



Three new species of *Pristionchus* (Nematoda: Diplogastridae) show morphological divergence through evolutionary intermediates of a novel feeding-structure polymorphism

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Developmental plasticity is often correlated with diversity and has been proposed as a facilitator of phenotypic novelty. Yet how a dimorphism arises or how additional morphs are added is not understood, and few systems provide experimental insight into the evolution of polyphenisms. Because plasticity correlates with structural diversity in *Pristionchus* nematodes, studies in this group can test the role of plasticity in facilitating novelty. Here, we describe three new species, *Pristionchus fukushimae* sp. nov., *Pristionchus hoplostomus* sp. nov., and the hermaphroditic *Pristionchus triformis* sp. nov., which are characterized by a novel polymorphism in their mouthparts. In addition to showing the canonical mouth dimorphism of diplogastrid nematodes, comprising a stenostomatous ('narrow-mouthed') and a eurystomatous ('wide-mouthed') form, the new species exhibit forms with six, 12, or intermediate numbers of cheilostomatal plates. Correlated with this polymorphism is another trait that varies among species: whereas divisions between plates are complete in *P. triformis* sp. nov., which is biased towards a novel 'megastomatous' form comprising 12 complete plates, the homologous divisions in the other new species are partial and of variable length. In a reconstruction of character evolution, a phylogeny inferred from 26 ribosomal protein genes and a partial small subunit rRNA gene supported the megastomatous form of *P. triformis* sp. nov. as the derived end of a series of split-plate forms. Although split-plate forms were normally only observed in eurystomatous nematodes, a single 12-plated stenostomatous individual of *P. hoplostomus* sp. nov. was also observed, suggesting independence of the two types of mouth plasticity. By introducing these new species to the *Pristionchus* model system, this study provides further insight into the evolution of polymorphisms and their evolutionary intermediates.

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INTRODUCTION

Developmental (phenotypic) plasticity is often correlated with diversity, and has thus been proposed as a

facilitator of phenotypic novelty (West-Eberhard, 2003). With the growing interest in the evolution of phenotypic plasticity over the last decade, the phenomenon has seen increasingly intense investigation, including work on its theoretical bases (Moczek *et al.*, 2011). For example, alternative morphologies that arise to exploit disparate food sources or different ecological niches have been proposed to be fittest as

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discontinuous polymorphisms (Smith & Skúlason, 1996), although the alternative forms may in fact arise from the extremes of a phenotypic continuum (Suzuki & Nijhout, 2006). However, few case studies thus far have allowed detailed mechanistic insight into the molecular and developmental mechanisms involved in the regulation of developmental plasticity. Nematodes of the genus *Pristionchus* Kreis, 1932, as well as other species of Diplogastridae Micoletzky, 1922, are characterized by a dimorphism in feeding structures (Fürst von Lieven & Sudhaus, 2000), which are among the most diverse characters in the genus (Kanzaki *et al.*, 2012a, b, 2013a, b). The dimorphism consists of a stenostomatous ('narrow-mouthed') and a eurystomatous ('wide-mouthed') form, and is, in *Pristionchus pacificus* Sommer, Carta, Kim & Sternberg, 1996, an environmentally influenced polyphenism rather than a polymorphism resulting from genetic heterozygosity (Bento, Ogawa & Sommer, 2010).

Developmental mechanisms for this 'canonical' mouth dimorphism have begun to be elucidated for *Pristionchus* nematodes, which are a tractable model system for comparative genetics (Sommer & McGaughan, 2013). For example, studies have identified $\Delta 7$ -dafachronic acid and the nuclear hormone receptor DAF-12 (Bento *et al.*, 2010), as well as the cyclic-GMP-dependent protein kinase EGL-4 (Kroetz *et al.*, 2012), to be co-opted to regulate dimorphism in evolution. However, there are no known intermediates between monomorphic outgroups and the most basal known diplogastrid genus, *Koerneria* (Mayer, Herrmann & Sommer, 2009), which exhibits stomatal dimorphism. Thus, the original formative processes of the dimorphism remain elusive.

As in many areas of evolutionary biology, the most important entry point towards a detailed understanding of developmental plasticity is taxonomy and phylogeny. To provide such groundwork, we describe three new *Pristionchus* species that exhibit an additional plasticity in their mouthparts, specifically in the walls of the stoma. The putatively recent origin of this plasticity gives the possibility of identifying transitional states in the evolution of plastic and ecologically relevant traits. Therefore, to detect possible differences in this plasticity among three closely related species, we undertook a detailed morphological characterization of their mouthparts.

To reconstruct the history of the plasticity, we included the three new species in an independently derived phylogeny of the genus inferred from 26 ribosomal protein-coding genes and a fragment of the small subunit (SSU) rRNA gene. Analysis included all original sequence data for *Pristionchus fukushimae* sp. nov. and *Pristionchus hoplostomus* sp. nov., and several newly sequenced loci for the close outgroup *Parapristionchus giblindavisi* Kanzaki, Ragsdale, Herrmann,

Mayer, Tanaka, and Sommer, 2012. The ancestral state reconstruction also included new observations and characterization of the stoma of a species putatively close to the three new species, *Pristionchus entomophagus* (Steiner, 1929) Sudhaus & Fürst von Lieven, 2003. Dense sampling within and among species can test for the presence of intermediate forms in dimorphic species and thereby shed light on the process of divergence within a plastic trait. To this end, we conducted a survey of polymorphic states to test for the increased canalization of homologous morphs across species, and thereby also test the link between plasticity and the radiation of forms.

MATERIAL AND METHODS

NEMATODE ISOLATION AND CULTIVATION

Pristionchus triformis sp. nov. was isolated as described by Herrmann, Mayer & Sommer (2006). *Pristionchus hoplostomus* sp. nov. was isolated from soil in a botanical garden in Tokyo, Japan. *Pristionchus fukushimae* sp. nov. was collected from an adult individual of *Lucanus maculifemoratus* Motschulsky, 1861 (Coleoptera: Lucanidae) in a forest in Fukushima, Japan. For *P. fukushimae* sp. nov., host beetles were dissected on 2.0% agar plates, after which the plates were kept at 20–25°C for several weeks. Nematodes proliferated on bacteria associated with the host beetle cadavers. After isolation, individuals of all species were transferred to nematode growth medium (NGM) agar plates seeded with *Escherichia coli* OP50 and since then have been kept in laboratory culture on this medium. The strain of *P. triformis* sp. nov. isolated from its type locality was frozen as a voucher (strain RS5101), and was also inbred for 20 generations, after which it was frozen as a separate voucher (RS5233). The inbred strain was designated as the type strain.

MORPHOLOGICAL OBSERVATIONS AND TYPE MATERIAL

Cultures (1–2 weeks old) of *P. triformis* sp. nov. (RS5232, RS5233), *P. hoplostomus* sp. nov. (JU1090), and *P. fukushimae* sp. nov. (RS5595) provided material for morphological observation. In *P. triformis* sp. nov. only a single male was found after examination of hundreds of individuals in culture. Observations by differential interference contrast (DIC) microscopy were conducted using live nematodes, which were hand-picked from culture plates. Close observation of mouthparts included pressing specimens to evert and dissect stomata from the bodies of the nematodes. Because of the clarity and integrity of its morphology, live material was used for morphometrics. For line drawings, specimens were mounted in water on slides and then relaxed by applying gentle heat. For morpho-

metrics, specimens were mounted on slides with pads of 5% agar noble and 0.15% sodium azide, and were additionally relaxed by heat when necessary. To prepare type material, nematodes were isolated from type strain cultures, rinsed in distilled water to remove bacteria, killed by heating at 65 °C, fixed in triethanolamine-formalin (TAF) to a final concentration of 1.5% triethanolamine and 5% formalin, and processed through a glycerol and ethanol series using Seinhorst's method (see Hooper, 1986). In addition to the newly described species, live specimens of *P. entomophagus* (RS0144) were observed by light microscopy (LM) for comparison.

SCANNING ELECTRON MICROSCOPY

Nematodes were prepared for scanning electron microscopy (SEM) by fixation in 2.5% glutaraldehyde in M9 buffer and then post-fixation 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 approximately 1-kb fragment of the SSU rRNA gene as described in Mayer, Herrmann & Sommer (2007). The SSU rRNA sequences obtained for *P. hoplostomus* sp. nov. and *P. fukushimae* sp. nov. are original to this study and have been deposited in the GenBank database under the accession codes KC819346 and KC819347, respectively. The final alignment of the SSU rRNA gene fragment consisted of 851 positions. For phylogenetic analysis, we also employed 26 ribosomal protein genes that have been used in previous studies of the genus (Mayer *et al.*, 2007; Kanzaki *et al.*, 2012a). Sequences were examined as trace files with FinchTV (Geospiza, Seattle, WA, USA) and were assembled using MEGA 5.05 (Tamura *et al.*, 2011). 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-30*, *rpl-31*, *rpl-32*, *rpl-34*, *rpl-35*, *rpl-38*, *rpl-39*, *rps-1*, *rps-8*, *rps-20*, *rps-21*, *rps-24*, *rps-25*, *rps-27*, and *rps-28*. All information regarding genes, primers, and PCR conditions is given in Mayer *et al.* (2007). All ribosomal protein gene sequences for *P. hoplostomus* sp. nov. and *P. fukushimae* sp. nov. were original in this study. Sequences of *rpl-26* and of 57- and 66-nucleotide fragments of *rpl-39* and *rps-28*, respectively, were not obtained for

P. fukushimae sp. nov., and were coded as missing data for that species. New sequences for *Parapristionchus giblindavisi* included *rpl-1*, *rpl-16*, *rpl-27*, *rpl-27a*, *rpl-28*, *rpl-30*, *rpl-31*, *rpl-32*, *rpl-34*, *rpl-38*, *rpl-39*, *rps-1*, *rps-8*, *rps-20*, *rps-21*, *rps-24*, *rps-25*, *rps-27*, and *rps-28*. Alternative primers to those used by Mayer *et al.* (2007) were designed for the amplification of *rpl-1*, *rpl-26*, *rps-1*, and *rps-24* from *Pa. giblindavisi* (see the Appendix). Sequences of *Pa. giblindavisi* not included in the final concatenated data set, and thus coded as missing data, were fragments of 64 bp from *rpl-27*, 75 bp from *rpl-34*, and 277 bp from *rps-8*. Original ribosomal protein gene sequences for all of the above taxa, except those of *rpl-38*, *rpl-39*, and *P. hoplostomus* sp. nov. *rps-28*, which were less than 200 nucleotides in length, have been deposited in GenBank under accession numbers KC819348–KC819409. Missing data for other species were: 152 bp of *rps-1*, *rps-8*, and 70 bp of *rps-1* for *Pristionchus bucculentus* Kanzaki, Ragsdale, Herrmann, Röseler & Sommer, 2013a; 182 bp of *rps-1*, 285 bp of *rps-8*, and *rps-14* for *Pristionchus maxplancki* Kanzaki, Ragsdale, Herrmann, Röseler & Sommer, 2013b. Ribosomal protein gene sequences for *P. triformis* sp. nov. and the other species included in the analysis have previously been published (Mayer *et al.*, 2007) and are thus available in GenBank.

The concatenated data set of the partial SSU rRNA and ribosomal protein genes was aligned using MUSCLE (Edgar, 2004), followed by manual alignment in MEGA, which included the removal of ambiguously aligned positions. The data set of ribosomal protein genes comprised a total of 10 767 characters. The alignment was partitioned into one subset for the partial SSU rRNA gene and one subset for each of the three codon positions for the concatenated ribosomal protein genes. Analyses by maximum likelihood (ML) and Bayesian inference, as implemented in RAxML 7.2.8 (Stamatakis, 2006) and MrBayes 3.2 (Ronquist *et al.*, 2012), respectively, were used to infer the phylogeny. Fifty independent runs were performed for the ML analysis, in which trees were inferred under a general time-reversible model, with a gamma-shaped distribution of rates across sites. Bootstrap support (BS) was calculated by 1000 pseudoreplicates on the most likely tree among all runs. Bayesian analyses were initiated with random starting trees and were run with four chains for 4×10^6 generations. Markov chains were sampled at intervals of 100 generations. Two independent runs were performed for the analysis. After confirming convergence of runs and discarding the first 2×10^6 generations as burn-in, the remaining topologies were used to generate a 50% majority-rule consensus tree with clade credibility values given as posterior probabilities (PPs). Bayesian analysis invoked a mixed model of substitution with

a gamma-shaped distribution across sites, and *Koerneria* sp. (RS1982) was specified as the outgroup. Model parameters were unlinked across character partitions in both ML and Bayesian analyses.

MATING EXPERIMENTS

Because preliminary diagnosis by SSU rRNA sequences revealed *P. hoplostomus* sp. nov. and *P. fukushimae* sp. nov. to be close to each other, mating experiments were performed between these two gonochoristic species to test whether they were each biologically unique. In the experiments, five virgin (J4) females of one strain were placed together with five males of the other strain on a plate maintained at 20 °C and containing a lawn grown from 25 µl of *E. coli* OP50 in Luria broth. Mating tests were performed in triplicate. All crosses were performed reciprocally. We considered strains to be reproductively isolated if no viable F₁ progeny resulted from crosses, which were checked for up to 3 weeks.

FREQUENCIES OF STOMATAL FORMS IN THE NEW SPECIES

To determine the spectrum of stomatal plasticity in *P. triformis* sp. nov. (RS5233), *P. hoplostomus* sp. nov., and *P. fukushimae* sp. nov., a survey of the relative abundance of forms and their intermediates in the three new species was conducted. In this survey, 300 females or hermaphrodites of each species were randomly picked from well-fed culture populations and screened for their mouth form. Additionally, 300 males each from *P. hoplostomus* sp. nov. and *P. fukushimae* sp. nov. were screened. The traits scored were: (1) the canonical mouth form (eurystomatous versus stenostomatous); and, for the eurystomatous form, (2) the number of lateral cheilostomatal plates present. For trait 1, the characters used to discriminate between eurystomatous and stenostomatous individuals, respectively, were: (1) the presence versus absence of a subventral tooth; (2) a claw-like versus flint-like dorsal tooth; and (3) a wide versus narrow stoma. For trait 2, cheilostomatal plates that were separated from each other by a clear incision for any length were counted as individual plates; plates split only at their tips (flaps) were counted as single plates. The number of lateral plates present thus ranged between four and eight. All phenotypes were scored using DIC microscopy.

RESULTS AND TAXONOMY

MOLECULAR CHARACTERIZATION AND PHYLOGENETIC ANALYSIS

The 830-bp SSU rRNA sequences obtained for *P. hoplostomus* sp. nov. and *P. fukushimae* sp. nov. differed

by five nucleotide positions, clearly supporting their identity as two unique species. Differences in five and ten positions in *P. hoplostomus* sp. nov. and *P. fukushimae* sp. nov., respectively, from *P. triformis* sp. nov. also supported their separation from the latter species. Phylogenetic analysis of sequences of the SSU and 26 ribosomal protein loci further revealed the divergence of *P. triformis* sp. nov., *P. hoplostomus* sp. nov., and *P. fukushimae* sp. nov. from other *Pristionchus* species (Fig. 1).

The final alignment comprised 2526 parsimony-informative sites. Only the ML tree is shown, as topologies inferred by ML and Bayesian analyses were almost identical. The only difference between the two analyses was the placement of *Pristionchus fissidentatus* Kanzaki, Ragsdale, Herrmann, Sommer, 2012b, which was not resolved in either tree: *P. fissidentatus* was negligibly supported either as sister to the *elegans* group in the ML topology (< 50% BS) or as sister to the rest of *Pristionchus* excepting the *elegans* group (66% posterior probability, PP; not shown). The phylogeny showed that the three new species constitute a well-supported monophyletic clade (100% BS and PP), herein designated the 'triformis' group of *Pristionchus* species. Within this clade, *P. triformis* sp. nov. and *P. hoplostomus* sp. nov. were moderately to well supported as sister taxa (78% BS, 100% PP) with *P. fukushimae* sp. nov. as the outgroup to these two species. The clade of the three new species was weakly supported (65% BS, 98% PP) as the sister group to the *lheritieri* group (Kanzaki *et al.*, in press), which includes *P. entomophagus* and was itself well supported as monophyletic (100% BS and PP). *Pristionchus* sp. 14 was variably supported (69% BS, 100% PP) as sister to the *pacificus* group (Kanzaki *et al.*, in press), and is herein designated a member of that group. *Pristionchus bucculentus* and *P. elegans* Kanzaki, Ragsdale, Herrmann & Sommer, 2012b were well supported as sister taxa, and are herein designated as the 'elegans' group of *Pristionchus* species.

MATING EXPERIMENTS

Reciprocal mating tests between *P. hoplostomus* sp. nov. and *P. fukushimae* sp. nov. did not result in any viable F₁ progeny. Therefore, each of the two species was considered to be biologically unique.

DESCRIPTION OF COMMON CHARACTERS

All three new species are phylogenetically close to each other (Fig. 1), and are thus alike in many diagnostic characters (Figs 2–12). Therefore, to avoid redundancy, characters common to both new species

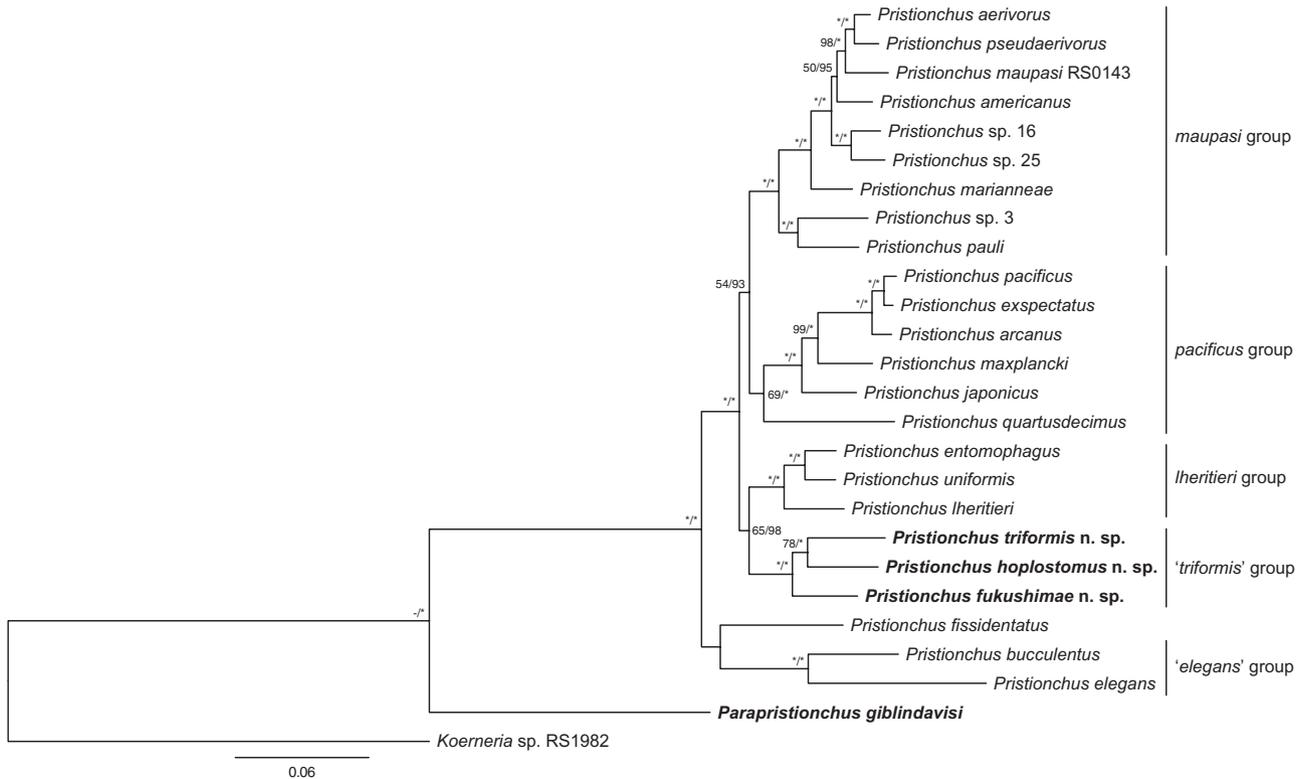


Figure 1. Phylogenetic relationships of *Pristionchus* species inferred by maximum likelihood (ML) analysis from a fragment of the small subunit (SSU) rRNA gene and 26 ribosomal protein coding genes. The tree with the highest log likelihood (−64246.134130) is shown. The percentage of trees in which the associated taxa clustered together in 1000 bootstrap pseudoreplicates is shown next to the nodes (left value). Posterior probabilities are additionally given (right value) for nodes that are also present in the topology inferred by Bayesian analysis; *100% support. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site.

are described first, followed by specific characters and diagnoses for each species.

Adults

Cuticle thick, with fine annulation and clear longitudinal striations (Figs 2D, 6B, E, 8A, 9D, 11B, C). Lateral field consisting of two lines, only weakly distinguishable from body striation. Head without apparent lips, and with six short and papilliform labial sensilla (Figs 3H, 7C). Four small, papilliform cephalic papillae present in males, as typical for diplogastrid nematodes (Fig. 7C). Amphidial apertures located at level of posterior end of cheilostomatal plates. Stenostomatous form occurring in both males and females. Eurystomatous form or forms not observed in males. Dorsal pharyngeal gland clearly observed, penetrating dorsal tooth to gland opening. Anterior part of pharynx (= pro- and metacarpus) 1.5 times as long as posterior part (isthmus and basal bulb) (Figs 2C, 8A, 9C). Procorpus muscular, stout, occupying half to two-thirds of corresponding body diameter. Metacarpus muscular, forming well-

developed median bulb. Isthmus narrow, not muscular. Basal bulb glandular. Pharyngo-intestinal junction clearly observed, well developed. Nerve ring usually surrounding anterior to middle region of isthmus (Figs 2C; 8A; 9C). Excretory pore not conspicuous, with ventral opening at level of posterior isthmus to basal bulb (Figs 2C, 8A, 9C). Hemizonid not clearly observed. Deirid observed laterally, at level of basal bulb to slightly posterior to pharyngo-intestinal junction (Figs 2D, 8A, 9D). 'Postdeirid' pores present and observed laterally, either slightly left or right of lateral midline (Figs 2D, 9D), with positions along body axis inconsistent among individuals, with between five and eight pores for males and nine or ten pores for females being confirmed by LM.

Stenostomatous form

Cheilostom consisting of six per- and interradiial plates (Figs 3E–G, 7D, E, 10A–D). Incisions between plates not easily distinguished by LM. Anterior end of each plate rounded and elongated to stick out from stomatal

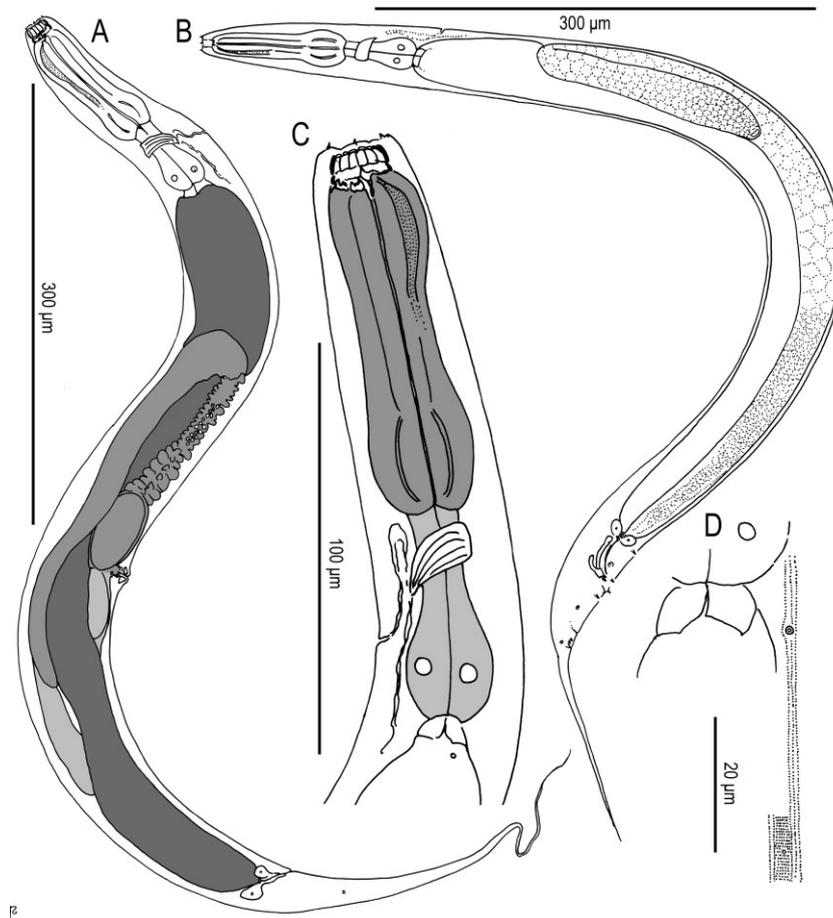


Figure 2. Adults of *Pristionchus triformis* sp. nov. A, whole body of megastomatous (i.e. 12-plated eury stomatous) hermaphrodite, right lateral view. B, whole body of stenostomatous male, right lateral view. C, neck region of megastomatous hermaphrodite, left lateral view. D, deirid (above) and 'postdeirid' (small pore, bottom left), right lateral view.

opening and form a small flap (Fig. 10A). Gymnostom short, shorter than cheilostom, cuticular ring-like anterior end overlapping cheilostom internally. Pro- and mesostegostom not obvious, ring-like, connecting gymn stomatal ring and metastegostom, i.e. not overlapping with gymn stomatal ring. Metastegostom bearing a conspicuous, flint-shaped (or inverted V-shaped) dorsal tooth (Figs 3E–G, 7D, E, 10A–D), sometimes with anterior curvature at apex (Figs 3E, G, 10C). Telostegostom not sclerotized, deeper in ventral side than dorsal side, posterior part narrowing to procorpus. Left and right subventral sectors differ among species. Dorsal tooth with strongly sclerotized surface. Dorsal tooth movable. Movement not observed for armature in left subventral and right subventral sectors. Additional stomatal morphology is given separately for individual species below. A 12-plated stenostomatous individual observed in *P. hoplostomus* sp. nov. is also described separately.

Eury stomatous form

Gymnostom with thick cuticle, forming a short, barrel-shaped tube (Figs 3C, D, 4C, 7G, H, 10E, F). Anterior end of gymn stom internally overlapping posterior end of cheilostomatal plates and lined with finely serrated ring at anterior margin (Figs 3C, D, 7G, H, 10E, F). Anterior margin of stegostom (pro- and mesostegostom) forming a conspicuously serrated ring (Figs 3C, D, 4B, 7G, H, 9E, F). Metastegostom bearing a large claw-like dorsal tooth (Figs 3C, D, 4C, 7G, H, 10E, F), and a large, claw-like or pointed right subventral tooth (Figs 3D, H, 7H, 10F). Left subventral sector of metastegostom differs among species. Dorsal tooth and right subventral tooth movable. Movement not observed in left subventral denticles. Telostegostom not sclerotized, deeper in ventral side than dorsal side, posterior part narrowing to procorpus. Additional stomatal morphology is given separately for individual species below.

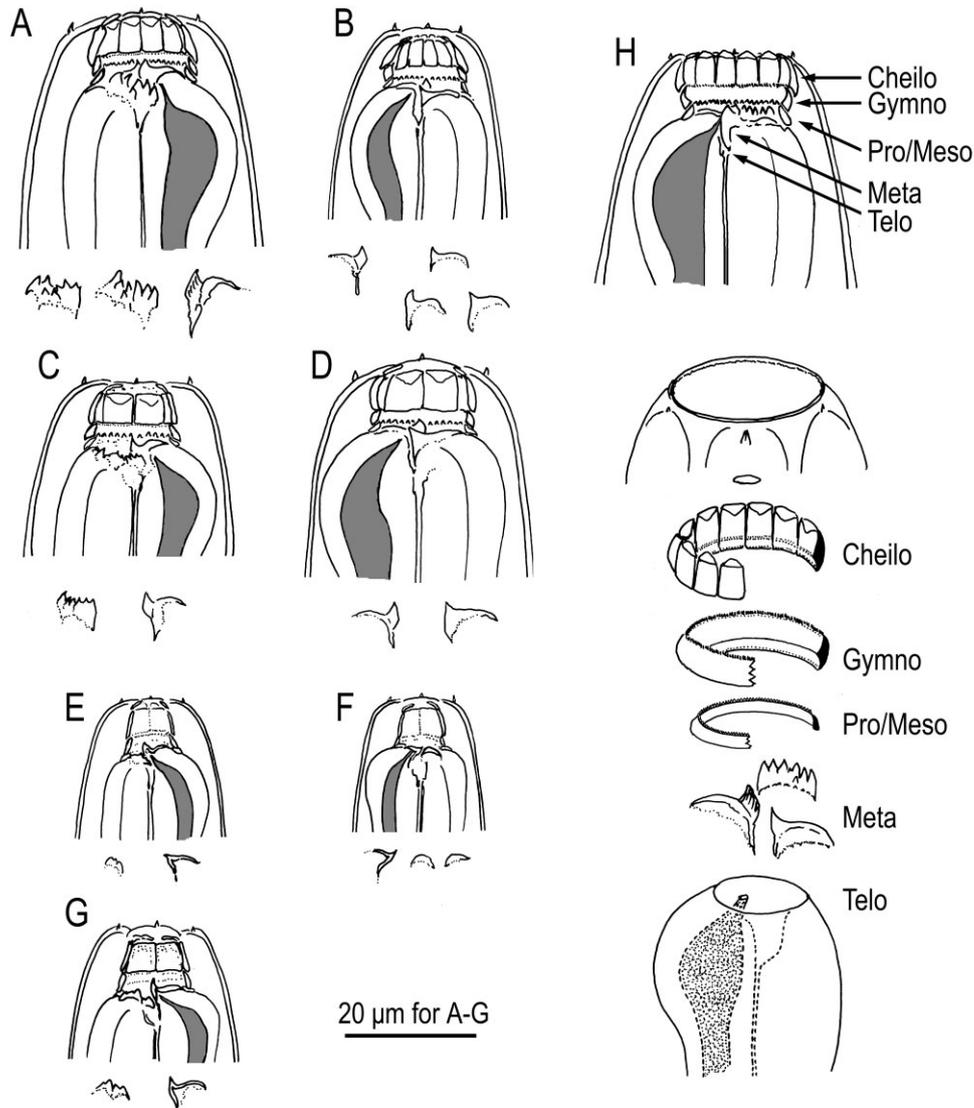
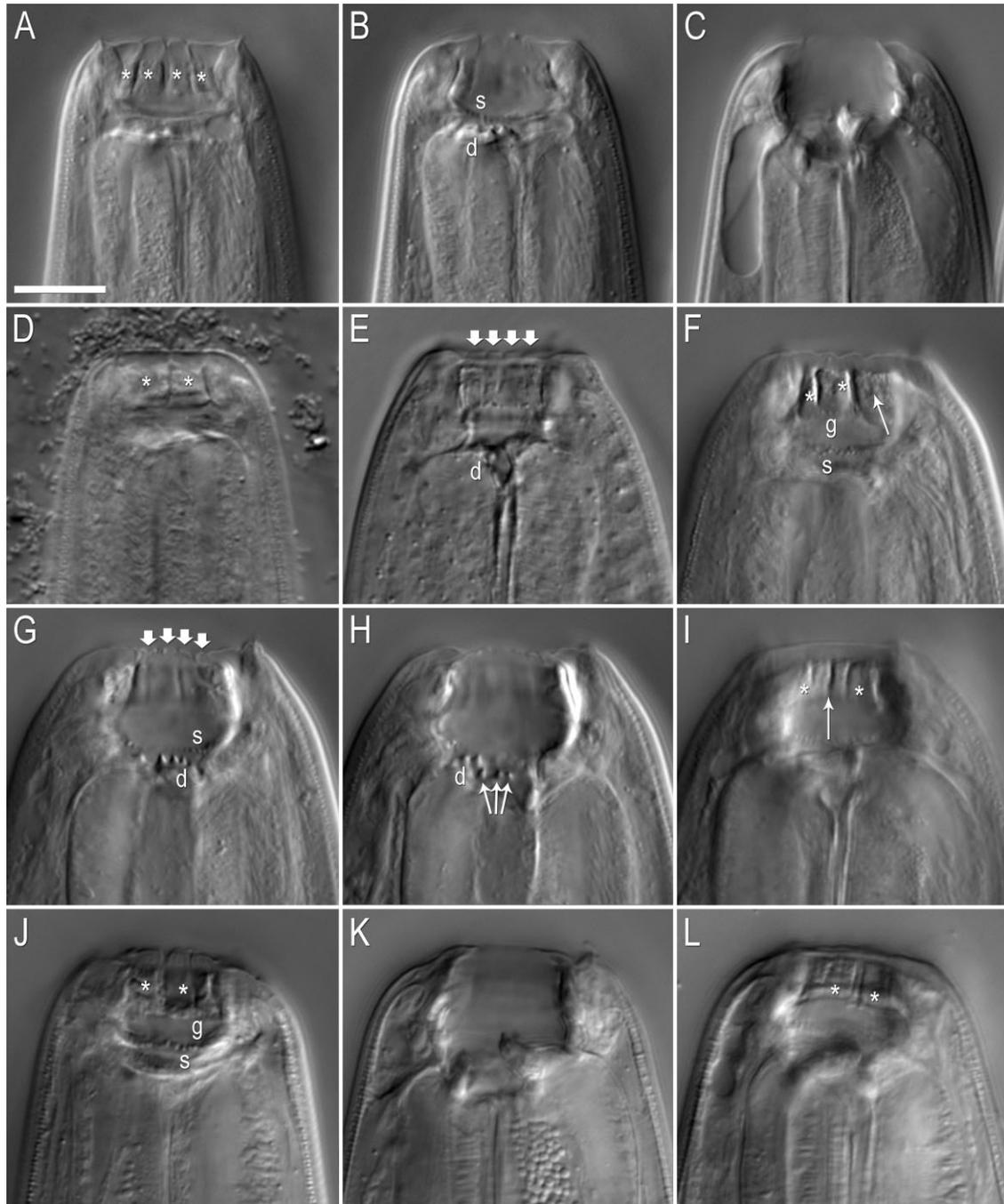


Figure 3. Stomatal region of adult hermaphrodites of *Pristionchus triformis* sp. nov. A, megastomatous (i.e. 12-plated eury stomatous) form, left lateral view, showing two variants of left subventral metastegostom (below, left) and a large variant of dorsal tooth (below, right). B, megastomatous form exhibiting a variation of head and stoma size, right lateral view, showing dorsal tooth (below, left) and three variants of right subventral tooth (below, right). C, canonical eury stomatous (i.e. six-plated eury stomatous) form, left lateral view, showing left subventral denticles (below, left) and dorsal tooth (below, right). D, canonical eury stomatous form exhibiting a variation of head and stoma size, right lateral view, showing dorsal tooth (below, left) and right subventral tooth (below, right). E, stenostomatous form, left lateral view, showing left subventral ridge (below, left) and dorsal tooth (below, right). F, stenostomatous form, right lateral view, showing dorsal tooth (below, left) and two variants of right subventral ridge (below, right). G, variant of stenostomatous form, left lateral view, showing left subventral ridge (below, left) and dorsal tooth (below, right). H, schematic representation of individual rhabdia of the metastomatous form, namely the cheilostom (Cheilo), gymnostom (Gymno), pro- and mesostegostom (Pro/Meso), metastegostom (Meta), and telostegostom (Telo). This diagram also applies to *Pristionchus hoplostomus* sp. nov. and *Pristionchus fukushimae* sp. nov., except for the differences in cheilostomatal plates and components of metastegostom described.

Male

Ventrally arcuate, strongly ventrally curved at tail region when killed by heat. Testis single, ventrally located, anterior part reflexed to right side. *Vas def-*

erens not clearly separated from other parts of gonad (Figs 2B, 7B, 9B). Three (two subventral and one dorsal) cloacal gland cells observed at distal end of testis and intestine (Figs 6F, 8F, 11E). Spicules



paired, separate (Figs 8E, 11F). Spicules smoothly curved in ventral view, adjacent to each other for distal third of their length, each smoothly tapering to pointed distal end (Figs 8E, 11F). Spicule in lateral view smoothly ventrally arcuate, giving spicule about 100° curvature, lamina/calomus complex expanded just posterior to manubrium, then smoothly tapering to pointed distal end (Figs 6G, 8G, 11G). Thick cuticle around tail region, sometimes falsely appearing like a narrow leptoderan bursa in ventral view (Figs 8E,

11F). Cloacal opening slit-like in ventral view (Figs 8E, 11F, 12B). One small, ventral, single genital papilla on anterior cloacal lip, nine pairs of genital papillae and a pair of phasmids present (Figs 6F, 8E, F, 11E, F, 12). P1–P4 papillae of almost equal size, rather large and conspicuous, P5d slightly smaller than P1–P4, P6 and P7 both 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 6F, 8E, F, 11E, F,

Figure 4. Nomarski micrographs of the stomatal region of *Pristionchus triformis* sp. nov., *Pristionchus hoplostomus* sp. nov., and *Pristionchus fukushimae* sp. nov. View is left lateral in A–C and E–L, and right lateral in D. Scale bar: 10 µm (all images are at same scale). Stars indicate cheilostomatal plates. The numbers of plates could be verified by rotating worms along their body axis for observation. A–C, a single megastomatous (12-plated euryatomatous) individual of *P. triformis* sp. nov. at serial focal planes. A, left lateral plane, showing four lateral, completely divided plates. B, plane medial of (A). C, sagittal plane, showing dorsal tooth. D, six-plated euryatomatous individual, showing two lateral plates. E, 12-plated stenostomatous individual of *P. hoplostomus* sp. nov., showing four lateral plates and flaps (arrows), dorsal tooth, and left subventral ridge. F–I, a single euryatomatous individual of *P. hoplostomus* sp. nov. in serial focal planes. F, left lateral plane, showing two partially split plates and a faint incision (arrow) of a lateral plate ending in two flaps (shown in G). G, plane medial of (F), showing four lateral cheilostomatal flaps (arrows). H, plane medial of (G), showing several adventitious denticles (arrows). I, right lateral plane, showing partially split plates separated by a complete incision (arrow). J–L, a single individual of *P. fukushimae* sp. nov. at serial focal planes. J, left lateral plane, showing an undivided lateral plate (left star) and partially split lateral plate (right star). K, sagittal plane, showing dorsal tooth. L, two partially split plates separated by a complete incision. Additional abbreviations: d, left subventral denticles; g, serrated ring of gymnostom; s, serrated ring of stegostom.

12). P6 and P7 papilliform and borne form socket-like base, P8 simple or typical thorn-like in shape (Fig. 8F). Tip of P7 papillae split into two small papilla-like projections (Fig. 8F). Detailed arrangement of paired papillae and phasmids is described for individual species below; comparative morphology of male tails is presented in Figure 12. Tail conical, about two to three cloacal body diameters long, with long spike, about three to four cloacal body diameters long (Figs 2B, 6F, 7B, 8E, F, 11E). Bursa or bursal flap absent.

Hermaphrodite/female

Relaxed or slightly ventrally arcuate when killed by heat. Gonad didelphic, amphidelphic. Each gonadal system arranged from vulva and vagina as uterus, oviduct, and ovary/ovotestis (Figs 6A, 8B, 11A). 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/ovotestis extending dorsally on left of intestine (Figs 2A, 7A, 9A). Oocytes mostly arranged in multiple, sometimes more than five, rows in distal two-thirds of ovary/ovotestis and in single row in remaining third of ovary/ovotestis, distal tips of each ovary/ovotestis reaching oviduct of opposite gonad branch (Figs 6A, 8B, 11A). Middle part of oviduct serving as spermatheca, which is not clearly delineated or offset, sperm observed in distal part of oviduct, close to ovary/ovotestis (Figs 6A, 8B, 11A). Eggs in single to multiple-cell stage or even further developed at proximal part of oviduct (= uterus). Dorsal wall of uterus at level of vulva thickened and appearing dark in LM observation. Four vaginal glands present but obscure (Fig. 11B). Vagina perpendicular to body surface, surrounded by sclerotized tissue. Vulva slightly protuberant in lateral view, pore-like in ventral view (Figs 6B, 11B). Rectum

about one anal body diameter long, intestinal–rectal junction surrounded by well-developed sphincter muscle. Three (two subventral and one dorsal) anal glands present at the intestinal–rectal junction, but not obvious (Figs 6C, 8C, 11D). Anus in form of dome-shaped slit (Figs 6D, E, 8D, 11C), posterior anal lip slightly protuberant (Figs. 6C, 8C, 11D). Phasmid about 1.5–2 times anal body diameter posterior to anus (Figs 6D, 8D, 11C). Tail long and conical, distal end filiform (Figs 2A, 6C, 8C, 9A, 11D).

Type strains

All three new species are available in living culture and as frozen stocks in the Department of Evolutionary Biology, Max Planck Institute for Developmental Biology, Tübingen, Germany, and can be provided to other researchers upon request.

***PRISTIONCHUS TRIFORMIS* SP. NOV.**

= *Pristionchus* sp. 6 *apud* Herrmann, Mayer & Sommer (2006); Mayer *et al.* (2007); Weller *et al.* (2010)

Description

Measurements: See Table 1.

Adults: Species androdioecious, with males and self-fertile hermaphrodites. Three different stomatal forms, i.e. stenostomatous, euryatomatous, and megastomatous, present, with known intermediates between the latter two forms.

Stenostomatous form: Metastegostom bearing: a crescent-shaped right subventral ridge varying between being transversely symmetrical and with ridge apex turning inwardly, ridge sometimes with one minute denticle (Fig. 3F); in the left subventral

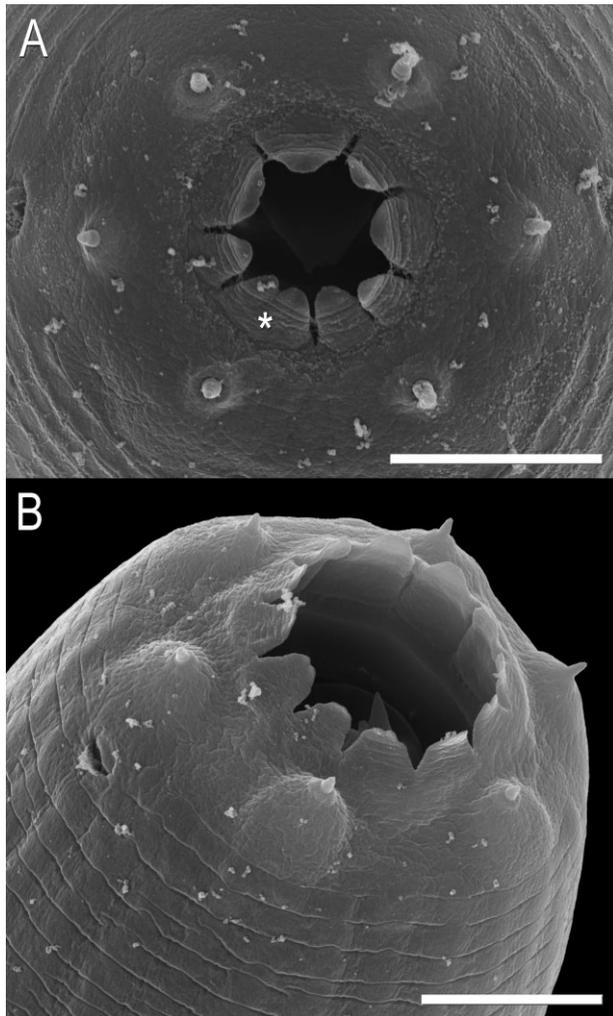


Figure 5. Scanning electron micrographs of the cheilostom plasticity of *Pristionchus hoplostomus* sp. nov. and *Pristionchus triformis* sp. nov. Scale bars: 5 µm. A, stoma of *P. hoplostomus* sp. nov., showing an irregular number of cheilostomatal flaps (eight), including one flap split only at the tip (star); en face view; top, dorsal. B, megastomatous form of *P. triformis* sp. nov., showing 12 completely divided cheilostomatal plates and flaps; en face view; left, subdorsal.

sector, three or four blunt or weakly pointed left subventral denticles apparently projecting from a common cuticular plate that sometimes appears split in lateral view (Fig. 3E, G).

Eurystomatous form: Cheilostom divided into six distinct per- and interradiial plates (Figs 3C, D, 4D). Anterior end of each plate rounded and elongated to stick out from stomatal opening and form a small flap (Fig. 3C, D). Gymnostom with thick cuticle, forming a short, barrel-shaped tube. Metastegostom bearing: a large claw-like dorsal tooth (Fig. 3C, D); a large, claw-

like or pointed right subventral tooth (Fig. 3D); a left subventral ridge of small denticles of varying shape, size, and number, i.e. between five and seven, arising from a split cuticular plate (Fig. 3C). Dorsal tooth and right subventral tooth movable. Movement not observed in left subventral denticles.

Megastomatous (12-plated eurystomatous) form: Cheilostom divided into 12 complete, distinct per- and interradiial plates (Figs 3A, B, H, 4A, 5B). Anterior end of each plate rounded and elongated to stick out from stomatal opening and form a small flap (Fig. 3H). Occasional intermediates between 12-plated and six-plated eurystomatous forms observed, e.g. where the common 12-plated form shows incomplete division of an individual plate, such that it culminates in a single flap, resulting in 11 flaps in total. Anterior margin of stegostom (pro- and mesostegostom) coarsely serrated (Figs 3A, B, H, 4B). Metastegostom bearing: a large and strongly sclerotized claw-like dorsal tooth (Figs 3A, B, H, 4C); a large, claw-like or pointed right subventral tooth (Fig. 3B, H), with shape varying from dorsoventrally symmetrical to strongly hooked (Fig. 3B); in the left subventral sector, a ridge of large denticles of varying shape, size, and number, i.e. between six and eight, arising from a split cuticular plate (Figs 3A, H, 4B), in addition to one or two posterior adventitious denticles (Fig. 3A, H). Although the number of denticles is variable, it commonly exceeds the extent of complexity observed in the six-plated eurystomatous form. Telostegostom not sclerotized, deeper in ventral side than dorsal side, posterior part narrowing to procorpus. Dorsal tooth and right subventral tooth movable. Movement not observed in left subventral denticles.

Male: Spontaneous (= fatherless) males very rare (< 0.5%) in culture. Squared manubrium at anterior end of spicule (Fig. 6G). Gubernaculum conspicuous, about 40% spicule length, slightly broader anteriorly such that dorsal wall is recurved, and that dorsal and ventral walls separate at a 30° angle at posterior end (Fig. 6G). In lateral view, anterior half of gubernaculum with two serial curves separated by anteriorly directed process, and with long terminal curvature about half of gubernaculum length and proximal curvature about one-third of gubernaculum length; posterior half forming a tube-like process enveloping spicules (Fig. 6G). Nine pairs of genital papillae and a pair of phasmids present, and arranged as <P1, P2d, P3, C, P4, P5d, Ph, (P6, P7, P8, P9d)> [= <v1, v2d, v3, C, v4, ad, Ph (5, v6, v7, pd)> in the nomenclature of Sudhaus & Fürst von Lieven, 2003] (Fig. 6F). Positions of P1 papillae are located about one cloacal body diameter posterior to cloacal slit, P2d equidistant between P1 and P4, P3 clearly posterior to P2d, P4

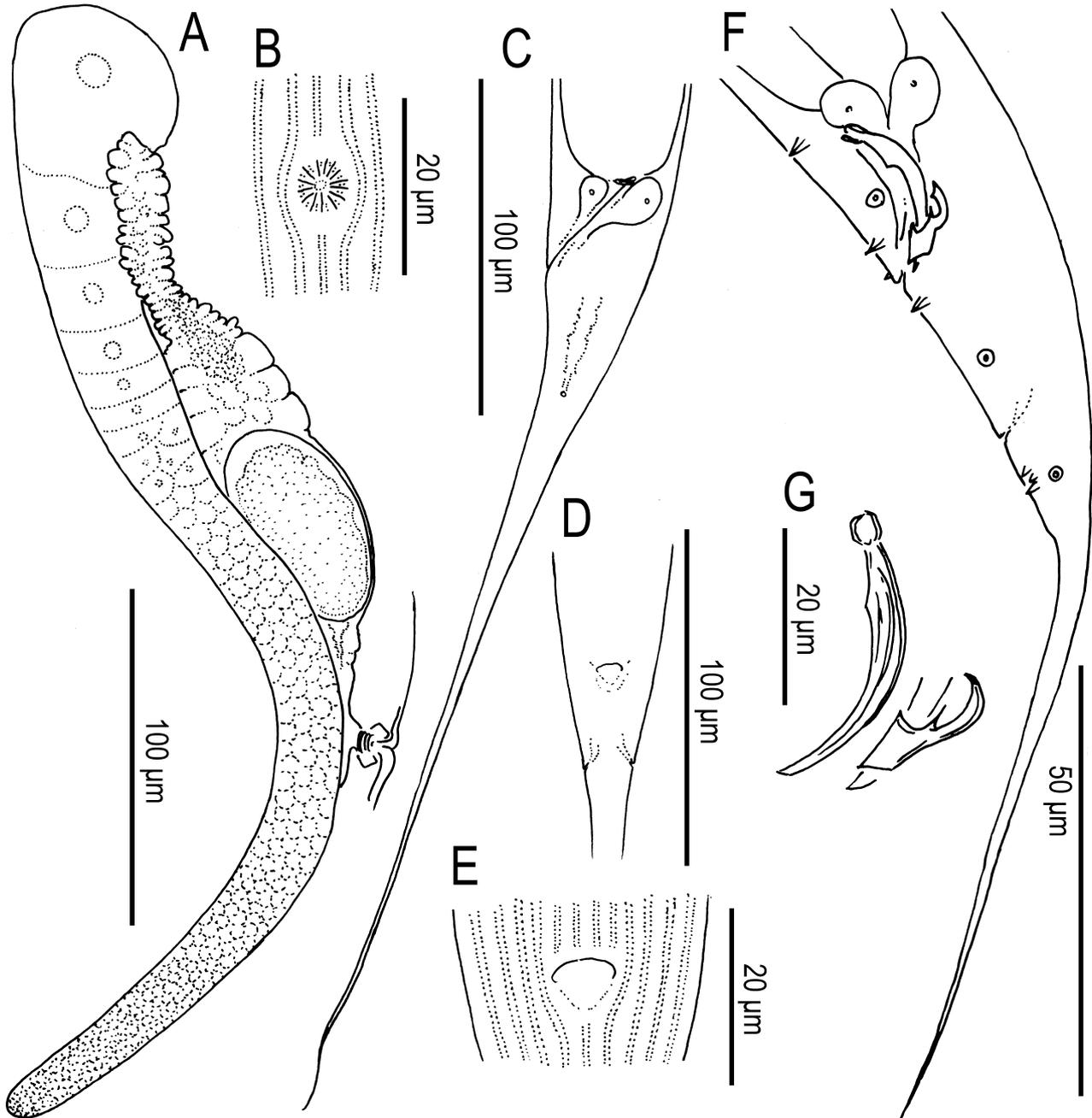


Figure 6. Adults of *Pristionchus triformis* sp. nov. A–E, hermaphrodite. F–G, male. A, anterior gonad branch, right lateral view. B, vulva, ventral view. C, tail region, left lateral view. D, E, anus, ventral view. F, tail region, left ventral view. G, spicule and gubernaculum, left lateral view.

within one-third of cloacal body diameter of C, P5d closer to P4 than to P6, and equidistant between P4 and Ph, Ph clearly anterior to P6, P6–P8 linearly arranged, and P9d overlapping P7–P8 (Figs 6F, 12A).

Differential diagnosis: *Pristionchus triformis* sp. nov. is diagnosed from all other species of *Pristionchus* and Diplogastridae by the presence versus absence of two

distinct eury stomatous forms: (1) a form with six cheilostomatal plates, putatively homologous with the eury stomatous form of other *Pristionchus* species; and (2) a ‘megastomatous’ form with 12 cheilostomatal plates or flaps, accompanied by a more complex left subventral ridge. It is further distinguished from all other species of *Pristionchus*, except *P. hoplostomus* sp. nov. and *P. fukushimae* sp. nov., by an

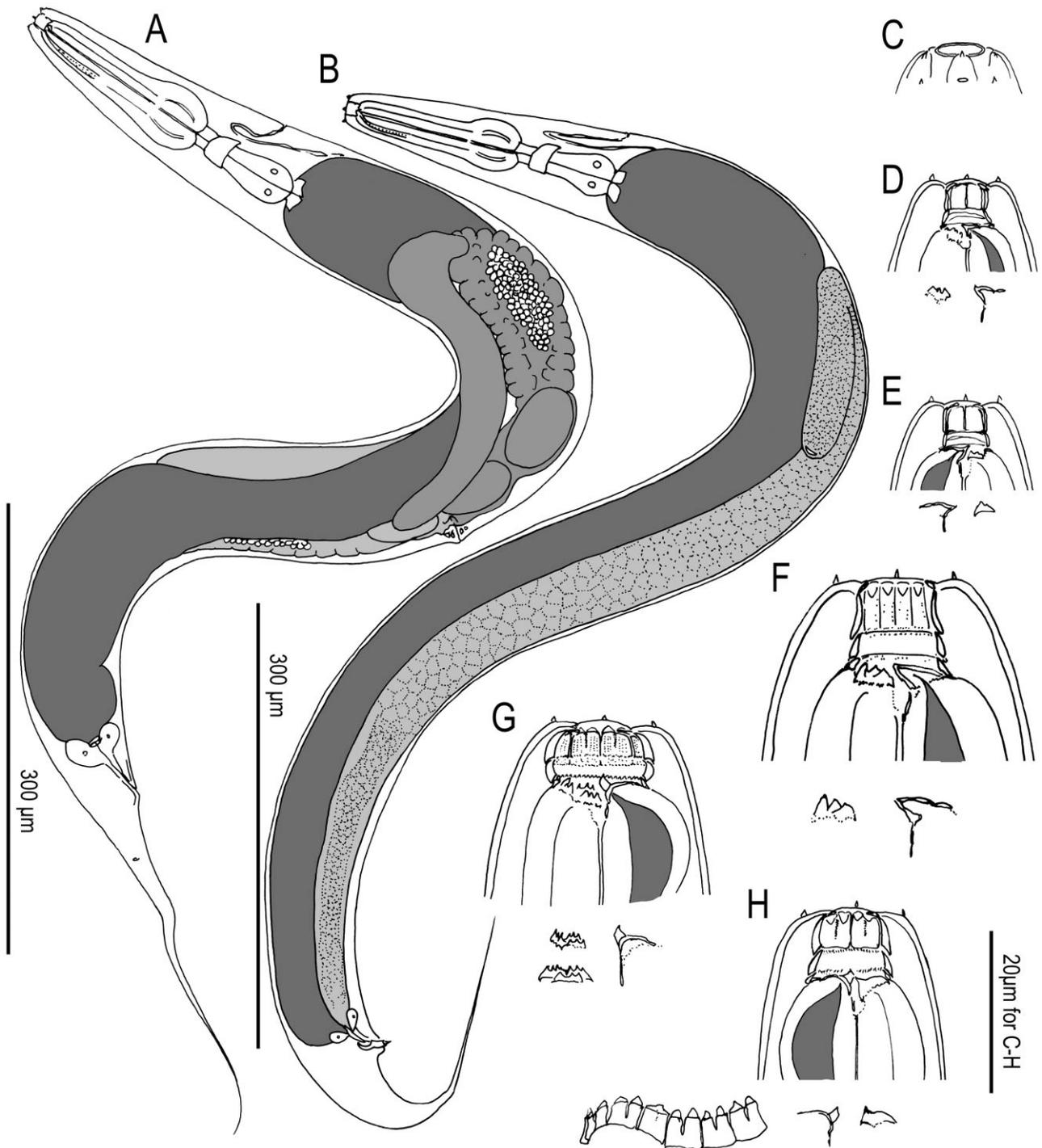


Figure 7. Adults of *Pristionchus hoplostomus* sp. nov. A, whole body of stenostomatous female, right lateral view. B, whole body of stenostomatous male, right lateral view. C, lip region of stenostomatous male, lateral view. D–H, stomatal region of female. D, stenostomatous form, left lateral view, showing left subventral ridge (below, left) and dorsal tooth (below, right). E, stenostomatous form, right lateral view, showing dorsal tooth (below, left) and right subventral ridge (below, right). F, 12-plated stenostomatous form, showing left subventral denticles (below, left) and dorsal tooth (below, right). G, eurystomatous form, showing two variants of left subventral metastegostom (below, left) and one variant of dorsal tooth (below, right). H, eurystomatous form, exhibiting a variation of stoma width, right lateral view, showing 11-plate variant of cheilostom dissected from body (below, far left), dorsal tooth (below, center), and right subventral tooth (below, right).

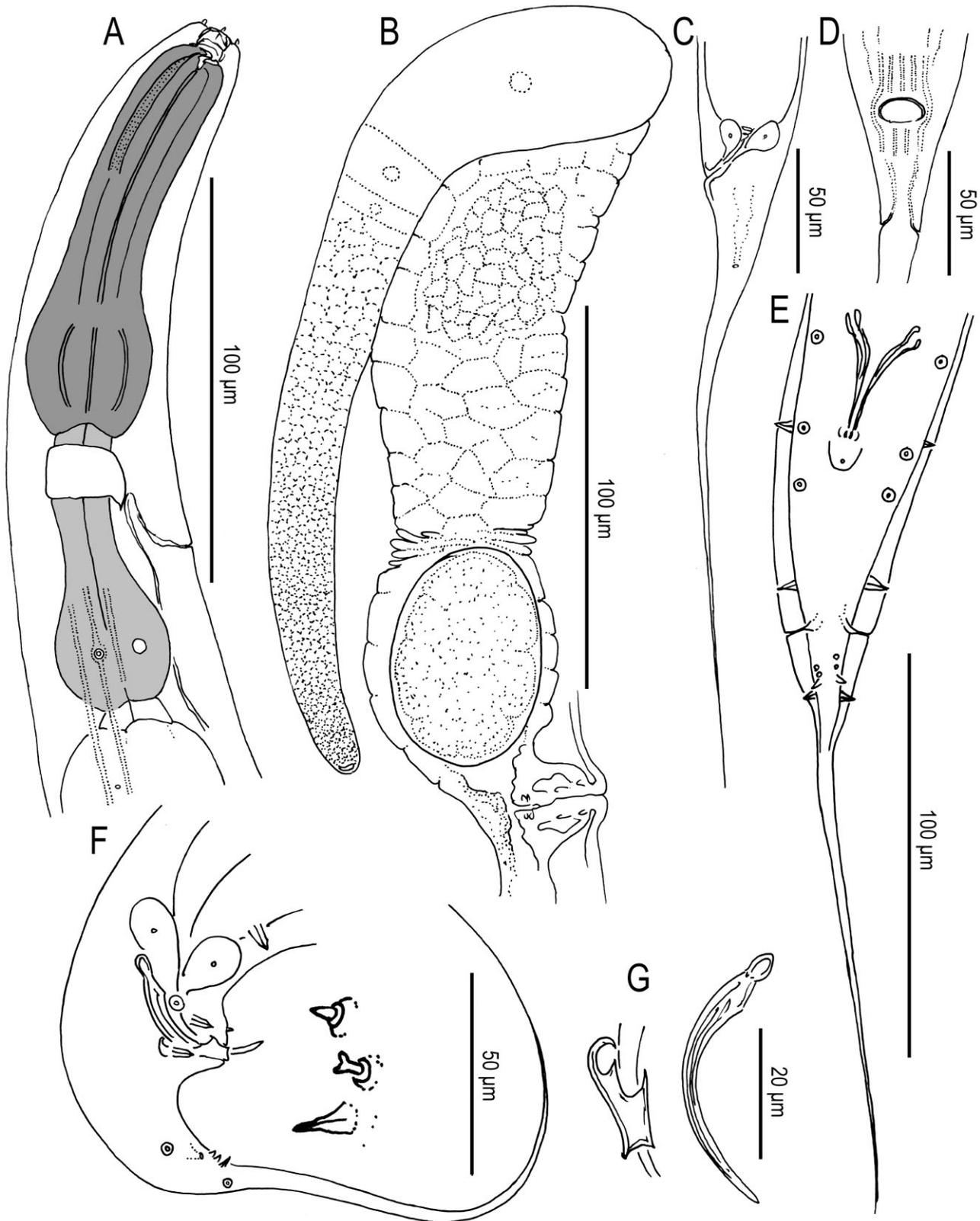


Figure 8. Adults of *Pristionchus hoplostomus* sp. nov. A–D, female; E–G, male. A, neck region of stenostomatous individual, right lateral view. B, anterior gonad branch, right lateral view. C, tail region, left lateral view. D, anus, ventral view. E, tail region, ventral view. F, tail region, right lateral view, including close-up (not to scale) of papilla P6–P8 in left lateral view. G, gubernaculum and spicule, right lateral view.

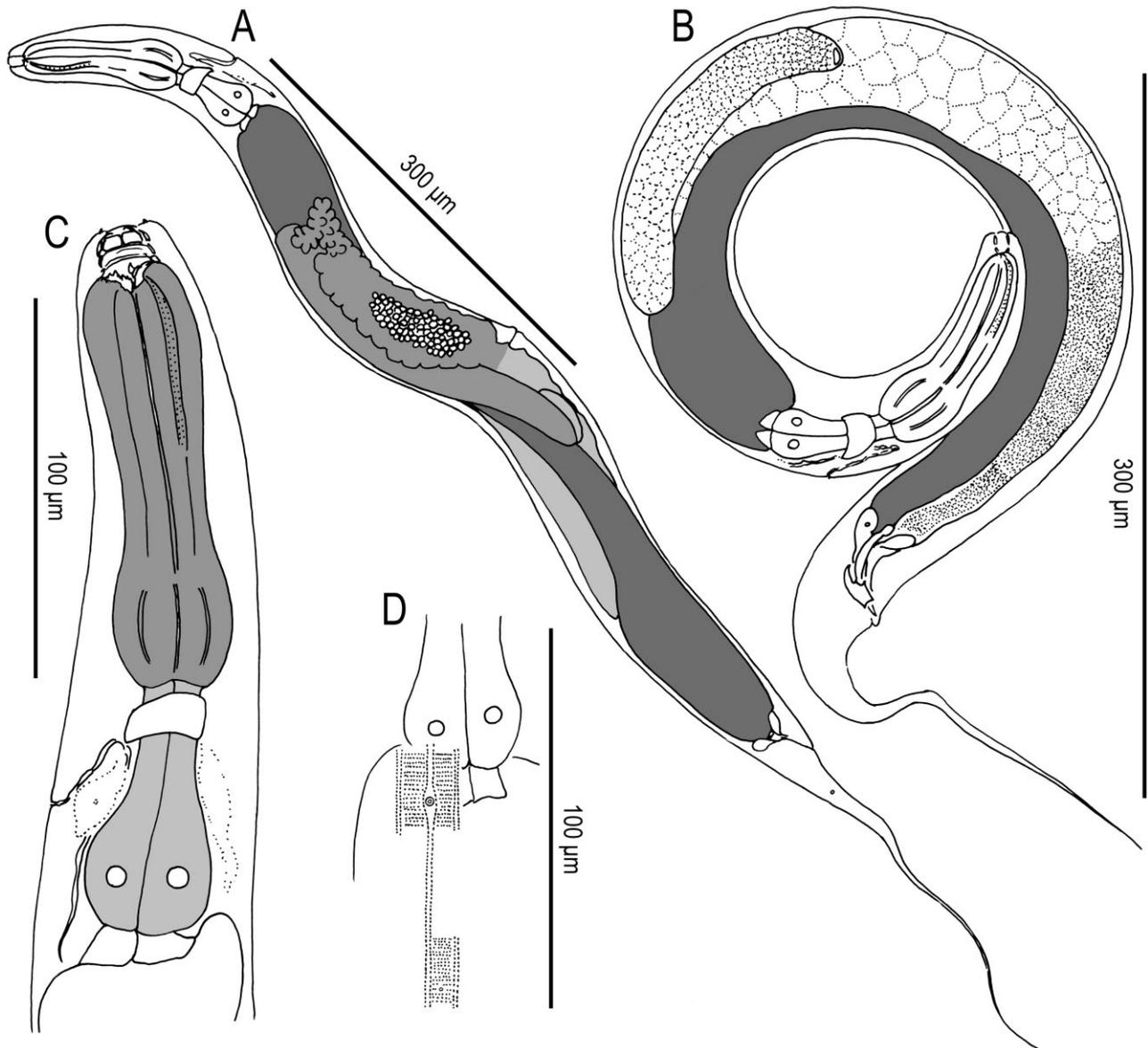
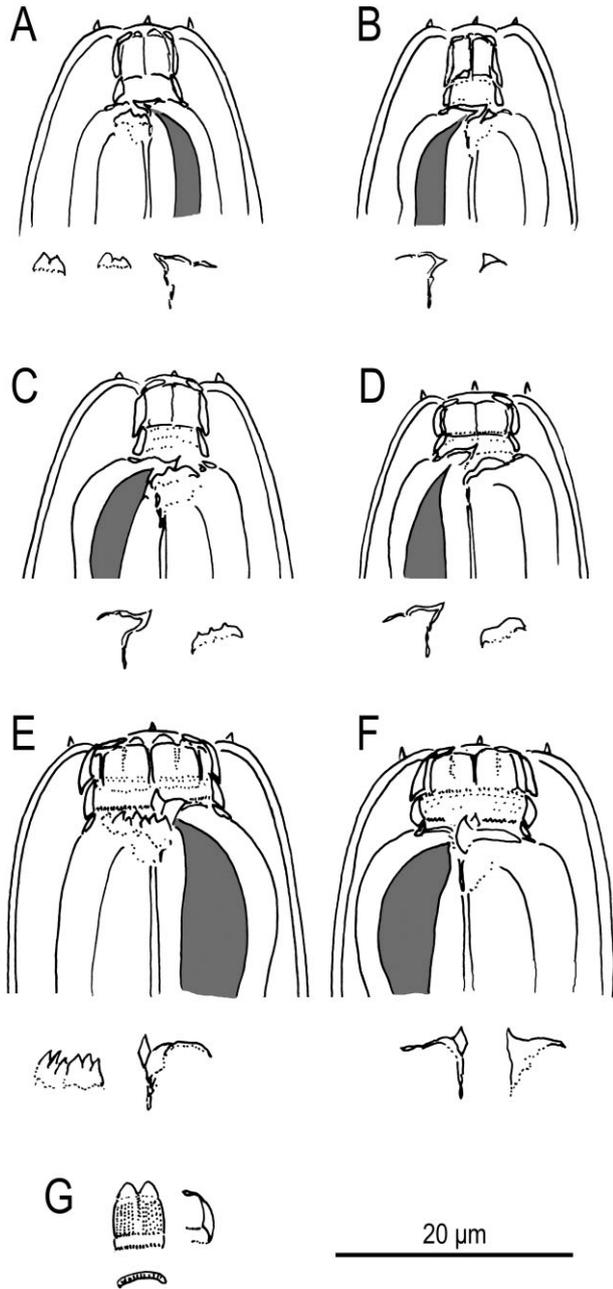


Figure 9. Adults of *Pristionchus fukushimae* sp. nov. A, whole body of stenostomatous female, right lateral view. B, whole body of stenostomatous male, right lateral view. C, neck region of eurystomatous female, left lateral view. D, deirid (above) and 'postdeirid' (small pore, bottom left), left lateral view.

anteriorly serrated versus smooth stegostomatal ring in the eurystomatous form, and by a finely serrated versus smooth anterior gymnostomatal ring of the eurystomatous form; *P. elegans* has a coarsely serrated anterior gymnostom in the stenostomatous form, although no eurystomatous form is known for this species. *Pristionchus triformis* is distinguished from all other *Pristionchus* species by having a mouth form with 12 separate cheilostomatal plates, and is thus distinguished from *P. hoplostomus* sp. nov. and *P. fukushimae* sp. nov. by the cheilostomatal plates of the megastomatous form being completely separated

into 12 plates versus fused for the posterior one-third of their length. Although 12 plates are found in the genus *Parapristionchus*, *P. triformis* sp. nov. is distinguished from *Parapristionchus* by having 12 plates in only one morph of the eurystomatous form versus constitutently having 12 plates in both the eurystomatous and stenostomatous forms; it is further distinguished from *Parapristionchus* by all other diagnostic generic characters, most notably by a flint-shaped versus claw-like dorsal tooth in the stenostomatous form. *Pristionchus triformis* sp. nov. is further distinguished from *P. hoplostomus* sp. nov. by the absence



versus presence of a distinct, additional left subventral plate with multiple denticles. The species is distinguished from *P. fukushimae* sp. nov. by a gubernaculum that broadens anteriorly at a 30° versus 15° angle, and by P4 being less than one-third versus more than one-half of a cloacal body diameter from the cloacal opening (C). *Pristionchus triformis* sp. nov. is tentatively distinguished from *P. hoplostomus* sp. nov. by: P2d clearly anterior to, versus slightly anterior to or overlapping, P3; P4 being equidistant between P3 and P5d; P9d overlapping versus being clearly posterior to P7–P8. It is also tentatively dis-

Figure 10. Stomatal region of adult females of *Pristionchus fukushimae* sp. nov. A, stenostomatous form, left lateral view, showing two variants of left subventral ridge (below, left) and one variant of dorsal tooth (below, right). B, stenostomatous form, right lateral view, showing dorsal tooth (below, left) and right subventral denticle (below, right). C, D, variants of stenostomatous form, right lateral view, showing dorsal tooth (below, left) and right subventral ridge (below, right). E, eurystomatous form, left lateral view, showing variant of left subventral denticles (below, left) and dorsal tooth (below, right). F, eurystomatous form, right lateral view, showing dorsal tooth (below, left) and right subventral tooth (below, right). G, detail of individual cheilostomatal plate dissected from body of eurystomatous form, showing inner view (top, left), view through section (top, right), and ventral view (below).

tinguished from both *P. hoplostomus* sp. nov. and *P. fukushimae* sp. nov. by P5d being equidistant between Ph and Ph versus closer to Ph than to P4. *Pristionchus triformis* sp. nov. is distinguished from all other species by its unique SSU rRNA sequence, an 830-bp fragment of which differs from the phylogenetically close species *P. hoplostomus* sp. nov. and *P. fukushimae* sp. nov. by 5 and 10 nucleotides, respectively. Finally, *P. triformis* sp. nov. is distinguished from *P. hoplostomus* sp. nov. and *P. fukushimae* sp. nov. by a hermaphroditic versus gonochoristic mode of reproduction.

Type host (carrier) and locality: The culture from which the type specimens were obtained was originally isolated from the body of an adult dung beetle, *Anoplotrupes stercorosus* (Hartmann in Scriba, 1791) (Coleoptera: Scarabaeidae), collected by M. Herrmann in the Schönbuch Forest near Tübingen, Germany, in July 2004.

Distribution: Besides its collection from the type locality and type host on several occasions (Herrmann *et al.*, 2006; Weller *et al.*, 2010), including strain RS5232, the species was isolated by M. Herrmann from soil containing parts of dead *Marronus borbonicus* Coquerel, 1866 (Coleoptera: Dynastidae) on La Réunion Island in January 2012 and by James H. Thomas from soil in Vancouver, BC, Canada. This species has been additionally identified by its SSU rRNA sequence from several localities in Great Britain (Robbie Rae, pers. comm.).

Type material and strain: Holotype megastomatous hermaphrodite and three paratype megastomatous hermaphrodites are deposited in the University of California Riverside Nematode Collection (UCRNC), CA, USA. Two paratype megastomatous hermaphrodites and one paratype stenostomatous

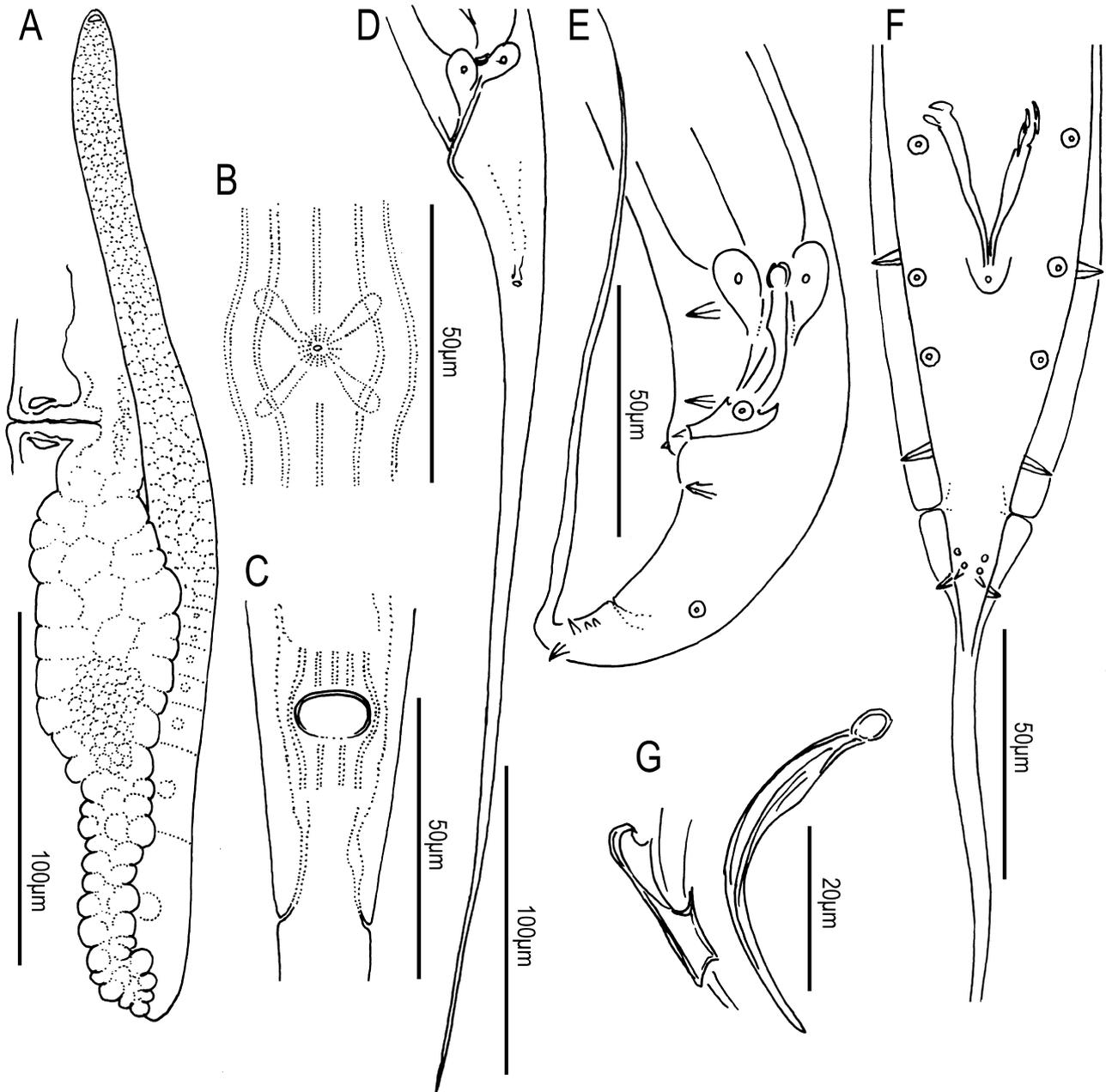


Figure 11. Adults of *Pristionchus fukushimae* sp. nov. A–D, female; E–G, male. A, anterior gonad branch, left lateral view. B, vulva, ventral view. C, anus, ventral view. D, tail region, left lateral view. E, tail region, left lateral view. F, tail region, ventral view. G, gubernaculum and spicule, right lateral view.

hermaphrodite are deposited in the Swedish Natural History Museum, Stockholm, Sweden. Two paratype megastomatous hermaphrodites and one paratype stenostomatous hermaphrodite are deposited in the Natural History Museum, Karlsruhe, Germany. The type strain is available under culture code RS5233.

Etymology: The species epithet is a Latin adjective meaning 'having three forms', and refers to the sten-

ostomatous, six-plated euryostomatous, and 'megastomatous' morphs of this species.

***PRISTIONCHUS HOPLOSTOMUS* SP. NOV.**

= *Pristionchus* sp. 17 apud Mayer et al. (2009)

Description

Measurements: See Table 2.

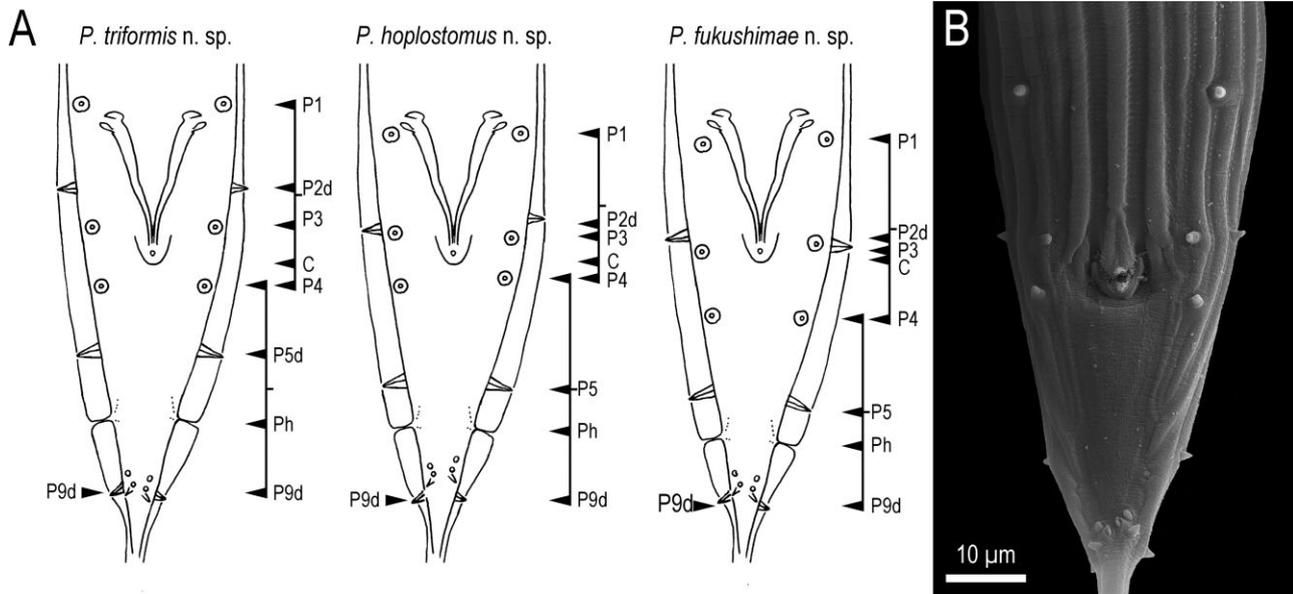


Figure 12. Comparative tail morphology of males of *Pristionchus triformis* sp. nov., *Pristionchus hoplostomus* sp. nov., and *Pristionchus fukushima* sp. nov. Views are ventral. A, line drawings of the male tail: the relative positions of the papillae, cloaca, and phasmid are indicated by the arrowheads on the vertical bars. Papilla characters in *P. triformis* sp. nov. males are considered tentative ($N=1$), and were interpreted from lateral view. Although the papillae arrangement is similar in the three species, the distinguishing characters are: P2d clearly separated from, versus overlapping, P3; P4 equidistant between P3 and P5d (*P. triformis* sp. nov. and *P. fukushima* sp. nov.) versus closer to P3 than to P5d (*P. hoplostomus* sp. nov.); P5d equidistant between P4 and Ph (*P. hoplostomus* sp. nov. and *P. fukushima* sp. nov.) versus closer to Ph than to P4 (*P. triformis* sp. nov.); position of P9d clearly posterior to (*P. hoplostomus* sp. nov.), slightly posterior to (*P. fukushima* sp. nov.), or overlapping (*P. triformis* sp. nov.) P7–P8. B, scanning electron micrograph of the male tail of *P. hoplostomus* sp. nov.

Adults: Species gonochoristic (i.e. dioecious), with males and females.

Stenostomatous form: Metastegostom bearing: crescent-shaped right subventral ridge, with two or three minute denticles, the denticle at the apex of the ridge being the most conspicuous (Fig. 7E); in the left subventral sector, three or four blunt or weakly pointed left subventral denticles, apparently projecting from a common cuticular plate (Fig. 7D).

Twelve-plated stenostomatous form: Only one individual observed, presumed rare. Cheilostom divided into 12 complete, distinct per- and interrational plates (Fig. 7E), some of which may be fused at bases (Fig. 4E). Anterior end of each plate rounded and elongated to stick out from stomatal opening and form a small flap (Figs 4E, 7F). Anterior margin of pro- and mesostegostom, smooth or irregular (Fig. 7F). Metastegostom bearing: a large, flint-shaped dorsal tooth (Fig. 7F); a crescent-shaped right subventral ridge, with three minute denticles, the denticle at the apex of the ridge being the most conspicuous;

three large, triangular left subventral denticles (Figs 4E, 7F).

Eurystomatous form: Cheilostom divided into six distinct per- and interrational plates, some plates incompletely split into two longitudinal strips, usually for about two-thirds the length of plate, division of plate often asymmetrical (Fig. 4F, I), incisions between strips sometimes difficult to observe by LM (Fig. 4I), resulting in between six and 12 strips in total (Figs 4F, G, I, 7G, H). Anterior end of each strip rounded and elongated to stick out from stomatal opening and form a small flap, i.e. constituting between six and 12 flaps in total (Figs 5A, 7G, H), although plates that are not split sometimes end in two flaps (Figs 4F, G, 5A). Metastegostom bearing: a large and strongly sclerotized claw-like dorsal tooth; a large, claw-like or pointed right subventral tooth (Fig. 7G, H); in the left subventral sector, a ridge of large denticles of varying shape, size, and number, i.e. between six and eight, arising from a split cuticular plate, in addition to an adventitious denticle and a separate plate with two or three denticles (Figs 4G, H, 7G).

Table 1. Morphometrics of megastomatous (12-plated eurytomatous) hermaphrodite holotype (in glycerin) and of hermaphrodite and male specimens of *Pristionchus triformis* sp. nov. (temporary water mounts)

Character	Megastomatous hermaphrodite		Six-plated eurytomatous hermaphrodite		Stenostomatous hermaphrodite		Stenostomatous male	
	Holotype	Temporary water mounts	Temporary water mounts	Temporary water mounts	Temporary water mounts	Temporary water mounts	Temporary water mounts	Temporary water mounts
<i>N</i>	–	15	6	10	1	1	1	1
<i>L</i>	883	1043 ± 182 (833–1349)	986 ± 47 (932–1016)	1175 ± 190 (924–1889)	903	903	903	903
<i>L'</i>	686	816 ± 173 (634–1105)	767 ± 36 (728–800)	944 ± 178 (695–1149)	753	753	753	753
<i>a</i>	18	16 ± 2.1 (13–20)	16 ± 1.9 (14–18)	14 ± 1.3 (12–17)	17	17	17	17
<i>b</i>	6.0	6.2 ± 0.9 (5.2–7.7)	6.2 ± 0.4 (5.8–6.5)	7.1 ± 0.9 (5.8–8.1)	6.0	6.0	6.0	6.0
<i>c</i>	4.5	4.6 ± 0.6 (3.7–5.5)	4.5 ± 0.2 (4.3–4.7)	5.1 ± 0.6 (4.0–5.8)	6.0	6.0	6.0	6.0
<i>c'</i>	8.6	7.7 ± 0.7 (6.7–8.9)	8.0 ± 0.4 (7.6–8.3)	7.5 ± 0.8 (6.2–8.7)	4.4	4.4	4.4	4.4
T or V	50	46 ± 1.5 (43–49)	47 ± 0.5 (47–48)	47 ± 1.4 (45–49)	58	58	58	58
Maximum body diameter	48	66 ± 18 (44–96)	64 ± 7.0 (57–71)	83 ± 16 (56–98)	52	52	52	52
Stoma width	11.4	12.7 ± 1.0 (11.1–14.8)	11.6 ± 1.0 (10.6–11.5)	6.1 ± 0.4 (5.4–6.9)	5.0	5.0	5.0	5.0
Stoma height (cheilo- + gymnostom)	9.0	8.8 ± 0.9 (7.6–10.5)	8.8 ± 1.3 (6.6–9.7)	8.6 ± 0.8 (7.2–9.9)	7.3	7.3	7.3	7.3
Stoma height (to base of stegostom)	14.0	14.8 ± 1.3 (12.9–17.2)	13.9 ± 1.6 (11.8–14.8)	12.8 ± 1.1 (11.4–14.6)	9.9	9.9	9.9	9.9
Neck length (head to base of pharynx)	143	161 ± 7.3 (149–172)	155 ± 4.6 (151–160)	161 ± 9.7 (141–176)	148	148	148	148
Anterior pharynx (pro- + metacarpus)	86	94 ± 4.7 (86–103)	91 ± 3.5 (88–95)	92 ± 4.7 (84–97)	92	92	92	92
Posterior pharynx (isthmus + basal bulb)	48	58 ± 2.8 (54–63)	56 ± 2.1 (54–58)	61 ± 5.3 (50–71)	49	49	49	49
Post./ant. pharynx ratio	56	61 ± 2.6 (56–66)	61 ± 2.9 (58–64)	66 ± 4.2 (60–74)	53	53	53	53
Excretory pore from ant. end	128	152 ± 23 (121–185)	139 ± 17 (122–155)	156 ± 17 (134–180)	164	164	164	164
Testis length	–	–	–	–	523	523	523	523
Ant. female gonad	203	194 ± 68 (123–312)	153 ± 11 (143–165)	230 ± 65 (135–292)	–	–	–	–
Post. female gonad	138	159 ± 55 (89–252)	138 ± 17 (120–153)	188 ± 62 (113–266)	–	–	–	–
Distance from vulva to anus	255	332 ± 80 (244–451)	302 ± 12 (293–315)	394 ± 87 (277–497)	–	–	–	–
Cloacal or anal body diameter	23	30 ± 3.7 (22–37)	27 ± 1.5 (26–29)	31 ± 3.6 (25–37)	34	34	34	34
Tail length	204	227 ± 17 (196–248)	219 ± 16 (204–236)	231 ± 18 (210–263)	150	150	150	150
Spicule length (curve)	–	–	–	–	36	36	36	36
Spicule length (chord)	–	–	–	–	31	31	31	31
Gubernaculum length	–	–	–	–	19	19	19	19

All measurements are in µm, and are presented as mean ± SD (range), except for the single male specimen, for which all measurements are given. *L*, total body length; *L'*, total body length to tail; *a*, body length/maximum body diameter; *b*, neck length/body length; *c*, body length/tail length; *c'*, tail length/anal body width; *T*, testis length/body length; *V*, vulva from anterior end/body length.

Table 2. Morphometrics of stenomatous male holotype (in glycerin) and of male and female specimens of *Pristionchus hoplostomus* sp. nov. (temporary water mounts)

Character	Stenostomatous male		Eurystomatous female		Stenostomatous female	
	Holotype	Temporary water mounts				
<i>N</i>	–	20	15	10	–	–
<i>L</i>	1116	953 ± 126 (786–1201)	1188 ± 204 (822–1476)	1375 ± 343 (998–1924)	–	–
<i>L'</i>	958	778 ± 116 (627–1007)	933 ± 184 (584–1202)	1096 ± 304 (759–1589)	–	–
<i>a</i>	16	17 ± 1.4 (14–19)	15 ± 1.7 (12–18)	15 ± 1.7 (11–17)	–	–
<i>b</i>	6.5	5.5 ± 0.8 (4.0–6.7)	5.6 ± 0.6 (4.2–6.6)	6.7 ± 1.3 (5.2–8.7)	–	–
<i>c</i>	7.1	5.4 ± 0.5 (4.8–6.4)	4.6 ± 0.5 (3.5–5.4)	4.9 ± 0.5 (4.2–5.8)	–	–
<i>c'</i>	4.9	5.5 ± 0.4 (4.9–6.2)	7.5 ± 0.6 (6.7–8.7)	7.3 ± 0.6 (6.3–8.0)	–	–
<i>T</i> or <i>V</i>	58	53 ± 5.5 (45–64)	46 ± 5.0 (32–57)	46 ± 0.9 (44–47)	–	–
Maximum body diameter	68	57 ± 10 (44–75)	81 ± 20 (49–111)	97 ± 35 (66–154)	–	–
Stoma width	6.0	5.5 ± 0.6 (4.7–7.0)	13.6 ± 0.8 (12.5–15.3)	7.2 ± 0.6 (6.3–8.4)	–	–
Stoma height (cheilo- + gymnostom)	8.6	9.3 ± 0.8 (8.1–10.7)	11.1 ± 1.1 (9.3–13.3)	11.0 ± 1.6 (9.5–14.2)	–	–
Stoma height (to base of stegostom)	12.7	13.4 ± 0.7 (11.9–14.3)	18.2 ± 1.5 (13.7–19.8)	16.4 ± 1.4 (14.0–18.6)	–	–
Neck length (head to base of pharynx)	167	169 ± 14 (149–207)	203 ± 17 (160–231)	298 ± 16 (178–217)	–	–
Anterior pharynx (pro- + metacarpus)	94	95 ± 9.4 (82–123)	113 ± 11 (90–135)	113 ± 7.0 (104–122)	–	–
Posterior pharynx (isthmus + basal bulb)	64	65 ± 5.7 (55–75)	79 ± 6.6 (61–87)	74 ± 8.3 (63–88)	–	–
Post./ant. pharynx ratio	68	69 ± 5.4 (59–79)	70 ± 4.2 (64–81)	65 ± 4.9 (59–77)	–	–
Excretory pore from ant. end	171	151 ± 15 (129–179)	169 ± 12 (153–191)	167 ± 10.3 (149–181)	–	–
Testis length	648	509 ± 120 (351–732)	–	–	–	–
Ant. female gonad	–	–	206 ± 78 (92–312)	264 ± 97 (147–464)	–	–
Post. female gonad	–	–	166 ± 40 (87–222)	248 ± 114 (138–415)	–	–
Vulva to anus distance	–	–	392 ± 79 (259–517)	470 ± 152 (307–729)	–	–
Cloacal or anal body diameter	32	32 ± 3.2 (27–39)	34 ± 4.7 (25–41)	38 ± 5.3 (31–46)	–	–
Tail length	158	175 ± 13 (153–201)	255 ± 26 (202–299)	279 ± 41 (237–347)	–	–
Spicule length (curve)	45	44 ± 2.6 (38–49)	–	–	–	–
Spicule length (chord)	37	36 ± 2.7 (31–41)	–	–	–	–
Gubernaculum length	16	17 ± 1.4 (14–19)	–	–	–	–

All measurements are in µm, and are presented as mean ± SD (range)

Male: Oval manubrium at anterior end of spicule (Fig. 8G). Gubernaculum conspicuous, about one-third spicule length, slightly broader anteriorly such that dorsal wall is recurved, and that dorsal and ventral walls separate at a 15° angle at posterior end (Fig. 8G). In lateral view, anterior half of gubernaculum with two serial curves separated by anteriorly directed process and with highly concave terminal curvature about one-quarter of gubernaculum length and proximal curvature about one-third of gubernaculum length; posterior half forming a tube-like process enveloping spicules (Fig. 8G). Nine pairs of genital papillae and a pair of phasmids present, and arranged as <P1 (P2d, P3), C, P4, P5d, Ph, (P6, P7, P8), P9d> [= <v1 (v2d, v3), C, v4, ad, Ph (5, v6, v7), pd> in the nomenclature of Sudhaus & Fürst von Lieven, 2003] (Figs 8E, F, 12B). Positions of P1 papillae located more than one cloacal body diameter posterior to cloacal slit, P2d and P3 closer to P4 than to P1, P2d at same level or slightly anterior to P3, P4 only slightly posterior to (i.e. within one-quarter of cloacal body diameter of) C, and closer to P2d than to P5d, P5d equidistant between P4 and P6, Ph clearly anterior to P6 and equidistant between P5d and P6, P6–P8 linearly arranged, and P9d clearly posterior to P7–P8 (Figs 8E, F, 12B).

Differential diagnosis: *Pristionchus hoplostomus* sp. nov. is diagnosed from all other species of *Pristionchus* and Diplogastridae, except *P. fukushimae* sp. nov., by a eury stomatous form with cheilostomatal plates variably and incompletely split versus being completely divided into six or 12 plates. It is further distinguished from all other species of *Pristionchus*, except *P. triformis* sp. nov. and *P. fukushimae* sp. nov., by an anteriorly serrated versus smooth stegostomatal ring in the eury stomatous form and by a finely serrated versus smooth anterior gymnostomatal ring of the eury stomatous form. It is distinguished from all other *Pristionchus* species, except *P. triformis* sp. nov. and *P. fukushimae* sp. nov., by a serrated versus smooth anterior gymnostomatal ring in the eury stomatous form. The species is distinguished from *P. triformis* sp. nov. and *P. fukushimae* sp. nov. by the presence versus absence of a distinct, additional left subventral plate with multiple denticles. *Pristionchus hoplostomus* sp. nov. is separated from *P. fukushimae* sp. nov. by reproductive isolation, namely by the inability to produce viable hybrid F₁ offspring. It is further distinguished from *P. fukushimae* sp. nov. by: male papilla P4 being closer to P3 than to P5d versus equidistant between P3 and P5d; the anterior curvature of gubernaculum being shallow versus deeply concave; the proximal curvature of gubernaculum being one-third versus one-half of gubernaculum length. It is tentatively also distinguished from *P. triformis* sp. nov. by: P2d slightly anterior to or overlap-

ping versus clearly anterior to P3; P4 being closer to P3 than to P5d versus equidistant between P3 and P5d; P5d being closer to Ph than to P4 versus equidistant between P4 and Ph; P9d being clearly posterior to, versus overlapping, P7–P8. *Pristionchus hoplostomus* sp. nov. is distinguished from all other species by its unique SSU rRNA sequence, an 830-bp fragment of which differs from each of the phylogenetically close species *P. triformis* sp. nov. and *P. fukushimae* sp. nov. by five nucleotides. Finally, *P. hoplostomus* sp. nov. is distinguished from *P. triformis* sp. nov. by a gonochoristic versus hermaphroditic mode of reproduction.

Type locality: The culture from which the type specimens were obtained was originally isolated by Marie-Anne Félix from soil from a botanical garden in Tokyo, Japan.

Type material and strain: Holotype stenostomatous male, two paratype stenostomatous males, and three paratype six-plated eury stomatous females are deposited in the UCRNC, CA, USA. Two paratypes each of stenostomatous males and eury stomatous females are deposited in the Swedish Natural History Museum, Stockholm, Sweden. Two paratypes each of stenostomatous males and eury stomatous females are deposited in the Natural History Museum, Karlsruhe, Germany. The type strain is available under culture code JU1090.

Etymology: The species epithet is derived from the Greek *όπλον* ('armor, tool') + *στόμα* ('mouth'), and refers to the stacked rows of left subventral denticles in the eury stomatous form.

***PRISTIONCHUS FUKUSHIMAE* SP. NOV.**

Description

Measurements: See Table 3.

Adults: Species gonochoristic (i.e. dioecious), with males and females.

Stenostomatous form: Metastegostom bearing: right subventral ridge of variable shape and armature, having a conspicuous triangular denticle, being rounded, or with two or three rounded to pointed denticles (Fig. 10B–D); two or three blunt or weakly pointed left subventral denticles apparently projecting from a common cuticular plate (Fig. 10A).

Eury stomatous form: Cheilostom divided into six distinct per- and interrational plates, some plates incompletely split into two longitudinal strips, for half to two-thirds length of plate, division of plate often asymmetrical, incisions between strips sometimes

Table 3. Morphometrics of stenomatous male holotype (in glycerin) and of male and female specimens of *Pristionchus fukushimae* sp. nov. (temporary water mounts)

Character	Stenomatous male			Eurystomatous female			Stenostomatous female		
	Holotype	Temporary water mounts		Holotype	Temporary water mounts		Holotype	Temporary water mounts	
		N	Mean ± SD		N	Mean ± SD		N	Mean ± SD
N	-	20	-	10	-	10	-	-	-
L	978	996 ± 205 (731-1473)	-	1173 ± 214 (931-1536)	-	1236 ± 241 (974-1651)	-	-	-
L'	762	770 ± 170 (535-1172)	-	879 ± 178 (676-1143)	-	929 ± 205 (734-1270)	-	-	-
a	18	16 ± 1.5 (13-19)	-	16 ± 1.1 (14-18)	-	16 ± 1.3 (13-17)	-	-	-
b	6.3	6.1 ± 0.6 (5.1-7.4)	-	6.2 ± 0.9 (5.0-7.6)	-	6.9 ± 0.9 (5.5-8.1)	-	-	-
c	4.5	4.4 ± 0.5 (3.7-5.3)	-	4.0 ± 0.4 (3.5-4.6)	-	4.0 ± 0.4 (3.5-4.6)	-	-	-
c'	7.2	6.9 ± 1.0 (5.3-9.3)	-	9.4 ± 1.4 (7.4-11.2)	-	8.9 ± 1.2 (7.6-11.1)	-	-	-
T or V	46	51 ± 5.1 (42-60)	-	45 ± 2.1 (43-49)	-	44 ± 1.4 (42-46)	-	-	-
Maximum body diameter	56	62 ± 14 (44-92)	-	74 ± 17 (57-98)	-	80 ± 20 (60-126)	-	-	-
Stoma width	5.1	73 ± 1.2 (5.0-10.1)	-	13.9 ± 1.6 (11.5-16.6)	-	7.8 ± 1.2 (5.5-9.2)	-	-	-
Stoma height (cheilo- + gymnostom)	8.2	9.8 ± 1.3 (7.6-12.4)	-	12.2 ± 1.1 (10.3-14.0)	-	10.2 ± 0.9 (8.6-11.9)	-	-	-
Stoma height (to base of stegostom)	13.2	13.5 ± 1.6 (9.9-15.8)	-	18.2 ± 1.1 (16.5-20.1)	-	14.8 ± 1.6 (12.2-16.8)	-	-	-
Neck length (head to base of pharynx)	150	159 ± 19 (132-212)	-	184 ± 8.5 (169-195)	-	174 ± 19 (149-206)	-	-	-
Anterior pharynx (pro- + metacarpus)	93	94 ± 12 (79-127)	-	107 ± 8.2 (98-120)	-	104 ± 12 (85-122)	-	-	-
Posterior pharynx (isthmus + basal bulb)	49	54 ± 6.7 (44-74)	-	64 ± 5.3 (56-70)	-	59 ± 7.4 (48-74)	-	-	-
Post./ant. pharynx ratio	53	58 ± 2.6 (53-64)	-	61 ± 7.7 (50-70)	-	57 ± 5.2 (49-65)	-	-	-
Excretory pore from ant. end	99	146 ± 22 (114-191)	-	163 ± 17 (144-195)	-	160 ± 25 (132-200)	-	-	-
Testis length	447	511 ± 144 (305-828)	-	-	-	-	-	-	-
Ant. female gonad	-	-	-	225 ± 73 (151-342)	-	238 ± 72 (160-355)	-	-	-
Post. female gonad	-	-	-	210 ± 72 (130-325)	-	207 ± 70 (120-323)	-	-	-
Vulva to anus distance	-	-	-	363 ± 92 (242-496)	-	387 ± 100 (273-570)	-	-	-
Cloacal or anal body diameter	30	33 ± 4.3 (25-40)	-	31 ± 4.4 (25-39)	-	35 ± 6.2 (29-50)	-	-	-
Tail length	216	226 ± 43 (173-301)	-	294 ± 45 (239-393)	-	307 ± 41 (239-381)	-	-	-
Spicule length (curve)	50	47 ± 3.7 (43-54)	-	-	-	-	-	-	-
Spicule length (chord)	41	38 ± 3.6 (32-45)	-	-	-	-	-	-	-
Gubernaculum length	18	19 ± 1.4 (17-22)	-	-	-	-	-	-	-

All measurements are in µm, and are presented as mean ± SD (range).

difficult to observe by LM (Fig. 4 L), resulting in between six and 12 strips in total (Figs 4J, L, 10E, F). Anterior end of each strip rounded and elongated to stick out from stomatal opening and form a small flap (Fig. 10E, G), i.e. constituting between six and 12 flaps in total, although plates that are not split sometimes end in two flaps. Metastegostom bearing: a large and strongly sclerotized claw-like dorsal tooth (Figs 4K, 10E, F); a large, claw-like or pointed right subventral tooth (Fig. 10F); in the left subventral sector, a ridge of large denticles of varying shape, size, and number, i.e. between six and eight, arising from a split cuticular plate, sometimes with one or two adventitious denticles (Fig. 10E).

Male: Oval manubrium at anterior end of spicule (Fig. 11G). Gubernaculum conspicuous, about 40% spicule length, narrow anteriorly such that dorsal wall is recurved, and that dorsal and ventral walls separate at a $< 15^\circ$ angle at posterior end (Fig. 11G). In lateral view, anterior half of gubernaculum with two serial curves separated by anteriorly directed process, and with a short, shallow, concave terminal curvature less than one fourth of gubernaculum length, and with a proximal curvature about half of gubernaculum length; posterior half forming a tube-like process enveloping spicules (Fig. 11G). Nine pairs of genital papillae and a pair of phasmids present and arranged as <P1 (P2d, P3, C), P4, P5d, Ph, (P6, P7, P8), P9d> [= <v1 (v2d, v3, C), v4, ad, Ph (5, v6, v7), pd> in the nomenclature of Sudhaus & Fürst von Lieven, 2003] (Fig. 11E, F). Positions of P1 papillae located one cloacal body diameter posterior to cloacal slit, P2d at same level or slightly anterior to P3, P2d and P3 closer to P4 than to P1, or P2 equidistant between P1 and P4, P4 one-half of cloacal body diameter posterior to C, and equidistant between P2d and P5d, P5d equidistant between P4 and P6, Ph clearly anterior to P6 and equidistant between P5d and P6, P6–P8 linearly arranged, and P9d slightly posterior to or overlapping P7–P8 (Fig. 11E, F).

Differential diagnosis: *Pristionchus fukushimae* sp. nov. is diagnosed from all other species of *Pristionchus* and Diplogastridae, except *P. hoplostomus* sp. nov., a eury stomatous form with cheilostomatal plates variably and incompletely split versus being completely divided into six or 12 plates. It is further distinguished from all other species of *Pristionchus* except *P. triformis* sp. nov. and *P. hoplostomus* sp. nov. by an anteriorly serrated versus smooth stegostomatal ring in the eury stomatous form, and by a finely serrated versus smooth anterior gymnostomatal ring of the eury stomatous form. It is distinguished from all other *Pristionchus* species except *P. triformis* sp. nov. and *P. hoplostomus* sp. nov. by a serrated versus

smooth anterior gymnostomatal ring in the eury stomatous form. It is further distinguished from *P. hoplostomus* sp. nov. by the absence versus presence of a distinct, additional left subventral plate with multiple denticles. *Pristionchus fukushimae* sp. nov. is separated from *P. hoplostomus* sp. nov. by reproductive isolation, namely the inability to produce viable hybrid F₁ offspring. It is further distinguished from *P. hoplostomus* sp. nov. by: male papilla P4 being closer to P2d than to P5d versus equidistant between P2d and P5d; the anterior curvature of gubernaculum being deeply concave versus shallow; the proximal curvature of gubernaculum being one-half versus one-third of gubernaculum length. It is tentatively also distinguished from *P. triformis* sp. nov. by P2d slightly anterior to or overlapping versus clearly overlapping P3, and by P5d being closer to Ph than to P4 versus equidistant between P4 and Ph. *Pristionchus fukushimae* sp. nov. is distinguished from all other species by its unique SSU rRNA sequence, an 830-bp fragment of which differs from the phylogenetically close species *P. triformis* sp. nov. and *P. hoplostomus* sp. nov. by ten and five nucleotides, respectively. Finally, *P. fukushimae* sp. nov. is distinguished from *P. triformis* sp. nov. by a gonochoristic versus hermaphroditic mode of reproduction.

Type host (carrier) and locality: The culture from which the type specimens were obtained was originally isolated from the body of an adult *Lucanus maculifemoratus* collected by N. Kanzaki and M. Herrmann in a forest in Tadami, Fukushima Prefecture, Japan, in June 2012.

Type material and strain: Holotype stenostomatous male, two paratype stenostomatous males, and three paratype six-plated eury stomatous females are deposited in the UCRNC, CA, USA. Two paratypes each of stenostomatous males and eury stomatous females are deposited in the Swedish Natural History Museum, Stockholm, Sweden. Two paratypes each of stenostomatous males and eury stomatous females are deposited in the Natural History Museum, Karlsruhe, Germany. The type strain is available under culture code RS5595.

Etymology: The species epithet is the Latin genitive of the feminized place-name 'Fukushima', and refers to the type locality of the species.

FREQUENCIES OF STOMATAL FORMS IN THE TRIFORMIS GROUP

A survey of stomatal phenotypes in the three new species by DIC microscopy revealed several patterns (Table 4). First, hermaphrodites of *P. triformis* sp.

Table 4. Frequencies of stomatal forms in *Pristionchus fukushimae* sp. nov., *Pristionchus hoplostomus* sp. nov., and *Pristionchus triformis* sp. nov. (RS5233) in well-fed culture

		Eu, by number of lateral cheilostomatal plates					St	% Eu
		4	5	6	7	8		
Females/hermaphrodites	<i>P. fukushimae</i> sp. nov.	9	6	12	18	51	204	32
	<i>P. hoplostomus</i> sp. nov.	0	2	5	7	10	276	8
	<i>P. triformis</i> sp. nov.	10	1	0	1	240	48	84
Males	<i>P. fukushimae</i> sp. nov.	0	0	0	0	0	300	0
	<i>P. hoplostomus</i> sp. nov.	0	0	0	0	0	300	0

Each of 300 individuals in each species was scored for: (1) whether it was stenostomatous (St) or eurystomatous (Eu); and, in the case of eurystomatous individuals, (2) the number of cheilostomatal plates could be counted in a series of focal planes through a lateral view of the individual. For examples of plates observable in the left- and right-lateral focal planes, see Figure 4A, D, F, G, I, J, L. Each number given as a column heading is the number of plates observable in the two focal planes, i.e. the sum of the countable plates with complete divisions (for *P. triformis* sp. nov.) or the sum of countable, undivided plates + countable, partially divided plates (for *P. fukushimae* sp. nov. and *P. hoplostomus* sp. nov.). This sum is referred to as the 'number of lateral plates' and ranges from four to eight. The number of plates on the dorsal and ventral margins of the cheilostom circumference was not discernible in lateral view, but each margin should include either one plate or two partially or completely divided plates. Only individuals with eight lateral, completely divided plates (between ten and 12 possible total plates) in *P. triformis* sp. nov. could be megastomatous. Eurystomatous frequency (% Eu) includes all variants of the eurystomatous form, including the megastomatous form of *P. triformis* sp. nov.

nov. (RS5233) were biased towards the eury- or megastomatous form (84% eury- or megastomatous), whereas females of *P. fukushimae* sp. nov. and *P. hoplostomus* sp. nov. were biased towards the stenostomatous form (32 and 8% eurystomatous, respectively). Second, within the eurystomatous spectrum, a form with eight observable plates (and between ten and 12 total possible plates) was the most common variant in all three species. Eury- or megastomatous hermaphrodities of *P. triformis* sp. nov. in particular were overwhelmingly represented by the morph with eight countable plates (240 out of 252), which was the only morph that could include the megastomatous form (i.e. 12 completely divided plates). Third, intermediates between the eurystomatous form with four observable plates and that with eight observable plates were relatively rare (two out of 252) in *P. triformis* sp. nov., whereas they were relatively diverse and common in both *P. fukushimae* sp. nov. and *P. hoplostomus* sp. nov. Nonetheless, the frequent occurrence of individuals with eight countable plates in all three species suggests that the 12-plated form is likely to be common in the three species. Finally, males of both gonochoristic species were completely stenostomatous.

ANCESTRAL STATE RECONSTRUCTION OF STOMATAL CHARACTERS

To reconstruct the history of novel character states in the three new species, we characterized a moderately

supported outgroup to these species (Fig. 1), *P. entomophagus*, which represents the *lheritieri* group of *Pristionchus* species. As typical of *Pristionchus* species in the *maupasi* and *pacificus* groups, and consistent with observations of *P. lheritieri* (Fürst von Lieven & Sudhaus, 2000), *P. entomophagus* was confirmed to have exactly six cheilostomatal plates and flaps (Fig. 13). In contrast to the three new species, the anterior gymnostom and anterior stegostom in *P. entomophagus* were smooth, not serrated. Lastly, the left subventral ridge was sometimes host to an adventitious denticle, although this denticle was close to the anterior level of other left subventral denticles (Fig. 13A).

Given the position of *P. entomophagus* as an outgroup to the three new species, the splitting of cheilostomatal plates evolved independently within the genus *Pristionchus* (Fig. 14), being convergent with the 12-plated state of both mouth forms in *Parapristionchus*. Incomplete division of the six basic plates, which characterizes *P. fukushimae* sp. nov. and *P. hoplostomus* sp. nov., is supported as an intermediate state with respect to their complete division in *P. triformis* sp. nov. (Fig. 14). The variable presence of secondary (i.e. incomplete) plate divisions in the former two species is also supported as an intermediate state to the consistent division of plates in *P. triformis*, in which the 12-plated form is more than ten times as common as all other eurystomatous morphs (Table 4).

The fine serration of the gymnostom and conspicuous serration of the stegostom of the eurystomatous

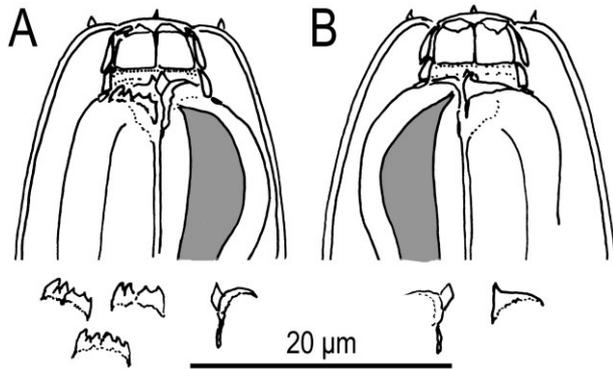


Figure 13. Eurystomatous stoma of *Pristionchus entomophagus* (Steiner, 1929) Sudhaus & Fürst von Lieven, 2003. A, left lateral view, showing three variations of left subventral denticles (below, left) and dorsal tooth (below, right). B, right lateral view, showing dorsal tooth (below, left) and right subventral tooth (below, right). As typical of other previously described *Pristionchus* species with a known eurystomatous form, the anterior margins of the gymnostom and stegostom are smooth, not serrated.

(and megastomatous) form are putative synapomorphies for the *triformis* group of species. The coarse serration observed in the gymnostom of *P. elegans*, a more distant outgroup, is therefore predicted to be non-homologous with the serration observed in the new species. The presence of up to multiple adventitious denticles in the eurystomatous form is a putative synapomorphy for the clade, whereby the inconsistent adventitious denticle in *P. entomophagus* is a predicted intermediate state with respect to other outgroups, including *P. fissidentatus* and most species characterized in the *elegans*, *pacificus*, and *maupasi* groups (but not *Pristionchus japonicus* Kanzaki, Ragsdale, Herrmann, Mayer & Sommer, 2012a). Still, because this character is plastic in all four of the above species, a greater degree of overlap in states across taxa may still be discovered.

DISCUSSION

NOVEL FEEDING STRUCTURES IN *PRISTIONCHUS*

This description of three new *Pristionchus* species has characterized feeding morphologies that are new for the genus, primarily the serrated stegostomatal ring and the division of cheilostomatal plates. Further analysis of cheilostomatal morphology in the three new species revealed a polymorphism defined by the presence of multiple types of intermediates. The cheilostom was characterized by two developmental traits: (1) the form of the plates; and (2) the number of plates. Forms of plates comprised: (1) an undivided wide plate with one flap, a state possible in all known *Pristionchus* species; (2) a partially divided wide

plate, usually with two flaps, known only in *P. fukushima* sp. nov. and *P. hoplostomus* sp. nov.; and (3) a narrow one-flap plate formed by a complete division, known only in *P. triformis* sp. nov. The second trait, plate number, comprised: (1) six wide, undivided plates, observed in all *Pristionchus* species; (2) 12 narrow plates formed by the complete divisions of wide plates, constituting the megastomatous form, and known only in *P. triformis* sp. nov.; or (3) many intermediate forms made up of combinations of undivided and partially divided plates (*P. fukushima* sp. nov. and *P. hoplostomus* sp. nov.) or of undivided and completely divided plates (*P. triformis* sp. nov.). Articulation of these two traits is necessary for predicting selection differentials or different developmental constraints. For example, achievable plate forms might depend on the ability to construct a suitable cytoskeletal scaffold to support divided plates, whereas the number of plates might depend additionally on the period of time programmed for the final moult or on the rate at which the epidermis can supply the required structural macromolecules. Although the mechanisms underlying cheilostomatal development necessarily remain speculative, the independence of the two traits demonstrates the potential for mouth development to respond to selection in multiple ways.

UNIQUE PLASTICITY IN A CLADE OF NEW *PRISTIONCHUS* SPECIES

The existence of the cheilostom polymorphism in the three new species shows how two types of plasticity interact in a single organ to generate novel morphology. To our knowledge, the presence of a third form has never been reported for a nematode exhibiting mouth plasticity. As combinations of individually plastic traits can confer adaptive differences (Griffith & Sultan, 2006), the composite spectrum of feeding forms in the new species could in principle respond to selection pressures differently than the canonical dimorphism of other diplogastrid species. Although the 12-plated and intermediate forms were normally only observed in eurystomatous mouth types, a single example of a 12-plated form was discovered in a stenostomatous individual in *P. hoplostomus* sp. nov. The occurrence of this form suggests that some developmental module of the 12-plated form operates independently of what regulates the canonical mouth dimorphism. In this case, the ability to produce six or 12 plates (or intermediate numbers of plates) could arise or become uninhibited in either the stenostomatous or eurystomatous developmental programme and thus also be selected upon within a single form. The presence of exaggerated armature, notably the larger left subventral denticles, in the observed

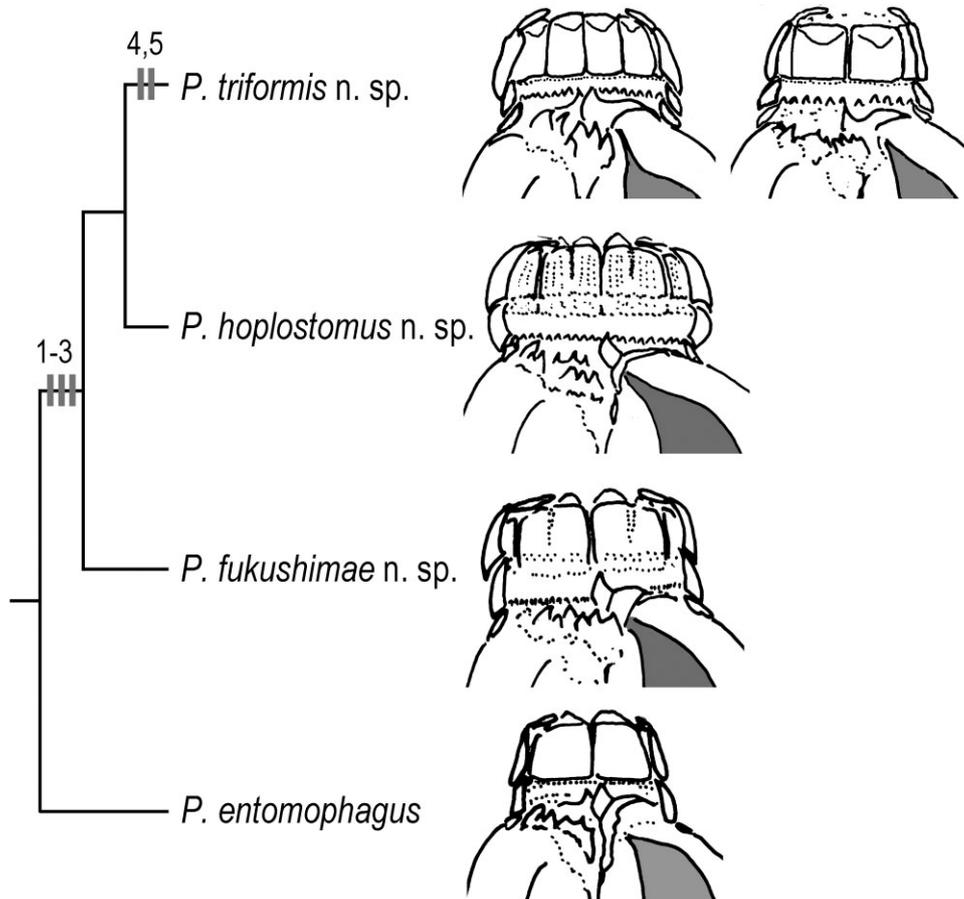


Figure 14. Comparative stomatal morphology of *Pristionchus fukushimae* sp. nov., *Pristionchus hoplostomus* sp. nov., and *Pristionchus triformis* sp. nov., with respect to putative outgroup *Pristionchus entomophagus*. The dendrogram is summarized from Figure 1. Putative synapomorphies for the three new species are: (1) the capability for partial splitting of cheilostomatal plates; (2) the conspicuously serrated ring of the pro- and mesostegostomum; (3) the finely serrated ring of the gymnostomum; (4) the complete division of the cheilostom into 12 plates is derived in *P. triformis* sp. nov., whereas the incomplete division of plates in *P. fukushimae* sp. nov. and *P. hoplostomus* sp. nov. are intermediate states; (5) the distinct bias toward a megastomatous form is putatively autapomorphic in *P. triformis* sp. nov. (Table 4).

12-plated stenostomatous individual further suggests an independent developmental module for the 12-plated condition, although the observation of only one individual prevents meaningful comparison with this form. Finally, independent selection on mouth-polymorphism traits may be possible between sexes. Consistent with findings in *P. pacificus* (Seroby et al., 2013), males were highly stenostomatous in the three new species, in contrast to the relative abundance of the eurystomatous form in females and hermaphrodites.

With a growing understanding of genetic mechanisms of the canonical mouth dimorphism in *Pristionchus*, developmental regulators of the megastomatous form of the new species can be studied empirically. For example, one could examine the extent to which

the megastomatous form differentially responds to cues that have been shown in *P. pacificus* to induce the eurystomatous form, such as starvation and pheromones (Bento et al., 2010; Bose et al., 2012; Seroby et al., 2013). Because the polymorphism was observed in a strain of *P. triformis* sp. nov. (RS5233) that has undergone extensive inbreeding and is putatively isogenic, it is likely that the plasticity in this species is not a result of genetic heterozygosity but is instead a polyphenism. In *P. fukushimae* sp. nov. and *P. hoplostomus* sp. nov., however, it is possible that genetic heterozygosity has persisted in culture populations and might account for the higher frequency of intermediate forms in those species. Artificial selection experiments in both hermaphroditic and gonochoristic species with the

plate polymorphism will thus be necessary to test whether either the six-plated or 12-plated forms can be fixed.

For now, the potential fitness advantages of the megastomatous form can only be hypothesized, but the typical six-plated eury stomatous form, although slower in its postembryonic development than the stenostomatous form, could confer the benefit of access to a broader diet (Seroby *et al.*, 2013). Namely, the eury stomatous form is associated with omnivory and predatory feeding (Kiontke & Fitch, 2010). It is possible that selection for these feeding modes would favour the evolution of the 12-plated form because of its greater flexibility, as would be offered by the doubled number of vertices in the cheilostom. Empirical investigation of the functional and fitness advantages of the dimorphism in *P. pacificus* as a model will provide the foundation for comparative studies, including the new polymorphic species.

INTERMEDIATE TAXA REVEAL ANCESTRAL STATES OF THE MEGASTOMATOUS FORM

Recognizing the convergence and evolution of novel character states can only be achieved through outgroup analysis (Watrous & Wheeler, 1981), as made possible by a phylogeny based on an independent character set (Patterson, 1982). Therefore, to test whether the complete 12-plated form evolved through a transformation series, we invoked a phylogeny inferred from 27 genetic loci that includes species with a 12-plated eury stomatous form (*P. triformis* sp. nov.), exclusively six-plated species (previously described *Pristionchus* species), and species with a putatively intermediate state, i.e. plates divided partially and for variable lengths (*P. fukushimae* sp. nov. and *P. hoplostomus* sp. nov.). Additionally, we included the closest known outgroup to *Pristionchus*, *Parapristionchus giblindavisi*, which is characterized by a 12-plated cheilostom in both mouth forms (Kanzaki *et al.*, 2012b). In the phylogeny, the *triformis* sp. nov. was nested within a clade of *Pristionchus* species with six undivided plates. Therefore, the presence of 12 plates in the *triformis* group is supported as being convergent with the complete 12-plated eury stomatous form of *Parapristionchus*. Furthermore, molecular phylogeny indicated that *P. hoplostomus* sp. nov. and *P. fukushimae* sp. nov. occupy branches between six-plated outgroups and *P. triformis* sp. nov. The placement of *P. fukushimae* sp. nov. as an outgroup to *P. hoplostomus* sp. nov. + *P. triformis* sp. nov., therefore, supports a transformation series from an ancestral six-plated eury stomatous form through the intermediate, split-plate eury stomatous form of the former two species, to a

eury stomatous form with completely divided plates, including the megastomatous form, in *P. triformis* sp. nov. (Fig. 14). Phylogenetic relationships also support the evolution of a bias towards the megastomatous form in *P. triformis* sp. nov. with respect to the other two new species. The lack of a 12-plated form in the closest predicted outgroups to *Pristionchus* + *Parapristionchus*, namely *Acrostichus* Rahm, 1928 and *Micoletzkyia* Weingärtner, 1955 (Mayer *et al.*, 2009; Kanzaki *et al.*, 2012b), suggests that the condition in *Parapristionchus* is also derived with respect to other Diplogastridae. Moreover, the presence of the 12-plated form in some (but not all) species of *Koerneria* Meyl, 1960, an outgroup to all of the above genera (Mayer *et al.*, 2009), suggests that this trait has at least three independent origins.

A NOVEL PLASTICITY IS CORRELATED WITH THE DIVERGENCE OF FORMS

Developmental plasticity is often correlated with diversity of form, and so has been proposed as a causal mechanism for morphological divergence (West-Eberhard, 2003; Pfennig *et al.*, 2010). In particular, the co-occurrence of resource polyphenisms, including dimorphisms, and species radiations in some well-sampled animal groups supports this link (Pfennig & McGee, 2010). Some recent studies have indicated that hormone signalling is crucial for the regulation of developmental plasticity in both insects and nematodes (for review see Simpson, Sword & Lo, 2011; Sommer & Ogawa, 2011), but how such dimorphisms evolve in the first place, and how they diversify further to result in polyphenisms, was previously little explored. In the present study, the description of three new species of *Pristionchus* with two eury stomatous forms revealed a correlation of novel plasticity and morphological divergence. The most obvious divergence observed was in the megastomatous form of *P. triformis* sp. nov., which showed the complete separation of cheilostomatal plates. In *P. triformis* sp. nov., the infrequency of forms with less than the maximum number of observable plates is consistent with the hypothesis that a new morph can be canalized from the extreme of what is originally a phenotypic spectrum (Nijhout, 2003; Suzuki & Nijhout, 2006). Furthermore, the higher frequency of the presumed megastomatous form (included in forms with eight lateral plates) over other morphs in all three new species suggests that plasticity has enabled the accommodation of a bias towards the novel, derived state, as predicted by theory (West-Eberhard, 2003; Moczek, 2007). A more thorough characterization of intermediates in *P. triformis* sp. nov. will be a first test of the degree to which the polar ends of the polymorphism are developmentally independent.

Putative functional or adaptive advantages that could facilitate the megastomatous form are also to be explored in future studies. Taken together, detailed molecular phylogenetic studies of *Pristionchus* and Diplogastridae, the genetic and molecular studies of the regulation and evolution of the mouth dimorphism in *P. pacificus*, and the diversification of plasticity in these new *Pristionchus* species make the mouth dimorphism of *Pristionchus* a powerful model for the evolution of developmental plasticity.

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APPENDIX

Supplementary primers (see Mayer *et al.*, 2007) for the amplification of ribosomal protein genes from *Parapristionchus giblindavisi*.

Designation	Gene	Strand	Sequence (5' → 3')
WM8116a	<i>rpl-1</i>	Antisense	YTGGAACTTGATGGTGGCACKGA
WM8220a	<i>rpl-26</i>	Sense	TGGACAATGACCGTAAAAAGA
WM12262a	<i>rps-1</i>	Antisense	ACGACATGCCGTGGAAGTTCGT
WM8267a	<i>rps-24</i>	Sense	AGAAGCTCTCCAGATGTACAA