, *RpS5*, and *RpL7A* on the chromosome confirmed the gene order found in previous studies by linkage mapping and two-color probe BAC-FISH (Yasukochi et al. 2006). The published figure (Fig. 4c in Yasukochi et al.

Fig. 2. Five-color BAC-FISH mapping of single-copy genes on LG15 of *Bombyx mori* (a) and *Manduca sexta* orthologs of *B. mori* genes belonging to LG15 (b). Pachytene oocyte of *B. mori* (a) showing 6 BAC probes that identify the bivalent of chromosome 15 and localize the genes in the proper sequence of loci on LG15. Colored abbreviations in the figure represent the genes carried on the BACs. Colors correspond to *B. mori* BAC signals from diethylaminocoumarin (18B2A, blue), SpectrumOrange (10C5D, orange), SpectrumGreen (15I9C, green), SpectrumOrange/SpectrumGreen (18D1A, yellow), Cy5 (20A1F, magenta), and SpectrumRed (10A11H, red). Three other bivalents are discriminated by DAPI counterstaining (cf. Sahara et al. 2003; Yoshido et al. 2005). In pachytene spermatocytes of *M. sexta* (b), 6 BAC probes carrying orthologs of the *B. mori* LG15 genes show clear signals on a bivalent (putative chromosome 15 of *M. sexta*). As in Fig. 2a, colored abbreviations represent the genes contained in the *M. sexta* BACs 02I22 (yellow), 02B17 (blue), 19P12 (magenta), 30J18 (red), 09A17 (green), and 30P01 (orange). Chr11, chromosome 11 bivalent; Chr24, chromosome 24 bivalent; N, nucleolus; WZ, sex-chromosome bivalent. Scale bars are 10 μ m.

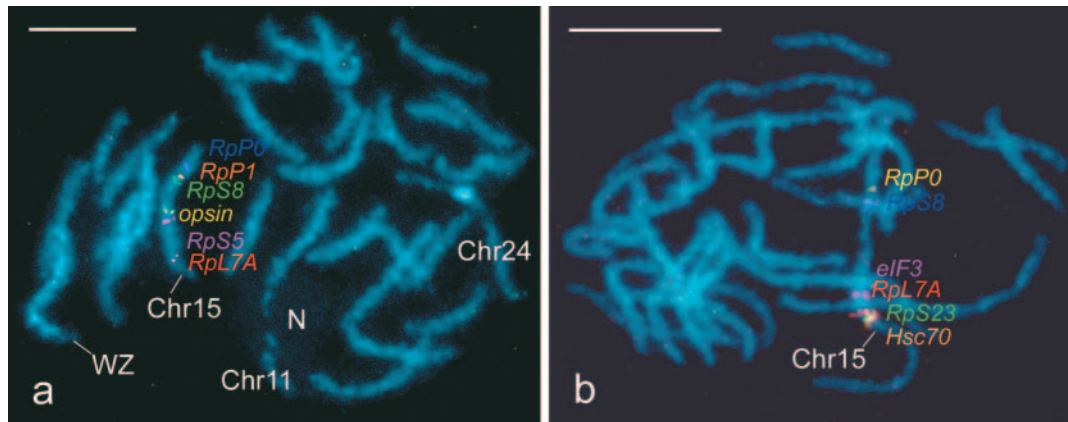
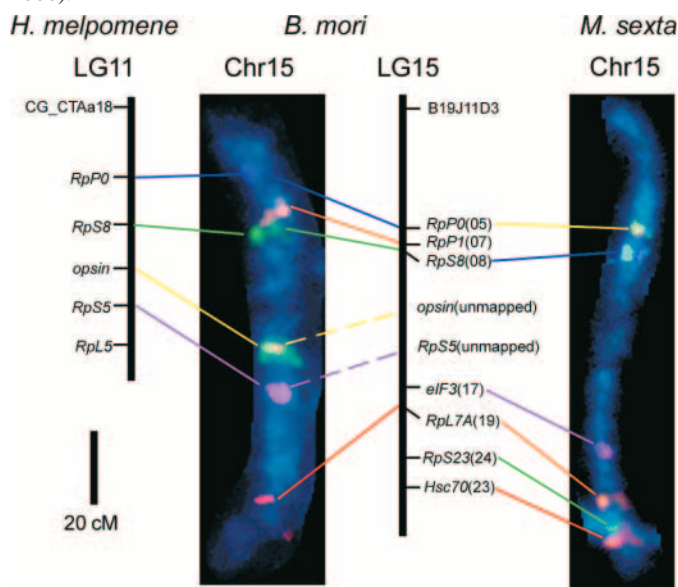


Fig. 3. Conserved chromosome synteny found in 3 lepidopteran species. It is apparent that the gene order in chromosome 15 of *Bombyx mori* (corresponding to LG15), the putative chromosome 15 of *Manduca sexta*, and a chromosome corresponding to LG11 of *Heliconius melpomene* (data taken from Yasukochi et al. 2006) is highly conserved. CG_CTAa18 in LG11 of *H. melpomene* and B19J11D3 in LG15 of *B. mori* represent markers corresponding to 0 cM of the linkage maps. Numbers in parentheses show BAC contig codes mapped on LG15 of *B. mori* (see Yasukochi et al. 2006).



2006), which showed that *RpP0* (BAC 18B2A) was located between *RpP1* (BAC 10C5D, not 10D5C as labeled) and *RpS8* (BAC 15I9C), was incorrectly labeled. Subsequent BAC-FISH analysis using different clones containing the

RpP0 (19F7D) and *RpP1* (19J8C) sequences confirmed that the gene order obtained in the present report is correct (data not shown). Since two-color BAC-FISH required at least 5 hybridization experiments to determine the sequence of 6 BAC probes on the chromosome, the five-color BAC-FISH with 6 probes spared a good number of chromosome preparations and significantly advanced BAC mapping, thus facilitating studies of gene organization and synteny between chromosomes.

To examine the synteny of *B. mori* chromosome 15 with the karyotype of *M. sexta*, we first identified *M. sexta* orthologs of *B. mori* genes, namely *RpP0*, *RpS8*, *eIF3*, *RpL7A*, *RpS23*, and *Hsc70*, previously mapped genetically to *B. mori* LG15. The *B. mori* genes were from BAC contigs 15_05, 15_08, 15_17, 15_19, 15_24, and 15_23, respectively (see supplemental Table 1 of Yasukochi et al. 2006). By PCR screening we then identified individual *M. sexta* BAC clones containing the corresponding orthologs and used them for five-color BAC-FISH with 6 probes. All the *M. sexta* BAC probes containing orthologs of the *B. mori* LG15 genes mapped to a single bivalent and in the same order as in *B. mori* (Figs. 2 and 3). These results suggest well-conserved synteny of *B. mori* LG15 between the two species. Hence the chromosome of *M. sexta* identified by BAC-FISH can be provisionally designated as chromosome 15.

Recently, conserved synteny and gene order were demonstrated between *B. mori* chromosome 15 and LG11 of a butterfly, *Heliconius melpomene* (L.) (Nymphalidae), using molecular linkage maps (see Jiggins et al. 2005) and BAC-FISH mapping (Yasukochi et al. 2006). A wider comparison of orthologous genes among linkage maps for the two species revealed synteny for all linkage groups except *B. mori* LG26 (Pringle et al. 2007). Our results extend the data on the conserved synteny of *B. mori* chromosome 15 in a lepi-

dopteran species for which no linkage maps have been developed. Figure 3 compiles the available synteny data on the positions of single-copy gene loci on chromosomes 15 of *B. mori* and *M. sexta* and LG11 of *H. melpomene*. These results suggest a high conservation of gene order in this chromosome among these three species, strongly indicating well-conserved synteny in 2 large groups of Lepidoptera, the Bombycoidea (*B. mori* and *M. sexta*) and the Nymphalidae (*H. melpomene*), lineages of which are estimated to have diverged approximately 103 Mya (Pringle et al. 2007).

Our study clearly shows that the increase of probe number significantly increases the efficiency of BAC-FISH for the detection of the conserved synteny between orthologous chromosomes in Lepidoptera. Five-color BAC-FISH to elongated pachytene bivalents is an excellent tool for gene mapping; further, it can be applied to any lepidopteran species. Use of this directly applicable approach, especially where detailed genetic studies are not feasible, can significantly contribute to our understanding of how the genomes of one of the largest animal groups have been conserved or remodeled during the course of evolution.

Acknowledgements

We are very grateful to Sandra Barns and Stuart Reynolds (Bath University, UK) for *Manduca sexta* cultures and for teaching us to rear these insects. We also thank Yasuhiro Yamada (Field Science Center for Northern Biosphere, Hokkaido University, Sapporo, Japan) and Marie Korchová (Institute of Entomology, České Budějovice, Czech Republic) for their kind technical assistance. This work was funded by the Program for Promotion of Basic Research Activities for Innovative Biosciences (PROBRAIN). K.S. and Y.Y. received grant 18380037 of the Japan Society for the Promotion of Science (JSPS). F.M. and I.F. were supported by grant 206/06/1860 of the Grant Agency of the Czech Republic (Prague) and by the Entomology Institute project Z50070508. M.R.G., C.W., and H.B.Z. were supported by US National Science Foundation (NSF) grant IBN0208388.

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