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Cultivation of the Rhabditid *Poikilolaimus oxycercus* as a Laboratory Nematode for Genetic Analyses

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ABSTRACT Vulva formation is a paradigm for evolutionary developmental biology in nematodes. Not only do the number of vulval precursor cells (VPCs) differ between members in the Rhabditidae and Diplogastridae, they are also sculpted via different developmental mechanisms, either by cell fusion in most Rhabditidae or by programmed cell death in the Diplogastridae. In this context, the species *Poikilolaimus oxycercus* is the only known species in the family Rhabditidae to have a subset of the P*n*.p cells commit programmed cell death during the patterning of the VPCs. Our current study introduces *P. oxycercus* as a new laboratory organism. There are discrete laboratory strains that are genetically polymorphic from each other as well as heterogeneous within each strain. In order to cultivate this gonochoristic nematode into an experimental model with a tractable genetic system, we produced two inbreeding tolerant, near-isogenic strains capable of producing viable progeny with each other. We also described *P. oxycera's* morphology by scanning electron microscopy (SEM), basic life history traits, hybrid viability, and mating behavior. *P. oxycercus* females have no preference for inter- or intra-strain matings, and can mate with multiple males in a relatively short time period, suggesting a propensity for maintaining heterozygosity through promiscuity. Interestingly, all sexes from three species in the genus *Poikilolaimus* show five 4′,6-diamidino-2-phenylindole (DAPI) staining bodies in their germ line cells. This could indicate that *Poikilolaimus* species possess five bivalent chromosomes in their germ lines, in contrast to the hermaphroditic *Caenorhabditis elegans* or *Pristionchus pacificus*, which have six chromosomes. *J. Exp. Zool. 303A:742–760, 2005.*

The study of developmental evolution requires detailed analyses among related species whose anatomy and genetic programs are similar enough to be directly comparable. Implicit in this agenda is the need to conduct in-depth investigations of pivotal clades that may represent informative additions to the known models. In the Nematoda, argued to be the most species-diverse phylum, the free-living nematode *Caenorhabditis elegans* serves as a model system (Brenner, '74). The wealth of tools available for *C. elegans* research includes cell lineage, genetics, genomics, molecular biology, and natural variations (Sulston and Horvitz, '77; Horvitz and Sulston, '80, Sternberg and Horvitz, '84; Davies et al., 2004). Comparisons between *C. elegans* and other members of the family Rhabditidae have been made, ranging from genome organization in *C. briggsae* to the phylogeny of the *Caenorhabditis* genus (Sudhaus and Kiontke, '96; Stein et al., '03; Kiontke et al., '02; Kiontke et al., '04). Additionally, detailed comparative analyses of vulva formation between *C. elegans* and major rhabditid clades such as *Oscheius*, *Rhabditoides*, *Mesorhabditis*, and *Pelodera* have collectively enhanced our understanding of the extent and context of evolutionary changes in developmental patterning (Sommer and Sternberg, '95, '96b; Felix et al., 2000b). Recently, further comparisons have been made with nematodes outside of the Rhabditidae. *Pristionchus pacificus* is a distant relative in the family Diplogastridae that represents even more extensive morphological and developmental differences in vulva and gonad formation (Sommer

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et al., '96, '99, 2000; Rudel et al., 2005). Comparisons of developmental processes even further outside of the Rhabditidae and Diplogastridae, however, such as in parasitic nematodes *Brugia malayi*, are incommensurable due to their disparate host-specific developmental life styles.

The vulva is the egg-laying structure of nematode females and hermaphrodites and is derived from the vulva precursor cells (VPCs) of the epidermis (Sulston and Horvitz, '77; Sternberg and Horvitz, '86). In *C. elegans*, these epidermal cells consist of 12 P*n*.p cells, called P(1–12).p, and are distributed from anterior to posterior in a linear array. The distal ends of this cell array P(1,2, 9–11).p, as well as the P12 descendent P12.pa, fuse early on in the L1 larval stage to the hypodermal syncytia hyp7 and hyp12, respectively. By the L3 larval stage, three of the six central VPCs P(5–7).p subsequently divide and differentiate to form the vulva, while the other VPCs, P(3,4,8).p, eventually also fuse with the surrounding hypodermis (Sternberg and Horvitz, '89). In a survey of vulva formation in free-living nematodes, it was shown that these non-specialized P*n*.p cells either fuse or undergo programmed cell death. In particular, the most striking difference was observed between *C. elegans* and *P. pacificus* vulva development. In *P. pacificus*, programmed cell death, or apoptosis, occurred to rid the non-VPCs in *P. pacificus* (Sommer and Sternberg, '96a). Within the Rhabditidae, only *Poikilolaimus oxycercus* has been observed to have the homologous non-VPCs undergo apoptosis, whereas these non-VPCs undergo apoptosis in all Dipgastridae species examined (Sommer and Sternberg, '96a; Sommer, '97). In *P. oxycercus* females, P(1–4).p and P(9–11).p undergo apoptosis in the first larval stage (L1), while a subset of the surviving P(5–8).p cells subsequently form the vulva. Outside of the Rhabditidae and Diplogastridae, in *Panagrolaimus* sp. in the family Panagrolaimidae, P(1–4).p also undergo apoptosis but the P(9–11).p survive in this case to attain neuron-like morphology (Sommer and Sternberg, '96a; Felix et al., 2000a).

The genus *Poikilolaimus* is considered to be an early branch of the Rhabditinae sensu lato using morphological characters, but separate from the Rhabditinae using the SSU rDNA molecular marker (Sudhaus, '76; Fitch et al., '95; Sudhaus and Fitch, 2001). Improved resolution of the molecular phylogeny of these species may eventually determine whether apoptosis in the ventral cord lineage is due to convergence or synapomorphy. Even without knowing the detailed phylogeny of *Poikilolaimus*, the value of *P. oxycercus* as a developmental model is that it shares morphological traits with Rhabitidae but displays a vulva developmental program akin to members of the Diplogastridae. Since morphological similarities can conceal changes in underlying genetic pathways, the study of developmental processes in distant family members can enlighten other aspects of rhabditid evolution. Therefore, *Poikilolaimus* has the potential to become an ''intermediate'' proxy between the more intensely studied members of the Rhabditidae and Diplogastridae.

Earlier attempts to establish *P. oxycercus* as a laboratory model for nematode biology capitalized on its virtues of long life span and reproductive period (2–3 months), as well as its tolerance to environmental toxins (Kampfe and Wagner, '64, Kampfe and Schmidt, '66; Sudhaus, '80; Kampfe et al., '93). However, natural variations in this cosmopolitan gonochoristic species may have confounded its domestication under various laboratory conditions, such as diet, temperature, and inbreeding. Such variations may have contributed to observed differences in *P. oxycercus* biology by individual researchers. To make experimentations more consistent, we have consolidated the available *P. oxycercus* strains in order to regiment an ideal culturing condition and to reduce heterogeneity within strains for genetic analysis of this species. We have also instituted standard *C. elegans* protocols for raising nematodes on OP50 *E. coli* on NGM agar plates and for utilizing molecular genetic markers (Wood, '88). When feasible, we also described *P. oxycercus* biology in the context of other members of its genus, such as *P. ernstmayri*, *P. piniperdae*, and *P. regenfussi*.

Our primary aim was to cultivate *P. oxycercus* from wild isolates as well as existing laboratory stocks, and to establish this species as a laboratory organism with tractable genetic and genomic resources. As a first step, we cultivated two laboratory *P. oxycercus* strains: a reference strain and a crossing/mapping strain. This is not trivial in the sexually reproducing *P. oxycercus* species since high DNA polymorphism is inherent within gonochronistic populations and inbreeding depression makes the generation of isogenic lines arduous. Nevertheless, high genetic homogeneity in model organisms is not only a prerequisite for genetic marker analyses but also for comparisons regarding intra-species and inter-species differences. We have considered the following

qualities to be important in new laboratory organisms:

(i) monophyly with multiple members in the genus,
(ii) high brood size with short generation time,
(iii) low intra-strain polymorphism and inbreeding tolerant strains,
(iv) high inter-strain polymorphism,
(v) inter-strain crosses are viable,
(vi) amenable to forward and reverse genetic tools.

With the exception of (vi), this study focuses on achieving these goals through inbreeding the hitherto available strains and ascertaining their degree of DNA polymorphism. In the process, we investigated the basic biology of this species for factors that may influence population diversity.

MATERIALS AND METHODS

Microscopy

Scanning electron microscopy (SEM) of *P. oxycercus* strain SB200 was prepared through graded ethanol series and sputter-coating, as described in Rudel et al. (2005). We made multiple attempts to ameliorate the possible fixation artifacts in *P. oxycercus* adult samples but did not observe samples that do not undergo the ''shrink-wrap'' effect on their cuticles; thus, this may reflect a high water content in the thick *P. oxycercus* cuticles. To visualize chromosomes, we fixed worms for 2 hr in Carnoy solution and stained DNA in the germ line with 1 μg/ml of 4′, 6-diamidino-2-phenylindole (DAPI) (D-1306, Molecular Probes, Eugene, OR) in VectaShield mounting medium (H-1000, Vector Laboratories Inc., Burlingame, CA) for 8 hr. Samples were viewed on an Axioplan 2 microscope (Zeiss, Goettingen, Germany) and a CCD camera (Sony DXC-950P). All pictures were also subsequently prepared with Photoshop® 7.0 for Macintosh (Adobe). A minimum of three individuals were analyzed for a given sex and strain or species, except for *P. ernstmayri* females, in which chromosomes from only one oocyte in diakinesis could be counted unambiguously.

Nematode husbandry and inbreeding

All laboratory *Poikilolaimus* strains were handled with standard *C. elegans* protocols on monoxenic OP50 *E. coli* agar plates, as described in *The nematode Caenorhabditis elegans* handbook (Wood, '88). All strains were acquired as gifts from Drs. W. Sudhaus (U. Berlin) and D. Fitch (NYU). The laboratory *P. oxycercus* strains are EUK103 and EUK106, northeastern India; SB200, derived from a cross between Belgian and German isolates; RS6877, Ithaca, NY; DF5058, Brooklyn, NY; and RS6756, Boston, NH. The related laboratory species are *P. piniperdae* SB352, Corsica (Fuchs, '30); *P. ernstmayri* SB346, Corsica (Sudhaus and Koch, 2004); *P. regenfussi*, Sumatra (Sudhaus, '80). Although *Poikilolaimus* species have an average generation time of 2–3 weeks, we were able to obtain worm-saturated plates at 20°C consistently. ''Chunking'' agar bits for transfers to new plates is feasible with *P. oxycercus* cultures. As a recently isolated *P. oxycercus* strain, RS6877 was immediately bottlenecked from a pair of male and female, and the next generation separated into RS6877α (kept at 20°C) and RS6877β (kept at 30°C). At the same time, we also tried to preserve the high level of natural variation in RS6877α and β strains by consistently picking large numbers of individuals onto fresh OP50 plates, as well as mixing individuals from several plates. Near-isogenic lines of RS6877 were derived from the RS6877α lineage. Most *P. oxycercus* strains, *P. regenfussi*, and *Panagrolaimus* sp. PS1159 can be maintained indefinitely at 30°C, but *P. piniperdae* SB352 and *P. ernstmayri* cannot tolerate 30°C and grow well only at 20°C. Since we obtained most strains previously grown on other food sources (e.g., fungi, potato, apple), we acclimated our strains to feed on OP50 for at least two generations before commencing our analyses.

In order to homogenize the inherent polymorphisms within each *P. oxycercus* strain, we began inbreeding four strains at 20°C—SB200, EUK103, EUK106, RS6877—by limiting the population size in each generation in closed isogenic populations. The first founder of each isogenic line contained a virgin mating pair that produced viable progeny, but subsequent generations contained two to six founders at various adult stages and both sexes. We set up founding populations again from the previous generation if certain lines did not produce progeny after 1 week. This process was repeated at least once up to generation 10 and as many times as possible after generation 10 to maximize viable inbred lines. Parents from the previous generation were killed as soon as progeny were observed to minimize inter-generational matings. Similarly, we also propagated recombinant inbred lines of inter-strain crosses for up to

generation 5 to determine the two ideal reference strains for future mapping and quantitative traint loci (QTL) projects. To solve for the expected value of inbreeding coefficient at generation 16, we used the formula $F_t = 1 - \prod_{i=1}^{t}[1-1/(2N_{ei})]$, where $N_{e1} = 2$ in generation 1 and $N_{e2-16} = 4$ in generations 2–16; thus $F_{16} = 0.90$.

Phylogenetic analysis

The 18S and 26S rDNA sequences were aligned separately, first with Clustal X (Thompson et al., '97) and then manually using Se–Al v2.0a11 (Andrew Rambaut, Oxford University, Oxford, UK). Each aligned 18S entry (477 characters) was subsequently concatemerized together with its respective 26S entry (276 characters) to form a dataset of 11 taxa, each with 753 characters, of which 459 are variable. The best-fit DNA substitution model GTR+G was selected by Akaike-Informational Criterion (AIC) based on log-likelihood scores produced by Modeltest v3.5 (Posada and Crandall, '98). This maximum likelihood substitution model was used to generate a neighbor-joining distance tree bootstrapped with 1,000 replicates in Paup4.0b10 (Swofford, '93). *Panagrolaimus* sp. SP1159 was designated as the outgroup and nodes with bootstrap values greater than 65 were retained. Similar trees were found using heuristic and branch-and-bound searches. The existing GenBank accession numbers used for this study are AF036640, AF083010, U81587, AF083022, U81579, AF083006, NR000054, and AF083023; the new accession numbers are DQ059054–DQ059066.

P. oxycercus life history traits

To mate virgin worms, two 3-day-old *P. oxycercus* larvae were picked onto agar plates containing OP50. When sexual morphologies could be determined, female–male pairs were left alone while the same-sex pairs were paired with their opposite sexes to commence recordings on brood size, egg hatching success, and life span of individual females. We never observed eggs in single *P. oxycercus* of either sex, confirming that *P. oxycercus* is a true gonochoristic species. Males were left on the plate for as long as the oldest progeny was still clearly smaller than both parents.

We defined the total number of eggs each female can lay in its lifetime as its brood size. When the oldest progeny of a gravid female became a young adult, but before she began to lay eggs, her mother was transferred onto a fresh OP50 plate and the number of larvae and eggs in her last plate was counted. The female life span was the length of time from hatching to natural death, while the generation time was the time between the hatching of the female to the hatching of her first progeny. After the mother was transferred onto new plates, the number of visible larvae on the previous plate after 2 weeks divided by the total number of eggs defined the hatching success rate. Statistical values were computed using Microsoft Excel for Macintosh.

Amplified fragment length polymorphism analysis

Disparate genomic DNAs from non-inbreeding populations of six *P. oxycercus* strains were isolated using an AquaPure Genomic Isolation Kit (Biorad). However, to avoid exaggerated high intra-strain polymorphism due to sampling errors, we performed whole genome amplification on the original six samples and also used these DNA sources in our amplified fragment length polymorphism (AFLP) analysis. Consequently, we struck a compromise between high intra-strain polymorphisms in heterogeneous populations and AFLP reproducibility by performing whole genome amplification using Genomify (Amersham, Freiburg, Germany) to obtain sufficient DNA from the same extractions for our present study. AFLP fingerprinting was done according to Srinivasan et al. (2001). With the exception of a few AFLP bands, most marker bands were the same between their original genomic DNAs and their corresponding whole genome amplified DNA (1st lane vs. 2nd–3rd lanes for each strain, Fig. 3) as well as between polymerase chain reactions (PCRs) for each amplified DNA (2nd vs. 3rd lanes for each strain). Four primer combinations were used for character analysis: E-TA/M-CAG, E-TG/M-CTG, and E-AC/M-CAA, E-AG/M-CAC. We visually scored for polymorphic bands that were reproduced from both DNA types and three separate PCRs. These markers are highly reliable and not exaggerated by intra-strain polymorphisms.

Molecular biology

PCR primers SSU 18A 5′-aaagattaagccatgcatg-3′, SSU 26R 5′-cattcttggcaaatgctttcg-3′ were used for PCR amplification and SSU 9R 5′-agctggaattaccgcggctg-3′ for direct sequencing of the 18S/

SSU rDNA; the 26S rDNA PCR products were amplified with IS807 5′-cgatagcgaacaagtaccgt-3′, IS809 5′-ctcaggcatagttcaccatc-3′, subcloned into TA-TOPO (Invitrogen, Paisley, UK), and sequenced with T3/T7 primers (Srinivasan et al., 2001, Floyd et al., 2002). The 18S sequences were also used to verify that all *P. oxycercus* strains have identical sequences while other *Poikilolaimus* species do not share 18S DNA sequences. Polymorphic marker PS30 with similarity to *C. elegans mlc-4* myosin light chain (C56G7; BLASTX $1e^{-78}$) was derived from a *P. oxycercus* cDNA library and used for mating choice experiments. The *P. oxycercus* RS6877 mixed-stage poly-A cDNA library was made according to the manufacturer's instructions (BD SMART PCR cDNA Synthesis, BD Biosciences, Palo Alto, CA), shotgun cloned into TA-TOPO, and end sequenced with T7. PS30 primers AV6797 5′-GACGACTTCCTC GAGGACATG-3′ and AV6798 5′-GCTCGTGA GTGTAGCGATC-3′ span ~250 bp in the putative coding region of the cDNA. SSCP was performed as described previously (Srinivasan et al., 2002).

Mating experiments

We verifed that females were virgins by singling young *Poikilolaimus* females overnight on individual agar plates before using them for mating experiments. We attempted inter-strain (*P. regenfussi* hermaphrodites × *P. oxycercus* SB200 males; *P. oxycercus* SB200 females × *P. ernstmayri* males; *P. oxycercus* SB200 females × *P. piniperdae* males) and intra-strain matings of all *P. oxycercus* strains to the previously characterized SB200 strain to confirm that the existing species and strain assignments are appropriate. Three males were usually incubated per female at 20°C for 5 days. To determine how many males *P. oxycercus* females can mate with in a period of 4–5 days at 25°C, we set up mating crosses between a single EUK106-G isogenic female with four males of different inter- and intra-strain genotypes. The genotypes of all parents and F_1 progeny were determined by the highly polymorphic SSCP marker PS30. PS30 is polymorphic not only between two isogenic lines EUK106-G and EUK106-T but also within an isogenic line RS6877-5. Thus, three known polymorphic banding patterns were possible between EUK106-G and RS6877-5 (genotypes A, B, C). Mating plug-like structures were observed on ~80% of recently mated females.

RESULTS

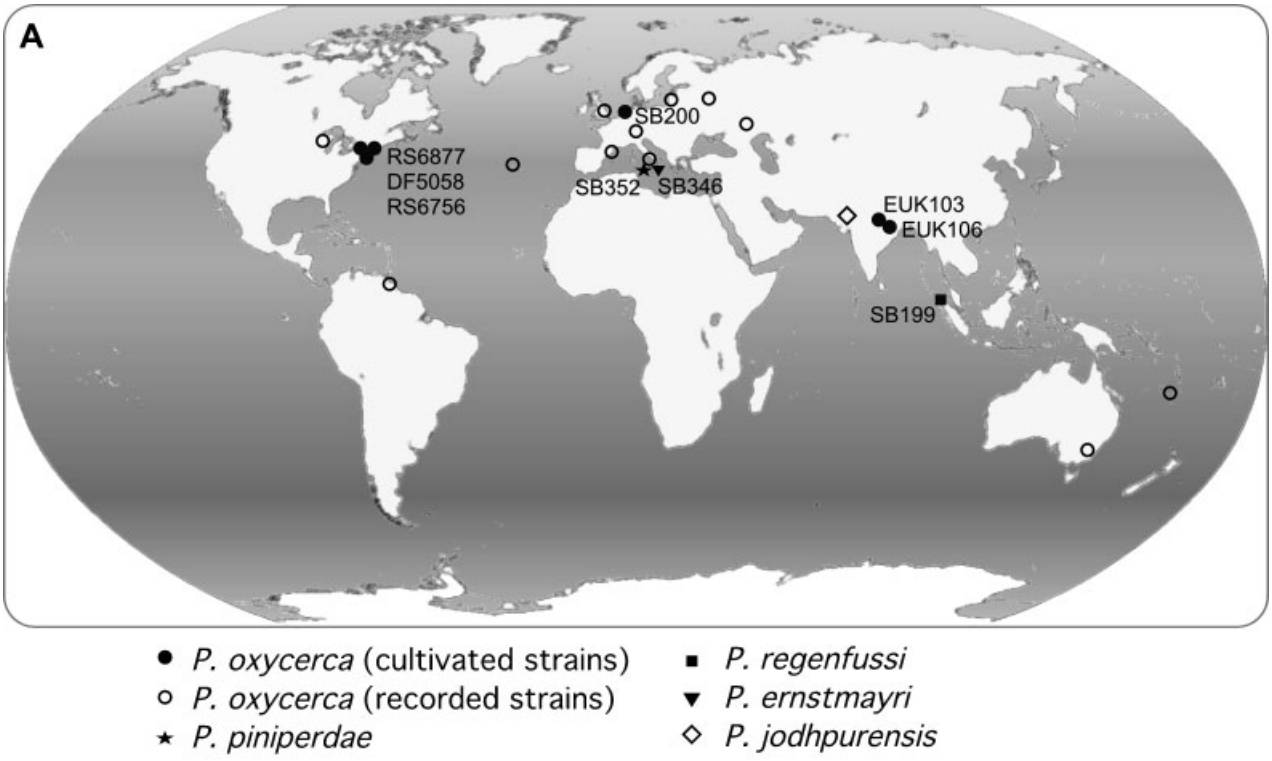
Biogeography

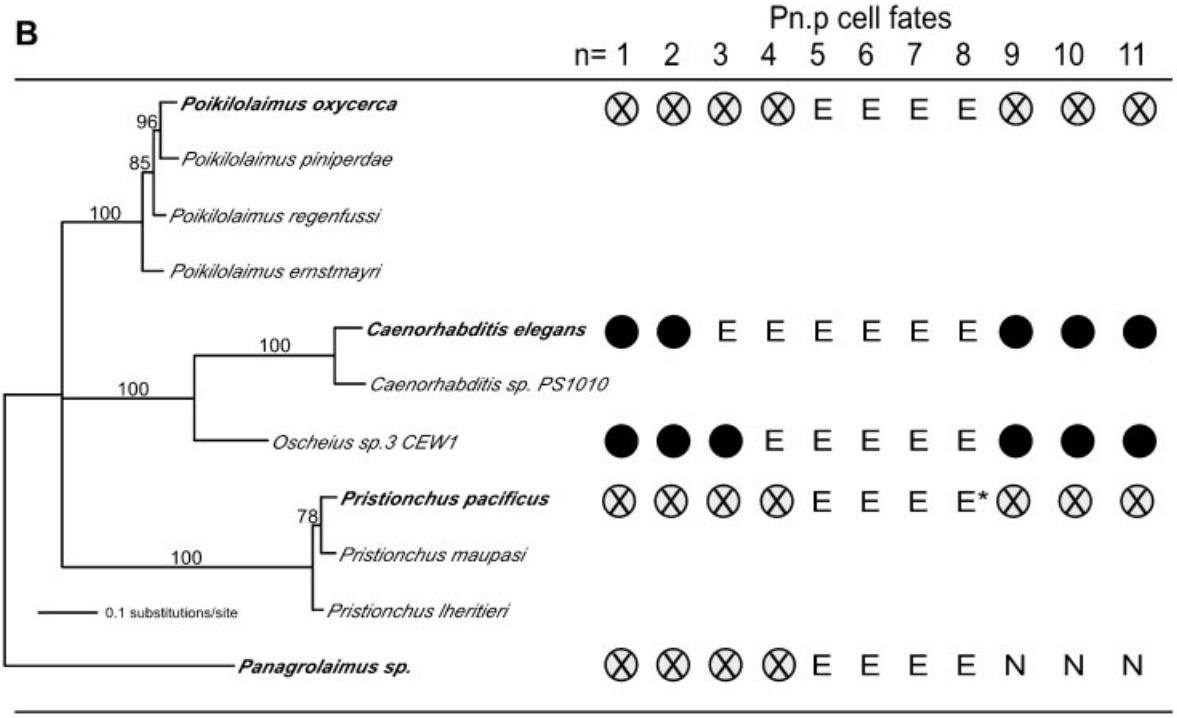
Poikilolaimus oxycercus was first described as *Rhabditis oxycerca* (de Man, 1895), then synonymously as *Poikilolaimus oxycerca* (Fuchs, '30) and *Cuticularia oxycerca* (van der Linde, '38), and most recently as *Poikilolaimus oxycercus* by Sudhaus and Koch ('04). Our laboratory *P. oxycercus* strains were originally isolated from northeastern India (EUK103, EUK106), northeastern United States (RS6877, DF5058, RS6756), and northern Europe (SB200), and they thereby represent the cosmopolitan distribution of recorded isolates on islands and five continents worldwide (Fig. 1A). Most strains were isolated from decomposing organic material on wood or in soil, but a few were also reported to have been isolated from Scarabaeidae beetles, suggesting phoretic associations (Poinar, '72; Sudhaus, '80). Our laboratory

Fig. 1. (**A**) World distribution of *Poikilolaimus* species. Labeled and filled shapes denote cultivated laboratory strains/species and unfilled shapes denote recorded isolates/species excerpted from Sudhaus ('80). The laboratory *P. oxycercus* strains are EUK103 and EUK106, northeastern India; SB200, derived from a cross between Belgian and German isolates; RS6877, Ithaca, NY; DF5058, Brooklyn, NY; RS6756, Boston, NH. The related laboratory species are *P. piniperdae* SB352, Corsica; *P. ernstmayri* SB346, Corsica (Sudhaus and Koch, 2004); *P. regenfussi*, Sumatra. Additional information regarding these strains is listed in Table 1. *P. oxycercus* have also been isolated from England (de Man, 1895), Hungary (Andrássy, '58), Lithuania (Slepetiene, '61), Moscow (Krylov, '62/'63; Sumenkowa, '63), Stalingrad/Volgograd (Ivanova, '60), Wisconsin (Thorne, '61), Trinidad (Goodey, '29), Canberra (Marchant and Nicholas, '74), as well as Sicily, Barcelona, Azores Islands, and New Hebrides (Sudhaus, '80). *P. jodhpurensis* (Khera, '69) is considered to belong to the *Poikilolaimus* genus (Sudhaus, '80). (**B**) Proposed phylogeny of *Poikilolaimus* and corresponding P*n*.p cell fates in major clades. A neighbor-joining tree was constructed using combined 18S(SSU) and 26S(LSU) sequences and the maximum-likelihood DNA substitution model GTR+G, with *Panagrolaimus* sp. as the designated outgroup. Bootstrap values from 1,000 replicates are indicated on each node. The polytomies among the three major clades represent <65% bootstrap support. The fates of non-VPC P*n*.p cells in major clades are illustrated for the corresponding representatives (in bold): *Poikilolaimus oxycercus* P(1–4, 9–11).p, *Caenorhabditis elegans* P(1–2, 9–11).p, *Oscheius tipulae* P(1–3, 9–11).p, *Pristionchus pacificus* P(1–4, 9–11).p, and *Panagrolaimus sp.* P(1–4, 9–11).p, where filled circles represent fusion cell fate, crossed circles represent programmed cell death, "E" represents VPCs, and "N" represents neuronal cell fate. P12.pa forms a special hypodermis, hyp12, in all species and is not shown. (P*n*.p fates based on Sommer and Sternberg, '96a, b; Sommer et al., '96; Sommer '97.)

P. piniperdae (Fuchs, '30) and *P. ernstmayri* strains (Sudhaus and Koch, 2004) were both similarly derived from wood debris, while the only strain of *P. regenfussi* (Sudhaus, '80) that has ever been isolated came from a forest compost.

To confirm that these laboratory *P. oxycercus* strains belong to a single species, we sequenced





the SSU 18S rDNA loci of each strain and found that they were exactly the same. Furthermore, we also successfully mated all strains to *P. oxycercus*, SB200. As expected for reproductively isolated species, the SSU sequences of *P. regenfussi*, *P. piniperdae*, and *P. ernstmayri* are all significantly different from those of *P. oxycercus*, and no two species were able to inter-breed under our laboratory conditions.

Nomarski and SEM

We made SEM and DIC micrographs of *Poikilolaimus* to highlight some distinguishing features in their body contour and sexual organs. Figure 2 shows comparisons between *C. elegans* and two *Poikilolaimus* species, *P. regenfussi* and *P. oxycercus* SB200. Both *Poikilolaimus* species have a short needlepoint-shaped tail—hence the name ''oxycercus'' given for the founder species (Fig. 2F, L). In addition, *P. oxycercus* male caudal papillae originate predominantly from the ventral side and are not connected by a clearly visible bursa velum, as opposed to the more lateral origins of the papillae in *C. elegans* with an elaborate bursa velum connecting the rays (Fig. 2C, L). In the *C. elegans* vulva, the apical side of the epithelial toroids is clearly visible at the vulval slit opening of hermaphrodites (Fig. 2B). These epithelial toroids are less pronounced in *P. oxycercus* females and not at all in *P. regenfussi* hermaphrodites, which has a simple circular vulval opening (Fig. 2H, E). In adults, both *C. elegans* and *P. oxycercus* display prominent cuticular alae situated laterally on both sides of the body. However, the alae of *C. elegans* are proportionally smaller and organized as three parallel structures compared with the alae of *P. oxycercus*, which is a single thickening along the entire body (Fig. 2A, G, J and insets). Such alae-like structures were never observed in *P. regenfussi* samples at any developmental stage (Fig. 2D). Other special morphological traits of *Poikilolaimus* are its heavily cuticularized excretory duct readily visible by DIC (not shown), and its pair of glands/pouches on the anterior and posterior sides of the vulva, at the proximal ends of both gonad arms in the vicinity of the uterus (Fig. 2K). In adult *P. oxycercus* females, these vulval glands are filled with viscous granular fluids but their function and organ ontology remain unknown. Similar vulval glands were also observed in one genus of the Diplogastridae, *Acrostichus* (previously named *Aduncospiculum*), but not in members of the Rhabditidae (von Lieven and Sudhaus, 2000).

Monophyly with multiple members

To ascertain *P. oxycercus*'s phylogenetic relationship with other nematodes and to determine if the genus is monophyletic, we utilized the SSU marker from the *Poikilolaimus* species to propose a molecular phylogeny (Blaxter et al., '98). Using both the concatemerized SSU and the LSU molecular phylogeny markers (Srinivasan et al., 2001) in a best-fit DNA substitution model, the resulting phylogeny does not resolve the polytomies among the *Poikilolaimus* group and the two major clades, Rhabditidae (represented by *C. elegans*, *Caenorhabditis* sp. *PS1010*, and *Oscheius*) and Diplogastridae (represented by *P. pacificus*, *P. maupasi*, and *P. lheritieri*), but does show that the molecular support is strong for *Poikilolaimus* as a monophyletic genus (Fig. 1B). This phylogeny could reflect an ancient divergence of the *Poikilolaimus* group in the Rhabditidae family and is not inconsistent with the phylogeny derived from independent morphological characters such as male bursa and buccal cavities (Fitch et al., '95, Sudhaus and Fitch, 2001). Thus, *Poikilolaimus* is a bona fide genus with several member species—*P. piniperdae* being the closest

Fig. 2. Nomarski and scanning electron micrographs. (**A–C**) *Caenorhabditis elegans*, (**D–F**) *Poikilolaimus regenfussi*, (**G–L**) *Poikilolaimus oxycercus*. (A) View of a whole adult *C. elegans* with prominent alae along its lateral sides; inset shows the characteristic triple parallel alae (B) lateral view of *C. elegans* vulva with lateral ''folds'' on both ends of the lip. (C) *C. elegans* male tail lateral view showing elaborate bursa ray papillae. (D) Lateral/ventral view of a whole adult *P. regenfussi* showing a smooth body contour and lateral groove without a visible alae. (E) *P. regenfussi* vulva with a simple circular opening. (F) *P. regenfussi* hermaphrodite tail and anal region with ''needle point'' tail characteristic of the genus. (G) Posterior–lateral and posterior–ventral view of two *P. oxycercus* female adults with alae along the lateral sides and a heavily cuticularized exterior accentuated after fixation; inset shows no substructure in the alae. (H) *P. oxycercus* vulva shows a simple left–right invagination. (I) *P. oxycercus* female tail. (J) View of *P. oxycercus* larva with visible alae; inset shows the detail of alae. (K) Arrowheads point to the two ''glands'' straddling the anterior and posterior sides of the *P. oxycercus* female vulva (down). (L) Ventral view of a young adult *P. oxycercus* male showing that most male papillae are orientated ventrally. Anterior is to the left in panels C, F, I, K, L. Scale bars in C, F, H, I, L 5 μm; in B, E 2 μm; in J, K 10 μm; in A, D, G 50 μm. Insets 2 × main panels.

relative of *P. oxycerca*, followed by *P. regenfussi*, and *P. ernstmayri* being the most basal member of the genus. This monophyly is in contrast to other groups in the Rhabditidae, such as the polyphyletic *Rhabditoides* and the monotypic genera *Cruznema tripatitum* (Linstow, '06), whose purported phylogeny may be obfuscated by the use of poorly discriminating or distorting characters. We could not examine the two other putative *Poikilolaimus* species, *Cuticularia* sp. and *Praeputirhabditis jodhpurensis* (Khera, '69). *Cuticularia* sp. is known only by its SSU rDNA sequence

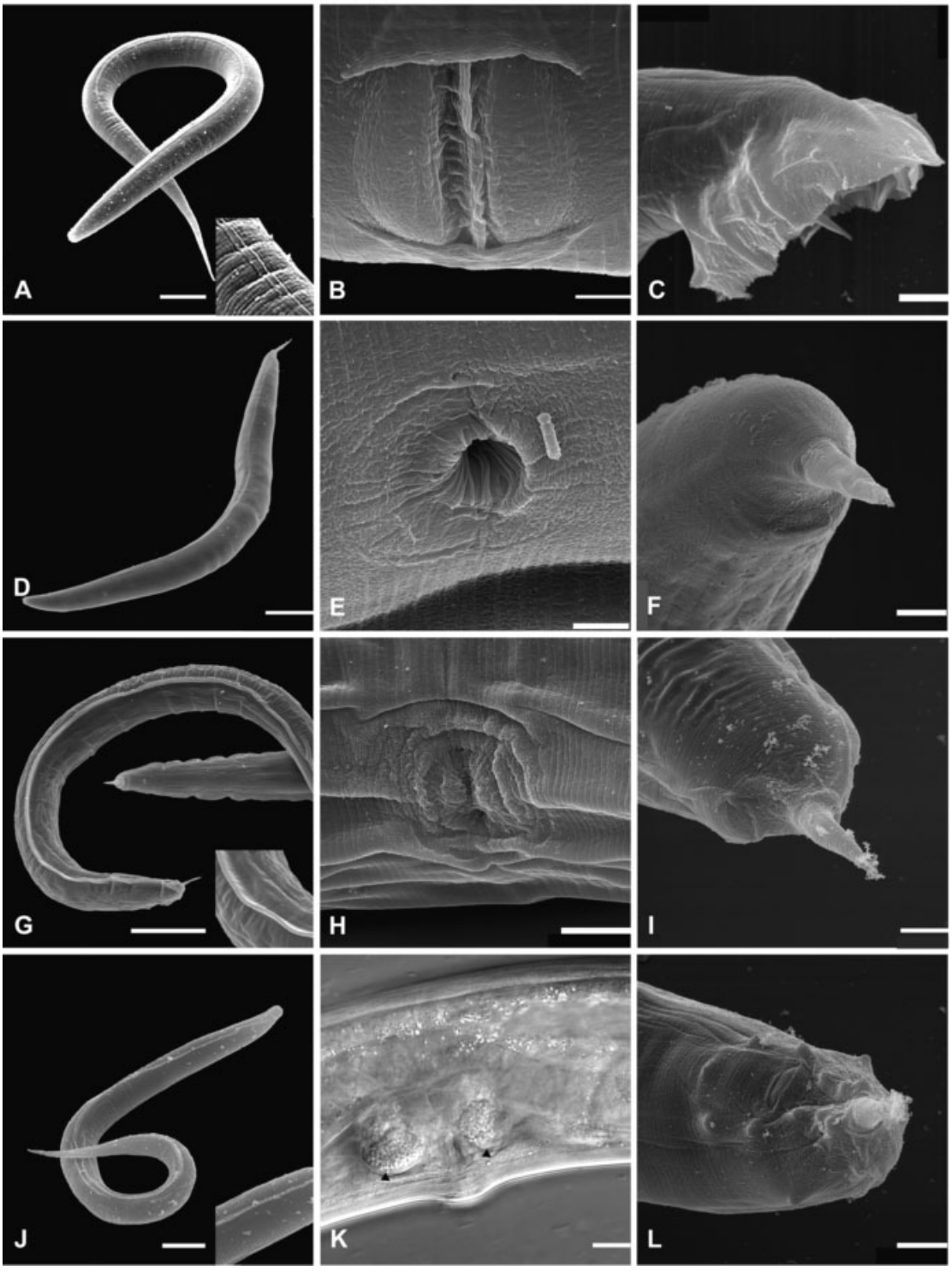


TABLE 1. Life history traits of Poikilolaimus strains

Species	Strain	Temperature	*n*	Egg count	Viable brood size (% hatch)	Generation time (days)	Life span (days)	Origin (researcher)
P. oxycercus	EUK106	20°C	22	101∗37	45 (45%)	21	92±31	India (E. Khan via D. Fitch)
P. oxycercus	RS6877	20°C	12	221±156	166 (75%)	16	69±46*	Ithaca, NY, USA (W. Sudhaus)
P. oxycercus	SB200	20°C	8	137±43	40 (29%)	16	50±9	Belgium × Germany, EU (W. Sudhaus)
		25°C	16	100±64	27 (27%)	15.6	69±29	
		30°C	16	113±67	—	—	30±10	
P. regenfussi	SB199	20°C	10	55±38	NA	17.6	23±4	Sumatra, Indonesia (W. Sudhaus)
		30°C	10	76±36	NA	—	13±2	

All values are for the original, heterogeneous laboratory (EUK106, SB200, SB199) or recently isolated wild populations (RS6877). Values for egg count, generation time, and life span of females are all averages. Viable brood size is the product of % eggs hatching and egg count, *P. oxycercus* is oviparous and gonochronistic while *P. regenfussi* is ovoviviparous and hermaphroditic (or parthenogenic). SB200 is the result of a cross between two isolates from Belgium and northern Germany. Detailed information can be found in Materials and Methods. *n*, number of females/pairs analyzed. *, omitting an outlier reduces the average to 54±24 days. —, not measured. NA, not applicable due to ovoviviparity.

(CSU81583) and *P. jodhpurensis* is known only by its morphological description in the aforementioned monograph.

Brood size and generation time

P. oxycercus is gonochoristic and significantly slower growing than the hermaphroditic *C. elegans* or *P. pacificus*. To choose ideal strains with short generation time and high viable brood size for genetic studies, we measured life history traits of three selected *P. oxycercus* strains—EUK106, RS6877, SB200 from Asia, North America, and Europe—as well as *P. regenfussi* as an intra-genus, hermaphroditic comparison (Table 1). These values represent females that were mated once to the same male for approximately 2 weeks. At 20°C, SB200 lays more eggs than EUK106 but has the lowest viable brood size (40 progeny/female). SB200 has the lowest viable brood size (40 progeny/female) while RS6877 has the highest (166 progeny/female). Both SB200 and RS6877 have short generation times of approximately 16 days, in contrast to EUK106 which requires the longest generation time among the three strains (21 days). The high embryonic lethality may be due to the initial response to unintended inbreeding depression in the case of RS6877 which was newly isolated from the wild, or to changes in laboratory conditions in the case of EUK106 and SB200. Strain EUK106 also lives the longest, with a mean of 92 days, compared with 69 days and 50 days in strains RS6877 and SB200, respectively. Unlike *C. elegans* or *Pristionchus pacificus*, the four *P. oxycera* strains that we tested (SB200, RS6877, EUK106, EUK103) could tolerate 30°C as their ambient growth temperature for many generations, with RS6877 being the most robust strain at this temperature. However, although the generation time of SB200 was reduced from 16 days at 20°C to 6 days at 30°C, SB200 suffers from a smaller brood size and shorter life span at 30°C. The hatching success rate of SB200 at 30°C, already the lowest among the three strains at 29%, was likely much lower but difficult to measure due to the OP50 being contaminated by more vigorous microorganisms at 30°C.

Interestingly, the related *P. regenfussi* actually showed an increase in brood size at 30°C compared with 20°C, along with a reduction in average life span. In summary, although an ambient temperature of 30°C reduced the generation time for strain SB200, its concomitant reduction in brood size is exacerbated by the difficulty in maintaining a monoxenic *E. coli* culture and the increased incidences of worms burrowing beneath the agar surfaces at 25–30°C. This rendered 20°C to be the optimal growth temperature for the three *P. oxycercus* strains studied herein. The high brood size and comparatively short generation time of RS6877 compares it favorably against those of EUK106 and SB200.

High inter-strain polymorphism

In addition to having a high brood size and a short generation time, the strains considered for genetic analyses should also have high inter-strain DNA polymorphism to facilitate future marker discovery. We therefore performed AFLP analysis

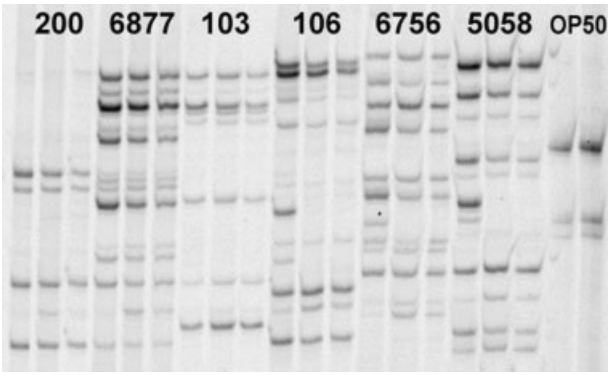


Fig. 3. A sample of an AFLP autoradiogram using selective primers E-AG/M-CAC. The six *P. oxycercus* strains used were not subjected to inbreeding. Independent PCR reactions were performed three times per strain, and only highly repeatable polymorphic markers were scored (see Materials and Methods for details). OP50 *E. coli* DNA was used as a control for nematode-only bands.

on genomic DNA to determine the degree of polymorphism among the six available *P. oxycercus* strains. AFLP generates a fingerprint of globally distributed dominant markers that can be scored as present or absent between any two strains (Vos et al., '95) (Fig. 3). Genomic DNA was extracted once from multiple culturing plates per strain and collected into one sample tube per strain (see Materials and Methods). The degree of pairwise differences in 265 AFLP markers ranged from 39% (RS6756 vs. DF5058) to 50% (SB200 vs. RS6877), with an approximate mean of 45% difference (Table 2). This is likely a conservative estimation of polymorphism level, since for each strain we used only one DNA sample from an otherwise highly polymorphic population. To our surprise, there appeared to be no correlation between DNA sequence diversity and geographical proximity, as neither the three northeastern US strains (RS6877, RS6756, DF5058) nor the two northeastern Indian strains (EUK103, EUK106) had a lower degree of polymorphism among themselves than to their trans-Pacific brethrens. We also noticed several strain-specific markers, with strain RS6756 having the most with 7% of the total markers scored (Table 2). On the other hand, even recently separated lineages RS6877α (kept at 20°C) and RS6877β (kept at 30°C) displayed 9% polymorphism with each other, suggesting that the common parents they descended from seven generations ago had a high level of allelic diversity or heterogeneity (197 markers scored, data not shown). In all, AFLP analysis showed that all strains are almost equally polymorphic from each other, and thus any two strains can potentially be chosen as the reference/mapping pair based on their degree of DNA polymorphism.

Low intra-strain polymorphism and inbreeding tolerant strains

In addition to measuring inherent life traits and DNA polymorphism, the successful isolation of homogeneous laboratory strains depends on cultivating inbred isogenic lines. Our inbreeding effort involved the random mating of small populations consisting of two to six individuals of both sexes, starting with a single mating pair in each line. Figure 4A shows the percentage of surviving isogenic lines of four strains over 16 generations. Inbreeding depression manifested as non-hatching eggs or non-mating individuals, and was a gradual process in strains EUK103, EUK106, and RS6877. These three strains also had similar percentages of surviving lines at the end of 16 generations, with

TABLE 2. Number of markers (parenthesis) and percentages of pairwise differences in AFLP markers among the six Poikilolaimus oxycercus laboratory strains using four primer pairs–E-TA/M-CAG, E-TG/M-CTG, E-AC/M-CAA, E-AG/M-CAC

Strains	SB200	RS6877	EUK103	EUK106	RS6756	DF5058
SB200		(133)	(110)	(130)	(121)	(102)
RS6877	50		(131)	(125)	(120)	(109)
EUK103	42	49		(118)	(123)	(97)
EUK106	49	47	45		(115)	(87)
RS6756	46	45	46	43		(97)
DF5058	46	49	44	39	44	
Unique markers	2 (5)	4 (8)	5 (12)	6 (14)	7 (16)	4 (9)

A total of 265 markers were scored for each strain, except for the DF5058 strain (222 markers). The lowest row indicates the percentage of markers (number of markers) unique to a given strain not present in the other five strains.

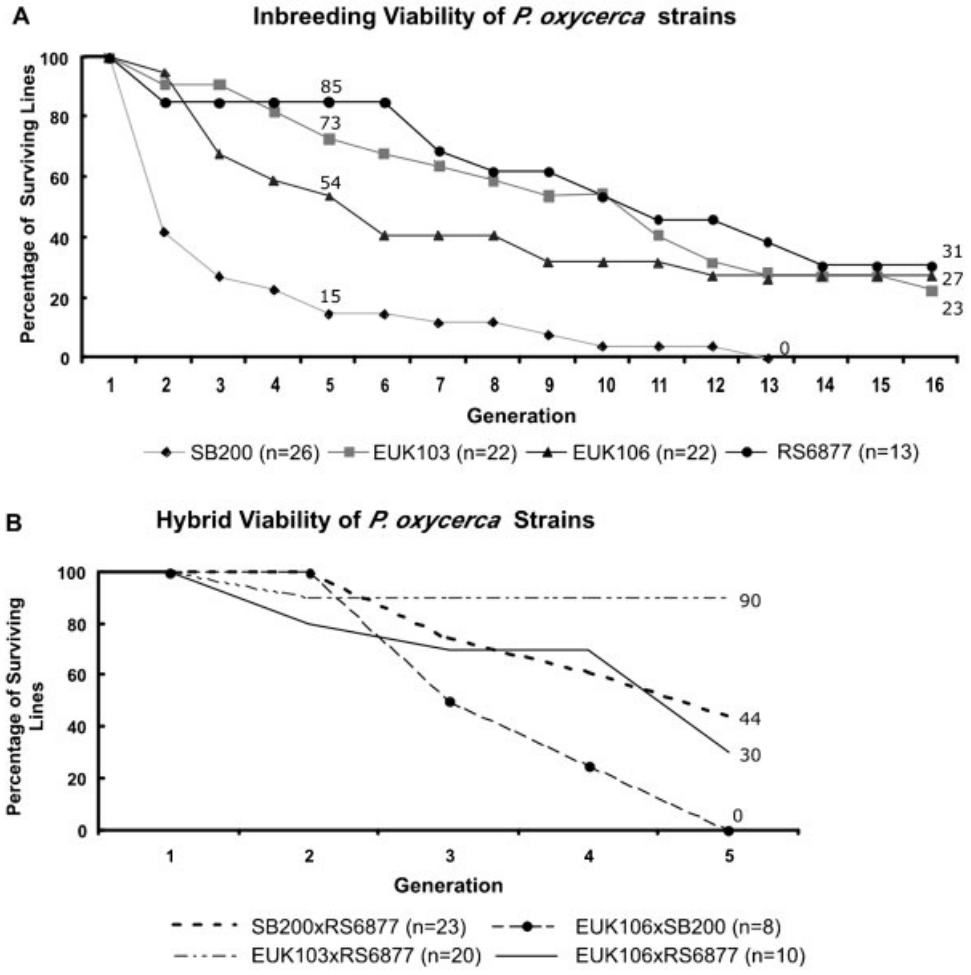


Fig. 4. (**A**) Inbreeding viability of *P. oxycercus* strains. Four *P. oxycercus* strains—SB200, EUK103, EUK106, and RS6877—were inbred for over 16 generations. ''*n*'' is the number of lines initiated. Values above generation 5 are shown for comparison to those of recombinant inbred lines at the same generation, while those values next to the last entries indicate the surviving lines after generation 16. SB200 inbred lines became extinct by generation 12. (**B**) Hybrid viability of *P. oxycercus* strains. Four recombinant inbred lines—SB200 × RS6877, EUK106 × SB200, EUK103 × RS6877, EUK106 × RS6877 (male × female)—were inbred for five generations. Values next to the last entries indicate the percentage of surviving lines after generation 5. Inbred lines of SB200 × RS6877 became extinct by generation 4.

EUK106 and RS6877 having the highest values at 27% and 31%, respectively. SB200, on the other hand, suffered from inbreeding depression immediately following the first generation, and despite starting with the most number of lines ($n = 26$), all lines became extinct by generation 13. No AFLPs or inbreeding depression were detected in the selfing relative *P. regensfussi* among five inbreeding lines after the same number of inbreeding generations (data not shown). Assuming that we started with two unrelated individuals in making our isogenic lines, inbreeding by limiting population sizes averaging four individuals for 16 generations will result in an inbreeding coefficient of $F_{16} = 0.90$, that is, 90% of the two alleles at a given locus will be autozygous (homozygous by common descent). This is approximately equivalent to three generations in a selfing species. This expected inbreeding coefficient is in agreement with our finding that only 10% of the 40 inter-strain polymorphic molecular markers are also intra-strain polymorphic after the 16th generation (R. Hong, unpublished results).

Inter-strain crosses are viable

In addition to cultivating inbreeding tolerant lines necessary for mutagenesis screens, inbred lines must also be hybrid compatible for mapping crosses and QTL analysis. Hence, we also plotted

survival curves of four inter-strain crosses: SB200 × RS6877, EUK106 × SB200, EUK103 × RS6877, and EUK106 × RS6877. After five generations, the SB200 × RS6877 recombinant inbred lines (RILs) became extinct, while the EUK103 × RS6877 RIL did not suffer noticeable inter-strain incompatibility, given that 90% of the lines survived (Fig. 4B). The numbers of surviving lines as isogenic lines and as RILs can be compared at generation 5 (Fig. 4A and B). Since future mapping efforts will require RIL viability only until the F_2 generation, we considered EUK106 × SB200, EUK103 × RS6877, and EUK106 × RS6877 crosses to be equally practical options, but not SB200 × RS6877, as the severe drop in survival value in F_3 was already apparent in F_2 as slow-growing non-mating worms. In total, strain SB200 segregates for many lethal incompatible loci (high genetic load) and could neither be maintained as inbreeding lines nor as an inter-strain hybrid to EUK106 under our culturing conditions. However, EUK106, EUK103, and RS6877 can survive as viable near-isogenic lines and certain inter-strain hybrids. Due to its high survival rate at generation 5, the EUK103 × RS6877 combination may be the best starting cross for future QTL studies. Given that our priority is to set up mapping crosses, we chose at this juncture two inbred tolerant isogenic lines, EUK106-G and RS6877-5, which grow robustly and have low intra-strain polymorphism (data not shown). Consequently, we constructed two strain-specific poly-A cDNA libraries from these reference lines and tested the resulting PCR-based markers for SSCP polymorphisms (R. Hong, unpublished results). We subsequently utilized one of these markers, a highly polymorphic marker PS30, for marking strains and individuals within the same strain.

Mating behavior

To determine if the high heterogeneity in *P. oxycercus* can be attributed to an outcrossing mating preference for less related individuals, we used the molecular marker PS30 to measure progeny genotypes. Individuals of sexually reproducing organisms have a tendency to outcross to avoid inbreeding, especially in small populations, and this behavior can thwart our efforts to cultivate inbreeding tolerant, homogeneous strains. To test whether isogenic *P. oxycercus* females prefer other isogenic strains or have no preference, we set up isogenic line EUK106-G females in polyandrous mating scenarios with four males—from the same isogenic line EUK106-G, a different isogenic line but separated by ten generations from the same ancestral strain, EUK106-T, or an altogether separate strain, RS6877-5 (Fig. 5). Furthermore, the RS6877-5 line used was still polymorphic for three different PS30 marker genotypes, thus allowing us to discriminate up to three possible progeny outcomes by different individuals of this isogenic strain. We determined the PS30 genotypes of all parents and viable F_1 offspring from six independent crosses. Mating worms were incubated for 4 or 5 days, that is, approximately 10% of their fecund period (assuming that females remain fertile for 10 weeks after sexual maturity at 3 weeks). We observed progeny with contributions from all three discernible genotypes of the four RS6877-5 males when EUK06-G females were presented with males from the RS6877-5 strain (crosses 1 and 2), demonstrating that females may mate with multiple males in a relatively short time. The lack of female preference to the ''self'' strain was again observed in matings to both EUK106-G (intra-strain) and RS6877-5 (inter-strain) males (crosses 3 and 4). Finally, the presence of the ''self'' isogenic brethrens EUK106-G, ''non-self'' isogenic EUK106-T, and inter-strain RS6877-5 all together similarly resulted in progeny from two or all three potential fathers (crosses 5 and 6). These mating experiments underscore the potential for *P. oxycercus* females, without female competitors, to mate and perhaps retain sperm from multiple males in a short period, and without noticeable preference to males from more distant strains.

DAPI staining

In order to estimate the number of potential linkage groups in *P. oxycercus*, we counted the number of chromosome pairs at diakinesis in the germ line of both sexes by DAPI staining whole worms. During diakinesis in the prophase of meiosis I, condensing cells in both *C. elegans* and *P. pacificus* hermaphrodite oocytes have six bivalents that represent six paired chromosomal homologs still attached by chiasmata (Villeneuve, '94). Male spermatocytes in these species, however, produce both X and O spermatocytes; thus, they are visible as six or five bivalents.

DAPI staining of *P. oxycercus* revealed that in EUK106 and RS6877 strains, both females (Fig. 6A, B) and males (Fig. 6E, F) have only five bivalents in their germ line. Five chromosomes

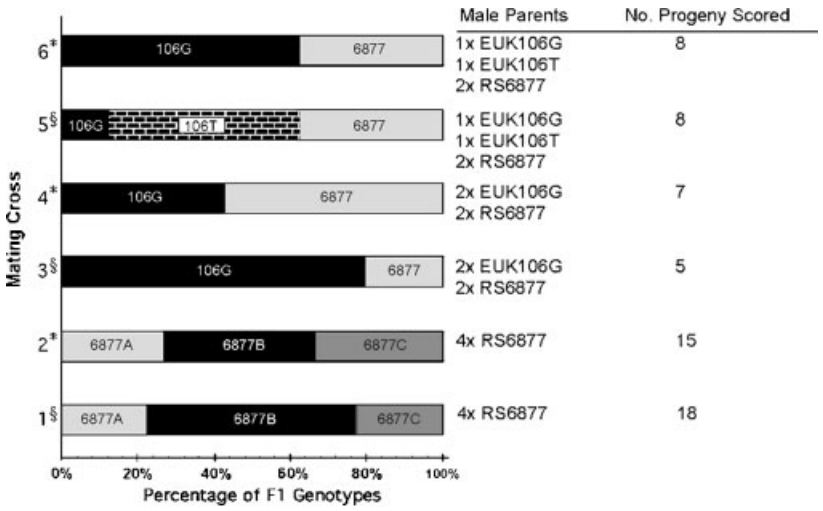


Fig. 5. The mating outcomes of EUK106 inbred line G females in the company of four males of different intra- or inter-strain genotypes as scored by the genotypes of F_1 progeny. Mating animals were incubated for either 4 days (§) or 5 days (*) at 25°C. Black patterns denote F_1 genotypes with EUK106-G male parentage and gray patterns denote F_1 genotypes with RS6877-5 male parentage. Males from EUK106 are either from isogenic lines EUK106-G or EUK106-T. The four RS6877-5 males in crosses 1 and 2 both consisted of two genotype B (6877B), one genotype A (6877A), and one genotype C (6877C) individuals. Refer to text for details.

were also observed in both sexes of the gonochoristic species *P. ernstmayri*, a basal species in the *Poikilolaimus* genus (Fig. 6C, G), as well as in the hermaphroditic-related species *P. regenfussi* (Fig. 6D; we never witnessed any *P. regenfussi* males). As a comparison, *P. pacificus* oocytes have six bivalents visible during diakinesis (Fig. 6H). Sudhaus ('80) reported that *P. regenfussi* may be a parthenogenic species but we observed sperm-like DAPI staining in their putative spermathecae (not shown); thus, *P. regenfussi* could be a true hermaphroditic species or a parthenogenic species that requires sperms to initiate oocyte progression. Most *P. oxycercus* oocytes in meiotic prophase show the expected temporal order of chromosome condensation along the distal–proximal axis of the gonads. We also analyzed SB200, EUK103, and DF5058 strains of *P. oxycercus* and observed five DAPI staining bodies in the germ lines of both sexes. Thus, the members of the *Poikilolaimus* genus may have five rather than the six chromosomes observed in *C. elegans* and *P. pacificus*, but the final confirmation of chromosome number will depend on additional independent assays (e.g., genetic linkage group analysis).

DISCUSSION

The current study incorporates the basic biology, inbreeding tolerance, DNA polymorphisms, and mating behavior of *Poikilolaimus oxycercus* as criteria for choosing the ideal conditions and strains for long-term genetic analyses. This species portrait of *P. oxycercus* was the summation of six strains and three related species. We consider our findings to be directly comparable and relevant to other laboratory nematode species given that our *Poikilolaimus* species were raised under standard *C. elegans* conditions.

In our effort to select the reference *P. oxycercus* strain, we compromised between provisions for inbreeding tolerance and shorter generation periods. In particular, we chose the isogenic line EUK106-G as our reference line because of this line's robust reproductive fitness under inbreeding conditions, albeit the ancestral EUK106 strain has the longest generation time of 21 days out of the three strains measured. The RS6877-5 isogenic line was selected as the mapping cross line, because of its short generation time and high brood size, but not as the reference line because it retained much more intra-strain DNA polymorphism than EUK106-G at the 16th inbred generation (data not shown). These two selected isogenic lines appear to be much more fit than their founders and some of their related lines as measured by the time required for four individuals to saturate 3.5 cm OP50 plates, so we believe that the culturing conditions are now optimal and sufficient for future studies. To our advantage, the gross body morphology of RS6877-5 is dumpy (short and thick) and thus is readily discernable

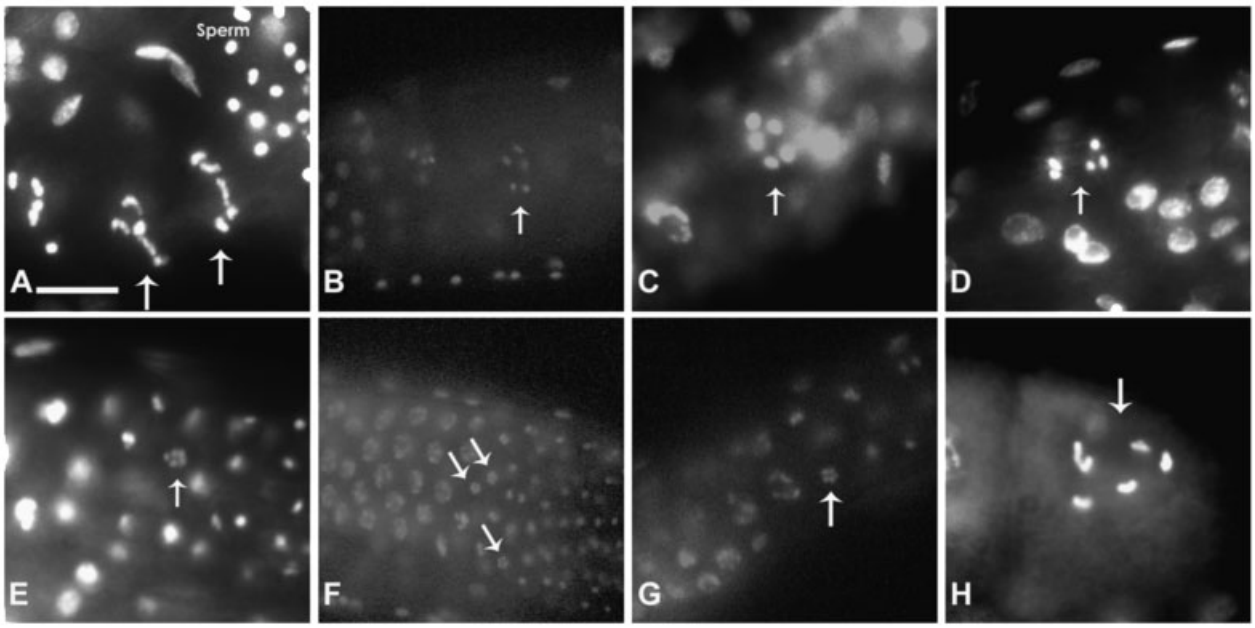


Fig. 6. DAPI-stained bodies show five chromosomes in both sexes in the germline of *Poikilolaimus* (A–C, E–G). Oocytes during prophase of meiosis I (arrows, A–D, H). Maturing male spermatocytes during prophase show that the condensed chromosomes are visible as five bivalents (arrows, E–G). (**A**) *P. oxycercus* strain EUK106 female oocytes in diplotene/diakinesis distal to sperms in the spermatheca. The chiasmata still attach the homologs together as the chromosomes continue to condense. (**B**) *P. oxycercus* strain RS6877 oocyte in diakinesis shows five condensed bivalents. (**C**) *P. ernstmayri*, the basal species of this genus, also possesses five bivalents in oocytes in diakinesis. (**D**) *P. regenfussi* hermaphrodite oocyte in diakinesis shows five condensed bivalents. (**E–G**) *P. oxycercus* EUK106, *P. oxycercus* RS6877, *P. ernstmayri* spermatocytes, respectively, in their highly condensed state all show five bivalents. (**H**) *Pristionchus pacificus*, an *XX*/*XO* hermaphroditic/male species, shows six visible bivalents in an oocyte in diakinesis. Anterior is to the left and ventral side is down in all panels. Scale bars denote 10 μm. (A) and (D) are derived from 13-μm-thick stacked images.

from the EUK106 strain. The body morphologies of several other RS6877 isogenic lines at generation 16 actually resemble the svelte EUK106 more than its brethren, RS6877-5. The reciprocal was not observed in the EUK106-G line in that all surviving EUK106 isogenic lines displayed similar body morphology. This suggests that certain quantitative morphological traits are preserved in our collection of isogenic lines and, as such, may be an asset for future QTL analyses. The ideal ambient temperature for maintaining *P. oxycercus* seemed to be 20–25°C owing to the increasing tendency at higher temperatures for contamination of OP50 by other microorganisms and in worm burrowing. Nevertheless, higher temperatures up to 30°C (*P. oxycercus* do not proliferate at all at 37°C) remain a viable option for accelerating *P. oxycercus*'s reproductive cycle when the cultures' hygiene and sample availability are not a priority.

Our values for life span and brood size at 20°C for *P. oxycercus* are somewhat different from previously published values for this species. In particular, the strains EUK106, RS6877, and SB200 are all lower than those of an early study on *P. oxycercus*, where it was found that paired *P. oxycercus* grown on oat gruel had 346 ± 145 progeny and lived for 138 days for both males and females (Kampfe and Schmidt, '66). In a later study, paired *P. oxycercus* grown on potato extract had an average of 219 eggs and an average 59-day life span (Sudhaus, '80). The primary difference in our brood size is likely due to allowing the males to mate only for one generation period rather than for their entire reproductive period, while another reason for the different values among all three studies may be due to diet (oat, potato, or *E. coli*). In any case, the higher range brood size of ~220 eggs per *Poikilolaimus oxycercus* female is similar to those of individual hermaphrodites of *Pristionchus pacificus* (~200 eggs) and *C. elegans* (~300 eggs). The difference in life span could also be attributed to natural variation, since SB200 shares a common ancestry with the population used in the Sudhaus ('80) study, and thus is similar to our average of a 50-day life span, while the provenance for the *P. oxycercus* studied by

Kampfe and Schmidt ('66) was not clear. Differences due to natural variation alone were strikingly evident in this study, in which a twofold difference in life span between EUK106 and SB200, and a fourfold difference in brood size between RS6877 and SB200 were observed. Consequently, our brood size and life span values are expected to be lower estimates for the reproductive potential of *P. oxycercus* as a species in culture, but a more accurate estimate for practical genetic studies involving single pair matings between specific isogenic lines.

Compared with members of *Caenorhabditis* or *Pristionchus* genera, the absolute generation time of *P. oxycercus* is much longer at 20°C, with a mean generation time between 16 and 21 days and a life span of 50–92 days. However, when measured in terms of average generation time as a proportion of the average life span, the relative generation time of *P. oxycercus* is comparable to that of *C. elegans*, which has a generation time of 3 days and lives for 11–17 days (Gems and Riddle, 2000). Thus, both *C. elegans* and *P. oxycercus* have average life spans roughly four times as long as their generation periods. *C. remanei* is the closest gonochoristic species related to *C. elegans* in the *Caenorhabditis* genus; it also has a similar generation time of 3 days, and is generally intolerant to ambient temperatures of 30°C (Gems, 2000). However, the long generation time and higher thermo-tolerance of *P. oxycercus* are within the range found for other rhabditids. For example, the free-living, gonochoristic nematode *Rhabditis tokai* has a female life span ranging from 92–97 days at 20°C to 30 days at 35°C on *E. coli*, and therefore is more similar to the life span and temperature tolerance range exhibited by *P. oxycercus* (Suzuki et al., '78). Finally, members of the *Poikilolaimus* genus itself exhibit different levels of thermo-tolerance, as both *P. oxycercus* and *P. regenfussi* can live blithely at 30°C whereas *P. piniperdae* and *P. ernstmayri* cannot.

The high DNA polymorphism of *P. oxycercus* prompted us to wonder if the females have a preference for less-related individuals. Using the molecular marker PS30, we were able to assign the paternity of offspring: we found that not only do *P. oxycercus* females mate with multiple males within 10% of their expected fecund period, they also have no measurable preference for less related suitors. This inclination for multiple matings was evident when controlled for males of only RS6877 background, and the comparatively high number of progeny scored in both replicates indicated an equal distribution of progeny from all three possible genotypes (crosses 1 and 2, Fig. 5). The mating strategy of *P. oxycercus* can thus warrant further investigation with molecular tools and aid in the understanding of the reproductive biology of free-living gonochoristic nematodes. The unusually lifetime long fecund period of *P. oxycercus*, when coupled with its promiscuous reproductive strategy, may be an asset for studying various aspects of mating behavior in free-living nematodes.

P. oxycercus individuals are genetically very different from each other. Wild isolates of gonochoristic nematodes have more genetic diversity than their wild hermaphroditic relatives, as exemplified by the higher DNA polymorphism of the cross-fertilizing *C. remanei* compared with their self-fertilizing *C. elegans* and *C. briggsae* sister species (Graustein et al., 2002). This genetic diversity is inclement to genetic mapping, which is contingent upon homogeneous isogenic laboratory strains. The cultivation of isogenic lines involves mating between related individuals, but the ensuing inbreeding depression causes a loss of reproductive fitness due to inbreeding. Although inbreeding depression has been well documented in dioecious metazoans and plants, and to a slight extent in *C. elegans*, the studies are scant for gonochoristic free-living nematodes (Charlesworth and Charlesworth, '87). The prevailing hypothesis for the genetic basis of inbreeding depression is the loss of heterozygosity at recessive lethal loci normally maintained through outbreeding, which results in the accumulation of lethal homozygous alleles that reduce fitness (Charlesworth and Charlesworth, '99). In this context, the cultivation of inbreeding tolerant lines can be thought of as the stochastic outcome of selecting for compatible homozygous alleles at lethal loci.

Our inbreeding program culminated in the selection of two near-isogenic lines for future analyses. We began cultivation with a single-pair bottleneck followed by closed small population inbreeding of four *P. oxycercus* strains: SB200, EUK103, EUK106, and RS6877. Strains SB200, EUK103, and EUK106 have been kept under laboratory conditions for some time, while strain RS6877 was freshly isolated from the wild. We expected that older laboratory strains have already gone through selective bottlenecks and therefore would exhibit less inbreeding depression than newly domesticated strains. Hence, we were intrigued to find that the established laboratory

strains EUK103 and EUK106 incurred similar levels of line loss during inbreeding (73% and 77% line loss) as the newly isolated RS6877 strain (69% line loss). This suggests that normal laboratory culturing methodology is not sufficient to ensure the generation of inbreeding tolerant lines, and that a stricter inbreeding regiment is required to expedite the process. We also found that SB200 carried a high negative genetic load and did not produce a viable isogenic line through inbreeding. Alternatively, since SB200 did not exhibit noticeable heterosis in SB200 × RS6877 and SB200 × EUK106 hybrid crosses with respect to surviving RILs (Fig. 4), it may bear dominant incompatible alleles from an older hybridization event (Belgian × German cross, see Materials and Methods) rather than inbreeding depression per se. Nevertheless, our cultivation effort yielded two isogenic lines, the Indian EUK106-G and the North American 6877-5, which are inbreeding and interbreeding tolerant as well as polymorphic to each other.

The well-known laboratory nematodes *C. elegans* and *P. pacificus* both have six chromosomes, including an X sex chromosome, whereas the *Poikilolaimus* genus is unusual in that all three member species we analyzed contained only five chromosomes. We have shown by counting DAPI-stained bodies in the spermatocytes and oocytes that *P. oxycercus* germ line cells contain five chromosome pairs, and that these chromosome numbers extend to two other members of the *Poikilolaimus* genus: the most basal and gonochoristic *P. ernstmayri* as well as the hermaphroditic/parthenogenic *P. regenfussi*. This finding is consistant with a previous cursory chromosome count in *P. oxycercus* and *P. regenfussi* (Schuster, '80). *P. oxycercus* is a gonochoristic species with almost equal segregation of male and female progeny. The male:female ratios of both EUK106 and RS6877 display a slightly higher proportion of females, although the ratios are not statistically significantly different from 1:1 (data not shown). We stress that DAPI staining is only one type of assay used to determine chromosome number, and the making of a genetic linkage map is further needed to independently confirm that *P. oxycercus* has five linkage groups and that no small chromosome body has escaped detection. Additionally, DAPI staining of somatic cells in both sexes is also needed to correlate chromosome number with sex phenotypes. If both sexes of *P. oxycercus* indeed possess one less chromosome than *C. elegans* and *P. pacificus* hermaphrodites, then it becomes tempting to speculate if *P. oxycercus* utilizes a non-*XX*/*XO* sex determination system. Hermaphroditic species such as *C. elegans* and *P. pacificus*, as well as the gonochoristic species *C. remanei*, all utilize the *XX*/*XO* sex determination system, consisting of *XX* hermaphrodites/females and *XO* males (Hodgkin and Brenner, '77; Haag and Kimble, 2000; Srinivasan et al., 2002). Although an *XX*/*XY* type of sex determination has not been genetically characterized in nematodes, an *X*-autosome fusion in the last common ancestor of *Poikilolaimus* and *Caenorhabditis*, or *Poikilolaimus* and *Pristionchus*, could conceivably convert an *XX*/*XO* type of sex determination to an *XX*/*XY* system. A similar phenomenon can occur in *C. elegans* due to the fusion of chromosomes *X* and *IV* to create the translocation *mnT12* (Sigurdson et al., '86). In this instance, matings between *mnT12*/*mnT12* hermaphrodites and *mnT12*/*IV* males are stable for many generations, and hence the *mnT12* can be thought of as the neo-*X* chromosome and *IV* as the neo-*Y* chromosome. Hence, the study of chromosome evolution in *Poikilolaimus* may also be an unexpected but promising avenue in future research.

Our primary intention for cultivating *P. oxycercus* as a laboratory organism is to conduct molecular genetic studies on vulva patterning, particularly to determine the molecular components necessary for apoptosis in P*n*.p cells. The Hox gene *lin-39* is the primary factor in the patterning of the vulva equivalence group in both *C. elegans* and *Pristionchus pacificus* (Clark et al., '93; Wang et al., '93; Eizinger and Sommer, '97). Specifically, in *lin-39* loss-of-function mutants, the VPCs which normally differentiate into vulval tissue instead adopt the non-VPC cell fates of their anterior and posterior P*n*.p counterparts, either by cell fusion or apoptosis. However, while *Cel*-LIN-39 prevents cell fusion and has an instructive role in these VPCs, *Ppa*-LIN-39 prevents programmed cell death in the VPCs but does not play an instructive role in the later step of vulval specification (Sommer et al., '98). The question remains: what accounts for the divergence of *lin-39* function in these two nematodes? One route towards answering this question may lie in dissecting the role of homologous vulva patterning genes in *P. oxycercus*. Forward genetics screens for vulvaless and egg-laying defective mutants in *P. oxycercus* may now be feasible with inbreeding tolerant lines EUK106-G and RS6877-5, especially with the advent of a meiotic linkage map. Furthermore, because a putative *P. oxycercus*

lin-39 gene has already been cloned (R. Hong, unpublished results), we can determine if *P. oxycercus lin-39* has an instructive or preventative role in vulval patterning by employing reverse genetic approaches such as RNAi and TILLING (*P. oxycercus* is amenable to conventional freezing storage) (Fire et al., '98; Kamath et al., 2001; Till et al., 2003). In conclusion, our initial motivation to cultivate *P. oxycercus* in the study of vulval patterning led us also to other aspects of nematode biology that would benefit from comparative analyses, namely reproductive behavior in gonochoristic species and chromosome evolution.

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