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# Characterization of species-specific repeated DNA sequences from *B. nigra*

**Vibha Gupta [1], G. LakshmiSita [2], M.S. Shaila [2], V. Jagannathan [1], and Malathi S. Lakshmikumaran [1]**

[1] Tata Energy Research Institute, 90 Jorbagh, New Delhi 110003, India
[2] Microbiology and Cell Biology Department, Indian Institute of Science, Bangalore 560012, India

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**Summary.** The construction and characterization of two genome-specific recombinant DNA clones from *B. nigra* are described. Southern analysis showed that the two clones belong to a dispersed repeat family. They differ from each other in their length, distribution and sequence, though the average GC content is nearly the same (45%). These B genome-specific repeats have been used to analyse the phylogenetic relationships between cultivated and wild species of the family *Brassicaceae*.

**Key words:** Repeated DNA – *Brassica* species – Genome specificity – Phylogenetic studies

## Introduction

Plant genomes contain a large proportion of repetitive DNA. Many of the repeated DNA sequences are common to the genomes of related species, but some of the repeated sequences have been shown to be species or genome specific (Crowhurst and Gardner 1991; Guidet et al. 1991; Shepherd et al. 1990; Sonina et al. 1989; Zhao et al. 1989; Junghans and Metzlaff 1988; Metzlaff et al. 1986). These genome-specific repetitive DNA sequences are of interest for analysing intergeneric and interspecific (Schweizer et al. 1988; Rayburn and Gill 1987) hybrids at very early stages of development. These sequences can also be used for the characterization of chromosome addition lines (Hosaka et al. 1990; McGrath et al. 1990).

The family *Brassicaceae* consists of 51 genera and about 218 species (Gomez-Campo 1980), many of which are important cultivated species. Significant variation has been observed between the highly repeated DNA sequences amongst the *Brassica* species. Such sequences and their divergence have been used as a means for elucidating the phylogenetic relationships within the family *Brassicaceae* (Hallden et al. 1987; Song et al. 1988, 1990) The most extensively studied 177-bp satellite DNA of the family *Brassicaceae* is present in two of the three monogenomic species, *B. campestris* and *B. oleracea*, and also in some of the wild relatives, but is absent from *B. nigra* (Lakshmikumaran et al. 1988). Similarly a 350 bp-tandem repeat from *B. nigra* hybridizes to *B. oleracea*, but is absent from *B. campestris* (Gupta et al. 1990). This paper describes the characterization of *B. nigra* repeated DNA sequences that can be used as species-specific probes. The distribution and organization of these sequences in other *Brassica* species is also discussed.

*Correspondence to:* S. Lakshmikumaran

## Materials and methods

### *Plant material and DNA isolation*

Leaves of the various *Brassica* species studied (Table 1) were collected from field-grown plants and used for the isolation of DNA (Dellaporta et al. 1984). Nuclear DNA of *B. nigra* (var 'IC 257') was prepared from frozen leaves according to the method of Malmberg et al. (1985). All DNA preparations were purified on a CsC1 density gradient.

### *Southern blotting and hybridization*

Genomic DNA was digested with various restriction enzymes as recommended by the suppliers and according to Maniatis et al. (1982). The digests were electrophoresed on neutral 1% agarose gels, and the DNA was transferred onto nitrocellulose membranes according to Southern (1975). DNA hybridizations were carried out following the procedures of Lakshmikumaran et al. (1985) and Witney and Furano (1984). The prehybridization, hybridization and washing of filters was carried out as described in Gupta et al. (1990). After washing, the filters were exposed to Kodak XAR5 films with intensifying screens.

**Table 1.** Cross hybridization of various *Brassica* species with clones pBNBH35 and pBNMbo5

| Taxon | Gametic chromosome number | Genome type | Presence/absence of repeats | | | |
|---|---|---|---|---|---|---|
| | | | pBNBH35 | | pBNMbo5 | |
| | | | Stringency | | Stringency | |
| | | | High | Low | High | Low |
| *B. nigra* | 8 | BB | + | + | + | + |
| *B. campestris* | 10 | AA | − | − | − | + |
| *B. oleracea* | 9 | CC | − | + | + | + |
| *B. juncea* var Pusabold (natural) | 18 | AABB | + | + | + | + |
| *B. campestris* cv Norinosa × *B. nigra* (Synthetic) | 18 | AABB | + | + | + | + |
| *B. campestris* cv Japonica × *B. nigra* (Synthetic) | 18 | AABB | + | + | + | + |
| *B. carinata* | 17 | BBCC | + | + | + | + |
| *B. napus* | 19 | AACC | − | − | + | + |
| *B. fruticulosa* | 8 | – | − | − | − | − |
| *B. oxyrrhina* | 9 | – | − | − | − | + |
| *B. tournefortii* | 10 | – | − | − | − | − |
| *B. spinescens* | 8 | – | − | − | − | − |
| *B. barrelieri* | 10 | – | − | Smear | − | + |
| *B. adpressa* | 7 | – | − | − | − | − |
| *Diplotaxis erucoides* | 7 | – | − | − | − | − |
| *D. tenuisiliqua* | 9 | – | − | − | − | − |
| *D. muralis* | 11 | – | − | − | − | − |
| *Eruca sativa* | 11 | – | − | − | − | − |
| *Erucastrum varium* | 7 | – | − | − | − | − |
| *Erucastrum leucanthum* | 8 | – | − | − | − | − |
| *Sinapis arvensis* | 9 | – | + | + | + | + |
| *Sinapis alba* | 12 | – | − | − | − | − |
| *Rahanus sativus* | 9 | – | − | − | − | − |

*Cloning of repeated DNA sequences*

For cloning repetitive DNA sequences, *Bam*HI digestion products of *B. nigra* ('IC257') nuclear DNA were separated on 1% low-melting agarose gel (Seakem FMC). DNA fragments in the range of 0.3 to 1 kb were eluted out from the gel and ligated to *Bam*HI-cleaved pUC 13 using T4 DNA ligase. *E. coli* JM 105 competent cells were transformed with the ligation mixture, and ampicillin-resistant white colonies were picked up at random. Plasmid DNA was isolated according to the method of Birnboim (1983), and these clones were screened for repetitive DNA sequences.

*Sequence analysis*

Sanger's dideoxy chain termination method was used to sequence plasmid DNA. Double-stranded DNA was directly sequenced as described by Kraft et al. (1988). Polyacrylamide gels (5%) were used to fractionate the reaction products. Sequence analysis was carried out using a computer software programme in BASIC (Wilbur and Lipman 1983).

## Results

*Identification of repeats in B. nigra*

To isolate repetitive DNA sequences unique to *B. nigra* genome, we cloned fragments as described in the materials and methods. Clones containing highly repeated DNA sequences were identified on the basis of their strong hybridization signals with *B. nigra* nuclear DNA as a probe. A number of plasmids were screened for their species specificity by hybridizing the clones with *B. campestris* and *B. oleracea* DNA as probes (data not shown). Two clones, pBNBH35 and pBNMbo5, showed little or no cross hybridization and were further characterized.

*Hybridization pattern in B. nigra*

The distribution pattern of the two clones pBNBH35 and pBNMbo5 in the *B. nigra* genome was obtained by restricting *B. nigra* nuclear DNA with various restriction enzymes and then probing it with these two clones. The hybridization patterns obtained are shown in Figs. 1A and 2. Isochizomer *Msp*I and *Hpa*II showed different hybridization patterns with both these clones: *Msp*I cut more frequently than *Hpa*II (Fig. 1A, lanes a and c; Fig. 2, lanes f and g), indicating methylation of some of the inner C residues in the site 5′-CCGG-3′.

The nature of the dispersion of these sequences in the genome was determined by subjecting the nuclear DNA to partial, time-course restriction. On hybridization with clone pBNBH35, the DNA remained either at the top of the gel or got restricted to 0.6 kb band (Fig. 1 B). Similarly, with pBNMbo5, no multimeric ladder of bands was obtained (data not shown).

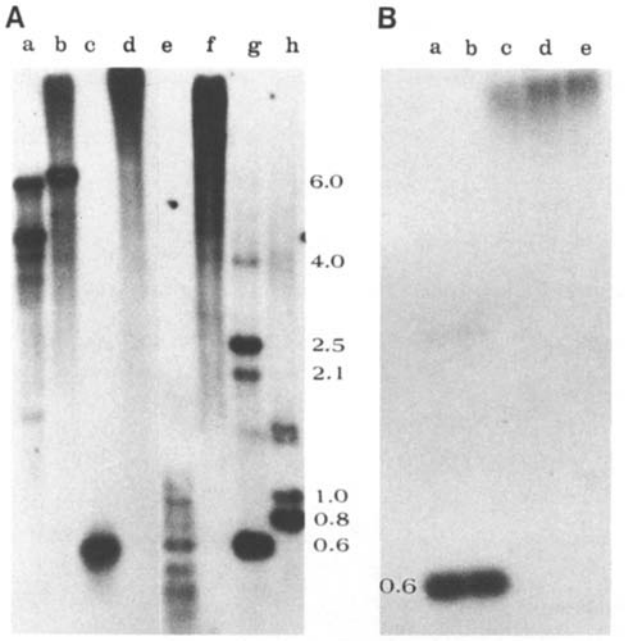


**Fig. 1.** **A** Hybridization pattern of *B. nigra* clone pBNBH35. Five micrograms *B. nigra* DNA was digested with *Msp*I (*lane a*), *Hpa*II (*lane b*), *Mbo*I (*lane c*), *Dpn*I (*lane d*), *Hin*fI (*lane e*), *Eco*RI (*lane f*) *Bam*HI (*lane g*) and *Hin*dIII (*lane h*) and blotted onto nitrocellulose filters. The filter was hybridized with nick-translated pBNBH35 clone. **B** Time-course reaction with *Bam*HI of *B. nigra* DNA probed with the nick-translated pBNBH35 clone. Twenty-five micrograms DNA was restricted with *Bam*HI (1 U/μg), and aliquots of 5 μg were taken out after 1 h (*lane a*), 30 min (*lane b*), 15 min (*lane c*), 10 min (*lane d*) and 5 min (*lane e*)

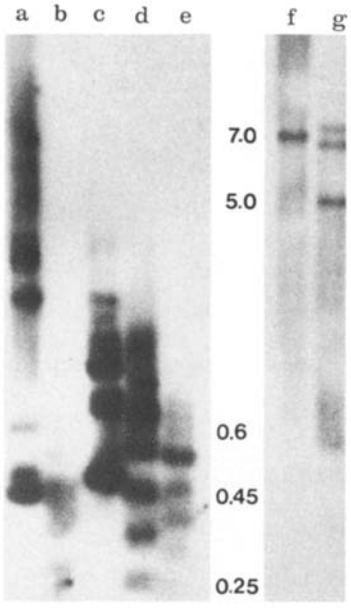


**Fig. 2.** Hybridization pattern of clone pBNMbo5. Five micrograms *B. nigra* nuclear DNA was digested with *Hin*dIII (*lane a*), *Alu*I (*lane b*), *Hae*III (*lane c*), *Hin*fI (*lane d*), *Sau*3A (*lane e*), *Hpa*II (*lane f*) and *Msp*I (*lane* g), and after Southern blotting was hybridized with nick-translated pBNMbo5

*Sequence analysis of clones*

The complete sequences and the restriction patterns of clones pBNBH35 and pBNMbo5 are shown in (Fig. 3 A and B). pBNBH35 is 496 bp in length, and its average GC content is 46%. The length and GC content of plasmid pBNMbo5 are 390 bp and 45%, respectively. No homology was observed among the two clones. An unusually large number of direct repeats of up to 9 bp were detected in pBNBH35. Clone pBNMbo5 also showed the presence of a few direct and inverted sub-repeats. These two cloned sequences showed no homology to any of the reported DNA sequences from the EMBL data bank.

*Determination of species specificity*

The specificity of clones pBNBH35 and pBNMbo5 was determined by hybridizing these clones to *Bam*HI-digested DNAs of the three monogenomic *Brassica* species, *B. nigra* (B genome, n = 8), *B. oleracea* (C genome, n = 9) and *B. campestris* (A genome, n = 10), the naturally occurring amphidiploids, *B. juncea* (AB genome, n = 18), *B. napus* (AC genome, n = 19) and *B. carinata* (BC genome, n = 17), and to some synthetic *B. juncea* (Fig. 4 A and B). The repeat pBNBH35 hybridized only to *B. nigra* (Fig. 4 A, lanes a–c), and clone pBNMbo5 hybridized both to *B. nigra* and *B. oleracea* under high stringency conditions (data not shown). Upon lowering of the stringency conditions, *B. oleracea* did show some faint bands (Fig. 4 B, lanes a–c) with pBNBH35, but the most prominent band of *B. nigra* at 0.6 kb was not seen. Under these conditions *B. campestris* did not show any signal with pBNBH35, but hybridized as a smear to pBNMbo5 (data not shown).

Clone pBNBH35 binds to the amphidiploids *B. juncea* and *B. carinata* but not to *B. napus* DNA (Fig. 4 A, lanes d–i) since *B. juncea* and *B. carinata* contain the *B. nigra* genome whereas *B. napus* does not. The hybridization of repeat clone pBNMbo5 to various amphidiploids is summarized in Table 1.

*Cross hybridization with other crucifers*

Total DNA from several accessions of wild species belonging to different genotypes were hybridized to the two

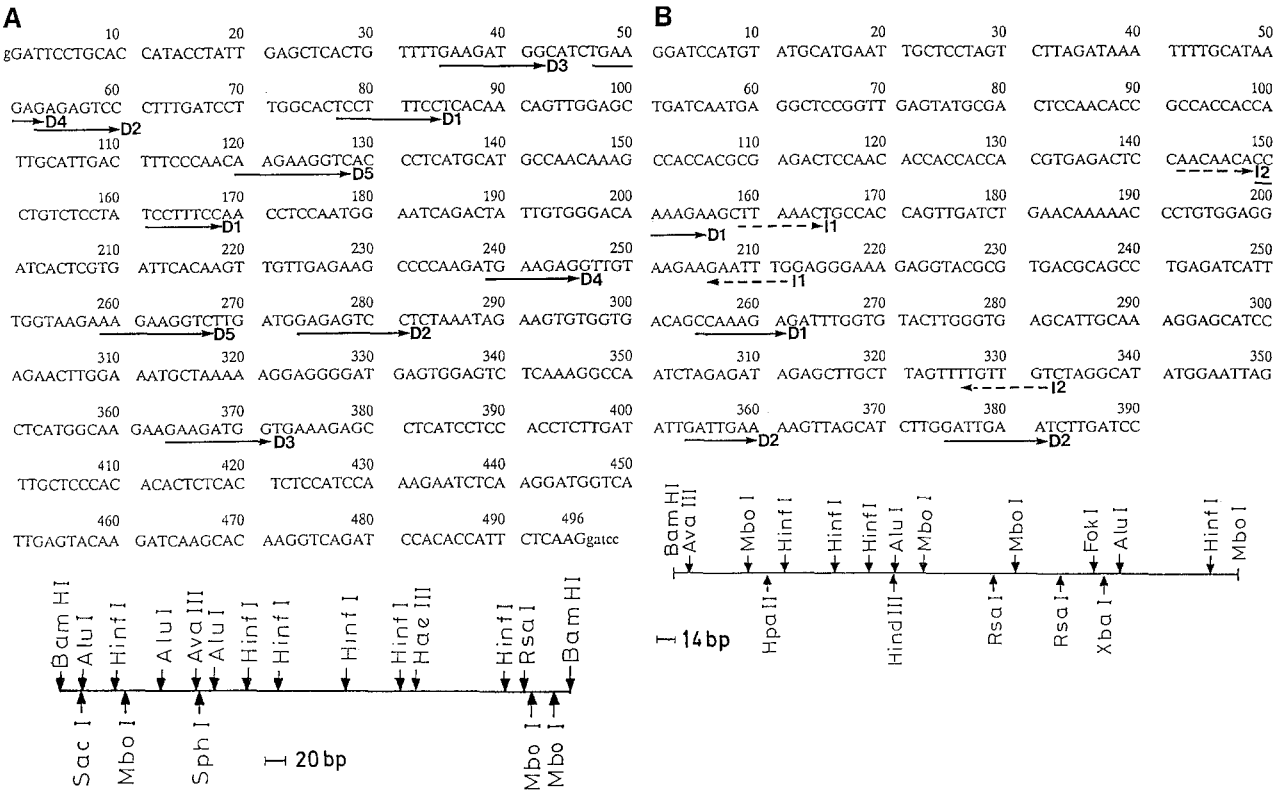


**Fig. 3.** **A** The nucleotide sequence and restriction map of the cloned pBNBH35 repeated DNA fragment. The complete sequence is shown in groups of ten nucleotides from 5′ to 3′. Direct (*D*) repeats are indicated by *arrows*. *Bam*HI sites which border the unit are in *small letters*. **B** The nucleotide sequence and restriction map of the clone pBNMbo5 repeated DNA fragment. The complete sequence is shown in groups of ten nucleotides from 5′ to 3′. Direct (*D*) and inverted (*I*) repeats are indicated by *arrows*

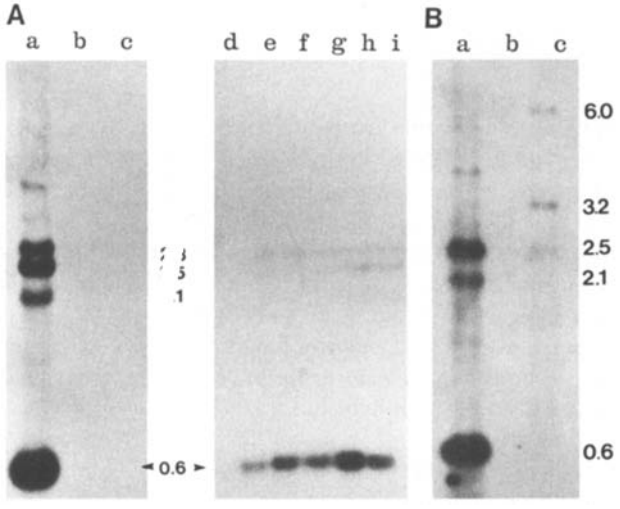


**Fig. 4.** **A** Hybridization of six basic *Brassica* species of U triangle to pBNBH35. Five micrograms of total DNAs of *B. nigra* (*lane a*), *B. campestris* (*lane b*), *B. oleracea* (*lane c*), *B. napus* (*lane d*), *B. carinata* (*lane e*), *B. juncea* (*lane f*) and three synthetic *B. juncea* Norinosa × *B. nigra* (*lane g*), Rapa × *B. nigra* (*lane h*) and Japonica × *B. nigra* (*lane i*) were restricted with *Bam*HI, and after Southern blotting hybridized with the probe pBNBH35 under high stringency conditions. **B** Hybridization of *Bam*HI-digested nuclear DNAs of *B. nigra* (*lane a*), *B. campestris* (*lane b*) and *B. oleracea* (*lane c*) with nick-translated pBNBH35 under low stringency conditions

clones under high and low stringency conditions. The results of these experiments are summarized in Table 1. Of all the wild species tested only *Sinapis arvensis* hybridized to both these clones under high stringency conditions. None of the other wild species tested showed any signal under high stringency conditions, but *B. oxyrrhina* and *B. barrelieri* did hybridize to plasmid pBNMbo5 under low stringency conditions; *B. barrelieri* showed unspecific signals with plasmid pBNBH35 under these conditions.

## Discussion

In this report the isolation and characterization of two repeated DNA sequence clones has been described of which one is *B. nigra* specific. The Southern analysis of the two clones, pBNBH35 and pBNMbo5, revealed that they belong to different repeated DNA families. The different hybridization patterns with various restriction enzymes and the partial restriction digests indicate the dispersed nature of these clones.

Sequence analysis of clones pBNBH35 and pBNMbo5 did not show any homology to any other reported plant repeated DNA sequences. A large number of direct

and inverted sub-repeats are present in both sequences suggesting that sequence duplications and rearrangements have occurred during their evolution. Methylation studies using isochizomers *Msp*I and *Hpa*II indicated that both sequence families are methylated at 'C' residues. Since lower molecular weight bands are obtained with *Msp*I, it is clear that the internal C is more methylated than the external C (Nelson and McClelland 1989). The average GC content of both sequences is nearly the same, i.e. 45%.

An interesting observation was the presence of a 2.8-kb band in *B. nigra* in Fig. 4A (lane a) that was absent in Fig. 1 (lane g) and Fig. 4B (lane a). The 2.8-kb band was obtained only when total DNA was used and was not observed when nuclear DNA was used for hybridization. This band may have arisen from the presence of this sequence in the organelle DNA.

The results described in this paper show that clone pBNBH35 can be used as a species-specific probe for *B. nigra*. Neither A- nor C-genome diploid species showed any hybridization signal, and only under lower stringency conditions could some homology be detected between B and C genomes. Clone pBNMbo5 hybridized to the C genome but not to the A genome, which shows some relatedness between B and C genomes. The presence of genome-specific markers indicates that these genomes have diverged enough to be distinguished from each other.

The characteristic banding pattern observed in *B. nigra* was retained when the B genome was in combination with other genomes such as A or C, which is shown by the similar hybridization patterns observed with synthetic and natural *B. juncea* and *B. carinata* (Fig. 4A) using probe pBNBH35.

The two clones from *B. nigra* which were tested did not reveal any homology with any of the wild relatives of family *Brassicaceae* except *Sinapis arvensis*, which shares some common fragments with *B. nigra*. The fact that the other genomes showed no hybridization to B genome-specific repetitive sequences indicates that there is a clear distinction at the DNA sequence level between the B genome and the other *Brassica* genomes. The hybridization signals that we observed with *Sinapis arvensis* revealed that this wild species and *B. nigra* have DNA sequence similarities. On the basis of data obtained with the two repeat DNA clones and from data of an earlier study (Gupta et al. 1990), *B. nigra* appears to be more closely related to *S. arvensis* than the A or C genomes. On the basis of their RFLP analysis of nuclear and chloroplast DNA Song et al. (1988) have suggested that *S. arvensis* could be the progenitor of *B. nigra*. Based on chloroplast DNA analysis, Yanagino et al. (1987) have also grouped *Sinapis arvensis* and *B. nigra* together. Evidence that *S. arvensis* is the progenitor of *B. nigra* has also been found in previous studies by Mizushima (1980), who reported a high degree of homeologous pairing in *B. nigra* × *S. arvensis* interspecific hybrids, and by Prakash and Hinata (1980), who indicated that both species have the same natural distribution.

The results of this study demonstrate that species relationship and genome divergence within the family *Brassicaceae* can be studied with these repeated DNA sequences. At the present time they are being used to screen intergeneric and interspecific hybrids and to identify *B. nigra* chromosome addition lines.

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