Intrinsic bent DNA colocalizes with the sequence involved in the Nd-s<sup>D</sup> mutation in the *Bombyx mori* fibroin light chain gene

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Multiple sequence alignments of the *Bombyx mori* fibroin light chain gene (fib-L) from hybrids and from Chinese and Japanese strains demonstrated that 51.6% of the fib-L third intron is conserved. One of these conserved segments, 41 bp long, contains the sequence CGTATTATACATATT, which is conserved. One of these conserved segments, 41 bp long, contains the sequence CGTATTATACATATT, which is duplicated in the *B. mori* Nd-s<sup>D</sup> mutant. In the present work, electrophoretic mobility assays and computational analyses revealed a major peak of intrinsic bent DNA within the segment that undergoes breakage in the previously-described Nd-s<sup>D</sup> mutation. This result suggested that this intrinsically-curved region might mediate DNA cleavage and enhance recombination events in the third intron of the *Bombyx mori* fib-L gene.

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INTRODUCTION

*Bombyx mori*, the mulberry silkworm, is a model organism for *Lepidoptera*, the second most numerous insect order and one that contains many species important for agriculture and forestry. *B. mori* has been domesticated for silk production for 5,000 years (1) and provides the major source of income for more than 3,000 families in China, India, Vietnam, Thailand, and Brazil. Advances in silkworm research have significantly improved sericulture and facilitated the development of new pest control strategies. In addition, with the development of biotechnology, *B. mori* has emerged as an important bioreactor for the production of recombinant proteins (2-4).

The silk moth domestication process has established more than 3,000 strains of *B. mori*. Besides the many different geographical and ancestral races, there are inbred and mutant lines that carry numerous genetic variants, some of which directly relate to the quality and yield of silk (5).

Speaking, *B. mori* strains of temperate geographic origin are good silk producers, while the tropical ones produce lower silk quantities but are more resistant to disease and adverse climatic conditions. Differences in silk production among *B. mori* strains have prompted studies of the genetic factors involved in the yield and quality of silk (5).

Silk fibroin is secreted into the lumen of the posterior silk gland (PSG) of the *B. mori* silkworm and is mainly composed of three polypeptides: a 350-kDa heavy chain (H-chain; 6), a 26-kDa light chain (L-chain; 7), and fibrohexamerin (fhx; 8). The fibroin light chain gene (fib-L) maps to chromosome 14, is 14,626 base pairs (bp) long, and contains seven exons with large introns (9). The first intron occupies about 60% of the gene, and the other introns together account for approximately 31% (9); therefore, 91% of the gene is composed of non-coding DNA.

The Nd-s and Nd-s<sup>D</sup> mutations are found in the third intron of the fib-L gene and result in a downstream deletion of exon III and enhanced aberrant recombination with downstream sequences (10). The Nd-s mutant was initially identified in 1960 in one specific strain of *B. mori*, and the Nd-s<sup>D</sup> mutant was identified four generations after injecting diethyl sulphate into a male pupa from a normal *B. mori* of unknown strain (11). These mutants have immature PSGs and secrete less than 1% of the normal level of fibroin, which leads to the production of a very thin, naked-pupa cocoon that consists mostly of sericin (10).

Detailed analysis of several known recombination sites has demonstrated that they are located at the bottom regions of DNA loops (12-14). Mutations, breakpoints, and recombination events are commonly associated with bent DNA, and they may preferentially occur at DNA loop anchorage sites, which could lead to deletion or repositioning of individual DNA loops (15). Intrinsic bends in the DNA sequence occur in 2- to 6-bp adenine-thymine tracts (A/T) at intervals of approximately 10 bp (or multiples of 10) (16). Bent DNA sites are involved in biological processes such as DNA recombination (15, 17), fragile sites (18), replication (19, 20), transcription (21-23), nucleosome formation (24), and scaffold/matrix attachment regions (S/MARs) (25).

This work described the physical structure of the fib-L gene and verified the presence of intrinsic bent DNA in the third intron, using electrophoretic mobility assays and 2D structures.

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of the 3D projection. The results showed that the main peak of intrinsic curvature lies within the fib-L gene segment containing the previously-described Nd-sD mutation. Therefore, we propose that this mutation could be related to the bent DNA site in the fib-L third intron.

RESULTS AND DISCUSSION

Electrophoretic mobility assay
Our research team previously conducted multiple alignments with Bombyx mori fib-L third intron sequences from hybrids and from Chinese and Japanese strains (Fig. 1; supplementary material 1 and 2; 26). These sequence alignments demonstrated that 51.6% of the sequence of this intron contains conserved regions, one of which is a 41-bp segment containing the CGTTAT TATACATATT sequence (positions 10973 to 10988; Fig. 1) that is duplicated in the Nd-sD mutant.

Since DNA fragments containing unusual structures generally show an anomalous mobility during polyacrylamide gel electrophoresis (25), electrophoretic mobility assays are a powerful tool to investigate the curvature of DNA segments. To analyze whether the structure of the fib-L third intron was curved, we cloned 903-bp amplified gene products from the Chinese C121A and C122B B. mori strains into the PCR® 2.1-TOPO® plasmid (Invitrogen). We then analyzed their migration patterns in agarose (AGA), polyacrylamide (PA), and ethidium bromide (EtBr) gels. AGA gels, ethidium bromide intercalates into the DNA molecule and opens up its structure, abolishing any altered PA migration. Therefore, a reversion of the PA migration in the PA gel (Fig. 2, black arrow), because this plasmid has a strong peak of curvature in the 5’ end. Fragments with faster mobility are characteristic of fragments with bent regions in one or both fragment ends (25). The plasmid pBluescript II (cleaved with BamHI restriction enzyme), which does not display mobility alterations with this technique, was included as a negative control (Fig. 2, dark gray arrow).

In silico analysis
To further characterize the structure of the fib-L gene third intron, we subjected the corresponding ~680 bp fragments from the C121A and C122B strains to theoretical 2D modeling and calculated the parameter helical ENDS ratio, a measure of DNA curvature in the 2D model. Fig. 3 shows the 2D projection of the 3D path and the ENDS ratio of the C121A (A) and C122B (B) fib-L third intron DNA fragments. Interestingly, the two sequences showed a stronger ENDS ratio value (1.5) in the region of the fib-L gene containing the previously-described Nd-sD mutation. The observed peak localized to the center of the most curved point in the structure (Fig. 3, small circle), regardless of variations in the angles (30 and 60 degrees) in the secondary structure (Fig. 3A and B, respectively). This result suggested that the intrinsic DNA bending site in this segment was important for fragment shape maintenance.

Fig. 4 shows the analysis of the 200 nucleotides surrounding the bent DNA site (rectangle) in the C121A and C122B third intron fib-L gene. This sequence analysis revealed dAdT tract distribution in 10-bp intervals or multiples thereof (bold), characteristic of curved DNA. There are four CanT motifs

![Fig. 1. Alignment of partial sequences of fib-L third intron genes from different B. mori strains. DNA sequence sources and geographical origins are provided in Supplementary Material 2. In this alignment, the Japanese strain M76430 was used as a reference. The dots represent homology to the M76430 sequence. Dashes indicate insertion/ deletion events. Nucleotide transitions and transversions are shown in bold, and the gray regions delimit the conserved regions. The sequence that is duplicated in Nd-sD mutation is boxed. A complete alignment of the fib-L third intron gene is shown in the Supplementary Material 1.](http://bmbreports.org)
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Fig. 2. Electrophoretic mobility assay of the ~900 bp fib-L third intron gene restriction fragments from C121A and C122B clones, utilizing 1.0% agarose gels (AGA) or 6% polyacrylamide gels without (PA) and with (PA + EtBr) ethidium bromide. Both fragments (gray arrows) showed reduced mobility in PA, with R-values of 1.64 for C121A and 1.59 for C122B. The black arrow indicates the PCR 2 TOPO plasmid (positive control) and the dark gray arrow the linearized pBluescript II plasmid (pBS; negative control). M, 1kb molecular weight marker (Invitrogen).

Fig. 3. ENDS ratio and 2D projection of 3D DNA path of the C121A (A) and C122B (B) fib-L third intron gene sequences. The two sequences showed stronger curvature, with an ENDS ratio of 1.50. Rotation of these fragments by 30 and 60 degrees confirmed that the bent regions (small circles) are important for maintaining the fragment shape.

(double underlined) in the C121A sequence and three in the C122B. This motif yields a stronger curvature, because the adenine tracts flanked by cytosine on the 5' end and thymidine on the 3' end induce local bends, which together produce a stronger global bend (22).

Intrinsic DNA curvature reportedly facilitates the binding of proteins such as DNA topoisomerase I and II (25). In principle, bent DNA might also facilitate the binding of diethyl sulphate or other compounds, such as insecticides, that are used in agriculture. Such compounds could produce breaks and recombination events, thereby inducing gene mutation.

Our results raise new questions about the structure of the fib-L third intron. Can this bent structure increase the likelihood of recombination in this chromosomal segment? Is it possible that in the Nd-sD mutation, the described 16-bp sequence nucleotide duplicates before the segment break? If so, is this duplication the reason for the breakage? Although previous reports suggested that the Nd-s mutant was achieved through population breeding and the Nd-sD was obtained from diethyl sulphate treatment, they did not find an additional 16-bp sequence in the downstream region of the chromosome (10), where the breakage and joint point for the new mutated sequence occurs. However, diethyl sulphate and related carcinogenic compounds might be able to duplicate segments and break the DNA molecule; and the intrinsic bent curvature of the DNA might enhance that ability, as reported elsewhere (17). Addressing these issues will require a search for the 16-bp sequence duplication prior to the third intron breakage event using B. mori strains that
are heterozygous for the fib-L gene and/or breeds treated with alkylating agents. Functional experiments with transgenic silk- 

worm lines in which the intrinsic bent structure of this intron is 

abolished by point mutations could also be helpful in answering 
such questions. Understanding more about the mechanisms 

involved in chromosomal recombination will expand our 

knowledge in this area, and these mechanisms may represent a 

powerful tool to further the understanding of mutation proc-

esses in eukaryotic cells.

**MATERIALS AND METHODS**

**Bombbyx mori** strains

*B. mori* strains were provided by COCAMAR (Cooperativa Agro-

industrial; Maringá, Parana State, Brazil) to the Universidade 

Estadual de Maringá, Parana State, Brazil. Silkworms were raised 

at 25°C with fresh mulberry leaves at a COCAMAR farm.

**DNA extraction**

Genomic DNA was extracted from the silk glands of five-day-

old fifth instar larvae using a modification of a previously-de-

scribed protocol (28). Briefly, pairs of silk glands were dis-

sected and incubated in 3 ml of extraction buffer (1.5% sarkosil, 

50 mM EDTA [pH 8.0], 10 mM NaCl, and 1 mg/ml proteinase K) 

for 2-3 hours at 50°C. The extract was subjected to one round 

of phenol (pH 8.0), ethanol precipitated with 0.2 M 

NaCl and 0.7 volume of isopropanol, and resuspended in TE 

buffer (10 mM Tris-HCl [pH 8.0] and 1 mM EDTA [pH 8.0]). 

DNA concentration was determined by spectrophotometry.

**PCR and cloning**

PCR primers for the amplification of the third intron of fib-L 

were constructed using FAST-PCR software (version 3.5-30 by 

Ruslan Kalendarr). The forward primer sequence (5'-ACCTGACGCCCCTCAGTT-3') and reverse primer sequence (5'-CGG 

ACCTGACGCCCCTCAGTT-3') are complementary to the third 

and fourth exons, respectively. PCR was performed routinely, 
in a final volume of 15 μl: 2.5 mM dNTPs, 100 ng of DNA, 25 

pmol of each primer, 1X PCR buffer (with 1.5 mM of MgCl₂), 

and 1 unit of Taq Polymerase (Invitrogen). The amplification 

conditions were 1 min at 94°C, 1 min at 58°C, and 1 min at 

72°C, for a total of 35 cycles, followed by a final extension 

of 10 min at 72°C. PCR products were purified using a PCR puri-

fication Kit (Qiagen) and cloned using a TOPO TA PCR Cloning Kit (Invitrogen). The PCR products, approximately 900 

bp long, were cloned from one individual of the strain C121A and one individual of the strain C122B, and subsequent analy-

ses were carried out with these PCR products.

**Sequencing**

Sequencing reactions were performed with the DYEnamic ET 

Dye Terminator Kit (Amersham Biosciences), and the reactions 

products were analyzed with a MegaBACE 1000 automated 

dNA sequencer (Amersham Biosciences).

**Electrophoresis mobility assay and computational analysis**

Fragments from recombinant pC121A and pC122B clones 

were obtained by digestion with the EcoRI restriction enzyme. 

The mobility of the fragments was compared under the follow-

ing conditions: electrophoresis in 1% agarose gels (control 

systems, an 

R-value (corresponding to the ratio of the observed 

length to the expected length) was calculated for each DNA 

fragment to determine the mobility alteration in the gels.

The nucleotide sequences for C121A and C122B (GenBank 

accession numbers EF505754 and EF505757, respectively) 

were analyzed by computational modelling using the Trifonov 

dinucleotide wedge model for curvature study. The projection 

of the three dimensional path and helical parameters were ob-

tained with Map15a and 3D15m1 software using the algo-

rithm of Eckdahl and Anderson (29) and the helical parameters 

of Bolshoy et al. (30), as previously described (31, 32).

Putative intrinsic bent DNA sites identified by the ENDS ra-

thm (Eckdahl and Anderson) of 150 bp width and a 10 bp step.

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