

A Bacterial Artificial Chromosome-Based Genetic Linkage Map of the Nematode *Pristionchus pacificus*

Jagan Srinivasan,* Waltraud Sinz,* Christa Lanz,[†] Alexandra Brand,* Ramkumar Nandakumar,[†] Günter Raddatz,[†] Hanh Witte,* Heike Keller,[†] Isabel Kipping,* André Pires-daSilva,* Taco Jesse,[‡] Jun Millare,[‡] Michiel de Both,[‡] Stephan C. Schuster[†] and Ralf J. Sommer^{*,1}

*Abteilung für Evolutionsbiologie, Max-Planck Institut für Entwicklungsbiologie, 72076 Tübingen, Germany, [†]Genomzentrum, Max-Planck Institut für Entwicklungsbiologie, 72076 Tübingen, Germany and [‡]Keygene N.V., 6708 AE Wageningen, The Netherlands

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ABSTRACT

To understand the evolution of developmental processes, nonmodel organisms in the nematodes, insects, and vertebrates are compared with established model systems. Often, these comparisons suffer from the inability to apply sophisticated technologies to these nonmodel species. In the nematode *Pristionchus pacificus*, cellular and genetic analyses are used to compare vulva development to that of *Caenorhabditis elegans*. However, substantial changes in gene function between *P. pacificus* and *C. elegans* limit the use of candidate gene approaches in studying *P. pacificus* mutations. To facilitate map-based cloning of mutations in *P. pacificus*, we constructed a BAC-based genetic linkage map. A BAC library of 13,440 clones was generated and completely end sequenced. By comparing BAC end and EST sequences between the “wild-type” strain *P. pacificus* var. California and the polymorphic strain *P. pacificus* var. Washington, 133 single-stranded conformational polymorphisms were identified. These markers were tested on a meiotic mapping panel of 46 randomly picked F₂ animals after a cross of the two strains, providing the first genetic linkage map of *P. pacificus*. A mapping strategy using two selected markers per chromosome was devised and the efficiency of this approach was illustrated by the mapping of the *Ppa-unc-1/Twitchin* gene.

RECENT advances in mapping and sequencing genomes are revolutionizing our knowledge of several model organisms including humans. These new technologies not only speed our understanding of model systems, but also bring together disparate branches and problems of biology. In evolutionary biology, for instance, a long-standing question is how changes in developmental processes translate into morphological diversity (RAFF 1996; GERHART and KIRSCHNER 1997). However, a detailed genetic and molecular understanding of developmental processes is currently restricted to a small number of model organisms such as *Caenorhabditis elegans*, *Drosophila*, or mouse. The availability of new DNA technologies will help extend our knowledge of nonmodel organisms and will facilitate the comparison of their development with that of phylogenetically related model systems.

Pristionchus pacificus is a free-living nematode of the Diplogastridae family and has recently been developed as a satellite organism for functional comparative studies in developmental biology (EIZINGER *et al.* 1999; SOMMER 2000). Like the model organism *C. elegans*, *P. pacificus* can be cultured easily in the laboratory. It has a 4-day

life cycle and propagates as self-fertilizing hermaphrodites or by outcrossing after the spontaneous generation of males (SOMMER *et al.* 1996). Many cellular, genetic, and molecular techniques successfully used in *C. elegans* are also applicable to *P. pacificus*, generating the basis for studying evolutionary developmental biology.

A developmental process studied in great detail is the development of the vulva, the egg-laying structure of nematodes. Comparative studies of vulva development between *C. elegans* and *P. pacificus* indicated that although homologous precursor cells are involved in vulva formation, the cell-cell interactions required to form a proper vulva vary greatly between both species (SOMMER 2001). Furthermore, the comparison of mutations in the homeotic genes *lin-39*, *mab-5*, and *vab-7* in both species revealed substantial changes in the function of these genes during vulva formation (EIZINGER and SOMMER 1997; JUNGBLUT and SOMMER 1998, 2001; SOMMER *et al.* 1998). Thus, a candidate gene approach or other techniques relying on *C. elegans* knowledge might be uninformative or even misleading in studying *P. pacificus* mutations. To overcome such limitations, map-based cloning offers an ideal alternative. Here, we describe a bacterial artificial chromosome (BAC)-based genetic linkage map of *P. pacificus* that allows map-based cloning of genes in this organism. A mapping strategy using two selected single-strand conformation polymorphism (SSCP) markers

¹Corresponding author: Department for Evolutionary Biology, Max-Planck Institute for Developmental Biology, Spemannstrasse 37, D-72076 Tübingen, Germany. E-mail: ralf.sommer@tuebingen.mpg.de

per chromosome has been devised and the efficiency of this approach is illustrated by the mapping of the *Ppa-unc-1/Twitchin* gene.

MATERIALS AND METHODS

BAC library construction: Agarose plugs containing high-molecular-weight DNA were prepared from first larval stage worms from synchronized cultures as described previously (OSOEGAWA *et al.* 1998, 2000). Plugs were partially digested with *Hind*III and size fractionated in two steps by pulsed-field gel electrophoresis. Several fractions of size-selected DNA fragments were electroeluted and cloned into the vector pIndigoBAC-536 (SHIZUYA *et al.* 1992). The ligation product was electroporated into *Escherichia coli* DH10B cells. After *Not*I sizing 96 white clones of each fraction, the four best fractions were picked by robot.

BAC end sequencing: To prepare BAC DNA for end sequencing, 4 ml of 2× YT medium-chloramphenicol (12.5 μg/μl) was inoculated with 4 μl of BAC freezer stocks, and cultures were grown for 20 hr at 37°. BAC-DNA was then prepared using the R.E.A.L. kit (QIAGEN, Valencia, CA) on a robotic platform (BioRobot 8000). Sequencing reactions were set up according to manufacturer's instructions for the Big Dye Terminator chemistry (Applied Biosystems, Foster City, CA). Samples were analyzed using capillary electrophoresis (Applied Biosystems, ABI Prism 3700). Base calling was performed by the software PHRED and vector sequences were masked with CrossMatch. Sequences containing at least 100 nonvector bases with Phred values >20 were used for further analysis. All programs are running under Solaris 5.7 on a Sun Sparc Ultra 10 workstation. The sequences are housed in a MySQL (v.3.23.33) database and are accessible online. The GenBank accession numbers of the sequences are 3576383–3595739. The program PrimeArray (RADDATZ *et al.* 2001) was used to design primers for testing conformational polymorphisms.

Preparation of meiotic mapping panel and PCR amplification: The meiotic mapping panel was prepared from 46 randomly picked F₂ animals from a cross between phenotypically marked hermaphrodites of the California strain and Washington strain males. F₂ animals were cloned and genomic DNA was extracted from them using the Bio-Rad (Richmond, CA) genomic DNA extraction kit. For a typical PCR assay, 1/1000 of this DNA preparation was used as a template. PCR amplification was pipetted in 96-well plates on a Beckman (Fullerton, CA) Biomek robot in 20-μl reaction volumes. A typical PCR reaction mixture consisted of 10 mM Tris-HCl, 50 mM KCl, 1.5 mM MgCl₂, 200 μM dNTP, 1 unit *Taq* polymerase, and 1 μM of each primer. Thermocycling was done in a Perkin-Elmer (Norwalk, CT) Gene Amp 9700 PCR machine under standard conditions consisting of an initial denaturation at 94° for 3 min; followed by 30 cycles of 94° for 1 min, 50° for 1 min, and 72° for 1 min; and a final incubation at 72° for 7 min. The PCR products were first confirmed on a 1% agarose gel before they were loaded onto SSCP gels.

Gel electrophoresis and SSCP detection: For SSCP detection, the samples were diluted 1:1 in denaturing solution (95% formamide, 0.1% xylene cyanol, 0.1% bromophenol blue, and 10 mM NaOH), denatured at 95° for 5 min, and loaded onto a GeneGel Excel prepoured 6% acrylamide gel (Pharmacia Biotech, Piscataway, NJ). Gels were fixed and silver stained to detect the DNA. To count for polymorphisms, mobility differences between the California and Washington PCR products were checked at both the single- and the double-stranded DNA levels. Wherever polymorphisms were detected between the two strains, the Washington bands were sequenced. Both the sequences were assembled using the program Sequencher

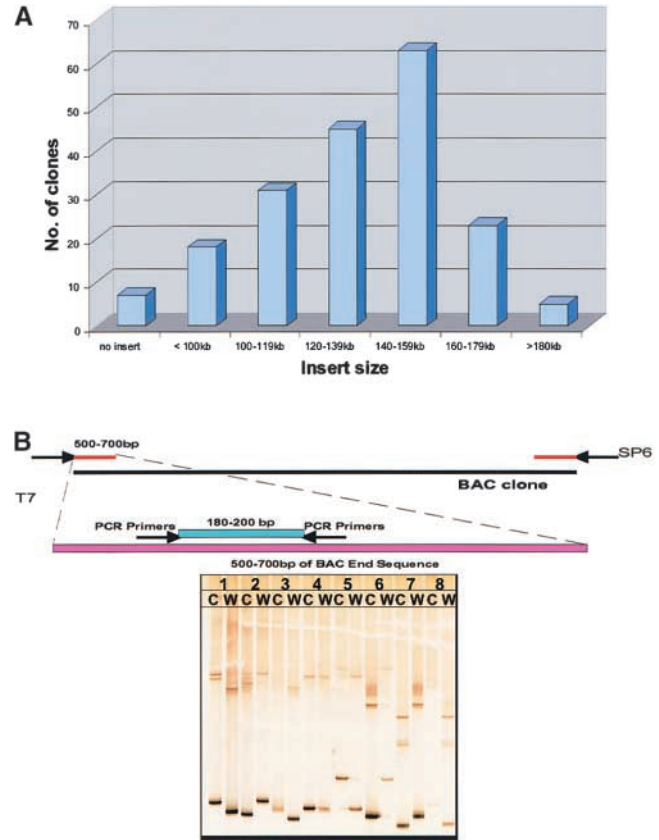


FIGURE 1.—(A) Insert size distribution of 192 randomly picked BAC clones. (B) Strategy for SSCP marker detection. PCR primers were designed to amplify a 180- to 200-bp fragment in BAC end sequences. California and Washington amplicons were tested for polymorphisms using the SSCP technique.

and the differences were tabulated. Ninety-three of the polymorphisms were characterized at the sequence level.

Data collection and linkage analysis: Data were collected using Map Manager software (<http://mcbio.med.buffalo.edu/mapmgr.html>) and analysis was conducted with Map Manager and MAPMAKER software (LANDER *et al.* 1987). Each locus was placed initially at the position that maximized its LOD score as indicated by the "Make Linkage Groups" command of Map Manager. Local order was then determined by manually placing the marker at the location that minimized the number of double recombinants. Data analysis ordered 122 loci into six linkage groups that were supported by LOD scores ≥ 5 . Map graphics were created with MAPMAKER using the Kosambi mapping function and later on modified for clarity with FreeHand 9.0.

RESULTS AND DISCUSSION

BAC library formation and BAC end sequencing: We constructed a BAC library of *P. pacificus* var. California in the pIndigoBAC-536 vector using *Hind*III partial digests (SHIZUYA *et al.* 1992). In total, 13,440 BAC clones were generated with insert sizes ranging from 100 to 180 kb and an average insert size of 128 kb (Figure 1a). All BAC clones were sequenced from both ends, yielding

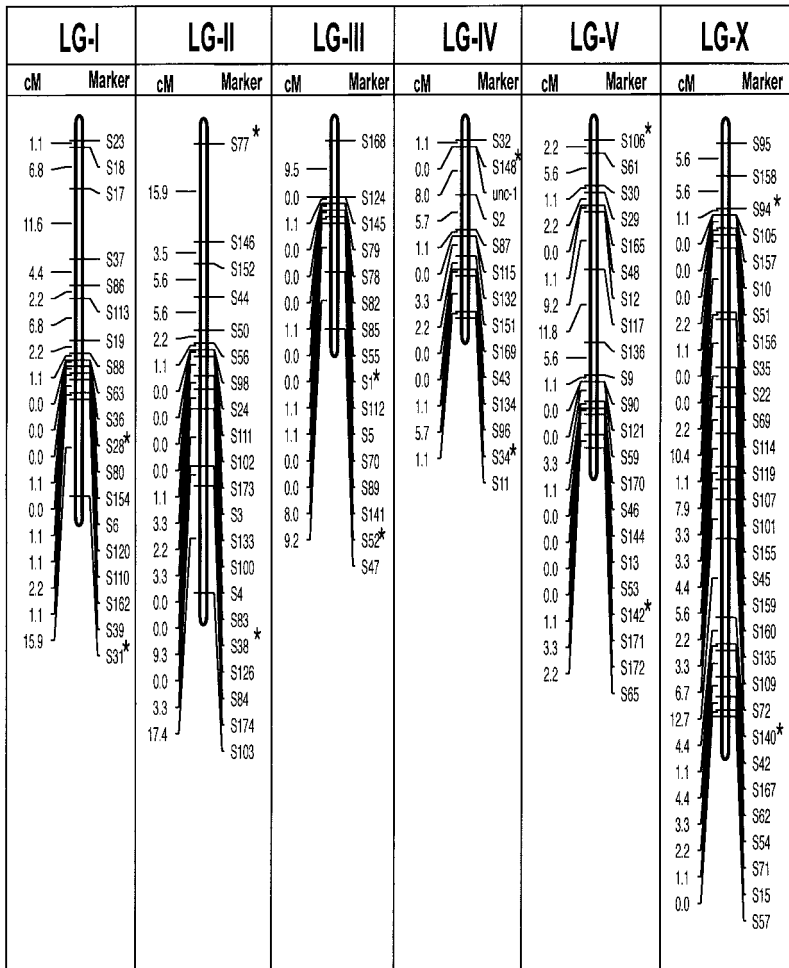


FIGURE 2.—A genetic linkage map of *P. pacificus*. DNA samples were prepared from 46 randomly picked F₂ progeny of an original cross of a hermaphrodite of the California strain and a male from the Washington strain. Amplified fragments were separated on SSCP gels. Linkage groups were identified with the program Map Manager (Stanford University). All map positions are supported by LOD scores ≥ 5 , using the Kosambi mapping function to estimate the occurrence of double crossovers. Asterisk indicates representative SSCP markers for mapping mutants to particular chromosomes.

18,133 good-quality sequences, 1400 of which have similarities to known *C. elegans* sequences. All sequences are accessible via GenBank (see MATERIALS AND METHODS).

Polymorphisms generated by SSCP analysis: Previous studies revealed substantial polymorphisms between various strains of *P. pacificus* (SCHLAK *et al.* 1997; SRINIVASAN *et al.* 2001). The highest degree of polymorphisms was observed between the wild-type strain from Pasadena, California and the strain from Port Angeles, Washington (SRINIVASAN *et al.* 2001). Our strategy involved designing PCR primers to randomly amplify a 180- to 200-bp amplicon (Figure 1b) in a subset of the sequenced BAC ends using PrimeArray (RADDATZ *et al.* 2001). The resulting PCR fragments were analyzed for the presence of polymorphisms using the SSCP technique (ORITA *et al.* 1989). From the 480 PCR reactions performed, 93 resulted in SSCP markers. These were confirmed by comparing the sequences of the PCR products of both strains. We observed 260 substitutions (73.7%) and 93 insertion/deletions (indels; 26.3%). *C. elegans* has slightly higher substitution rates (75.1%) and correspondingly lower indels (24.9%) between the most variable strains (KOCH *et al.* 2000; WICKS *et al.* 2001). Of the observed substitutions in *P. pacificus*, 47.3% were

transitions and 52.7% were transversions, which is exactly the converse of that in *C. elegans* (KOCH *et al.* 2000; WICKS *et al.* 2001).

The BAC-end-based SSCP analysis was complemented by a search for SSCP markers in a subset of the existing *P. pacificus* expressed sequence tag (EST) clones and previously cloned vulval patterning genes. In a total of 119 ESTs and genes tested by SSCP analysis, 40 (33%) were polymorphic. Together, 133 SSCP markers were generated from both BAC end and EST sequences.

A genetic linkage map of *P. pacificus*: To construct a genetic linkage map, we scored the 133 SSCP markers on a meiotic mapping panel. After crossing a genetically marked California hermaphrodite with Washington males, 46 random F₂ individuals were cloned, constituting the meiotic mapping panel. Linkage analyses assembled 122 of the 133 SSCP markers on a map with six linkage groups, which corresponds to the observation of six chromosomes after 4',6-diamidino-2-phenylindole (DAPI) staining of *P. pacificus* oocytes (Figure 2; SOMMER *et al.* 1996). All map positions were supported by LOD scores ≥ 5 and there were no gaps ≥ 20 cM (Figure 2). The remaining unassigned SSCPs are mostly unlinked to one another. The 122 assigned SSCP markers occur

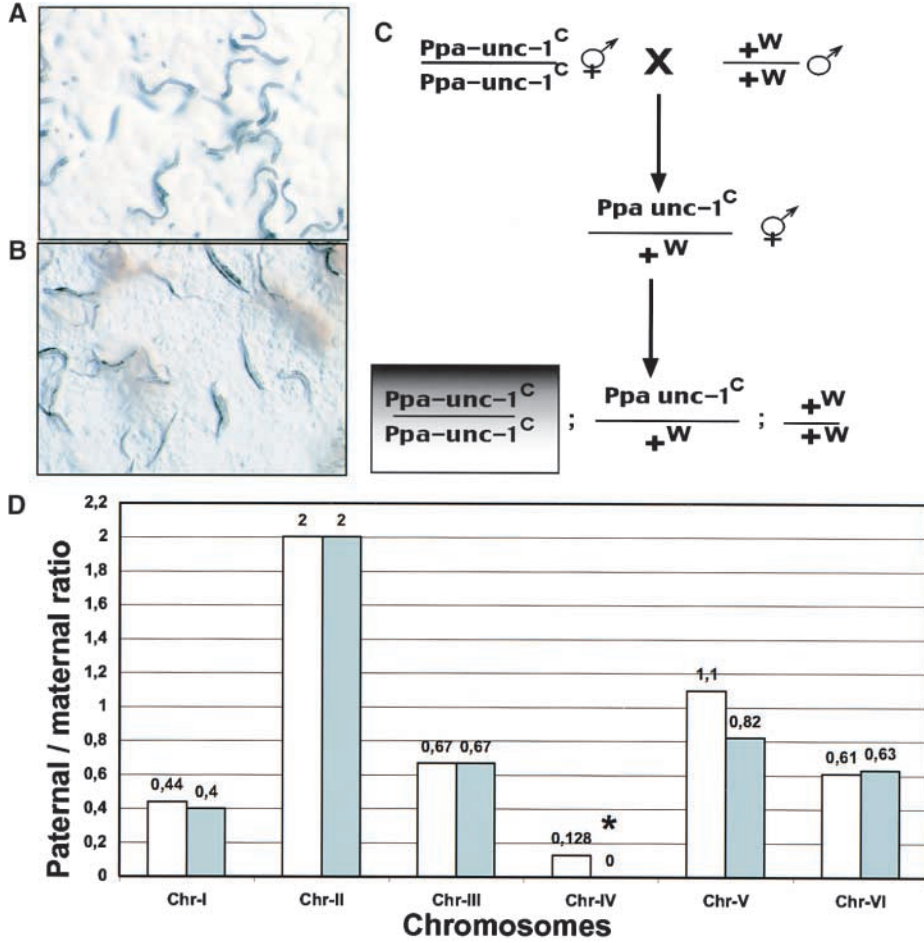


FIGURE 3.—(A and B) Photographs of *P. pacificus* wild-type (A) and *Ppa-unc-1* mutant animals (B). (C) Crossing scheme for obtaining Washington-backcrossed mutant animals for mapping. (D) Chromosome localization for *Ppa-unc-1*. One of the two markers on chromosome IV gives a 0 W/C ratio (*).

ped 88 unique map positions. In total, the linkage groups spanned 338.6 cM.

Mapping of *Ppa-unc-1*: Our SSCP-based genetic linkage map provides a unique source of information to facilitate mapping of experimentally derived mutations

in *P. pacificus*. To demonstrate the efficiency of map-based cloning, we mapped *Ppa-unc-1*, which has previously been suggested to represent the homolog of *Cel-unc-22/Twitchin* based on the unique phenotype of mutant animals (Figure 3, a and b; SOMMER *et al.* 1996). California-derived mutant hermaphrodites were crossed with Washington males and 21 clones of mutant F₂ animals were established (Figure 3c). Two representative SSCP markers were selected per chromosome (Figure 2). The selected markers were tested on these 21 clones for segregation of the paternal Washington (W) *vs.* maternal California (C) pattern. The segregation was measured as a unitless map ratio, W/C. Unlinked markers are expected to be represented equally and should have a ratio of ~1. In contrast, linked markers have a predominant maternal segregation pattern and therefore yield a value close to 0. Our analysis of Washington-backcrossed *Ppa-unc-1* animals showed clear linkage to chromosome IV (Figure 3d). Specifically, all 21 clones segregated the maternal pattern of the marker S148 (Figure 3d).

To verify this result, we cloned a genomic fragment of the *Cel-unc-22/Twitchin* ortholog from *P. pacificus* and generated an SSCP marker in this gene fragment. We mapped the *Ppa-unc-1*-obtained SSCP marker on the

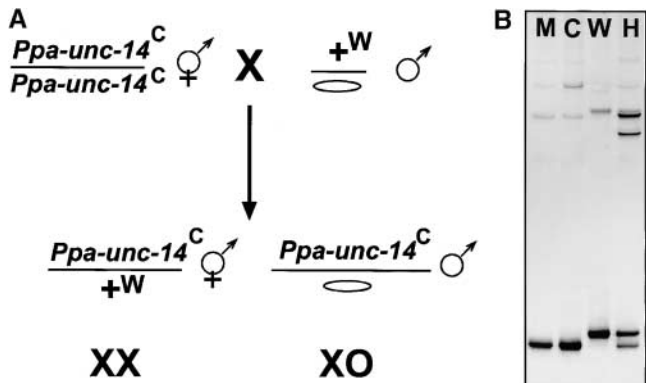


FIGURE 4.—(A) Crossing scheme for obtaining hemizygous F₁ males. (B) SSCP analysis of hemizygous F₁ males with the marker S105. Only the California variant was amplified, indicating that S105 represents an X-chromosomal marker. Other SSCP markers tested were S140 and S155. M, pooled F₁ males; C, W, and H, California, Washington, and heterozygous control DNAs, respectively.

genetic linkage map and found that it had a position identical to S148 (Figure 2). This result confirms that *Ppa-unc-1* represents the homolog of *Cel-unc-22* and also indicates that *P. pacificus* mutations can be successfully mapped using the existing genetic linkage map.

Mapping of the *P. pacificus* X chromosome: Previous studies revealed that *P. pacificus* males are induced by meiotic nondisjunction of the X chromosome (SOMMER *et al.* 1996). To identify which of the linkage groups corresponds to the X chromosome, we looked for linkage of the representative SSCP markers to the X-linked genetic marker *Ppa-unc-14* (SOMMER *et al.* 1996). California-derived mutant hermaphrodites were crossed with Washington males and F₁ males were pooled for further analysis (Figure 4a). These males are heterozygous for all autosomes but hemizygous for the California-derived X chromosome. S105 and other SSCP markers on the same chromosome segregated only the maternal pattern, thereby specifying this chromosome as the X chromosome (Figure 4b).

Comparison to *C. elegans*: This study provides a genetic linkage map of *P. pacificus* and lays the foundation for positional cloning. We mapped 133 SSCP markers on a meiotic mapping panel composed of 46 randomly picked F₂ *P. pacificus* animals. The average interval between markers is between 2 and 3 cM/SSCP and is similar on all linkage groups. However, it remains unknown if these SSCP markers provide an equal representation of the genome. As most SSCP markers are BAC derived, a potential underrepresentation of certain chromosomal regions in the BAC library might cause these regions to be excluded from the map.

A comparison of the genetic linkage map of *P. pacificus* with that of *C. elegans* indicates no overall synteny between the *P. pacificus* and the *C. elegans* genomes. For example, the cell death regulator *ced-3* is located on chromosome IV in *C. elegans* together with *unc-22* and *let-60*. In contrast, *Ppa-ced-3* (S173) is located on chromosome II, whereas *Ppa-unc-1* and *Ppa-let-60* (S2) are located on chromosome IV (Figure 2). Similar results have been obtained during mapping and cloning of vulval patterning genes (J. SRINIVASAN and R. J. SOMMER, unpublished observation). However, our analysis does not rule out the existence of small-scale synteny, which will be revealed by future sequencing efforts. These findings are in agreement with studies on *C. elegans* and *C. briggsae*, also indicating a violation of synteny (KENT and ZAHLER 2000).

Another striking feature of the *C. elegans* genome is the central clustering of genes (*C. ELEGANS SEQUENCING CONSORTIUM* 1998). However, as most SSCP markers of *P. pacificus* were generated from BAC ends, no exact information is available on the distribution of genes along the chromosomes. Similarly, the relationship between the recombination rates and gene density and the gene number per chromosome awaits further analysis.

Toward an integrated genome map of *P. pacificus*: Our genetic linkage map is built mostly on BAC-end-derived SSCP markers. Currently, this study is complemented by a physical mapping project of the complete BAC library using amplified fragment length polymorphism fingerprinting (T. JESSE, M. DE BOTH and R. J. SOMMER, unpublished data). Contigs generated in the physical map project can be anchored on the genetic linkage map by generating one SSCP marker per contig and testing it on the mapping panel. Together, the genetic and physical maps will provide an integrated genome map of *P. pacificus* because both are BAC clone based, a feature that allows the compatibility of BAC markers and clones used in both projects.

Furthermore, this work establishes *P. pacificus* as the primary candidate for comparative nematode genomics, with the long-term goal of sequencing its genome and comparing it to *C. elegans*. The comparison of the genome sequence of two distantly related free-living nematodes is also a prerequisite for studying and understanding the genome sequence of parasitic nematodes, many of which cause major diseases in humans and animals.

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