Description of Three *Pristionchus* Species (Nematoda: Diplogastridae) from Japan that Form a Cryptic Species Complex with the Model Organism *P. pacificus*

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Three new species of *Pristionchus* (*P. exspectatus*, *P. arcanus*, and *P. japonicus*) are described from Japan. They are morphologically similar, with *P. exspectatus* and *P. arcanus* being almost indistinguishable from the model organism *P. pacificus*. Reproductive isolation, namely the inability to produce interfertile F1 hybrids, separates all species pairs in the species complex. Additionally, all three new species are distinguished from *P. pacificus* Sommer, Carta, Kim, and Sternberg, 1996 by having a gonochoristic instead of hermaphroditic mode of reproduction. In addition to its reproductive isolation, *P. japonicus* is distinct from other *Pristionchus* species by its arrangement of genital papillae. All species in the complex are separated from each other by molecular sequence divergence, as indicated by analysis of 27 nuclear protein-coding genes and unique sequences of the small subunit ribosomal RNA gene. The identification of a species complex that includes *P. pacificus* is invaluable for studies of population genetics, speciation, and macroevolution, particularly the evolution of hermaphroditism in the genus.

Key words: hermaphroditism, insects, morphology, phylogeny, reproductive isolation, species concepts, taxonomy

INTRODUCTION

The nematode *Pristionchus pacificus* Sommer et al., 1996 is a well-established model system for evolutionary developmental biology (Sommer, 2009), drawing strength as a satellite model to that of *Caenorhabditis elegans* (Maupas, 1899) Dougherty, 1953. A suite of analytical tools available for *P. pacificus* enables comparative, mechanistic studies of genetics (e.g., Eizinger and Sommer, 1997; Zauner and Sommer, 2007; Rudel et al., 2008) and include an extensive genetic linkage map and physical map (Srinivasan et al., 2002, 2003), a completely sequenced genome (Dieterich et al., 2008), forward and reverse genetics capabilities (Zheng

et al., 2005; Schlager et al., 2006; Tian et al., 2008), and DNA-mediated transformation (Schlager et al., 2009). The isolation of hundreds of worldwide strains of *P. pacificus* (Herrmann et al., 2010) and the support of a robust phylogenetic infrastructure (Mayer et al., 2007, 2009) now offer the potential to study the evolution of mechanistic traits within and among species. Invaluable for linking processes of micro- and macroevolution is knowledge of closely related species, although those close enough to be considered sister species to either *C. elegans* or *P. pacificus* have been thus far unknown. To address this gap, we report and describe three new species of *Pristionchus* Kreis, 1932 from Japan that form a species complex with *P. pacificus*.

The genus *Pristionchus*, of the family Diplogastridae Micoletzky, 1922, is distributed globally and is particularly well known throughout the Holarctic ecozone. *Pristionchus* species have been often collected from soil and decaying organic matter (Sudhaus and Fürst von Lieven, 2003), although many species are known to be associates of insects, especially beetles (Völk, 1950; Fedorko and Stanuszek, 1971; Herrmann et al., 2006a, b, 2007; Kanzaki et al., 2011). Directly targeting insects for nematode sampling has thus led to a boom in the number of *Pristionchus* strains recovered (Herrmann et al., 2006a, 2010). Reproductive biology within *Pristionchus* is diverse, as hermaphroditism has arisen independently at least five times in the genus (Mayer et al., 2007). Thus with respect to their trac-

tability in the laboratory, *Pristionchus* nematodes constitute a prime model for the evolution of sex strategies. The discovery of gonochoristic species close to *P. pacificus* would therefore make basic genetics studies in this area feasible.

Despite increasing research on the biology of *Pristionchus* nematodes, several problems beset taxonomic description of the genus. Such problems include insufficient typological characters, intraspecific variation in qualitative and quantitative characters, and many species with unclear descriptions. Herrmann et al. (2006b) pointed out these problems and distinguished several cryptic species from closely related nominal species based on molecular sequence divergence and phylogenetic hypotheses. On this evidence they described four species: *P. marianneae* Herrmann et al., 2006b, *P. pauli* Herrmann et al., 2006b, *P. americanus* Herrmann et al., 2006b and *P. pseudaerivorus* Herrmann et al., 2006b.

Currently, the genus *Pristionchus* contains 31 recognized species (Sudhaus and Fürst von Lieven, 2003; Herrmann et al., 2006b) as well as many undescribed species that have been referred to in recently published works under tentative species codes (Mayer et al., 2007, 2009). In the present study, three new *Pristionchus* species, including two morphologically almost indistinguishable species (= cryptic species), are described based on their morphology, molecular sequences, inferred phylogenetic separation, and biological characters.

MATERIALS AND METHODS

Nematode isolation and cultivation

Pristionchus exspectatus was isolated from an adult of *Prismognathus angularis* Waterhouse, 1874 (Coleoptera: Lucanidae) collected at Mt. Shibi, Kagoshima, Japan in September 2010. The strain has been kept in laboratory culture on NGM agar plates seeded with *Escherichia coli* strain OP50, under the culture code and freezing voucher number RS5522. Detailed isolation conditions for the strain are described in Kanzaki et al. (2011).

Pristionchus arcanus was isolated from a colony of *Odontotermes formosanus* Shiraki, 1909 (Isoptera: Termitidae) collected from Iriomote Island, Okinawa, Japan in September 2009. Thirty termite workers were dissected on a 2.0% agar plate, after which the agar plate was kept at room temperature for one month. Nematodes proliferated on bacteria associated with the termite cadaver. Individuals were thereafter transferred to NGM agar plates seeded with *E. coli* OP50, and have been since kept in laboratory culture under the culture code and freezing voucher number RS5527.

Pristionchus japonicus was isolated from a dead earthworm collected from Enoshima Island, Kanagawa, Japan in September 2005. The strain has been kept as a laboratory culture on NGM agar plates seeded with *E. coli* OP50, under the culture code and freezing voucher number SB393.

Morphological observation and preparation of type material

One- to two-week-old cultures of these three species and *P. pacificus* strain RS2333 were prepared for morphological observation. Light microscopic observations, including those used as the basis for drawings and morphometrics, were conducted using live nematode material, which was hand-picked from culture plates. To prepare type material, nematodes were isolated from cultures, rinsed in distilled water to remove bacteria, heat killed at 65°C, fixed in 5% formalin, and processed through a glycerol and ethanol series using Seinhorst's method (see Hooper, 1986).

Scanning electron microscopy

In preparation for scanning electron microscopy (SEM), nematodes were fixed in 2.5% glutaraldehyde in M9 buffer and then post-fixed with 1% osmium tetroxide. After several rinses with water, samples were dehydrated through a graded ethanol series, followed by critical point drying from carbon dioxide. Specimens were mounted on polylysine-coated coverslips, sputter-coated with 20 nm gold/palladium, and then imaged with a Hitachi S-800 field emission scanning electron microscope operating at 20 kV.

Molecular characterization and phylogenetic analysis

To diagnose individual species, we amplified and sequenced an 830-bp fragment of the SSU rRNA gene. For phylogenetic analysis we employed 27 ribosomal protein genes, which were originally developed as marker loci based on their consistent presence in EST libraries of various species (see Mayer et al., 2007). The dataset of ribosomal protein genes comprised a total of 10,971 aligned coding nucleotides. Genes included in analysis were: *rpl-1*, *rpl-2*, *rpl-10*, *rpl-14*, *rpl-16*, *rpl-23*, *rpl-26*, *rpl-27*, *rpl-27a*, *rpl-28*, *rpl-29*, *rpl-30*, *rpl-31*, *rpl-32*, *rpl-34*, *rpl-35*, *rpl-38*, *rpl-39*, *rps-1*, *rps-8*, *rps-14*, *rps-20*, *rps-21*, *rps-24*, *rps-25*, *rps-27*, and *rps-28*. Ribosomal protein genes sequenced for *P. exspectatus* and *P. arcanus* have been deposited in GenBank under accession numbers JQ399909–JQ399956. Ribosomal protein gene sequences for *P. japonicus* were previously published (Mayer et al., 2007). All information regarding genes, primers, and PCR conditions is given in Mayer et al. (2007). New sequences generated in the present study are those for *P. exspectatus* and *P. arcanus*, and *P. japonicus*. All other sequences in analysis were published by Mayer et al. (2007) and thus previously available in the GenBank database. Species selected for analysis include three undescribed species isolated from Japan (*Pristionchus* sp. 10, *Pristionchus* sp. 14, and *Pristionchus* sp. 15) as well as all nominal, morphologically characterized *Pristionchus* species with available gene sequence data. Strain codes for species included in analysis are shown in the tree figure (Fig. 1). Isolation details for all included species other than those described herein are given in Mayer et al. (2007).

The concatenated dataset of ribosomal protein genes was aligned in ClustalX and then analyzed in MEGA5.05 (Tamura et al., 2011) after complete deletion of gapped or ambiguous positions. The substitution model with the best fit, GTR + I + G, was selected by hierarchical likelihood ratio tests and the Akaike information criterion in Modeltest (Posada and Crandall, 1998). Phylogenetic analysis was performed in MEGA5 under the maximum likelihood (ML) criterion and using the data specific model (Nei and Kumar, 2000). Node support was evaluated by 500 bootstrap pseudoreplicates.

Mating experiments

Mating experiments were performed according to standard protocols (Herrmann et al., 2006b). In short, five virgin females of a strain were tested with five males of another strain on a mating plate that contained a small amount of *E. coli* OP50 bacteria. All crosses were performed reciprocally. To test for the production of viable F1 hybrids, crosses were set up between all possible pairs of *P. pacificus*, *P. exspectatus*, *P. arcanus*, and *P. japonicus*. Where available, F1 hybrids were selfed and backcrossed to their parents to test their ability to produce F2 progeny. We considered strains to belong to the same species if they produced viable and fertile offspring.

RESULTS

Molecular characterization and phylogenetic analysis

Sequences of an 830-bp fragment of the SSU rRNA gene were unique for each new species described herein (Supplementary Fig. S1 online). Analysis of the concatenated set of 27 ribosomal protein genes revealed separation of all species in the *Pristionchus pacificus* species complex (Fig. 1). The three new species described herein and *P. pacificus* constitute a strongly supported (100% bootstrap support, BS) monophyletic group. Within this group, *P.*
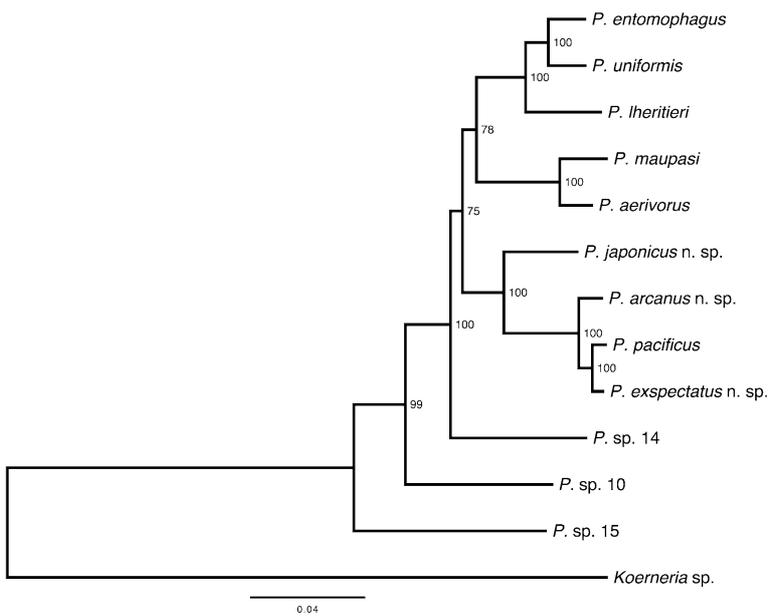
Fig. 1. Phylogenetic relationships of *Pristionchus* species inferred from 27 ribosomal protein-coding genes by maximum likelihood (ML). Analysis was performed using the data specific model (Nei and Kumar, 2000) and a substitution model of GTR + I + G. The tree with the highest log likelihood (–41226.8630) is shown. The percentage of trees in which the associated taxa clustered together in 500 bootstrap pseudoreplicates is shown next to the nodes. A discrete gamma distribution was used to model evolutionary rate differences among sites (five categories (+ G, parameter = 0.4389)). The rate variation model allowed for some sites to be evolutionarily invariable ([+ I], 32.9540% sites). The tree is drawn to scale, with branch lengths measured in the number of substitutions per site.

respective parents yielded only limited F2 progeny. These results suggest that *P. pacificus*, *P. exspectatus*, and *P. arcanus* are more closely related to each other than any of them are to *P. japonicus*. This finding is congruent with the results of detailed molecular phylogenetic analysis (see above). The observed viability and partial fertility of F1 hybrids between all pairwise combinations of *P. pacificus*, *P. exspectatus*, and *P. arcanus* is of potential interest and will be followed up by more detailed analysis following the logic of similar experiments performed between *Caenorhabditis briggsae* (Dougherty and Nigon, 1949) Dougherty, 1953 and *Caenorhabditis* sp. 9 (Woodruff et al., 2010).

TAXONOMY

General morphological characters of the three new species described herein and *P. pacificus* are very similar across species. The three species and *P. pacificus* form a species complex including several "cryptic species" which are distinguished mainly by molecular sequence characters and biological characters. To avoid redundancy, morphology common to all three species is described first, followed by species-specific characters and diagnoses for each species. Finally, relationships among these four species are discussed.

Description of common morphological characters

Adults. Cuticle thick, with fine annulation and clear longitudinal striations. Lateral field consisting of two lines, only weakly distinguishable from body striation. Head narrowly rounded, without apparent lips, and with six short and papilliform labial sensillae (Fig. 2A, B). Four small, papilliform cephalic papillae present in males, as typical for diplogastrid nematodes (Fig. 2B). Amphidial apertures located at level of posterior end of cheilostomatal plates. Stomatal dimorphism present, with stenostomatous (narrow mouth) and eurystomatous (wide mouth) forms occurring in both males and females. Detailed stomatal morphology is described for each species below. Dorsal pharyngeal gland clearly observed, penetrating dorsal tooth to gland opening. Anterior part of pharynx (= pro- and metacorpus) 1.5 times as long as posterior part (isthmus and basal bulb). Procorpus very muscular, stout, occupying half to two-thirds of corresponding body diameter. Metacorpus very muscular, forming well-developed median bulb. Isthmus narrow, not muscular. Basal bulb glandular. Pharyngo-intestinal junction clearly observed, well developed. Nerve ring usually surrounding posterior region of isthmus. Excretory pore not conspicuous, ventrally located at level of basal bulb to pharyngo-intestinal junction. Hemizonid not clearly observed. Deirid observed laterally (Fig. 2G), slightly posterior to pharyngo-intestinal junction. Postdeirids present and observed laterally (Fig. 2G), with positions inconsistent among individuals, numbering 5–8 for males and 9–13 for females.

Stenostomatous form. Cheilostom consisting of six per- and interradial plates. Incision between plates not easily distinguished by light microscopic observation. Anterior end of

exspectatus, *P. arcanus*, and *P. pacificus* form a strongly supported (100% BS) subclade with respect to *P. japonicus* and more distant outgroups. Furthermore, *P. exspectatus* and *P. pacificus* are confirmed as sister taxa (100% BS) with respect to all other known, sequenced *Pristionchus* species. The entire species complex forms a sister clade (75% BS) to all other nominal *Pristionchus* species included in analysis, with the outgroup to both clades being *Pristionchus* sp. 14 (100% BS) from Japan.

Mating experiments

We performed mating experiments to confirm the results of our original species identifications by molecular sequence analysis. As observed in previous studies of the genus *Pristionchus*, the results of mating experiments fully correlate with the SSU sequence analysis. Specifically, both strains of *P. japonicus* produced offspring in reciprocal crossing experiments. Similarly, offspring were observed in reciprocal crosses between strains of *P. exspectatus*. In both cases, the F1 progeny resulting from these crosses were viable and fertile, resulting in viable F2 progeny as well.

When we performed crosses between the reference strains of the four different species, we saw hybridization in the F1 generation, resulting in infertile animals for some of the crosses. While *P. japonicus* did not hybridize with any of the other species, crosses between all possible pairs of *P. pacificus*, *P. exspectatus*, and *P. arcanus* formed viable F1 hybrids, often with equal sex ratios. However, these F1 hybrids were infertile when selfed; backcrosses to their
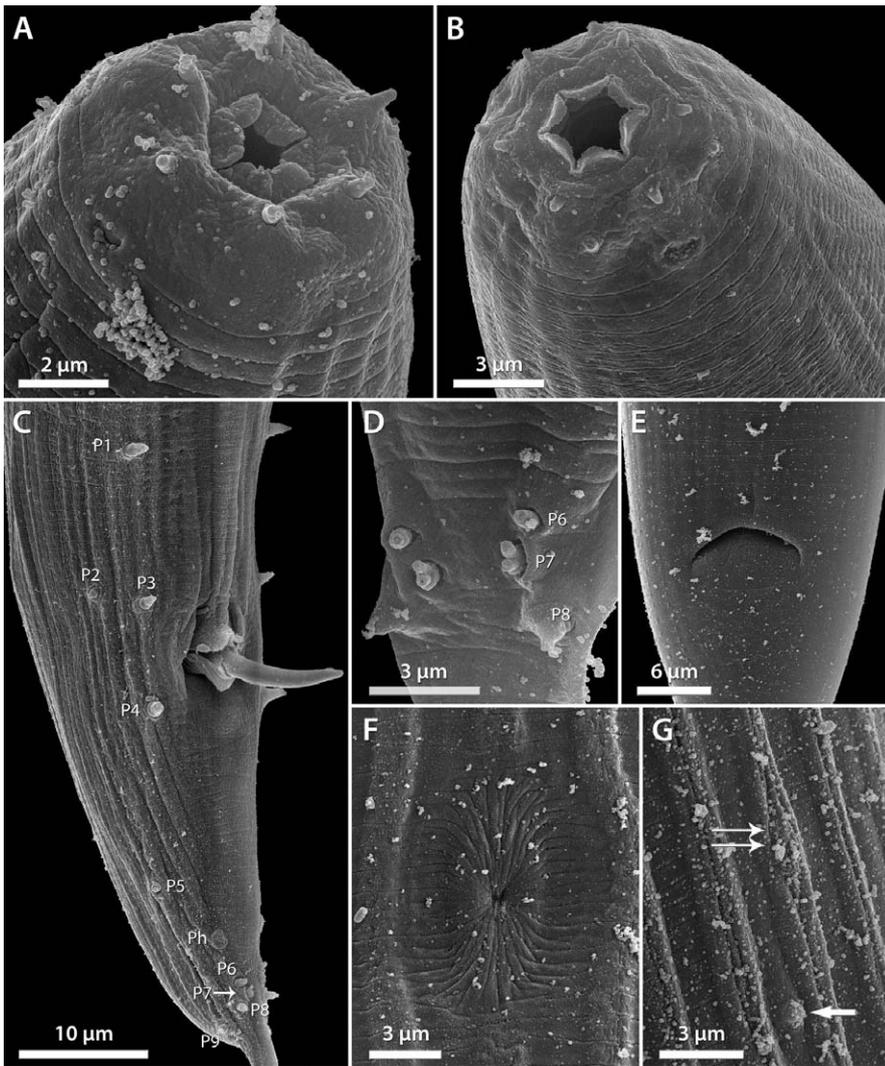
Fig. 2. Scanning electron micrographs of *Pristionchus exspectatus* n. sp., *P. arcanus* n. sp., and *P. japonicus* n. sp. **(A)** Lip region of stenostomatous *P. exspectatus* n. sp. female. **(B)** Lip region of stenostomatous *P. arcanus* n. sp. male. **(C)** Tail region of *P. arcanus* n. sp. male. **(D)** Tail region of *P. japonicus* n. sp. male, showing bifurcate P7 papilla. **(E)** Anus of *P. japonicus* n. sp. female. **(F)** Pore-like vulva of *P. japonicus* n. sp. female. **(G)** Deirid (double-arrow) and postdeirid (single arrow) openings (plugged) of *P. japonicus* n. sp. Deirid opens within mid-lateral line. Postdeirid opens just ventral of mid-lateral line.

anterior part reflexed to right side (Fig. 3B). Spermatogonia arranged in two or three rows in reflexed part; well-developed spermatocytes arranged as two to three rows in anterior two-thirds of main branch; mature amoeboid spermatids arranged in multiple rows in remaining, proximal part of gonad (Fig. 3B). *Vas deferens* not clearly separated from other parts of gonad. Spicules paired, separate. Spicules smoothly curved in ventral view, adjacent to each other for distal third of their length, each smoothly tapering to pointed distal end (Fig. 4E, F). Spicule in lateral view smoothly arcuate ventrally, giving spicule about 100° curvature, rounded manubrium present at anterior end, lamina/calomus complex clearly expanded just posterior to manubrium, then smoothly tapering to pointed distal end (Fig. 4C, E, F). Gubernaculum conspicuous, about half of spicule in length, anterior half with ear-like shape in lateral view (Fig. 4D), posterior half forming a tube-like process enveloping spicules (Fig. 4F). Dorsal side of gubernaculum possessing a single, membranous, anteriorly directed process and a lateral pair of more sclerotized, anteriorly directed processes. Tail conical, with long spike, which has filiform distal end (Figs. 4E, F; 5C, D; 6F, G). Thick cuticle around tail region, sometimes appearing like a narrow leptoderan bursa in ventral view. Cloacal opening slit-like in ventral view. One small, ventral, single genital papilla on the anterior cloacal lip. Nine pairs of genital papillae and a pair of phasmids present. P1–P4 papillae of almost equal size, rather large and conspicuous; P5d slightly smaller than P1–P4; P6 and P7 very small, sometimes difficult to observe with light microscope; P8 and P9d small, but larger than P6 and P7, i.e. intermediate between P5d and P6/P7 in size (Figs. 2C; 4E, F; 5C, D; 6F, G). P6 and P7 papilliform and borne from socket-like base (Fig. 2D); tip of P7 papillae split into two small papilla-like projections (Fig. 2D); P8 simple or typical thorn-like in shape (Figs. 2D; 4F; 5D; 6F). Detailed arrangement of paired papillae and phasmids is described for individual species below. Tail spike about three to four cloacal body-diameter long. Bursa or bursal flap absent.

Female. Relaxed or slightly ventrally arcuate when killed by heat. Gonad didelphic, amphidelphic. Each gonadal system arranged from vulva/vagina as uterus, oviduct, and ovary. Anterior gonad right of intestine, with uterus and oviduct extending ventrally and anteriorly on right of intestine and with a totally reflexed (= antidromous reflexion) ovary

each plate rounded and elongated to project from stomatal opening and form a small flap (Fig. 3F, G). Gymnostom short, cuticular ring-like anterior end overlapping with cheilostom internally (Fig. 3F, G). Dorsal gymnostomatal wall slightly thickened compared to ventral side. Stegostomatal morphology is given for individual species below.

Eurystomatous form. Cheilostom divided into six distinctive per- and interradial plates. Anterior end of each plate rounded and elongated to project from stomatal opening and form a small flap (Fig. 3D, E). Gymnostom with thick cuticle, forming a short, ring-like tube (Fig. 3D, E). Anterior end of gymnostom overlapping internally with the posterior end of cheilostomatal plates. Stegostomatal morphology is given for individual species below.

Male. Ventrally arcuate, strongly curved ventrally at tail region when killed by heat. Testis single, located ventrally,
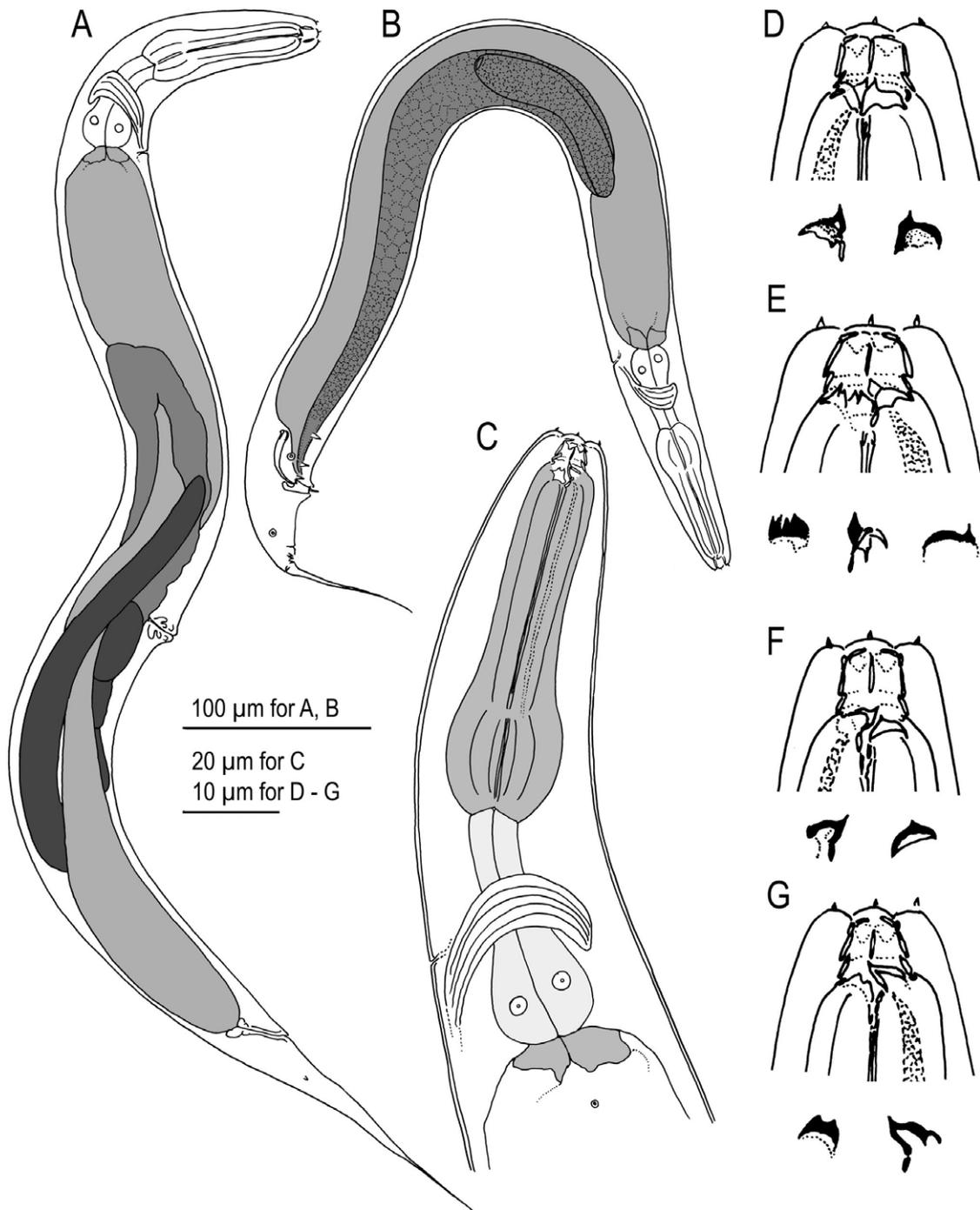
Fig. 3. *Pristionchus exspectatus* n. sp. Drawings are of live specimens (non-types) from temporary mounts. **(A)** Stenostomatous female, right lateral view. **(B)** Stenostomatous male, right lateral view. **(C)** Anterior end of stenostomatous male, left lateral view. **(D)** Stomatal region of eurystomatous female, right lateral view; below (from left to right) are dorsal tooth and right subventral tooth. **(E)** Stomatal region of eurystomatous female, left lateral view; below are left subventral ridge, dorsal tooth, and right subventral tooth. **(F)** Stomatal region of stenostomatous female, right lateral view; below are dorsal tooth and right subventral denticle. **(G)** Stomatal region of stenostomatous female, left lateral view; below are left subventral ridge and dorsal tooth.

extending dorsally on left of intestine (Figs. 3A; 4A). Oocytes mostly arranged in two to four rows in distal half of ovary and in single row in rest of ovary, one well-developed oocyte present at level just anterior to junction of ovary and oviduct, distal tips of each ovary reaching the oviduct of opposite gonad branch (Fig. 4A). Middle part of oviduct serving as spermatheca. Eggs in single to multiple-cell stage or even further developed at posterior part of oviduct (= uterus). *Receptaculum seminis* not observed. Vaginal glands present but obscure. Vagina perpendicular to body surface, surrounded by sclerotized tissue. Vulva slightly protuberant in lateral view (Figs. 3A; 4A), pore-like in ventral view (Fig. 2F).
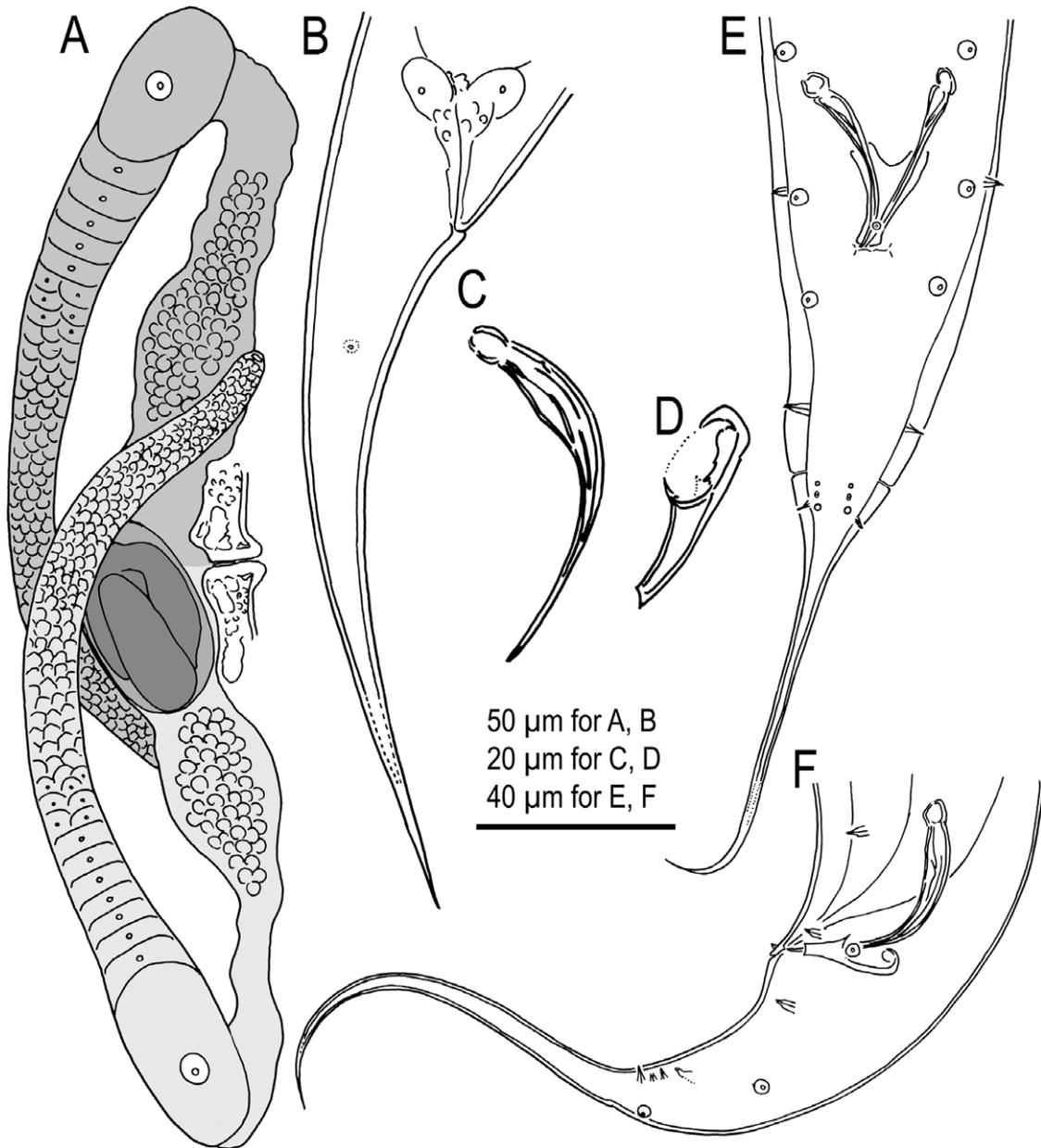
Fig. 4. *Pristionchus exspectatus* n. sp., female **(A–B)** and male **(C–F)**. Drawings are of live specimens (non-types) from temporary mounts. **(A)** Reproductive tract, left lateral view. **(B)** Tail region, right lateral view. **(C)** Spicule, left lateral view. **(D)** Gubernaculum, left lateral view. **(E)** Tail region, ventral view. **(F)** Tail region, left lateral view.

Rectum about one anal body-diameter long, intestine/rectum junction surrounded by well-developed sphincter muscle. Three anal glands present but not obvious. Anus in form of dome-shaped slit (Fig. 2E), posterior anal lip slightly protuberant. Phasmid about one to two anal body-diameter posterior to anus (Figs. 4B; 5B; 6B). Tail long, distal end variable from filiform to long and conical (Figs. 4B; 5B; 6B).

Species descriptions based on species-specific characters

Pristionchus exspectatus n. sp.
(Figs. 2, 3, 4, S1)
Pristionchus cf. *pacificus*: Kanzaki et al., 2011.

Measurements. See Table 1.

Description. *Stenostomatous form.* Stegostom bearing a conspicuous and movable dorsal triangular or diamond-shaped tooth, two bump-like (blunt) left subventral denticles apparently projecting from a common cuticular plate, and a small, short, and pointed right subventral denticle (Fig. 3F, G). Dorsal tooth with strongly sclerotized surface, appearing as an inverted "V" shape in light microscopic observation.

Eurystomatous form. Stegostom bearing a large claw-like dorsal tooth, a large claw-like right subventral tooth, and a row of left subventral denticles of varying numbers and size, i.e. two large denticles to four small denticles (Fig. 3D, E). Dorsal and right subventral teeth movable. Left subven-

Fig. 5. *Pristionchus arcanus* n. sp. Drawings are of live specimens (non-types) from temporary mounts. Regions not shown are considered to be identical with *P. exspectatus* n. sp. **(A)** Anterior end of eurystomatous female, right lateral view. **(B)** Female tail region, right lateral view. **(C)** Male tail region, ventral view. **(D)** Male tail region, left lateral view.

tral denticles immovable.

Male. Nine pairs of genital papillae arranged as ⟨P1, (P2d, P3), C, P4, P5d, Ph, (P6, P7, P8), P9d⟩ (Fig. 4E, F), where, in many individuals, phasmid (Ph) and P6 are close to each other. Ph and P6–P8 linearly arranged, and P9d located at the level of P7 or P8.

Female. Tail smoothly tapered (Fig. 3A), with distal end filiform or elongated conical.

Diagnosis. Besides its generic characters, *Pristionchus exspectatus* n. sp. is diagnosed by its size and arrangement of male genital papillae as described above. The species is also characterized by an 830-bp fragment of the SSU rRNA gene (GenBank accession number JQ399906), the sequence of which is distinct from that of all other *Pristion-*
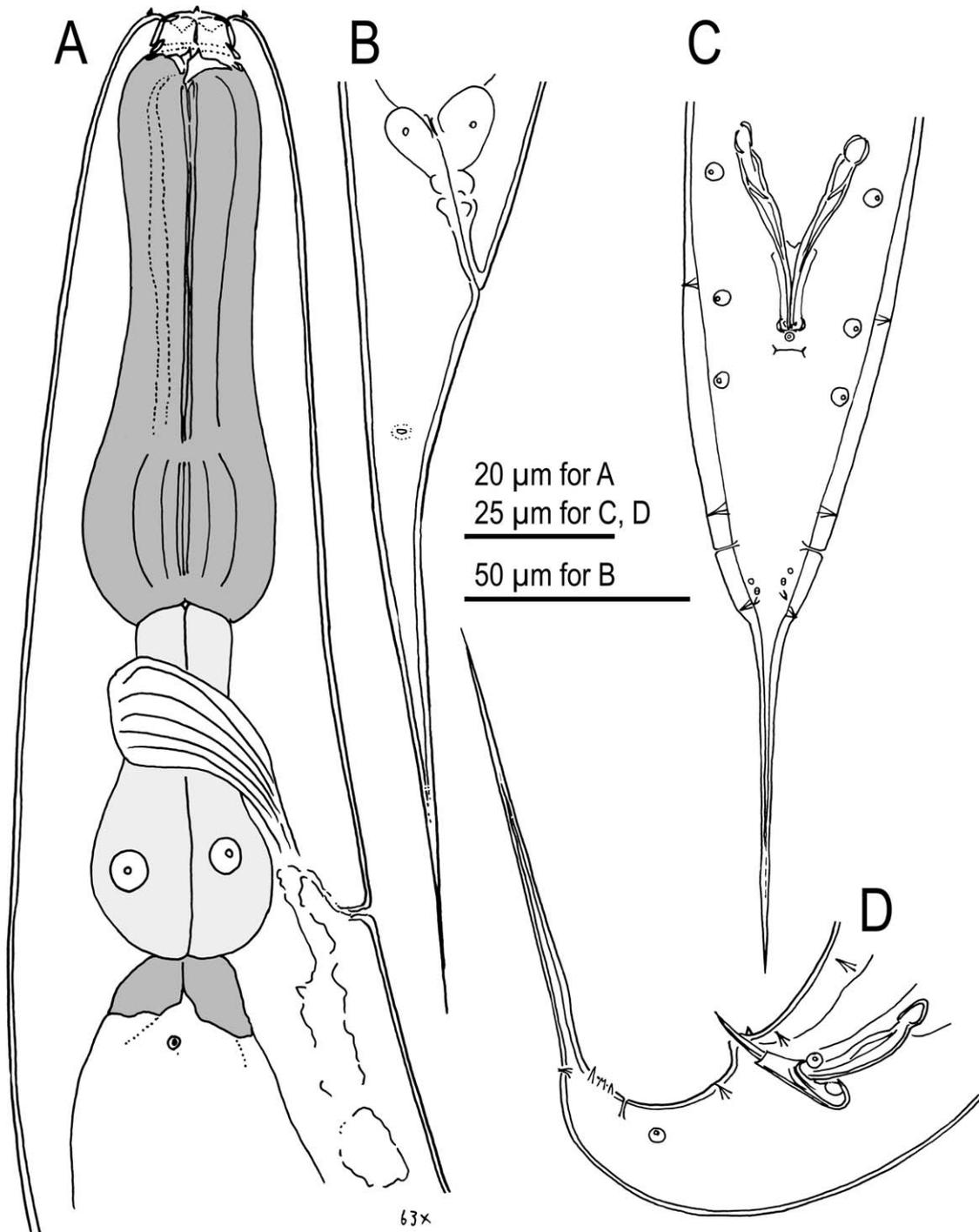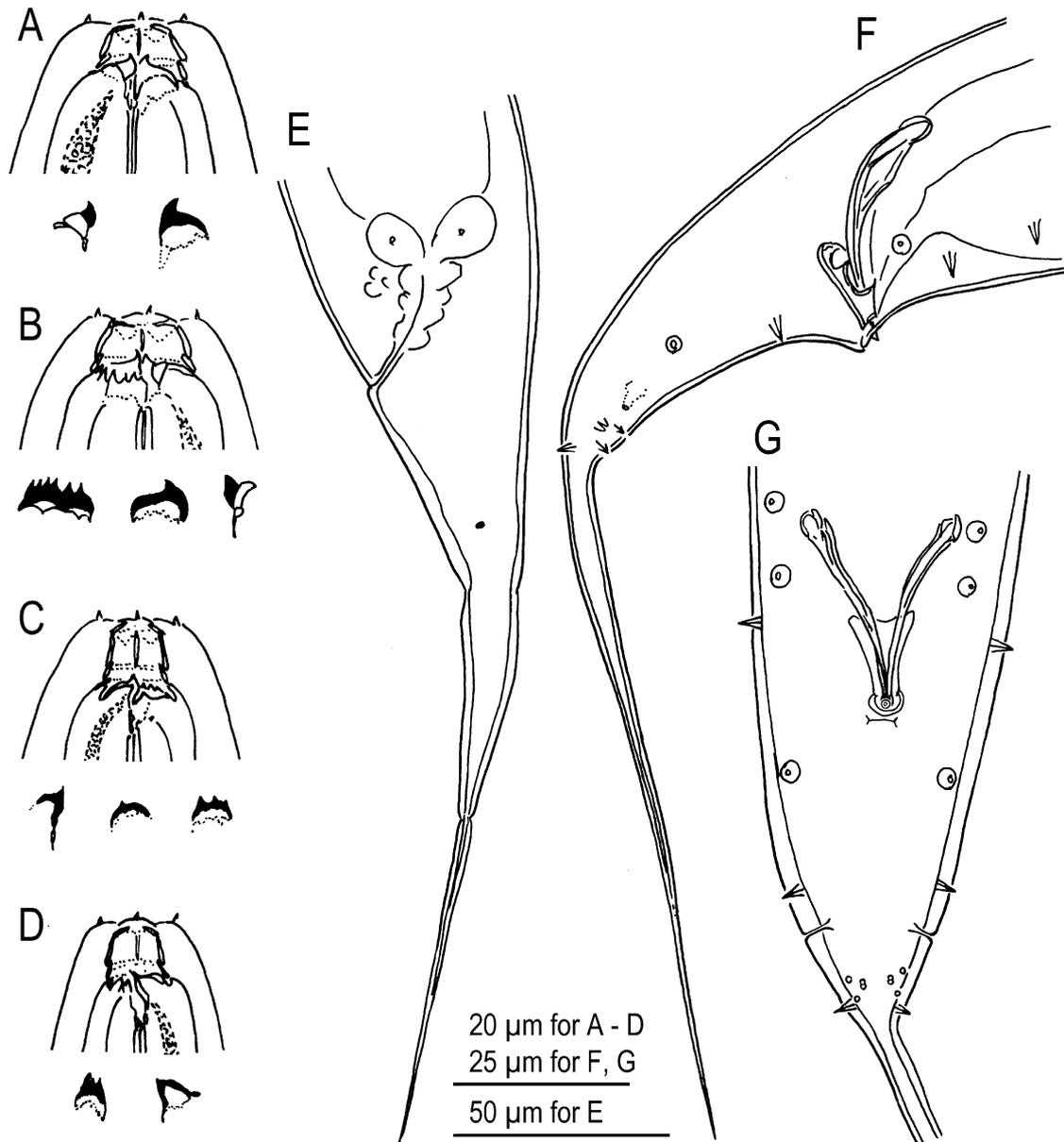
Fig. 6. *Pristionchus japonicus* n. sp. Drawings are of live specimens (non-types) from temporary mounts. Regions not shown are considered to be identical with *P. exspectatus* n. sp. **(A)** Stomatal region of eurystomatous female, right lateral view; below (from left to right) are dorsal tooth and right subventral tooth. **(B)** Stomatal region of eurystomatous female, left lateral view; below are left subventral ridge, right subventral tooth, and dorsal tooth. **(C)** Stomatal region of stenostomatous female, right lateral view; below are dorsal tooth, right subventral denticle, and left subventral ridge. **(D)** Stomatal region of stenostomatous female, left subventral view; below are left subventral ridge and dorsal tooth. **(E)** Female tail region, left lateral view. **(F)** Male tail region, right lateral view. **(G)** Male tail region, ventral view.

chus species, and by a gonochoristic reproductive mode.

Type host and locality. The culture from which the type specimens were obtained was originally isolated from the body of an adult of *Prismognathus angularis* (Coleoptera: Lucanidae) collected by Kyohei Nakamura and Mutsuhiro Yoshida at Mt. Shibi, Kagoshima, Japan in September 2010.

Type material. Holotype stenostomatous male (slide accession number 30622), seven paratype stenostomatous males, two paratype eurystomatous males, four paratype stenostomatous females, and four paratype eurystomatous females (30625–30641) deposited in the University of

California Riverside Nematode Collection (UCRNC), Riverside, CA, USA. Five paratype stenostomatous males, one paratype eurystomatous male, three paratype stenostomatous females, and three paratype eurystomatous females (SMNK-Nema-T 0119 – SMNK-Nema-T 0130) deposited in the Natural History Museum Karlsruhe, Germany. Five paratype stenostomatous males, one paratype eurystomatous male, three paratype stenostomatous females, and three paratype eurystomatous females (SMNH Type-8244 – SMNH Type-8255) deposited in the Swedish Natural History Museum, Stockholm, Sweden.

Table 1. Morphometrics of stenostomatous male holotype (in glycerin) and male and female specimens of *Pristionchus exspectatus* n. sp. (temporary water mounts). All measurements made in μm and given in the form: mean ± sd (range).

Character	Stenostomatous male	Eurystomatous male	Stenostomatous female	Eurystomatous female
	Holotype (UCRNC #30622)	Temporary water mounts	Temporary water mounts	Temporary water mounts	Temporary water mounts
n	–	10	10	10	10
L	774	625 ± 37 (554–676)	571 ± 54 (504–656)	853 ± 175 (735–1281)	789 ± 86 (678–963)
L'	699	551 ± 36 (478–592)	481 ± 45 (415–548)	667 ± 123 (566–977)	593 ± 83 (491–780)
a	13	13 ± 0.9 (12–15)	15 ± 1.4 (13–18)	14 ± 1.1 (12–16)	15 ± 2.1 (13–19)
b	6.4	5.8 ± 0.7 (5.1–7.2)	4.8 ± 0.4 (3.8–5.5)	5.8 ± 0.7 (5.1–7.2)	5.6 ± 0.8 (3.8–6.6)
c	10.3	8.7 ± 1.3 (7.3–11)	6.4 ± 0.9 (5.2–7.6)	4.7 ± 0.5 (4.0–5.5)	4.1 ± 0.6 (3.3–5.3)
c'	2.4	3.0 ± 0.5 (2.0–3.4)	4.0 ± 0.7 (3.1–5.5)	7.0 ± 1.2 (4.4–8.4)	7.5 ± 1.2 (6.1–10)
T or V	63	61 ± 5.2 (55–70)	63 ± 5.4 (54–71)	47 ± 3.1 (42–51)	42 ± 3.4 (37–47)
Maximum body diam.	62	28 ± 3.9 (23–34)	38 ± 6.2 (31–50)	62 ± 17 (52–107)	53 ± 10 (38–76)
Pharynx length (head to base of pharynx)	125	129 ± 8.3 (118–140)	120 ± 12 (103–132)	146 ± 16 (122–177)	143 ± 20 (124–191)
Anterior pharynx (pro– + metacorpus)	79	81 ± 4.8 (74–88)	81 ± 8.7 (66–92)	91 ± 8.2 (81–104)	83 ± 12 (58–97)
Posterior pharynx (isthmus + basal bulb)	43	48 ± 6.6 (39–56)	45 ± 6.9 (33–55)	55 ± 8.7 (42–73)	56 ± 12 (41–85)
Post./ant. pharynx ratio	54	59 ± 9.2 (49–74)	55 ± 5.4 (47–64)	61 ± 8.3 (47–72)	62 ± 10 (46–76)
Excretory pore from ant. end	99	108 ± 6.0 (98–115)	105 ± 14 (80–124)	129 ± 14 (109–152)	123 ± 15 (104–156)
Testis length	488	382 ± 35 (339–448)	358 ± 45 (302–451)	–	–
Ant. female gonad length (with flexure)	–	–	–	386 ± 106 (283–637)	341 ± 78 (221–463)
Post. female gonad length (with flexure)	–	–	–	424 ± 123 (258–704)	341 ± 52.1 (258–409)
Vulva to anus distance	–	–	–	274 ± 42 (237–381)	258 ± 40 (171–326)
Cloacal or anal body diam.	33	28 ± 3.9 (23–34)	23 ± 3.6 (19–30)	27 ± 5.7 (18–39)	26 ± 3.6 (22–32)
Tail length	75	73 ± 11 (56–91)	91 ± 17 (70–119)	186 ± 55 (133–304)	196 ± 29 164–245
Spicule length (curve)	46	36 ± 3.6 (32–43)	32 ± 3.8 (26–37)	–	–
Spicule length (chord)	35	19 ± 2.8 (16–23)	18 ± 3.6 (15–26)	–	–
Gubernaculum length	15	13 ± 1.5 (11–15)	14 ± 2.7 (9–17)	–	–

Type strain culture. Available as living cultures and frozen stocks under culture code RS5522 in the Department of Evolutionary Biology, Max Planck Institute for Developmental Biology, Tübingen, Germany and can be provided to other researchers upon request.

Etymology. The species epithet, a Latin participial adjective meaning "longed for, anxiously expected," denotes the awaited discovery of a putative sister species for *P. pacificus*.

Pristionchus arcanus n. sp.
(Figs. 2, 5, S1)

Measurements. See Table 2.

Description. *Stenostomatous form.* Stegostom bearing conspicuous and movable dorsal triangular or diamond-shaped tooth, two bump-like (blunt) left subventral denticles apparently projecting from a common cuticular plate, and a small, short, and pointed right subventral denticle. Dorsal tooth with strongly sclerotized surface, appearing as an inverted "V" shape in light microscopic observation.

Eurystomatous form. Stegostom bearing a large claw-like dorsal tooth, a large claw-like right subventral tooth, and a row of left subventral denticles of varying numbers and size, i.e. two large denticles to four small denticles. Dorsal and right subventral teeth movable. Left subventral denticles immovable.

Male. Nine pairs of genital papillae arranged as ⟨P1, (P2d, P3), C, P4, P5d, Ph, (P6, P7, P8), P9d⟩ (Figs. 2C; 5C, D), where, in many individuals, phasmid (Ph) and P6 are clearly apart from each other. P6–P8 arranged in a triangle, P9d located at the level of or posterior to P8.

Female. Tail smoothly tapered, with distal end filiform or elongated conical (Fig. 5B).

Diagnosis. Besides its generic characters, *Pristionchus arcanus* n. sp. is diagnosed by its size and arrangement of male genital papillae as described above. The species is also characterized by an 830-bp fragment of the SSU rRNA gene (GenBank accession number JQ399907), the sequence of which is distinct from that of all other *Pristionchus* species, and by a gonochoristic reproductive mode.

Type host and locality. The culture from which the type specimens were obtained was originally isolated from the bodies of adult *Odontotermes formasanus* (Isoptera: Termitidae) collected by N. Kanzaki on Iriomote Island, Okinawa, Japan in September 2009.

Type material. Holotype stenostomatous male (slide accession number 30623), seven paratype stenostomatous males, two paratype eurystomatous males, four paratype stenostomatous females, and four paratype eurystomatous females (30642–30658) deposited in the UCRNC, Riverside, CA, USA. Five paratype stenostomatous males, three paratype stenostomatous females, and three paratype eurystomatous females (SMNK-Nema-T 0131 – SMNK-Nema-T 0141) deposited in the Natural History Museum Karlsruhe, Germany. Five paratype stenostomatous males, one paratype eurystomatous male, three

Table 2. Morphometrics of stenostomatous male holotype (in glycerin) and male and female specimens of *Pristionchus arcanus* n. sp. (temporary water mounts). All measurements made in μm and given in the form: mean ± sd (range).

Character	Stenostomatous male	Eurystomatous male	Stenostomatous female	Eurystomatous female
	Holotype (UCRNC #30623)	Temporary water mounts	Temporary water mounts	Temporary water mounts	Temporary water mounts
n	–	10	10	10	10
L	672	679 ± 42 (615–751)	582 ± 57 (516–719)	881 ± 116 (782–1182)	853 ± 86 (769–1056)
L'	594	575 ± 35 (529–634)	504 ± 58 (446–641)	704 ± 63 (640–820)	670 ± 52 (609–795)
a	16	16 ± 1.7 (13–19)	14 ± 1.7 (11–16)	16 ± 1.5 (14–18)	16 ± 2.1 (13–20)
b	5.6	5.5 ± 0.5 (4.9–6.6)	4.9 ± 0.3 (4.6–5.5)	6.2 ± 0.7 (5.4–7.7)	6.1 ± 0.5 (5.2–6.9)
c	8.6	6.6 ± 0.6 (5.8–7.5)	7.7 ± 1.5 (5.3–10)	5.3 ± 0.9 (3.3–6.6)	4.9 ± 1.0 (3.1–6.5)
c'	2.9	3.8 ± 0.7 (2.5–4.9)	3.2 ± 0.5 (2.3–4.2)	5.9 ± 1.3 (4.6–8.8)	6.6 ± 2.0 (3.9–11.3)
T or V	57	65 ± 4.4 (54–71)	65 ± 6.5 (56–76)	46 ± 3.4 (37–49)	46 ± 3.2 (41–52)
Maximum body diam.	41	44 ± 4.6 (39–55)	41 ± 5.5 (34–50)	57 ± 11 (46–84)	54 ± 6.3 (45–63)
Pharynx length (head to base of pharynx)	121	124 ± 10 (100–133)	118 ± 8.7 (104–132)	143 ± 12 (129–163)	141 ± 14 (122–166)
Anterior pharynx (pro– + metacorpus)	78	81 ± 6.2 (69–92)	77 ± 7.4 (70–92)	91 ± 8.5 (76–104)	87 ± 8.3 (77–102)
Posterior pharynx (isthmus + basal bulb)	47	43 ± 5.8 (33–51)	43 ± 7.7 (33–55)	53 ± 7.1 (40–61)	54 ± 7.0 (43–67)
Post./ant. pharynx ratio	60	54 ± 6.6 (43–61)	56 ± 8.3 (46–69)	59 ± 8.9 (43–74)	62 ± 6.4 (55–74)
Excretory pore from ant. end	110	105 ± 12 (77–120)	96 ± 12 (80–124)	122 ± 13 (107–151)	119 ± 16 (98–152)
Testis length	385	440 ± 46 (373–530)	378 ± 66 (312–516)	–	–
Ant. female gonad length (with flexure)	–	–	–	415 ± 74.3 (335–551)	379 ± 25 (348–414)
Post. female gonad length (with flexure)	–	–	–	422 ± 82.8 (319–586)	400 ± 52 (320–468)
Vulva to anus distace	–	–	–	290 ± 55 (213–428)	276 ± 18 (253–305)
Cloacal or anal body diam.	27	28 ± 2.9 (24–33)	25 ± 3.6 (20–31)	29 ± 5.5 (22–41)	28 ± 3.4 (22–33)
Tail length	78	104 ± 12 (84–117)	78 ± 14 (54–106)	177 ± 67 (132–362)	183 ± 56 (123–294)
Spicule length (curve)	41	38 ± 4.6 (32–46)	34 ± 3.3 (29–38)	–	–
Spicule length (chord)	31	26 ± 3.4 (21–30)	18 ± 3.4 (14–24)	–	–
Gubernaculum length	18	14 ± 2.2 (11–17)	12 ± 1.8 (9.4–14)	–	–

paratype stenostomatous females, and three paratype eurystomatous females (SMNH-Type-8256 – SMNH-Type-8267) deposited in the Swedish Natural History Museum, Stockholm, Sweden.

Type strain culture. Available as living cultures and frozen stocks under culture code RS5527 in the Department of Evolutionary Biology at the Max Planck Institute for Developmental Biology and can be provided to other researchers upon request.

Etymology. The species epithet, a Latin adjective meaning "secret, mysterious," refers to the enigmatic nature of this species, namely in its diagnosis with respect to *P. pacificus* and *P. exspectatus*.

Pristionchus japonicus n. sp.
(Figs. 2, 6, S1)
Pristionchus sp. 11: Herrmann et al., 2007; Mayer et al., 2007, 2009.

Measurements. See Table 3.

Description. *Stenostomatous form.* Stegostom bearing conspicuous and movable dorsal triangular or diamond-shaped tooth, two to three bump-like (blunt) left subventral denticles apparently projecting from a common cuticular plate, and a small, short, and pointed right subventral denticle (Fig. 6C, D). Dorsal tooth with strongly sclerotized surface, appearing as an inverted "V" shape in light microscopic observation.

Eurystomatous form. Stegostom bearing a large claw-like dorsal tooth, a large claw-like right subventral tooth, and a row of left subventral denticles with varying numbers and size, i.e. four large denticles to six small denticles (Fig. 6A, B). Dorsal and right subventral teeth movable. Left subventral denticles immovable.

Male. Nine pairs of genital papillae arranged as ⟨P1, P2, P3d, C, P4, P5d, Ph, (P6, P7, P8), P9d⟩ (Fig. 6F, G), where, in many individuals, phasmid (Ph) and P6 are clearly apart from each other, P6–P8 arranged in a triangle (Fig. 2D), P9d overlaps with or is further posterior than P8.

Female. Tail tapered, with distal end filiform or elongated conical. One or two constrictions observed in the tail of many females (Fig. 6E).

Diagnosis. Besides its generic characters, *Pristionchus japonicus* n. sp. is diagnosed by its size and arrangement of male genital papillae as described above. The species is also characterized by an 830-bp fragment of the SSU rRNA gene (GenBank accession number JQ399908), the sequence of which is distinct from that of all other *Pristionchus* species, and by a gonochoristic reproductive mode.

Type host and locality. The culture from which the type specimens were obtained was originally isolated from a dead earthworm collected from Enoshima Island, Kanagawa, Japan in September 2005. The isolate was collected by Chiharu Kato and provided to us by Dr. Walter Sudhaus.

Type material. Holotype stenostomatous male (slide accession number 30624), four paratype stenostomatous males, four paratype eurystomatous males, four paratype stenostomatous females, and four paratype eurystomatous females (30659–30674) deposited in the UCRNC, River-

Table 3. Morphometrics of stenostomatous male holotype (in glycerin) and male and female specimens of *Pristionchus japonicus* n. sp. (temporary water mounts). All measurements made in μm and given in the form: mean ± sd (range).

Character	Stenostomatous male Holotype (UCRNC #30624)	Eurystomatous male Temporary water mounts	Stenostomatous female Temporary water mounts	Eurystomatous female Temporary water mounts
n	–	10	10	10	10
L	716	689 ± 112 (531–874)	749 ± 110 (535–881)	960 ± 224 (758–1255)	778 ± 140 (609–1071)
L'	622	575 ± 105 (435–741)	639 ± 96 (474–755)	759 ± 178 (581–1001)	614 ± 120 (464–864)
a	18	16 ± 2.0 (14–20)	15 ± 1.2 (13–18)	15 ± 1.6 (12–17)	16 ± 1.5 (14–19)
b	5.6	5.2 ± 0.5 (4.8–6.0)	5.6 ± 0.7 (4.5–6.7)	6.3 ± 0.9 (5.1–7.7)	5.6 ± 0.7 (4.8–6.8)
c	7.6	6.1 ± 0.8 (5.1–7.0)	6.9 ± 1.1 (5.4–8.8)	4.9 ± 0.7 (4.0–6.1)	4.8 ± 0.6 (3.9–5.7)
c'	3.2	3.8 ± 0.4 (3.4–4.7)	3.6 ± 0.8 (2.4–5.0)	6.1 ± 1.1 (4.0–8.0)	6.0 ± 1.0 (3.9–7.1)
T or V	58	61 ± 5.5 (54–70)	65 ± 10 (46–77)	47 ± 2.7 (42–52)	46 ± 3.4 (40–51)
Maximum body diam.	40	43 ± 10 (32–60)	52 ± 10 (30–65)	67 ± 20 (48–98)	49 ± 11 (37–75)
Pharynx length (head to base of pharynx)	128	132 ± 12 (107–146)	134 ± 9.0 (119–145)	152 ± 18 (120–177)	138 ± 11 (126–157)
Anterior pharynx (pro– + metacorpus)	84	80 ± 8.7 (60–94)	80 ± 4.1 (73–86)	93 ± 15 (66–109)	84 ± 7.8 (73–94)
Posterior pharynx (isthmus + basal bulb)	45	56 ± 6.4 (45–66)	55 ± 7.5 (42–66)	60 ± 7.5 (47–72)	55 ± 3.8 (49–61)
Post./ant. pharynx ratio	54	69 ± 5.7 (61–77)	68 ± 8.3 (58–80)	66 ± 12 (51–83)	66 ± 4.7 (61–74)
Excretory pore from ant. end	111	116 ± 10 (93–129)	113 ± 10 (94–121)	130 ± 19 (104–153)	118 ± 12 (107–142)
Testis length	415	425 ± 105 (320–609)	493 ± 133 (302–634)	–	–
Ant. female gonad length (with flexure)	–	–	–	468 ± 200 (209–739)	293 ± 132 (171–630)
Post. female gonad length (with flexure)	–	–	–	493 ± 205 (276–778)	299 ± 110 (202–556)
Vulva to anus distace	–	–	–	304 ± 81 (215–464)	261 ± 48 (214–339)
Cloacal or anal body diam.	29	30 ± 3.5 (27–38)	31 ± 4.8 (25–38)	33 ± 7.6 (25–47)	28 ± 4.1 (22–37)
Tail length	94	114 ± 12 (96–133)	110 ± 22 (61–133)	201 ± 56 (139–282)	164 ± 28 (118–207)
Spicule length (curve)	39	39 ± 3.2 (34–43)	38 ± 3.2 (33–43)	–	–
Spicule length (chord)	31	31 ± 2.1 (28–35)	32 ± 2.4 (27–35)	–	–
Gubernaculum length	14	15 ± 1.8 (13–18)	15 ± 2.7 (11–19)	–	–

side, CA, USA. Three paratypes each of stenostomatous males, eurystomatous males, stenostomatous females, and eurystomatous females deposited (SMNK-Nema-T 0142 – SMNK-Nema-T 0153) in the Natural History Museum Karlsruhe, Germany. Three paratypes each of stenostomatous males, eurystomatous males, stenostomatous females, and eurystomatous females (SMNH-Type-8268 – SMNH-Type-8279) deposited in the Swedish Natural History Museum, Stockholm, Sweden.

Type strain culture. Available as living cultures and frozen stocks under culture code SB393 in the Department of Evolutionary Biology at the Max Planck Institute for Developmental Biology, and can be provided to other researchers upon request.

Etymology. The species epithet, an adjective, denotes the type locality of the species.

Relationships among the three new species and *P. pacificus*

As mentioned above, three new species described herein and *P. pacificus* form a species complex. These four species are morphologically almost indistinguishable, although there are several minor differences among them. As in other diplogastrid species, morphometric values vary widely within species (e.g. Fürst von Lieven and Sudhaus, 2000; Herrmann et al., 2006a). Therefore, biological and molecular characters are necessary to separate these four species.

Pristionchus exspectatus is distinguished from *P. pacificus* by the arrangement of its genital papillae and phasmid. The phasmid (Ph) and P6 are closer to each other than in *P. pacificus*. It also differs from *P. pacificus* in that the phasmid opening and P6–P8 are linearly arranged and P9d is located at the level of P7 or P8 in many individuals *vs.* the phasmid and P6 being clearly apart from each other, P6–P8 being linearly arranged, and P9d being located at the level of P8 or further posterior (Sommer et al., 1996; present observation by NK). *Pristionchus exspectatus* is distinguished from *P. pacificus* by its unique SSU rRNA sequence, differing in at least four non-polymorphic nucleotide positions (Fig. S1), by gonochoristic vs. hermaphroditic reproduction, and by its reproductive isolation from the latter species, namely by inability to produce interfertile F1 hybrids.

Pristionchus exspectatus is distinguished from *P. arcanus* by the arrangement of its genital papillae and phasmid. In *P. exspectatus*, the phasmid (Ph) and P6 are close to each other, the phasmid opening and P6–P8 are arranged linearly, and P9d is located at the level of P7 or P8 in many individuals vs. the phasmid and P6 being clearly apart from each other, P6–P8 being arranged in a triangle, and P9d being located at the level of P8 or further posterior in *P. pacificus* (see species description above). *Pristionchus exspectatus* is also distinguished from *P. arcanus* by its unique SSU rRNA sequence, differing in at least five non-polymorphic positions (Fig. S1), and its reproductive isolation from the latter species, namely by inability to produce interfertile F1 hybrids.

Pristionchus exspectatus is distinguished from *P. japonicus* by its stomatal morphology, in that the left subventral stegostom possesses two blunt, bump-like denticles in the stenostomatous form and two to four pointed denticles in the eurystomatous form vs. two to three bump-like denticles in the stenostomatous form and four to six pointed denticles in the eurystomatous form. It differs from *P. japonicus* in the arrangement of its genital papillae and phasmid, ⟨P1, (P2d, P3), C, P4, P5d, Ph, (P6, P7, P8), P9d⟩, wherein P2d and P3 are located at almost the same level, or P2d is slightly anterior to P3, and P6–P8 are arranged linearly vs. ⟨P1, P2, P3d, C, P4, P5d, Ph, (P6, P7, P8), P9d⟩, wherein P2 is clearly anterior to P3d and P6–P8 are arranged in a triangle (see species description above). Also distinguishing *P. exspectatus* from *P. japonicus* is the female tail, which is smoothly tapered vs. sometimes possessing two constrictions. Finally, the species is distinguished from *P. japonicus* by its unique SSU rRNA sequence, differing in at least four non-polymorphic positions (Fig. S1), and its reproductive isolation from the latter species, namely by failure to produce viable hybrid F1.

Pristionchus arcanus is morphologically almost identical to *P. pacificus* (Sommer et al., 1996; present observation by NK), but is distinguished from *P. pacificus* by having tail papillae P6–P8 arranged in a triangle *vs.* arranged linearly. Also distinct in *P. arcanus* is its unique SSU rRNA sequence, differing in at least four positions (Supplementary material online Fig. S1). It differs from *P. pacificus* by having a gonochoristic vs. hermaphroditic reproduction and by its reproductive isolation from the latter species, namely by inability to produce interfertile F1 hybrids.

Pristionchus arcanus is distinguished from *P. japonicus* by its stomatal morphology, such that the left subventral stegostom possesses two blunt, bump-like denticles in the stenostomatous form and two to four pointed denticles in the eurystomatous form vs. two to three bump-like denticles in the stenostomatous form and four to six pointed denticles in the eurystomatous form. *Pristionchus arcanus* differs from *P. japonicus* in the arrangement of its genital papillae and phasmid, ⟨P1, (P2d, P3), C, P4, P5d, Ph, (P6, P7, P8), P9d⟩, whereby P2d and P3 are located at almost the same level or P2d is slightly anterior to P3 vs. ⟨P1, P2, P3d, C, P4, P5d, Ph, (P6, P7, P8), P9d⟩, whereby P2 is clearly anterior to P3d. Female tail morphology is smoothly tapered in *P. arcanus* vs. sometimes possessing one or two constrictions in *P. japonicus* (see species descriptions above). *Pristionchus arcanus* is also distinguished from *P. japonicus* by its unique SSU rRNA sequence, differing in at least three positions (Fig. S1), and its reproductive isolation from the latter species, namely by failing to produce viable hybrid F1.

Pristionchus japonicus is distinguished from *P. pacificus* by its stomatal morphology in that the left subventral stegostom possesses two to three bump-like denticles in the stenostomatous form and four to six pointed denticles in the eurystomatous form vs. two blunt bump-like denticles in the stenostomatous form and two to four pointed denticles in the eurystomatous form. In *P. japonicus* the arrangement of genital papillae and phasmid is ⟨P1, P2, P3d, C, P4, P5d, Ph, (P6, P7, P8), P9d⟩, where P2 is clearly anterior to P3d and P6–P8 are arranged in a triangle vs. ⟨P1, (P2d, P3), C, P4, P5d, Ph, (P6, P7, P8), P9d⟩, wherein P2d and P3 are located at almost the same level, or P2d is slightly anterior to P3, and P6–P8 are linearly arranged in *P. pacificus*. *Pristionchus japonicus* is distinguished from *P. pacificus* by the female tail morphology, which sometimes shows two constrictions vs. being always smoothly tapered (see above descriptions). It is distinct from *P. pacificus* by its unique SSU rRNA sequence, differing in at least four positions (Fig. S1), gonochoristic vs. hermaphroditic reproduction, and by its reproductive isolation from the latter species, namely by failure to produce viable hybrid F1.

Remarks on morphological characters

In the present study, the four examined species of the species complex were most strongly separated by biological characters, namely modes of reproduction and reproductive isolation. Although several morphological characters, including those of stomatal morphology, arrangement of male genital papillae, and female tail morphology, showed some differences among species, characters other than the arrangement of P1–P3 papillae vary within species and overlap among them.

Original descriptions of all other 31 valid species of *Pristionchus* reveal differences in the arrangement of P1–P3, which has been shown for several species. The pattern ⟨P1, (P2d, P3)⟩ was observed in *P. biformis* (Hirschmann, 1951) Sudhaus and Fürst von Lieven, 2003, *P. lheritieri* (Maupas, 1919) Paramonov, 1952, *P. linstowi* (Potts, 1910) Paramonov, 1952, and *P. maupasi* (Potts, 1910) Paramonov, 1952. In contrast, the arrangement ⟨P1, P2d, P3⟩ (i.e. P2d and P3 are clearly separate) was observed in *P. vidalae* (Stock, 1993) Sudhaus and Fürst von Lieven, 2003, *P. aerivorus* (Cobb in Merrill and Ford, 1916) Chitwood, 1937 (see Poinar, 1990), and *P. eurycephalus* Völk, 1950. *Pristionchus uniformis* Fedorko and Stanuszek, 1971 showed ⟨P1, P2, P3d⟩. In other species, these characters were not described or were obviously incorrectly illustrated. Although genital papilla arrangement does not necessarily correlate with phylogenetic groupings above the species level, such characters could be useful for reconstructing the taxonomy of the genus, following re-isolation and molecular identification of *Pristionchus* species.

SEM observation of all species described herein revealed a common morphological character, namely the tip of each P7 papilla being terminally split into two small papilla-like projections. In light microscopic observation, the tip of the P7 papilla appears somewhat flattened because of these two projections. This feature was also previously shown in *P. lheritieri* (strain code not specified), evident in published SEM and drawings of *P. lheritieri* although not explicitly mentioned (Kiontke and Sudhaus, 2000). Following our discovery of this character in the three new species, we closely examined the character in representatives of all major lineages of *Pristionchus* (Mayer et al., 2007), namely *P. lheritieri* (strain code SB245) (also see Kiontke and Sudhaus, 2000), *P. uniformis* (RS0141), *P. marianneae* (RS5108), *P. pauli* (RS5130), *P. pseudaerivorus* (RS5139), *Pristionchus* sp. 10 (RS5133), *Pristionchus* sp. 13 (RS5231), *Pristionchus* sp. 15 (RS5229), and *Pristionchus* sp. 17 (JU1090). All *Pristionchus* species examined were confirmed to share this character (NK, unpubl.; detailed morphological comparison among these species will be pre-

sented elsewhere). Although papilla P7 is trifurcate in *Diplogasteroides nasuensis* Takaki, 1941 (Kiontke et al., 2001) and P6–P8 are all modified from simple termini in *Koerneria* spp. (NK, MH, EJR, unpubl.; R. Giblin-Davis, pers. comm.), the unique bifurcate morphology observed in P7 in *Pristionchus* spp. is not reported for other diplogastrid genera. Therefore we propose a bifurcate P7 papillae to be an additional diagnostic character of the genus.

DISCUSSION

The discovery of a species complex and putative sister species for the model organism *Pristionchus pacificus* has tremendous implications for evolutionary biology of the genus. The partial ability to cross *P. pacificus* and *P. exspectatus* enables tools of classical genetics for the species pair. Outgroup species make polarization of characters variable within *P. pacificus* possible. An example of such variability within *P. pacificus* is in chemoattraction phenotypes, which may reflect incipient speciation. For studies of macroevolution, the availability of a species complex facilitates more precise ancestral-state reconstructions through closer intermediates.

The known geographical range of the species complex roots the biogeography of *P. pacificus*. The species *P. pacificus* has a cosmopolitan distribution, reported to date from all continents except Australia and Antarctica. The ranges of *P. exspectatus*, *P. arcanus*, and *P. japonicus* are presently only known to include Japan. Along with the presence of a major "clade" of populations of *P. pacificus* with a high proportion of East Asian membership (Herrmann et al., 2010), the geographic ranges of outgroup species are strong evidence for an East Asian origin of *P. pacificus*. Although sampling bias is by nature difficult to rule out in the case of negative host-range evidence, the overwhelming success with which *P. pacificus* strains have been isolated in comparison to those of *P. exspectatus*, *P. arcanus*, and *P. japonicus* suggests a relatively restricted distribution of the latter nematodes. In a rooted phylogeny of the genus, the closest known outgroup to a clade incuding the new species complex and several nominal *Pristionchus* species is *Pristionchus* sp. 14 (Fig. 1). This undescribed species was also isolated from Japan, suggesting that an East Asian origin is plesiomorphic for that clade and therefore also for the species complex. This will be tested further with more inclusive phylogeny, namely one that includes additional close outgroup *Pristionchus* species.

The correlation between a broad geographic distribution and hermaphroditic reproduction in *P. pacificus* may be explained by more efficient dispersal by a species that requires only a single individual to propagate (Herrmann et al., 2010). Population genetics studies on La Réunion Island in the Indian Ocean confirm multiple colonizations of the island by *P. pacificus* (Morgan et al., 2012), indicating the relative ease with which the species can disperse. Supporting this correlation are the limited known ranges of *P. exspectatus*, *P. arcanus*, and *P. japonicus*, all of which are gonochoristic species but otherwise very closely related. This dispersal phenomenon for *P. pacificus* is echoed by another widespread hermaphroditic nematode in the genus, *P. entomophagus* (Steiner, 1928) Sudhaus and Fürst von Lieven, 2003, which is commonly found in Europe (Steiner,

1928; Herrmann et al., 2006a) but also in North America (Herrmann et al., 2006b) and on La Réunion Island (Herrmann and Sommer, unpubl.).

While the ongoing discovery of nematodes in the genus *Pristionchus* has been a boon for the taxonomy of the group, the density of known species is accompanied by a decrease in characters available to diagnose them. It has been noted that morphology other than in the stoma and pharynx are relatively uniform across the family Diplogastridae (Sudhaus and Fürst von Lieven, 2003). Likewise, it is not unexpected that the discovery of more species that are clearly distinct by biological, molecular, and phylogenetic criteria should exhaust even the most diverse classes of typically used characters. The lack of morphological distinctions among close species of *Pristionchus* has already been observed in other lineages of the genus (Herrmann et al., 2006b). Older species descriptions that are incomplete by present standards and a previous unavailability of molecular sequence data may also have confounded the proper delineation and description of separate biological species of *Pristionchus*. We suspect that the apparent similarity in defining mouthpart morphology between *P. lheritieri* and *P. maupasi* (Fürst von Lieven and Sudhaus, 2000), which are clearly distinct in their molecular divergence (Mayer et al., 2007, 2009), may have led to the historical lumping of separate species. Namely, mode of reproduction seems to have been a primary criterion for synonymization with either of these species (Meyl, 1960; Andrássy, 1984), although hermaphroditism is convergent across several lineages of the genus (Mayer et al., 2007). We nevertheless argue that the paucity of classical characters should not inhibit taxonomy. Evolutionarily unique species, which exist independently of the characters circumscribing them (Adams, 1998), deserve labels. To this end, we have followed an integrative approach based on reproductive isolation, molecular sequence divergence, and, where possible, morphological characters.

Mating tests clearly show clear reproductive isolation in the form of mostly infertile F1 hybrids between all but one pair of species in the *P. pacificus* species complex. The putative sister species *P. pacificus* and *P. exspectatus* show some fertile F1, although differing modes of reproduction distinguish the two species. Independence of evolutionary trajectories, another criterion for delimiting species (Wiley, 1978), has also been tested by divergence of sequences in multiple (28) genetic loci, including a gene (SSU rRNA) operationally common in taxonomy and diagnostics. The 830-bp fragment of SSU rRNA used is highly conserved in rhabditid nematodes and can be identical in closely related species, for example in some *Caenorhabditis* species (Kiontke et al., 2011). The observed pairwise differences of 3–5 nucleotide positions is thus strong evidence for separation of all described species in the *P. pacificus* complex. Typological species concepts are satisfied in the case of *P. japonicus*, which is distinguished from *P. pacificus*, *P. exspectatus*, and *P. arcanus* by male sex-specific (genital papilla) characters.

Definitive morphological distinctions among *P. pacificus*, *P. exspectatus*, and *P. arcanus* are not apparent at present. Closer examination with transmission electron microscopy (TEM) or other techniques typically left out of routine

description may reveal differences at the level of homologous cells or nuclei, as grossly similar organs in nematodes can have variable underlying cellular architectures (Fitch and Emmons, 1995; Ragsdale et al., 2011). The emerging abundance of TEM data for *P. pacificus* (Bumbarger and Sommer, unpublished) makes such an approach realistic for *P. pacificus* and close relatives. Of course, such morphological differentiation would aid in diagnostics. However, simple recognition of the three new species herein described opens up exciting avenues of research only possible in model systems with known close species.

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REFERENCES

Adams BJ (1998) Species concepts and the evolutionary paradigm in modern nematology. J Nematol 30: 1–21

Andrássy I (1984) Klasse Nematoda. Akademie-Verlag, Berlin

Chitwood BG (1937) Cephalic structure and stoma. In "An Introduction to Nematology" Ed by BG Chitwood, MB Chitwood, Monumental Printing, Baltimore, p 53

Dieterich C, Clifton SW, Schuster LN, Chinwalla A, Delehaunty K, Dinkelacker I, et al. (2008) The *Pristionchus pacificus* genome provides a unique perspective on nematode lifestyle and parasitism. Nature Genet 40: 1193–1198

Dougherty EC (1953) The genera of the subfamily Rhabditinae Micoletzky, 1922 (Nematoda). Thapar Commemoration Volume 1953, pp 69–76

Dougherty EC, Nigon V (1949) A new species of the free-living nematode genus *Rhabditis* of interest in comparative physiology and genetics. J Parasitol (Suppl) 35: 11

Eizinger A, Sommer RJ (1997) The homeotic gene *lin-39* and the evolution of nematode epidermal cell fates. Science 278: 452–455

Fedorko A, Stanuszek S (1971) *Pristionchus uniformis* sp. n. (Nematoda, Rhabditida, Diplogasteridae), a facultative parasite of *Leptinotarsa decemlineata* Say and *Melolontha melolontha* L. in Poland. Morphology and biology. Acta Parastiol 19: 95–112

Fitch DHA, Emmons SW (1995) Variable cell positions and cell contacts underlie morphological evolution of the rays in the male tails of nematodes related to *Caenorhabditis elegans*. Dev Biol 170: 564–582

Fürst von Lieven A, Sudhaus W (2000) Comparative and functional morphology of the buccal cavity of Diplogastrina (Nematoda) and a first outline of the phylogeny of this taxon. J Zool Syst Evol Research 38: 37–63

Herrmann M, Mayer WE, Sommer RJ (2006a) Nematodes of the genus *Pristionchus* are closely associated with scarab beetles and the Colorado potato beetle in Western Europe. Zoology 109: 96–108

Herrmann M, Mayer WE, Sommer RJ (2006b) Sex, bugs and Haldane's rule: the nematode genus *Pristionchus* in the United States. Front Zool 3: 14

Herrmann M, Mayer WE, Hong RL, Kienle S, Minasaki R, Sommer RJ (2007) The nematode *Pristionchus pacificus* (Nematoda: Diplogastridae) is associated with the Oriental beetle *Exomala orientalis* (Coleoptera Scarabaeidae) in Japan. Zool Sci 24: 883–889

Herrmann M, Kienle S, Rochat J, Mayer WE, Sommer RJ (2010) Haplotype diversity of the nematode *Pristionchus pacificus* on Réunion in the Indian Ocean suggests multiple independent invasions. Biol J Linn Soc 100: 170–179

Hirschmann H (1951) Über das Vorkommen zweier Mundhöhlentypen bei *Diplogaster lheritieri* Maupas und *Diplogaster biformis* n. sp. und die Entstehung dieser hermaphroditischen Art aus *Diplogaster lheritieri*. Zool Jahrb Abf f Syst 80: 132–170

Hooper DJ (1986) Handling, fixing, staining and mounting nematodes. In "Laboratory Methods for Work with Plant and Soil Nematodes" Ed by JF Southey, Her Majesty's Stationary Office, London, pp 59–80

Kanzaki N, Taki H, Masuya H, Okabe K, Tanaka R, Abe F (2011) Diversity of stag beetle-associated nematodes in Japan. Environ Entomol 40: 281–288

Kiontke K, Sudhaus W (2000) Phasmids in male Rhabditida and other secernentean nematodes. J Nem Morph Syst 3: 1–37

Kiontke K, Mangold A, Sudhaus W (2001) Redescription of *Diplogasteroides nasuensis* Takaki, 1941 and *D. magnus* Völk, 1950 (Nematoda: Diplogastrina) associated with Scarabaeidae (Coleoptera). Nematology 3: 817–832

Kiontke KC, Felix MA, Ailion M, Rockman MV, Braendle C, Penigault JB, et al. (2011) A phylogeny and molecular barcodes for *Caenorhabditis*, with numerous new species from rotting fruits. BMC Evol Biol 11: 339

Kreis HA (1932) Beiträge zur Kenntnis pflanzenparasitischer Nematoden. Z f Parasitenkunde 5: 184–194

Maupas E (1899) La mue et l'enkystement chez les nématodes. Arch Zool Expér Gén 7: 563–628

Maupas E (1919) Essais d'hybridation chez les nématodes. Bull Biol Fr Belg 52: 466–498

Mayer WE, Herrmann M, Sommer RJ (2007) Phylogeny of the nematode genus *Pristionchus* and implications for biodiversity, biogeography and the evolution of hermaphroditism. BMC Evol Biol 7: 104

Mayer WE, Herrmann M, Sommer RJ (2009) Molecular phylogeny of beetle associated diplogastrid nematodes suggests host switching rather than nematode-beetle coevolution. BMC Evol Biol 9: 212

Merrill JH, Ford AL (1916) Life history and habits of two new nematodes parasitic in insects. J Agric Res 6: 115–127

Meyl AH (1960) Freilebende Nematoden. In "Die Tierwelt Mitteleuropas: Freilebende Nematoden" Ed by P Brohmer, P Ehrmann, G Ulmer. Quelle & Meyer, Leipzig. 1 (5a) pp 1–164

Micoletzky H (1922) Die freilebenden Erd-Nematoden. Arch Naturg Abt A 87: 1–650

Morgan K, McGaughran A, Villate L, Herrmann M, Witte H, Bartelmes G, et al. (2012) Multi-locus analysis of *Pristionchus pacificus* on La Réunion Island reveals an evolutionary history shaped by multiple introductions, constrained dispersal events, and rare out-crossing. Mol Ecol 21: 250–266

Nei M, Kumar S (2000) Molecular Evolution and Phylogenetics. Oxford University Press, New York

Paramonov AA (1952) Opyt ekologicheskoi klassifikatsii fitonematod. Trudy Gelmintol Lab Akad Nauk SSSR (Moskva) 6: 338–369

Poinar GO (1990) Redescription of *Chroniodiplogaster aerivora* (Cobb) gen. n., comb. n. (Rhabditida: Diplogasteridae) from termites. J Helminthol Soc Wash 57: 14–16

Posada D, Crandall KA (1998) Modeltest: testing the model of DNA substitution. Bioinformatics 14: 817–818

Potts FA (1910) Notes on the free-living nematodes. Quart J Micr Sci 55: 433–484

Ragsdale EJ, Ngo PT, Crum J, Ellisman MH, Baldwin JG (2011) Reconstruction of the pharyngeal corpus of *Aphelenchus avenae* (Nematoda: Tylenchomorpha), with implications for phylogenetic congruence. Zool J Linn Soc 161: 1–30

Rudel D, Tian HY, Sommer RJ (2008) The evolution of morphological novelties: an essential role for Wnt signaling in *P. pacificus* gonadal arm extension. Proc Natl Acad Sci USA 105: 10826–10831

Schlager B, Röseler W, Zheng M, Gutierrez A, Sommer RJ (2006) HAIRY-like transcription factors and the evolution of the nematode vulva equivalence group. Curr Biol 16: 1386–1394

Schlager B, Wang XY, Braach G, Sommer RJ (2009) Molecular cloning of a dominant roller mutant and establishment of DNA-mediated transformation in the nematode model *Pristionchus pacificus*. Genesis 47: 300–304

Shiraki T (1909) Japanese termites. Trans Entomol Soc Japan 2: 229–242 (In Japanese)

Sommer RJ (2009) The future of evo-devo: model systems and evolutionary theory. Nature Rev Genet 10: 416–422

Sommer RJ, Carta LK, Kim SY, Sternberg PW (1996) Morphological, genetic and molecular description of *Pristionchus pacificus* n. sp. (Nematoda: Neodiplogastridae). Fundam Appl Nematol 19: 511–521

Srinivasan J, Sinz W, Lanz C, Brand A, Nandakumar R, Raddatz G, et al. (2002) A bacterial artificial chromosome-based genetic linkage map of the nematode *Pristionchus pacificus*. Genetics 162: 129–134

Srinivasan J, Sinz W, Jesse T, Wiggers-Perebolte L, Jansen K, Buntjer J, et al. (2003) An integrated physical and genetic map of the nematode *Pristionchus pacificus*. Mol Genet Genomics 269: 715–722

Steiner G (1928) *Diplogaster entomophaga* n. sp., a new *Diplogaster* (Diplogasteridae, Nematodes) found on a *Pamphilius stellatus* (Christ) (Tenthredinidae, Hymenoptera). Zool Anz 80: 143–145

Stock SP (1993) *Micoletzkya vidalae* n. sp. (Nematoda: Diplogasteridae), a facultative parasite of *Diabrotica speciosa* (Coleoptera: Crysomelidae) from Argentina. Res Rev Parasitol 53: 109–112

Sudhaus W, Fürst von Lieven A (2003) A phylogenetic classification and catalogue of the Diplogastridae (Secernentea, Nematoda). J Nem Morph Syst 6: 43–90

Takaki SI (1941) Studies on a nematode parasitic of the common beetle *Anomala testaceipes*. J Coll Agr Imp Univ Tokyo 15: 1–11

Tamura K, Peterson D, Peterson N, Stecher G, Nei M, Kumar S (2011) MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Mol Biol Evol 28: 2731–2739

Tian H, Schlager B, Xiao H, Sommer RJ (2008) Wnt signaling by differentially expressed Wnt ligands induces vulva development in *Pristionchus pacificus*. Curr Biol 18: 142–146

Völk J (1950) Die Nematoden der Regenwürmer und aasbesuchenden Käfer. Zool Jb Syst 79: 1–70

Waterhouse CO (1874) Descriptions of five new lucanoid Coleoptera. Entomol Mon Mag 11: 6–8

Wiley EO (1978) The evolutionary species concept reconsidered. Syst Zool 27: 17–26

Woodruff GC, Eke O, Baird SE, Félix MA, Haag ES (2010) Insights into species divergence and the evolution of hermaphroditism from fertile interspecies hybrids of *Caenorhabditis* nematodes. Genetics 186: 997–1012

Zauner H, Sommer RJ (2007) Evolution of robustness in the signaling network of *Pristionchus* vulva development. Proc Natl Acad Sci USA 104: 10086–10091

Zheng M, Messerschmidt D, Jungblut B, Sommer RJ (2005) Conservation and diversification of Wnt signaling function during the evolution of nematode vulva development. Nature Genet 37: 300–304