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Morphological, genetic and molecular description of *Pristionchus pacificus* sp. n. (Nematoda : Neodiplogastridae)

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Summary – We describe a new free-living hermaphroditic nematode, *Pristionchus pacificus* sp. n. (Neodiplogastridae) that will be useful for genetic, developmental and molecular biological studies. *P. pacificus* sp. n. has six chromosomes, a three day generation time and is easily cultured. Forty-eight morphological mutations are described indicating the genetic accessibility. Molecular studies have been initiated with the generation of a genomic and a cDNA library and the cloning of the homologue of the *Caenorhabditis elegans let-60 ras* gene.

Résumé – ***Description morphologique, génétique et moléculaire de* Pristionchus pacificus *sp. n. (Nematoda : Neodiplogastridae)*** – Description est donnée d'une nouvelle espèce hermaphrodite de nématode libre, *Pristionchus pacificus* sp. n. (Neodiplogastridae), qui pourra être utilisée pour des études de génétique, de développement et de biologie moléculaire. *P. pacificus* sp. n. possède six chromosomes, un cycle de reproduction de trois jours et s'élève facilement. Quarante-huit mutations morphologiques sont décrites, ce qui indique l'accessibilité à l'étude génétique. Des études moléculaires ont débuté par l'établissement de banques d'ADN génomique et d'ADNc et par le clonage de l'homologue du gène *let-60 ras* de *Caenorhabditis elegans*.

Key-words : evolution, development, genetics, let-60 *ras gene, Pristionchus,* nematodes.

Comparative approaches can be used in many fields of biology including non-phylogenetic disciplines such as molecular or developmental biology to reveal general mechanisms. The power of this approach depends on the tools available, and the extent of knowledge of organisms in question. For the free-living nematode *Caenorhabditis elegans,* the combination of genetics, cell lineage analysis and molecular biology has generated a detailed understanding of many aspects of developmental and neurobiology. Here, we describe a new free-living hermaphroditic nematode, *Pristionchus pacificus* sp. n. (Neodiplogastridae), that will be useful for genetic, developmental and molecular comparative studies. In addition to morphological characters we provide genetic and molecular data indicating that this organism can be used in a way similar to *C. elegans*. We describe 48 morphological mutations that can be used for genetic mapping. We have initiated molecular analysis by the construction of a genomic and a cDNA library, and the cloning of the homologue of the *C. elegans let-60 ras* gene, a molecular switch gene involved in vulval development.

Materials and methods

NEMATODE CULTURE IN THE LABORATORY

Unless otherwise noted, *C. elegans* standard techniques have been used according to Wood (1988).

MAINTENANCE OF STOCKS

Stocks are maintained on 5 cm NG agar plates seeded with OP50, a uracil requiring mutant of *E. coli* (Brenner, 1974; Wood, 1988). Worms for daily use were grown at 20 °C, whereas they were incubated at 15 °C for long-term culture. Cultures are maintained by transferring several hermaphrodites. Male cultures were obtained by seeding plates with several hundred animals until bacteria is depleted, and then transferring to a new plate. Handling of the culture of *Pristionchus pacificus* sp. n. is very similar to *C. elegans,* so that all general features described for *C. elegans,* apply to the new *Pristionchus* species (Brenner, 1974; Wood, 1988).

MEASUREMENTS

Morphometric measurements were made on both heat killed and formalin-fixed, glycerin processed

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worms. The glycerin processed type specimens were prepared by the method of Seinhorst (1959). Heat processed worms were microwaved in 2 ml. M9 buffer at 50 % power in a Hotpoint® 1.45 kw microwave oven for 13-15 s. All specimens were measured on an agar pad with an ocular micrometer. Drawings were made from live worms relaxed with 100 mM azide in the agar pad with a Zeiss microscope, Nomarski optics and camera lucida.

STAINING

Nematodes were stained with 1 % Coomassie blue and observed within an hour of sealing with nail polish to highlight phasmids and body pores (Premachandran *et al.*, 1988).

LIFE-CYCLE

The life cycle of *P. pacificus* sp. n. was measured at 20 ° and 25 °C by transferring freshly laid eggs to new plates and observing the appearance of eggs of the next generation after self fertilization.

MATINGS

Two plates each containing five young males and one hermaphrodite were set up for each crossed pair of isolates and observed after 4-7 days. A cross was deemed successful if more than two males, but typically up to 50 % of the population, occurred in the F1 generation.

BROOD SIZE

The brood size was determined for self-fertilizing hermaphrodites by transferring L4 animals to individual plates at 20 °C.

GENETIC ANALYSIS

Chromosome staining

The chromosome number was determined by Hoechst 33258 staining of oocytes in gravid, squashed hermaphrodites. Adult worms are washed off plates in M9 buffer and transferred into a small volume. Specimens were fixed in 4 % formaldehyde solution in M9 buffer for 15 min and Hoechst 33258 (final concentration 0.1 µg/ml) stained in M9 buffer for 5 min. Worms were immediately transferred to slides and analyzed under epifluorescence.

Mutagenesis

Mixed stage animals were washed off the plates in M9 buffer, and ethyl methanesulphonate (EMS) added to a final concentration of 50 mM for 4 h at 20 °C. The suspension was washed in M9 five times and the worms were spotted onto the surface of two NG plates. After 1 h excess liquid had been absorbed and individual motile L4 hermaphrodites were picked individually to plates (9 cm plates). The number of eggs laid by the parent was counted; the parent was removed after 20 to 30 eggs were laid. Plates were incubated at 20 °C and young adult animals of the F2 generation were observed for mutant phenotypes as described below. To ensure independence of mutations, only one clonal culture of a particular phenotype was kept per parent.

Analysis of mutants

Mutants were backcrossed using five wild-type males and two mutant hermaphrodites per cross. Autosomal recessive mutants generate wild-type F1 progeny, which were picked one per plate. Their self progeny include one quarter mutant animals if the phenotype was due to a single mutation. More than 80 % of the mutants backcrossed successfully. Permanent cultures were established from single mutant hermaphrodites. Sex-linkage was indicated by the F1 male progeny of the backcross displaying the mutant phenotype.

Nomenclature

C. elegans nomenclature was adopted (Horvitz *et al.*, 1979), with the prefix "*Ppa*" indicating the species. Dumpy alleles are named *Ppa-dpy*, uncoordinated movement alleles are named *Ppa-unc* and roller alleles are named *Ppa-rol*. Each mutation is assigned a unique allele number (e.g. *sy#* for Sternberg lab; *tu#* for Sommer lab).

MOLECULAR BIOLOGY

Standard techniques were used according to Sambrook *et al.* (1989) unless otherwise noted.

Genomic library

Genomic DNA (80 µg) of *P. pacificus* sp. n. was partially digested with Sau 3A and 600 ng of large DNA fragments cloned into Lambda Fix II using the partial fill-in-technique and the corresponding Stratagene Vector Kit (Stratagene, La Jolla). Packaging and titering were done according to standard procedures (Sambrook *et al.*, 1989).

cDNA library

For RNA isolation worms from approximately hundred 5 cm plates were harvested as mixed stage cultures yielding approximately 500 µl of packed worms. Worms were washed in M9 several times for a 3 h period to digest all OP50 bacteria. The supernatant was eliminated and the worms were frozen at – 80 °C. Total RNA was isolated in homogenization buffer comprising 3 M LiCl, 6 M urea, 0.1 % sodium dodecyl sulfate, 10 mM sodium acetate, pH 5.0), and was dissolved in 400 µl water (DEPC treated). mRNA was isolated using the mRNA Isolation Kit from Pharmacia (Uppsala). A total of 5 µg mRNA was obtained. cDNA was generated and cloned using the ZAP-cDNA Synthesis Kit from Stratagene.

PCR/Screening of libraries

A PCR fragment of the *P. pacificus* sp. n. *ras* gene, encoding a region of 41 amino-acids, was used for screening. PCR was performed according to standard technology (Sommer & Tautz, 1991). The following degenerate primers were used : RS9, 5′ ATCTCACK-TACGARNGTRTA 3′ (amino-acid position 157-163

in Fig. 4, sequence underlined); RS11, 5′ CCTATGG-TRYTRGTYGGNAA 3′ (amino-acid position 110-116 in Fig. 4, sequence underlined). Both libraries were screened for a homologue of the *C. elegans let-60 ras* gene (Han & Sternberg, 1990). Ten positives were obtained from a screen of 60 000 plaque forming units (pfu) from the genomic library. Two of these have been analyzed further and the clone 22 was subcloned into bluescript and M13 for sequencing. Twenty-five positives were obtained from a screen of 7×10^6 pfu from the cDNA library. In a secondary screen of eight of these positive plaques, four have been recovered. These clones (148, 141, 113 and 236) have an insert size between 500 bp and 1.4 kb. The longest cDNA, clone 141 was sequenced using the T3, T7 and internal primers.

Genome blotting

To estimate the genome size of *P. pacificus* sp. n., 1000 ng of genomic DNA was completely digested with EcoRI and was hybridized with a 220 bp fragment of the *P. pacificus* sp. n. *let-60 ras* gene. The signal was compared to control lanes containing 10 pg, 100 pg and 1 ng of fragment used for hybridization.

***Pristionchus pacificus* sp. n.**

(Fig. 1)

MEASUREMENTS

Holotype, hermaphrodites, dauer larvae and *males :* See Table 1.

DESCRIPTION

Hermaphrodite : Body slightly arcuate to straight when heat-relaxed. Cuticle with faint annulations (ten annules = 6.9 µm long) beginning behind the amphid at the level of the metastom, where nine rows of superficial bilinear punctations present (lateral view) increasing to twelve rows at midbody. One or two faint longitudinally oriented lateral incisures occasionally visible. Eleven lateral pores, each connected to an underlying glandular cell. Seven pores anterior to vulva, beginning at nerve ring level, and four posterior, above the phasmid. Six deeply divided lip sectors each bearing a single outer labial papilla, and connected by an anteriorly flattened, thin surrounding cuticle. Two faint oval-shaped amphid openings at the base of the non-indented lip region. Head width at mid stoma level/ width of stoma 2-2.8. Eurystom form of stoma relatively short and wide (length = 7-11 µm, width = 7-11 µm), with a ratio of stoma length to width ranging from 0.9-1.6. Stenostom form not observed in either California (PS312) or Washington (PS1843) adult populations. Cheilostom represented by a thin-walled arch above the prostom. Six prorhabdions in prostom dividing a basally rugose wall. Mesorhabdions attached to anterior procorpus. A large dorsal retrorse, shark-like tooth on the metarhabdion and smaller retrorse triangular right subventral tooth visible laterally, and four smaller teeth connected at their base (often only two or three are visible) left subventrally. Short telostom not cuticularized. Anterior (corpus) to posterior esophagus (postcorpus) ratio 1.5-1.8. Corpus median bulb muscular with oval-shaped valve. Basal bulb glandular with faint medial anchoring strands perpendicular to the pharynx lumen. Oval excretory pore near the basal bulb of the postcorpus at 10-20 % of body length from anterior end, or 75-108 % of pharynx length. Hemizonid just anterior to the excretory pore. Vulva transverse, occasionally protruding. Gonads opposed, antidromously reflexed (Lorenzen, 1978) after spermatheca, with ovary extending beyond vulva. Anterior genital branch positioned to the right of intestine; posterior to the left. Anterior genital branch from vulva to reflex slightly longer than posterior branch (anterior : 150-440 µm, 16-34 % of body length; posterior : 120-330 µm, 13-29 % of body length). As many as twelve eggs of up to eight cells are seen in egg-laying competent hermaphrodites. Rectum length approximately equal to anal body diameter. Phasmid 33-58 µm posterior to the anus, 20-30 % of tail length. Tail acute and long, tapering gradually from anus to a slender terminus.

Male : Body J-shaped when heat-relaxed. Cuticle with twelve bilinear punctate longitudinal lines per side of body, without lateral pores. Stomas as in hermaphrodite in 55 of 60 specimens, eurystom, with a stoma length to width ratio of 0.7-1.6. Five males with a narrow stenostom-type stoma have a length to width ratio of 1.7-4.5. Oval amphids at lip base, excretory pore at base of oesophagus (distance from pore to anterior end represents 10-20 % of body length) and phasmids 28-45 µm posterior to the anus (30-40 % of tail length) and between genital papillae 5 and 7. Testis single and recurved (proximal arm to reflex : 330-610 µm, 50-63 % of body length, distal reflex : 109-179 µm). Testis ventral relative to intestine, with the distal reflex either lateral or ventral to proximal arm. Male tail with a narrow bursa. Genital papillae 1, 3, 5, 6 oriented laterally, with papilla 6 just beyond papilla 9 in longitudinal sequence (numbering after Potts, 1910). Oval spicule head knob is positioned relative to the distal pointed, unfused tips approximately at a right angle. Gubernaculum slipper-shaped, with an open " toe " and " heel ", plus a " donkey "-shaped upper shield with a horn on the " forehead ". At the side of the gubernaculum the " ears " of the shield form a sharp, posteriorly-directed prominence which varies somewhat in size and lateral position.

Dauer larvae : J2 cuticle sheath present. Cuticle with chain-like superficial stipples forming four longitudinal lines per lateral view. Longitudinal lines extend from behind amphid to tail, merging behind phasmid. Lumen of buccal capsule closed, with plug present from cheilostom, often to telostom or anterior intestine. Intestine

Table 1. *Morphometrics for* Pristionchus pacificus *n. sp. strain PS312* (All measurements in μm; parameter ranges in parentheses).

	Hermaphrodites			Males		Dauer larvae	
	Glycerine *		Water *	Glycerine	Water	Glycerine	Water
	(Holotype)	(Paratypes)					
n		20	20	20	20	20	20
Length	1075	1075 ± 108 (750-1210)	1181 ± 155 (785-1410)	690 ± 27 (650-730)	802 ± 86 (700-1010)	265 ± 14 (240-296)	253 ± 22 (203-300)
a	17	13.4 ± 0.9 (12.1-16.8)	14.6 ± 1.4 (12.3-17.4)	15.2 ± 2.1 (12.5-18.9)	14.2 ± 1.0 (12.3-15.8)	24.1 ± 2.2 (19.9-27.6)	17.6 ± 1.9 (12.7-21.0)
b	8.0	7.8 ± 0.6 (6.3-8.6)	7.7 ± 0.8 (5.6-9.1)	5.6 ± 0.3 (5.1-6.1)	5.9 ± 0.4 (5.2-6.6)	3.1 ± 0.1 (2.9-3.4)	2.9 ± 0.2 (2.4-3.4)
c	4.7	5.2 ± 0.5 (3.8-6.0)	5.2 ± 0.7 (4.1-6.7)	6.0 ± 0.4 (5.3-6.7)	7.2 ± 0.7 (6.4-9.4)	7.6 ± 0.7 (6.9-9.9)	7.1 ± 0.5 (5.2-7.7)
V	0.43	0.45 ± 0.03 (0.42-0.59)	0.46 ± 0.03 (0.42-0.55)				
c'	9.6	7.6 ± 0.6 (6.7-9.6)	6.1 ± 0.7 (5.0-7.2)	3.7 ± 0.3 (2.9-4.0)	3.0 ± 0.4 (2.1-3.6)	4.1 ± 0.4 (3.6-5.0)	3.6 ± 0.4 (2.8-4.4)
Width	64	81 ± 10 (60-100)	82 ± 14 (60-105)	46 ± 6 (35-55)	57 ± 6 (45-70)	11 ± 1 (10-13)	15 ± 2 (11-19)
Oesophagus length	135	138 ± 7 (120-150)	152 ± 10 (140-170)	123 ± 5 (110-130)	137 ± 12 (125-170)	85 ± 3 (75-90)	86 ± 6 (75-98)
Vulva (for dauers, genital primorduim)	465	484 ± 37 (410-560)	541 ± 72 (430-670)			153 ± 9 (134-165)	150 ± 11 (125-176)
Tail	230	208 ± 21 (170-250)	229 ± 38 (171-302)	115 ± 7 (101-124)	113 ± 17 (80-157)	35 ± 3 (27-40)	36 ± 2 (31-40)
Anal body diam.	24	27 ± 3 (22-32)	35 ± 4 (28-43)	32 ± 2 (28-37)	37 ± 3 (30-45)	9 ± 1 (7-10)	10 ± 1 (8-13)
Phasmid	42	40 ± 4 (33-50)	45 ± 6 (28-52)	29 ± 3 (23-34)	34 ± 4 (28-45)	16 ± 2 (11-19)	15 ± 1 (12-16)
Stoma length	10	11 ± 1 (8-12)	13 ± 1 (10-15)	10 ± 1 (9-11)	9 ± 1 (7-12)		
Stoma width	9	9 ± 1 (7-10)	10 ± 1 (9-13)	6 ± 1 (5-8)	7 ± 2 (2-11)		
Excret. pore to ant. end	120	129 ± 13 (100-165)	147 ± 11 (134-171)	116 ± 7 (105-125)	132 ± 13 (115-151)		
Anterior oesophagus	85	86 ± 3 (80-94)	93 ± 6 (85-105)	75 ± 2 (72-80)	86 ± 7 (78-106)		
Posterior oesophagus	50	52 ± 3 (45-56)	59 ± 5 (50-70)	48 ± 2 (44-50)	51 ± 6 (42-66)		
Spicule				32 ± 1 (30-35)	33 ± 2 (29-36)		
Gubernaculum				16 ± 1 (15-17)	16 ± 1 (15-17)		

* Glycerine, water : specimens mounted in glycerine or water respectively.

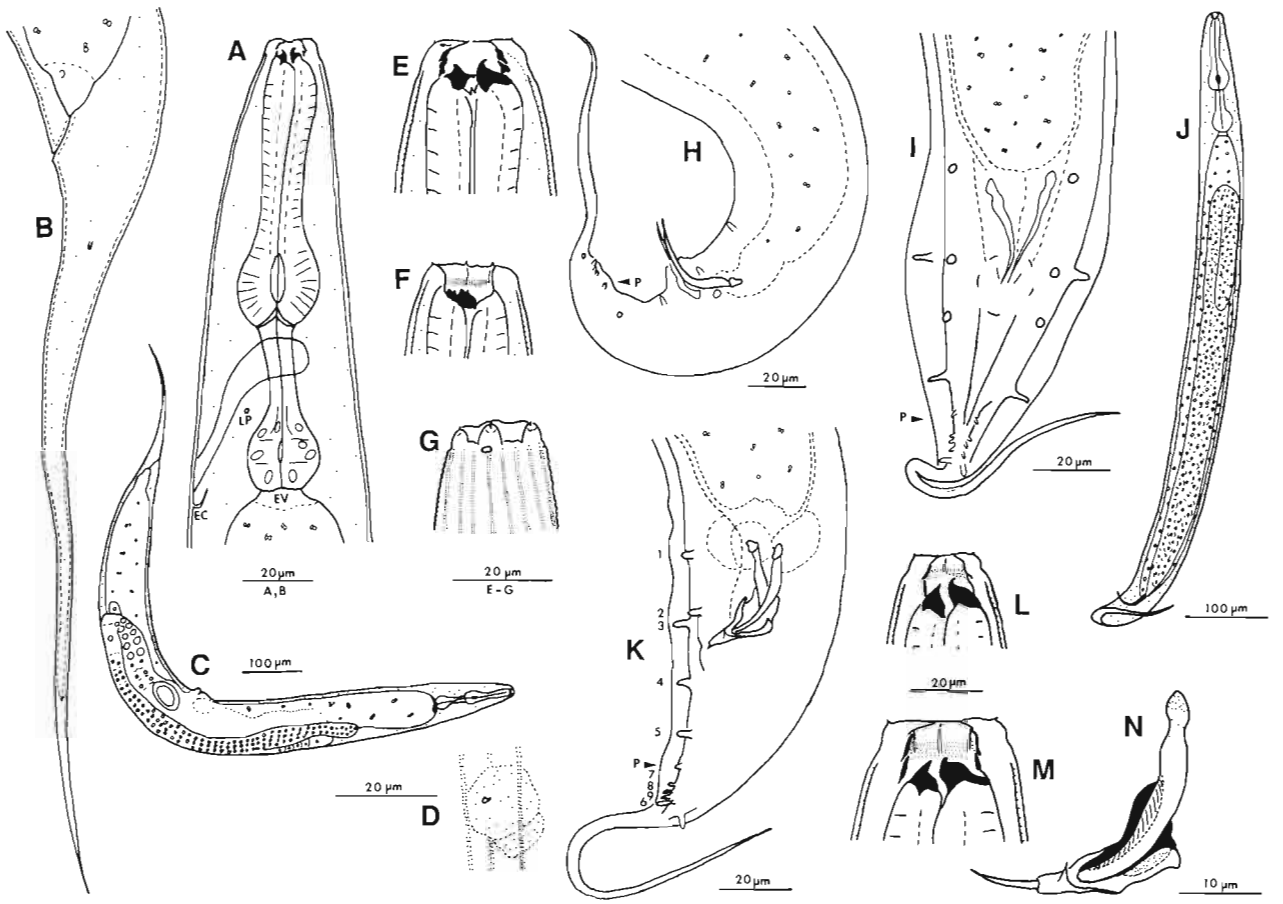

Fig. 1. Pristionchus pacificus *sp. n. Hermaphrodite. A : Oesophagus with excretory canal (EC), lateral pore (LP), oesophago-intestinal valve (EV), muscular anterior oesophagus, glandular posterior oesophagus; B : Tail with rectum and phasmid; C : Full body showing paired, antidromously reflexed gonads; D : Lateral pore and underlying glandular cell; E : Lateral view of stoma, largest tooth dorsal; F : Ventral view of smallest teeth; G : Cuticle at lip region showing lip papillae, amphid, stippled longitudinal lines – Male. H : Lateral view tail, with nine papillae, phasmid (P), spicules, and gubernaculum; I : Ventral view tail; J : Whole body with testis; K : Ventro-lateral view tail with rectal glands, spicule, gubernaculum, phasmid (P), nine papillae (papillae numbering system after Potts, 1910); L : Stenostom-type stoma; M : Eurystom-type stoma; N : Spicule and gubernaculum, lateral.*

with dense lipid granules and occluded intestinal lumen. Lip region strongly indented with amphids at indent. Amphids oval, framed by a thickened cuticular rim. Distinct papillar phasmid. Two small oval gonad arms each containing six to thirteen cells. Pharynx with median bulb and slender corpus and isthmus. Under a dissecting microscope dauers appearing slender, with very dark intestine and relatively rapid movement.

Type locality

Soil in a flower garden at the north end of Blair H. S., Pasadena by C. Tang and C. Lamb and obtained through Dr. G. Nelson, JPL, Pasadena, in 1988 (PS312). Other locality : Hurricane Ridge, Port Angeles, Washington (PS1843) sampled by A. Whittlesey in 1994.

Type specimens

Holotype and paratype slides are deposited in the nematode collection at the Department of Nematology, University of California, Riverside, CA 92521 as 30004-30007. Other paratype slides available from the USDA. Nematode Collection, Beltsville, MD, USA, Muséum National d'Histoire Naturelle, Paris, France, and the German Nematode Collection (DNST), Münster, Germany. Frozen stock available from the *Caenorhabditis* Genetics Center, 250 Biological Sciences Center, University of Minnesota, 1445 Gortner Ave., St. Paul, MN 55108-1095, USA.

Table 2. *Morphometric ranges for* Pristionchus *spp. (after Andrássy, 1984).*

	pacificus sp. n.	*maupasi*	*lheritieri*	*sexdentati*	*aequidentatus*	*robustus*
HERMAPHRODITES						
n	20	?	3	50	1	1
Length (µm)	750-1210	700-1260	940-1840	770-1340	1300	2490
a	12-17	11-21	16-17	16-26	19	20
b	6-9	5-8	6-7	5-8	7.5	10
c	4-6	4-6	4-5	12-23	9	8
V	42-59	45-59	43-50	50-64	55	50
ant/post esophagus	1.5-1.8	1.4-1.8 *	1.6-2.0	1	1.2	1.6
MALES						
n	20	?	2	50	4	1
Length (µm)	650-730	540-820	560-1260	600-1130	860-920	1400
Spicule (µm)	30-35	32-53 *	37-43	36-48	46	47
a	13-19	14-24	13-20	17-34	13-14	20
b	5-6	4-6	5-6	5-8	6	7
c	5-7	4-6	5-9	5-6	9	10

* Heat-treated nematodes (n = 10).

DIAGNOSIS AND RELATIONSHIPS

P. pacificus sp. n. hermaphrodites and males are of relatively short length, medium width and long tail for the genus. Massive stomatal teeth with triangular subdorsal teeth having acute, retrorse apices, oriented upward in the stoma. Prominent bilinear longitudinal lines are superficially stippled. Faint annulations along the cuticle. Hermaphrodite tail gradually tapers to a thread-like terminus. Male tail papilla 6 positioned at or beyond papilla 9. Males produce cross-progeny with hermaphrodites.

Table 2 compares *Pristionchus pacificus* sp. n. with the five other species of genus *Pristionchus* Kreis, 1932, which are keyed in Andrássy's (1984) monograph.

P. pacificus sp. n. hermaphrodites have a stouter body, longer anterior oesophagus relative to posterior, shorter spicules, and much longer tail than *P. aequidentatus* (Schuurmans Stekhoven & Teunissen, 1938) Andrássy, 1984, *P. robustus* (Maupas, 1900) Paramonov, 1952 and *P. sexdentati* (Blinova & Vosilite, 1978) Andrássy, 1984 females.

P. pacificus sp. n. shares a median vulva, slight lip indentation and hermaphroditism with *P. maupasi*. However, unlike *P. pacificus* sp. n., *P. maupasi* (Potts, 1910) Paramonov, 1952 has less acute tooth apices, with a basally wider, less upright dorsal tooth. Compared to the gradual tail taper of *P. pacificus* sp. n., live and published descriptions of *P. maupasi* (Steiner, 1929; Weingärtner, 1955) have a dramatic change in tail diameter over a shorter length. There is an asymmetric crook below the small phasmid and a very short distance where the tail is thread-like. *P. maupasi* also has more prominent annulations and no superficial longitudinal stippling. Even in live material, its phasmids and excretory pores are difficult to see due to the dense surrounding tissue. *P. maupasi* males do not produce cross-progeny when mated with conspecific hermaphrodites.

P. pacificus sp. n. shares certain features with *P. lheritieri*, such as a long hermaphrodite tail with a long thread-like distal region, and bilinear punctate longitudinal lines. Unlike *P. pacificus* sp. n., *P. lheritieri* (Maupas, 1919) Paramonov, 1952 is amphimictic. *P. pacificus* sp. n. has a slightly indented lip, unlike the unindented anterior head region of *P. lheritieri*. In *P. pacificus* the subdorsal teeth are more robust and their apices more upright within the stoma than in *P. lheritieri*. The ratio of the male gonad arm to its reflex is 2.0-4.4 *vs* 1.9-2.8 in live *P. lheritieri*. The gubernaculum of live *P. lheritieri* is significantly larger than *P. pacificus* (18 ± 1 [n = 10] *vs* 16 ± 1 µm, [n = 20]).

Juvenile hermaphrodites have a narrow stenostom mouth, unlike the strictly eurystomal adult hermaphrodites. The width dimensions of the five males with visually distinctive stenostom type stomas (1.7-4.5 l/w ratio) transition without a gap to the predominant eurystom dimensions (0.7-1.6) of the remaining fifteen in the sample. One subsample of 20 PS312 males showed a significantly discrete ($P < 5$ %) intermediate stoma width (7.4 ± 2 µm) between the fully eurystom (9.3 ± 1 µm [n = 20] 5/93) and stenostom (3.8 ± 1.3 µm [n = 5] 4/95) forms. A significant difference in mounted male paratype stoma widths (6.1 ± 1 µm [n = 20]) was found compared to each heat-treated subpopulation ($P < 5$ %, 4/95, $P < 0.1$ %, 5/93). There was no significant difference in hermaphrodite stoma widths between heat-treated and mounted paratypes, or among heat-treated samples.

Two discrete mouth width groups were reported in *P. maupasi* and *P. lheritieri* (Hirschmann *in* Weingärtner, 1955). Each population had a unique ratio of mouth forms. The proportion of stenostom to eurystom individuals was constant over many generations within each population studied (Hirschmann, 1951). In our lab this population of *P. lheritieri* had primarily stenostom stomas and *P. maupasi* had strictly eurystom stomas under the same conditions as for PS312.

The predominantly eurystomal pattern of adults in *P. pacificus* sp. n. is different than that in the dioplogasterid *Aduncospiculum halicti* (Giblin & Kaya, 1984). In *A. halicti,* all males were always stenostomatous, and only a small proportion of the females were eurystomatous. The eurystomatous proportion of the population decreased dramatically with culture time within a few months after being isolated as dauers from the phoretic bee host (Giblin & Kaya, 1984).

Biological data

All isolated strains of *P. pacificus* sp. n. grow on *E. coli* OP50. The life cycle is 3.5 days at 20 °C and 2.5 days at 25 °C. Self-fertilizing hermaphrodites lay an average of 198 eggs (150-250) over a period of several days with a maximum at the second day of adulthood. Adult hermaphrodites are larger than males. In unstarved populations males occur only at a very low frequency of *ca* 0.1 %. However, large numbers of males can easily be obtained if worms are continually starved (mass-transfer from plate to plate over many generations). Following this transfer procedure, hundreds of males have been generated daily by culturing around ten plates.

In squashed preparations six chromosomes are visible that are indistinghuisable in diakinesis (Fig. 2). However, we cannot rule out that additional small chromosomes exist which are invisible using squashed stained oocytes.

Mating experiments

Matings of the *P. pacificus* sp. n. type population, PS312, were successfully made with another population from Washington (PS1843). Matings of *P. pacificus* sp. n. with *P. lheritieri* and *P. maupasi* did not yield cross progeny. The mating was reciprocal for males and females of *P. lheritieri* with both males and hermaphrodites of *P. pacificus* sp. n. Males of *P. pacificus* sp. n. were mated unsuccessfully with hermaphrodites of *P. maupasi.* The three rare males of *P. maupasi,* which were crossed with conspecific hermaphrodites, were incapable of producing cross male progeny, as previously described (Potts, 1910).

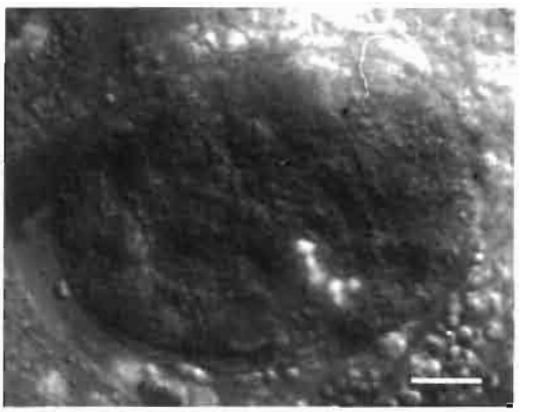

Fig. 2. *Photomicrograph of* Pristionchus pacificus *sp. n. oocytes stained with Hoechst 33258 and vizualized under epifluorescence* (Scale bar : 10 μm).

Genetics

P. pacificus sp. n. has self-fertilizing hermaphrodites and cross-fertilizing males, similar to *C. elegans.* Thus, a genetic analysis can be carried out using the procedure developed for *C. elegans* (Brenner, 1974).

Isolation of mutants

Genetic mutations have been isolated with ethyl methane sulfonate (EMS) in F2 clonal screens standard to *C. elegans* (Brenner, 1974). Single mutagenized L4 worms were picked onto 9 cm plates and were removed from the plates after laying 20-30 eggs. These F1 progeny will not include homozygous recessive mutant animals because at the stage of EMS treatment in the P0 no cells that give rise to both sperm and eggs are present. Single heterozygous F1 animals will produce one quarter homozygous mutant F2 progeny after self fertilization. At this stage, the plate that was initiated by a single parent contains between 10^4 and 10^5 individuals. In practice, plates were screened for mutant phenotypes two or three times over a 24 h period in early adulthood of the F2 generation to ensure that slower growing mutants are also observed. Nonetheless, a bias against particular mutants, *i.e.,* those that express a phenotype only in later adult stages, cannot be ruled out.

160 plates with an average of 20 F1 progeny per plate have been screened, giving altogether a total of approximately 6400 gametes. Ultimately just one mutant with a particular phenotype was kept per plate. A total of 147 mutants have been isolated, and can be grouped according to their phenotypic morphological abnormalities. In the following we describe the different groups of phenotypes, their frequencies and in some cases, specific characters of individual mutants.

Characterization of mutants

Originally more than 300 mutants were picked; half were sterile. These, and all additional mutants that did

not breed true or had a variable phenotype were discarded. Altogether 23 uncoordinated *(Unc)* mutants, 24 dumpy *(Dpy)* mutants and one roller *(Rol)* mutant were isolated and backcrossed as described above.

Phenotypes of mutants

Mutants have been selected by inspection only. Our primary interest was collecting mutants with morphological abnormalities that can be used as markers. Three classes of morphological mutants have been observed. In addition, mutant phenotypes which might be related to the development of the vulva have been isolated.

Uncoordinated mutants

The *P. pacificus* sp. n. wild-type displays a sinuous movement on agar surfaces as does *C. elegans* (*e.g.*, Mendel *et al.*, 1995) but the movement appears slower than in *C. elegans* presumably because of their different body shape. *P. pacificus* sp. n. has nearly the same size as *C. elegans*, but has a larger diameter that could influence movement. Animals move in both directions and reverse motion can be stimulated by touching as in *C. elegans*.

Twenty three *Unc* mutants with different movement defect phenotypes have been isolated (Fig. 3, C, D). Some mutants also display an egg-laying defect so that progeny hatch inside the mother. The different movement phenotypes observed in *P. pacificus* sp. n. correlate well with *Unc* mutants in *C. elegans*. It is very likely that a particular group of mutant phenotypes represents different genes, as it is known in *C. elegans* from genetic and molecular analysis. Thus, in most cases no conclusion on the molecular nature causing the mutant phenotype can be drawn. By contrast, there is one particular Unc mutant in *C. elegans* that represents a unique phenotype; *unc-22* mutant animals of *C. elegans* show a constant twitching of their body muscles, and these animals have a diameter smaller than wild-type (Benian *et al.*, 1989). A large number of *unc-22* mutants have been isolated consistent with the unusually large protein encoded by the *unc-22* gene. The polypeptide has a size of 6839 amino-acids and is involved in the regulation of myosin activity (Benian *et al.*, 1989, 1993). In *P. pacificus* sp. n., mutants with a very similar phenotype have also been isolated with a very high frequency (Fig. 3 B). The mutant *Ppa-unc(sy306)* of *P. pacificus* sp. n. has a small diameter and the body muscles twitch constantly. The uniqueness of the *C. elegans* mutant phenotype and the high frequency of mutants of this phenotype isolated in *C. elegans* and *P. pacificus* sp. n. suggests that the mutations causing the *unc-22* phenotype in *C. elegans* and the phenotype of *Ppa-unc(sy306)* are mutations in homologous genes. Therefore, we give the *sy306* allele the gene name *Ppa-unc-1* in agreement with suggested nomenclature (Bird & Riddle, 1994).

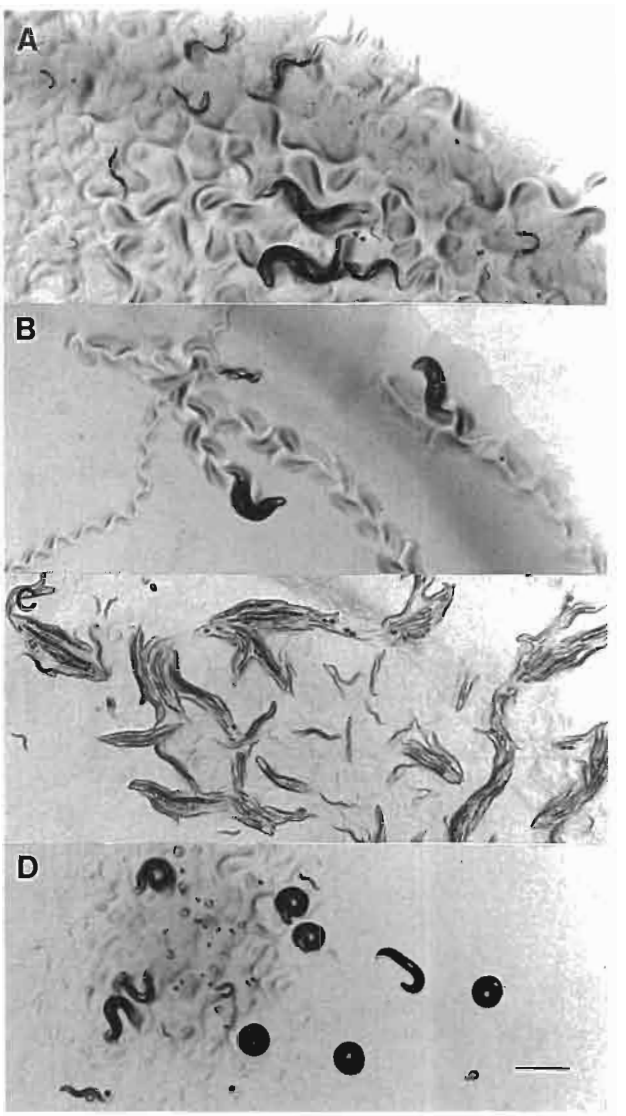

Fig. 3. *Photomicrographs of* Pristionchus pacificus *sp. n. and some of its mutants-A : wild-type; B :* Ppa-dpy(sy305); *C :* Ppa-unc-1; *D :* Ppa-unc(sy389) (Scale bar = 5 mm).

Dumpy mutants

Animals of this phenotype are shorter than wild-type reaching between one-third and two-thirds the size of a wild-type animal, but have the same diameter as wild-type animals. Twenty-four mutants with a dumpy phenotype have been isolated, most of which are one-third in size of wild-type animals. Several *Dpy* mutants are also defective in egg-laying. None of the dumpy mutations were sex-linked.

Roller mutants

Animals of this phenotype rotate around their long axis as they move. The phenotype is only clearly ex-

pressed in the adult stage. Just one mutant of this phenotype has been observed. The low frequency in comparison to *Unc* and *Dpy* mutants is similar to results in *C. elegans* where just a small number of *Rol* mutants have been isolated.

Other mutants

Additionally 99 egg-laying defective mutants have been isolated and partially characterized. Some of these mutants are defective in the development of the vulva and their characterization and phenotypes will be described elsewhere (Sommer & Sternberg, 1996). Only a subset of these mutants have been backcrossed as described above. The rest were discarded.

Initiation of linkage analysis

Backcrossing of the morphological mutants described above revealed that all but two are autosomal recessive. The two Unc mutants, *Ppa-unc(sy361)* and *Ppa-unc (sy384)* are sex-linked. We have not yet analyzed whether mutants with similar phenotypes belong to different complementation groups. Systematic complementation tests have not been done because of the large number of primary isolated mutants. Location of mutants on linkage groups has been initiated for some *Unc* and *Dpy* mutants and will be carried on for those mutants of further special interest. After linkage analysis is finished, complementation tests will be carried out in those cases where it is necessary.

Molecular biology

PCR analysis

Specific primers were designed to amplify a fragment of the *P. pacificus* sp. n. *let-60 ras* gene. In *C. elegans, let-60 ras* is a molecular switch gene during vulva development that influences the choice of undifferentiated precursor cells between a vulva and an epidermal fate (for review : Sternberg, 1993). Molecular cloning in *C. elegans* revealed high sequence similarity to the *ras* gene product of many different organisms (Han & Sternberg, 1990). A fragment encoding amino acids 110 to 162 of Fig. 4 was cloned into M13 and sequenced.

Screening of genomic and cDNA libraries

A genomic library and mixed stage cDNA library were generated as described above. The cDNA library comprises 10^{11} plaque forming units (pfu) with an estimated complexity around 10^6-10^7. Both libraries were screened with the PCR fragment of the *let-60 ras* gene described above. Ten primary signals have been obtained by screening 60 000 pfu of the genomic library, and 25 primary signals have been obtained by screening 7×10^6 pfu of the cDNA library. Of these 25 primary signals four have been further analyzed. One of the longest cDNAs was sequenced. This cDNA was full length based on homology with other *ras* proteins and the amino acid sequence inferred by conceptual translation is shown in Fig. 4. In addition, nearly the complete coding region and the first four of five observed introns were also sequenced from a genomic clone (see intron discussion below).

The overall sequence similarity between the *C. elegans* and the *P. pacificus* sp. n. *let-60 ras* gene is high. Two major regions of sequence divergency exist at the C-terminus and between position 120 and 140 (Fig. 4). Both regions are also most divergent among *ras* genes of species from different taxa, such as chordates and arthropods. Only limited phylogenetic conclusions can be drawn from this sequence comparison between *C. elegans* and *P. pacificus* sp. n. The sequence divergency in the region between amino acid 120 and 140 is nearly identical between the two nematodes and between a nematode, human or *Drosophila,* suggesting a large phylogenetic separation time for *Caenorhabditis* and *Pristionchus,* presumably in the range of 100 million years.

Based on the genomic sequence the position of the introns can be compared between the two nematodes. The position of the three *C. elegans* introns known in the coding sequence (intron 1,3,5 in Fig. 4) is conserved in *P. pacificus* sp. n. although the intron length differs. Two new introns are present in *P. pacificus* sp. n. compared to *C. elegans* (intron 2, 4 in Fig. 4) which are 44 and 89 bp long. The presence and positions of introns in the *ras* genes in the other species shown are not known because these genes were sequenced from cDNAs. Differences in exon-intron structure are common among nematodes and are also known between the closely related species *C. elegans* and *C. briggsae* (Kennedy *et al.,* 1993). Furthermore, PCR cloning of fragments of the Hom-C genes from *P. pacificus* sp. n. also revealed exon-intron structures different from *C. elegans* (Sommer & Sternberg, unpubl.).

Genome size

The genome size of *P. pacificus* sp. n. was estimated by genomic southern blotting against a 220 bp fragment of the *P. pacificus* sp. n. *let-60 ras* gene. The hybridization signal was compared to signals obtained from 1 ng, 100 pg and 10 pg of the fragment used for hybridization. One μg of genomic DNA gave a signal similar to 100 pg of the *let-60 ras* fragments, suggesting a genome size in the range of 10^4 to 10^5 kb (data not shown). This result is consistent with the number of positive clones observed after screening the genomic *P. pacificus* sp. n. library. Ten signals were obtained screening 60 000 pfu (average insert size about 15-20 kb), calculating a genome size of 10^4 to 10^5 kb. Although this method is only approximate, it is clear that *P. pacificus* sp. n. does not have an unusually large genome that is hard to work with molecularly. Furthermore, the fact that we isolated ten genomic clones suggests that this library contains a high proportion of the *P. pacificus* sp. n. genome.

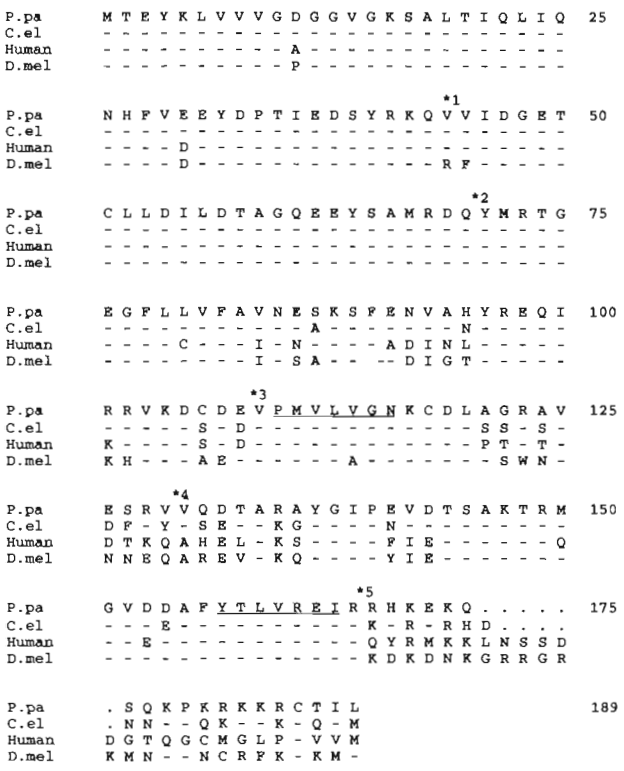

Fig. 4. *Comparison of the inferred amino-acid sequence of the* Pristionchus pacificus *sp. n.* let-60 ras *homologue with other known* ras *proteins. Dashes indicate identical amino acids. Dots indicate a sequence gap. The sequence used to create the primers for the PCR experiment are underlined. The position of the introns is indicated by numbered asterisks for the two nematode sequences. (P.pa :* Pristionchus pacificus *sp. n; C.el :* Caenorhabditis elegans *[Han & Sternberg, 1990]; Human [Taparowsky* et al., *1983]; D.mel :* Drosophila melanogaster *[Neumann-Silberberg* et al., *1984]).*

Conclusions

The data presented in this paper show that genetics and molecular biology can be used very intensively in this newly described free-living diplogasterid. In addition, cell lineage studies and cell ablation experiments standard to *C. elegans,* can be used in *P. pacificus* sp. n. as well (Sommer & Sternberg, 1996). Thus, *P. pacificus* sp. n. can serve as a " satellite " organism for comparison to the model system *C. elegans*. Our longterm goal is a detailed understanding of vulva development in *P. pacificus* sp. n., a process that is studied intensively in *C. elegans* (for review : Sternberg, 1993).

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