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Two New *Pristionchus* Species (Nematoda: Diplogastridae) from Taiwan are Part of a Species-cluster Representing the Closest Known Relatives of the Model Organism *P. pacificus*

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Matthias Herrmann, Christian Weiler, Christian Rödelsperger, Natsumi Kanzaki, and Ralf J. Sommer (2016) *Pristionchus pacificus* is an important model organism in evolutionary biology and has been used to establish integrative studies that link developmental biology with ecology and population genetics. This species is part of the *P. pacificus* species-complex of the genus, many members of which occur in East Asia. While *P. pacificus* is hermaphroditic, the two most closely related known species are *P. exspectatus* and *P. arcanus* from Japan, both of which are gonochorists. *P. exspectatus* is so far the closest known relative of *P. pacificus* and thus, considered to represent the sister species. Here, we describe two new species of *Pristionchus*, *P. taiwanensis* and *P. occultus* from Taiwan using morphology, morphometrics, mating experiments and genome-wide sequence analysis. Both species are gonochorists and they are morphologically indistinguishable from *P. exspectatus*, *P. arcanus* and *P. pacificus*. However, reproductive isolation, namely the inability to produce interfertile hybrids, separates all species pairs in the species-complex. Phylogeny inferred from more than 700,000 genome-wide variable sites that were genotyped in all species suggest that *P. taiwanensis* and *P. occultus* are the sister species of *P. arcanus* and *P. exspectatus*, respectively. *P. taiwanensis* and *P. occultus* together with *P. exspectatus* and *P. arcanus* form a species-cluster with *P. pacificus*. The identification of these two novel gonochoristic species is invaluable for studies of population genetics, speciation, and macroevolution in the genus. We discuss the biogeography of *Pristionchus* in East Asia and the origin of hermaphroditism in the *P. pacificus* species-complex.

Key words: *Pristionchus*, Hermaphroditism, Cryptic species, Taxonomy, Taiwan.

BACKGROUND

The nematode *Pristionchus pacificus* Sommer, Carta, Kim, and Sternberg (1996) is a well-established model system for evolutionary developmental biology (Sommer 2009; Sommer 2015). A suite of analytical tools available for *P. pacificus* enable comparative and mechanistic studies building on a completely sequenced genome (Dieterich et al. 2008), DNA-mediated transformation (Schlager et al. 2009), and forward and reverse genetics including the CRISPR/Cas9 technology (Sommer et al. 1996; Tian et al. 2008; Witte et al. 2015). The isolation of hundreds of worldwide strains of *P. pacificus* (Morgan et al. 2012) and the support of a robust phylogenetic framework (Susoy et al. 2015, 2016) offer the potential to study the evolution of morphological, behavioral and life history traits within and among *Pristionchus* (Kreis 1932) species. Linking processes of micro- and macroevolution require knowledge of closely related species, a pre-requirement that is hard to reach in many animal taxa. In the genus *Pristionchus*, species close

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enough to be considered sister species of *P. pacificus* have been isolated in the South-Western part of Japan in 2010 (Kanzaki et al. 2012a). Specifically, *P. exspectatus* and *P. arcanus* were isolated from beetles and termites, respectively, and characterized by morphological and morphometric data, DNA sequence analysis and mating experiments.

In general, the isolation of new *Pristionchus* species was fostered by the discovery of *Pristionchus* nematodes often being associated with scarab and related beetles (Herrmann et al. 2006a, 2006b). Directly targeting beetles for nematode sampling has thus led to a boom in the number of *Pristionchus* strains and species recovered (Kanzaki et al. 2012a b, 2013a b c, 2014; Ragsdale et al. 2013). Currently, 28 *Pristionchus* species have been described in this context, all of which are cultured in the laboratory and are thus, available for biological research (Fig. 1S) (Ragsdale et al. 2015). An overview about their morphology, biology, phylogeny and distribution is provided by the new online platform *Pristionchus* Scratchpads (Herrmann et al. 2015). Reproductive biology within *Pristionchus* is diverse, as hermaphroditism has arisen independently at least five times (Ragsdale et al. 2015; Weadick and Sommer 2016). In the *P. pacificus* species-complex, *P. pacificus* is the only hermaphroditic species whereas the remaining five species, including the two closest relatives, *P. exspectatus* and *P. arcanus*, are gonochorists. Mating system transitions as seen between *P. pacificus* and *P. exspectatus/P. arcanus* provide a powerful tool for comparative studies, as recently shown in the context of the evolution of life span and aging (Weadick and Sommer 2016). Therefore, the isolation and characterization of additional *Pristionchus* species is not only of taxonomic and systematic value but offers unique opportunities for research in various areas of biology.

Biogeographic studies suggested East Asia to represent the potential origin of the *P. pacificus* species-complex, but a sampling bias towards northern areas make current knowledge incomplete (Kanzaki et al. 2012a, 2013a; Ragsdale et al. 2015). Here, we describe the results of scarab beetle and *Pristionchus* sampling from Taiwan in 2015. We describe two new *Pristionchus* species, *P. taiwanensis* and *P. occultus* by morphology, morphometrics, mating experiments and genome-wide sequence analysis. Specifically, the use of around 700,000 variable sites allowed the inference of the phylogenetic relationship of these extremely closely related species suggesting *P. taiwanensis* to represent the sister species of *P. arcanus*, whereas *P. occultus* is the sister species of *P. exspectatus.* Mating experiments indicate the two new and the previously known gonochoristic species to be reproductively isolated. Together, these four gonochoristic species form a species cluster with *P. pacificus* and thus, they represent the most closely known relatives of *P. pacificus* known today.

MATERIALS AND METHODS

Beetle sampling and nematode isolation

A field trip to Taiwan was conducted from August 18th to September 1st, 2015. We sampled the areas of Kenting National Park, Li-lung Shan, Hui-sun Forest and Ren-ai Township and collected 83 specimens of scarab beetles belonging to 18 species and 16 genera. Nematodes were isolated using standard procedures as previously described (Herrmann et al. 2006a). In total, we isolated and characterized 34 strains of the Diplogastridae family with 30 of these 34 strains representing members of the genus *Pristionchus*. The majority of *Pristionchus* isolates are hermaphrodites and were shown by sequencing of the SSU and mating experiments (see below) to represent new isolates of *P. pacificus*. High frequency of hermaphrodites is typical for Island systems (Herrmann et al. 2010). The new *P. pacificus* isolates are available 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. However, these strains are not further considered in this study. Four isolates showed a gonochoristic mode of reproduction and are the subject of this analysis.

Nematode cultivation

Three strains of *Pristionchus taiwanensis* were isolated from an adult of *Onthophagus* sp. Latreille (1802) (Coleoptera: Scarabaeidae) and an *Apogonia* sp. Kirby, 1818 (Coleoptera: Melolonthidae) collected at Kenting National Park and an adult of *Prospocoilus formosanus* Hope et Westwood (1848) (Coleoptera: Lucanidae), collected at Li-lung Shan. All strains have been kept in laboratory culture on NGM agar plates seeded with *Escherichia coli* strain OP50 under the culture code and freezing voucher numbers

RS5797 (from *Onthophagus*), RS5800 (from *Apogonia*) and RS5815 (from *Prospocoilus*). We designated RS5797 to represent the reference strain for future studies.

Pristionchus occultus was isolated from a rose beetle grub (Coleoptera: Cetonidae) collected from Huisun, Taiwan. The strain has been kept in laboratory culture on NGM agar plates seeded with *E. coli* OP50 under the culture code and freezing voucher number RS5811.

Morphological observation and preparation of type material

One- to two-week-old cultures of all species were prepared for morphological observation. Light-microscopic observations for drawings and morphometrics were conducted using live nematode material, which was hand-picked from culture plates (Kanzaki 2013). 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 (Hooper 1986).

Molecular characterization and phylogenetic analysis

To diagnose individual species, we amplified and sequenced a 1598-bp fragment of the SSU rRNA gene as previously described (Ragsdale et al. 2015). GenBank accession numbers are found under KX113517 and KX113518.

In addition, 27 ribosomal proteins that have been previously used for reconstructing phylogenies of the *Pristionchus* genus (Ragsdale et al. 2013; Susoy et al. 2016) and genomic libraries of both new species were sequenced. Sanger sequencing data of the 27 ribosomal proteins was corrected using Illumina data and aligned to corresponding data for all known *Pristionchus* species (Susoy et al. 2016) using MUSCLE (version 3.8.31). Maximum likelihood trees for various substitution models were generated using the phangorn R package. Genomic libraries for both species were generated as described previously (Rödelsperger et al. 2014) and were sequenced as 10-plex on a single lane of an Illumina HiSeq3000 (2x151bp). Illumina data for both new species have been submitted to the NCBI sequence read archive and are available under the accession numbers SRX2136100, SRX2136054, SRX2135726, SRX2135725. Whole genome sequencing data was aligned to the *P. pacificus* reference genome as previously described (Rödelsperger et al. 2014). We extracted 725,846 variable positions that are called as homozygous sites in all analyzed species using samtools (version 0.1.18). Variants used in this analysis are only from those regions of the two novel species that can be clearly aligned with *P. pacificus*. As monomorphic sites were ignored in order to reduce data complexity, substitution rates cannot reliably be estimated by probabilistic methods. We thus relied on simple distance based approach (Neighbor joining) that was already previously applied to *P. pacificus* data (Baskaran and Rödelsperger 2015).

Mating experiments

Mating experiments were performed according to standard protocols (Herrmann et al. 2006b). In short, a virgin female of a strain was tested with a male of another strain on a mating plate that contained a small amount of *E. coli* OP50 bacteria. All crosses were performed reciprocally and at least in triplicate. To test for the production of viable F1 hybrids, crosses were set up between all possible pairs of *P. exspectatus*, *P. arcanus*, *P. taiwanensis* and *P. occultus*. F1 hybrids were tested for their ability to produce F2 progeny. We considered strains to belong to the same species if they produced viable and fertile offspring.

RESULTS

Scarab beetle sampling resulted in the isolation of 30 *Pristionchus* strains

A field trip to Kenting National Park, Li-lung Shan, Hui-sun Forest and Ren-ai Township in Taiwan was conducted in August 2015. From 83 specimens of scarab beetles belonging to 16 genera, we isolated 34 strains of nematodes belonging to the Diplogastridae family. Thirty of these 34 strains are members of the genus *Pristionchus*. The majority of isolates (26/30) are hermaphroditic strains that were shown by sequencing of the SSU and mating experiments to represent new isolates of *P. pacificus*. These strains are not considered further in this study. Four isolates showed a gonochoristic mode of reproduction and are the subject of this analysis. Molecular sequence analysis and mating experiments, all of which are described below, indicate that these four strains represent two novel species of *Pristionchus*, which are described thereafter as novel species.

Molecular characterization and phylogenetic analysis

Sequences of an 1598-bp fragment of the SSU rRNA gene were unique for both new species described herein (Fig. 2S), similar to observations made for all other *Pristionchus* species described previously (Kanzaki et al. 2012 a b, 2013 a b c, 2014; Ragsdale et al. 2013). The analysis of a concatenated set of 27 ribosomal protein genes that had fully separated all previously known *Pristionchus* species, including those in the *P. pacificus* species-complex (Fig. 1S), provided further support that both new species are members of the *P. pacificus* species-complex, but could not unambiguously resolve the exact phylogenetic relationships within this complex. However, a phylogeny based on whole-genome sequencing data, following an approach that was used successfully in the analysis of the population structure and microevolution of *P. pacificus* (Rödelsperger et al. 2014; Baskaran and Rödelsperger 2015), could resolve these ambiguities (Fig. 1). In addition, down sampling of the genome-wide data could show that the differences between trees were due to a combination of low number of informative sites and extremely close phylogenetic relationships in the *P. pacificus* species-complex. The phylogeny in figure 1 has 100% bootstrap support at all internal nodes. It places the two new species described here in monophyletic groups with *P. exspectatus* and *P. arcanus*, respectively. Specifically, *P. taiwanensis* is the sister species of *P. arcanus* and *P. occultus* the sister species of *P. exspectatus* (Fig. 1).

Mating experiments

We performed mating experiments to confirm results of our original species identifications by the SSU rRNA gene and genome-wide sequence analyses. As observed in previous studies of the genus *Pristionchus*, the results of mating experiments fully correlate with the SSU sequence analysis. Specifically, all three strains of *P. taiwanensis* produced viable and fertile offspring in reciprocal crossing experiments. The F1 progeny resulting from these crosses were fertile, resulting in viable F2 progeny.

When we performed crosses between the reference strains of the two new species with *P. exspectatus* and *P. arcanus*, we saw hybridization in the F1 generation in a few of these crosses mostly in an inconsistent manner. For example,

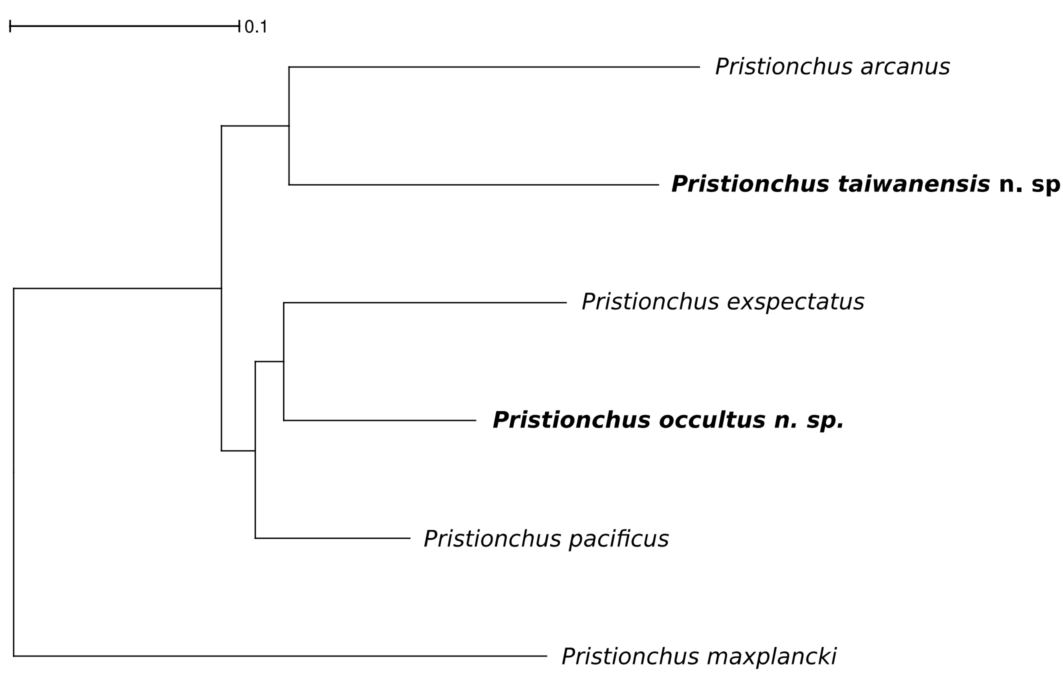

Fig. 1. Phylogenetic relationship of the newly described species and *P. pacificus*, *P. exspectatus*, *P. arcanus* and *P. maxplancki*. The tree represents a neighbor joining tree of 725,846 variable sites that are covered in whole-genome sequencing data of all species. Distances represent Hamming distances among all variable sites. All internal nodes have 100% bootstrap support.

from crosses performed in triplicate, single crossing plates gave F1 hybrid progeny for a cross between a *P. occultus* male and a *P. arcanus* female. However, crosses between F1 hybrid females and males never resulted in progeny. Similarly, parental backcrosses of F1 hybrid females and males did not result in any progeny suggesting that both hybrid genders are sterile. The absence of hybridization confirms the molecular sequence analysis indicating that *P. exspectatus*, *P. arcanus*, *P. taiwanensis* and *P. occultus* are closely related members of the *P. pacificus*-complex. It is important to note that the extent to which F1 hybrids were generated does not strictly correlate with the phylogenetic distance between species as inferred from genome-wide variation data. For example, *P. exspectatus* forms more F1 hybrids with *P. pacificus* and others than with *P. occultus*, although it is most closely related to the latter species. In summary, mating experiments confirm molecular sequence analysis and indicate that we have isolated two new *Pristionchus* species from Taiwan, both of which are taxonomically described below.

TAXONOMY

General morphological characters of the two new species described herein and other members of the *P. pacificus* species-complex are very similar to each other, *i.e.*, *P. pacificus*, *P. exspectatus*, *P. arcanus*, *P. taiwanensis* and *P. occultus* are typologically cryptic, and are mostly distinguished by molecular sequences and biological characters. To avoid redundancy, morphology common to both species is described first, followed by species-specific characters and diagnoses for each species. Finally, relationships among 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 striations based mostly on the presence of deirids. Head narrowly rounded, without apparent lips, and with six short and papilliform labial sensillae. Four small, papilliform cephalic papillae present in males, as typical for diplogastrid nematodes. Amphidial apertures located at level of posterior end of cheilostomatal plates, slightly dorsally shifted (*i.e.* Fig. 2C). Stomatal dimorphism present, with stenostomatous (narrow mouth-form) and eurystomatous (wide mouth-form) morphs 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 well developed. Nerve ring usually surrounding posterior region of isthmus. Excretory pore ventrally located at level of basal bulb to pharyngo-intestinal junction. Hemizonid not clearly observed. Deirid observed laterally, slightly posterior to pharyngo-intestinal junction. Postdeirids present and observed laterally around the anterior part of *vas deferens* (male) and posterior end of posterior gonad (female).

Stenostomatous form: Cheilostom consisting of six per- and interradial plates. Incision between plates not easily distinguished by light microscopic observation. Anterior end of each plate rounded and elongated to project from stomatal opening and forming a small flap. Gymnostom short, cuticular ring-like anterior end overlapping with cheilostom internally. Dorsal gymnostomatal wall slightly thickened compared to ventral side. Stegostom bearing a conspicuous and movable dorsal triangular or diamond-shaped tooth with strongly sclerotized surface appearing as an inverted “V” shape in light microscopic observation, three left subventral denticles apparently projecting from a common cuticular plate, and a small, short, and weakly pointed right subventral denticle.

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. Gymnostom with thick cuticle, forming a short, ring-like tube. Anterior end of gymnostom overlapping internally with the posterior end of cheilostomatal plates. Stegostom bearing a large claw-like dorsal tooth, a large claw-like right subventral tooth, and a row of left subventral-ventral denticles of varying numbers and size, *i.e.* the denticule plate is separated into three subsections, and each forms a large and triangular denticle, and its tip sometimes split into two or three. Dorsal and right subventral teeth movable. Left subventral denticles immovable.

Male: Ventrally arcuate, strongly curved ventrally at tail region (when killed by heat). Testis single, located ventrally, anterior part typically reflexed to right side, but to the left side in some individuals. 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. *Vas deferens* consisting of long cells, occupying ca 1/5-1/6 of total gonad. Spicules paired, separate and smoothly curved in ventral view, adjacent to each other for distal third of their length, each smoothly tapering to pointed distal end. 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. Gubernaculum conspicuous, about half of spicule in length, anterior half with ear-like shape in lateral view, posterior half forming a tube-like process enveloping spicules. 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. 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, a pair of phasmids and a ventral precloacal hook present with an arrangement of <v1, v2d, v3, vh, c, v4, ad, ph, v5, v6, v7, pd> from anterior where v, vh, c and ph mean laterally/ventrally directed papillae, ventral hook, cloacal opening and phasmid, respectively. The v1–v4 papillae of almost equal size, rather large and conspicuous; ad slightly smaller than v1–v4; v5 and v6 very small, sometimes difficult to observe with light microscope; v7 and pd small, but larger than v5 and v5, *i.e.* intermediate between ad and v5/v6 in size. The v5 and v6 papilliform and borne from socket-like base; tip of v6 papillae split into two small papilla-like projections; v7 simple or typical thorn-like in shape. Detailed arrangement of paired papillae and phasmids is described for individual species below. Tail spike about two 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 extending dorsally on left of intestine. 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. Middle part of oviduct serving as spermatheca. *Receptaculum seminis* not observed. Vaginal glands present but obscure. Vagina perpendicular to body surface, surrounded by sclerotized tissue. Vulva slightly protuberant in lateral view, pore-like in ventral 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, posterior anal lip slightly protuberant. Phasmid about one to two anal-body-diameters posterior to anus. Tail long, distal end variable from filiform to long and conical.

Species descriptions based on species-specific characters

***Pristionchus taiwanensis* n. sp.**
urn:lsid:zoobank.org:act:FD5B892C-E0E4-44F2-82DB-15E0A74E9DAE
(Figs. 2-3; Fig. 2S)

Measurements: See Table 1.

Description: *Stenostomatous form*. Stegostom bearing a conspicuous and movable dorsal triangular or diamond-shaped tooth, three bump-like (blunt) left subventral denticles apparently projecting from a common cuticular plate, and a small, short, and pointed right subventral denticle bearing from the reclangular plate (Fig. 2E). Dorsal tooth with strongly sclerotized surface, appearing as an inverted “V” shape in light microscopic observation.

Eurystomatous form: Clear species-specific characters were not observed.

Male: Nine pairs of genital papillae arranged as above, where, in many individuals, phasmid (ph) and v5 are close to each other. ph and v5-7 linearly arranged, and pd located slightly posterior to v7.

Female: Clear species-specific characters were not observed.

Diagnosis: Besides its generic (or *P. pacificus*

Table 1. Morphometrics of stenostomatous male and female specimens of *Pristionchus taiwaniensis* n. sp. RS5797 (temporary water mounts). All measurements made in μm and given in the form: mean ± SD (range)

Character	Stenostomatous males	Stenostomatous females
n	10	10
L	1016 ± 147 (735-1238)	1035 ± 114.2 (780-1207)
L’	891 ± 139 (623-1098)	866 ± 66.4 (780-987)
a	14 ± 2.2 (12-18)	14 ± 0.8 (13-16)
b	6.8 ± 0.8 (5.3-8.1)	6.9 ± 0.6 (6.1-7.9)
c	8.2 ± 1.3 (6.6-10.3)	5.7 ± 0.6 (4.9-6.9)
c’	3.1 ± 0.4 (2.5-3.6)	5.8 ± 0.6 (5.1-7.1)
T or V	60 ± 5.5 (53-71)	48 ± 3.6 (41-52)
Maximum body diam.	74 ± 14.5 (52-97)	75 ± 7.1 (63-90)
Stoma length	10.2 ± 1.1 (9-12.8)	11.2 ± 1.3 (9.7-13.6)
Stoma diam.	6.5 ± 0.5 (5.4-7.4)	6.9 ± 0.7 (5.7-8.2)
Pharynx length (head to base of pharynx)	139 ± 5.7 (129-146)	146 ± 6.3 (138-157)
Anterior pharynx (pro- + metacorpus)	83 ± 3.6 (78-90)	90 ± 5.1 (83-98)
Posterior pharynx (ithmus + basal bulb)	55 ± 4.4 (48-61)	56 ± 3.7 (51-62)
ant/total pharynx %	± 0.0 (1)	61 ± 2 (60-65)
Median bulb diam.	27.0 ± 1.2 (25-29)	30 ± 3.8 (25-36)
Terminal bulb diam.	23 ± 2.2 (20-26)	26 ± 3.2 (21-31)
Testis length	608 ± 126 (412-808)	-
Ant. end to vulva	-	503 ± 51.5 (445-615)
Vulva to anus distance	-	361 ± 40.1 (305-433)
Cloacal or anal body diam.	41 ± 5.9 (33-49)	32 ± 3.6 (29-40)
Tail length	124 ± 18 (94-156)	187 ± 24.6 (151-222)
Spicule length (curve)	48 ± 4.8 (40-53)	-
Spicule length (chord)	41 ± 3.9 (34-49)	-
Gubernaculum length	20 ± 0.8 (15-24)	-

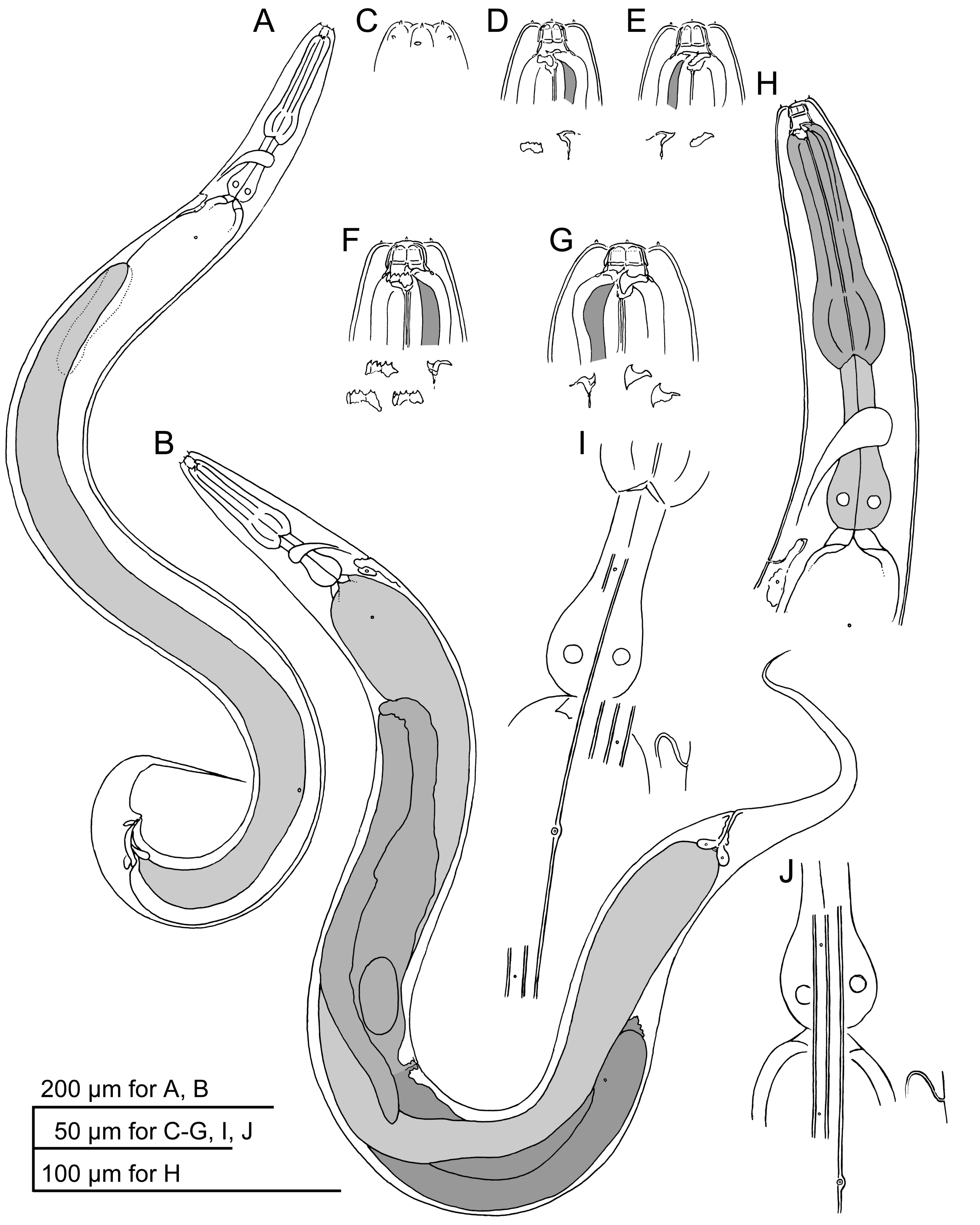

Fig. 2. *Pristionchus taiwanensis* n. sp. Drawings are of live specimens (non-types) from temporary mounts. (A) Stenostomatous male, left lateral view. (B) Stenostomatous female, right lateral view. (C) Anterior end of stenostomatous male, right lateral view. (D) Stomatal region of eurystomatous female, left lateral view; below (from left to right) are left subventral ridge and dorsal tooth. (E) Stomatal region of stenostomatous female, right lateral view: below are right subventral denticle and dorsal tooth. (F) Stomatal region of eurystomatous female, left lateral view; below are left subventral ridge and dorsal tooth. (G) Stomatal region of eurystomatous female, right lateral view; below are dorsal tooth and right subventraltooth . (H) Pharyngeal region of stenostomatous female, left lateral view. (I-J) Body surface showing longitudial striations and relative position of deirid and secretory pores.

species-complex) characters, *P. taiwanensis* n. sp. is diagnosed by its morphology of right subventral ridge in the stenostomatous form. The species is also characterized by an 830-bp fragment of the SSU rRNA gene (GenBank accession number KX113517), 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 (strain RS5797) from which the type specimens were obtained was originally isolated from the body of an adult *Onthophagus* sp. collected at Kenting

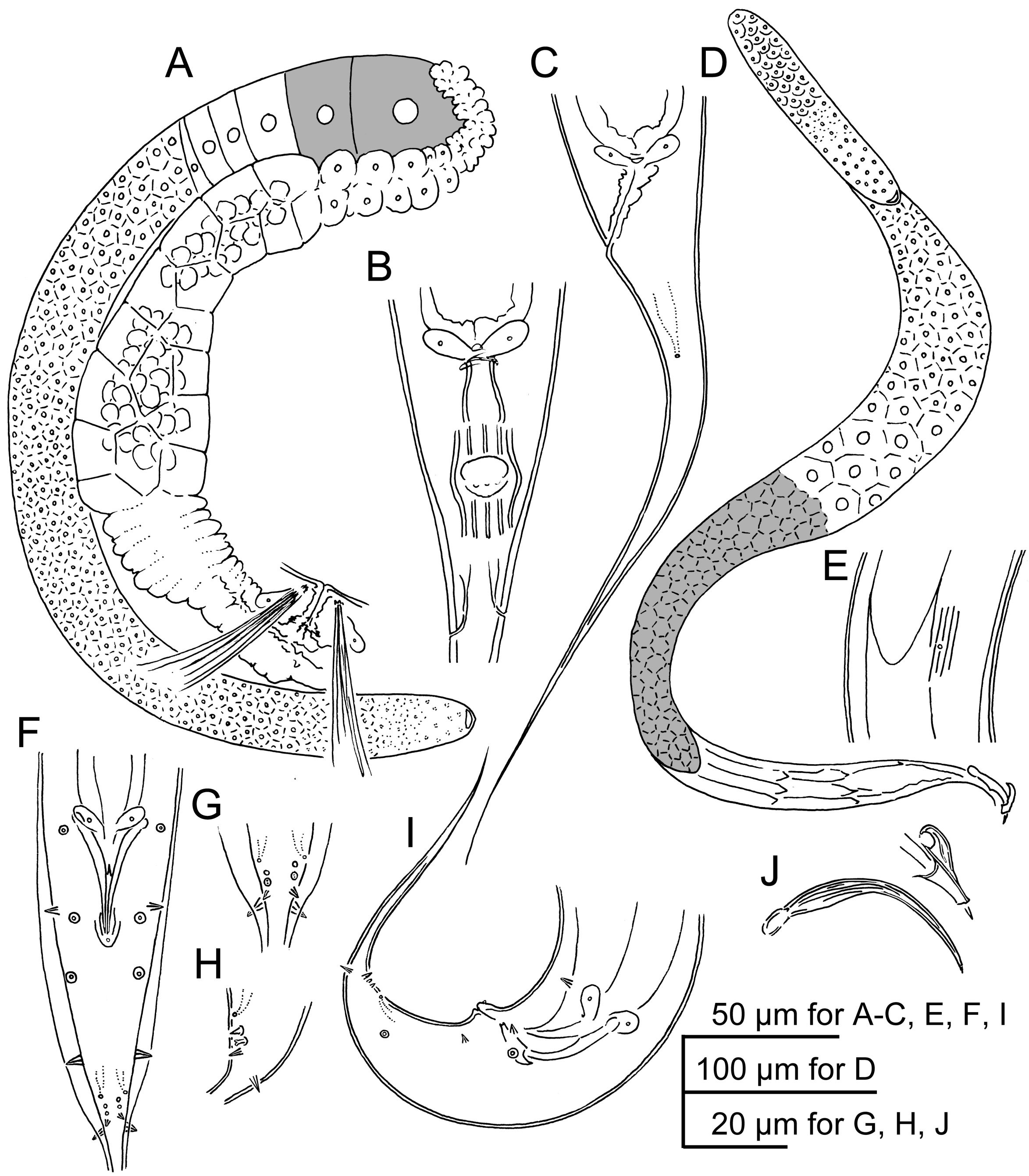

Fig. 3. *Pristionchus taiwaniensis* n. sp. (A) Anterior gonad of female, right lateral view. (B) Female anal region, ventral view. (C) Female tail, left lateral view. (D) Male gonad, left lateral view. (E) Position of male postdeirid. (F) Male tail region, ventral view. (G) Male phasmid and genital papillae v5-7 and pd, ventral view. (H) Male phasmid and genital papillae v5-7 and pd, left lateral view. (I) Male tail region, left lateral view. (J) Spicule and gubernaculum, left lateral view).

National Park in August 2015.

Type material: Holotype stenostomatous male (slide accession number 31486), three paratype stenostomatous males and three paratype stenostomatous females (31487-31492) deposited in the University of California Riverside Nematode Collection (UCRNC), Riverside, CA, USA. Three paratype stenostomatous males and three stenostomatous females (SMNK-Nema-T0119– SMNK-Nema-T0124) deposited in the Natural History Museum Karlsruhe, Germany. Three paratype stenostomatous males and three paratype stenostomatous females (SMNH Type-8791 – SMNH Type-8796) deposited in the Swedish Natural History Museum, Stockholm, Sweden.

Type strain culture: All strains are available as living cultures and frozen stocks under culture code RS5797, RS5800 and RS5815 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 adjective denotes the type locality of the species.

Pristionchus occultus n. sp.

urn:lsid:zoobank.org:act: F6ECE842-DFAE-4ECA-9B21-BDD46FBB71A2

(Figs. 4, 5, Fig. 2S)

Measurements: See Table 2.

Description: *Stenostomatous form*. Stegostom bearing conspicuous and movable dorsal triangular or diamond-shaped tooth, three bump-like (blunt) left subventral denticles apparently projecting from a common cuticular plate, and a small, short, and pointed right subventral denticle baring from flattened-triangular plate.

Eurystomatous form: Clear species-specific characters were not observed.

Male: Nine pairs of genital papillae arranged as above, where, in many individuals, pd located at the level of or slightly posterior to v7.

Female: As described above in common characters. Clear species-specific character was not observed.

Diagnosis: *Pristionchus occultus* n. sp. is a cryptic species of *P. pacificus*, *P. exspectatus* and *P. arcanus*, *i.e.*, the species does not show a clear species-specific morphological character to distinguish it clearly from the other species. The species is also characterized by an 830-bp fragment of the SSU rRNA gene (GenBank accession number KX113518), 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 body of a rose beetle grub (Coleoptera: Cetonidae) collected at Huisun, Taiwan in August 2015.

Type material: Holotype stenostomatous male (slide accession number 31493), three paratype stenostomatous males and three paratype stenostomatous females (31494-31499) deposited in the UCRNC, Riverside, CA, USA. Three paratype stenostomatous males and three paratype stenostomatous females (SMNK-Nema-T0125– SMNK-Nema-T0130) deposited in the Natural History Museum Karlsruhe, Germany. Three paratype stenostomatous males and three paratype stenostomatous females (SMNH-Type-8797 – SMNH-Type-8802) deposited in the Swedish Natural History Museum, Stockholm, Sweden.

Type strain culture: Available as living cultures and frozen stocks under culture code RS5811 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 denotes the formerly hidden nature of the species.

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

As mentioned above, the two new species described herein and *P. pacificus*, *P. exspectatus* and *P. arcanus* form a species-complex. Basically, all five species are morphologically almost indistinguishable. *P. pacificus* is separated from all other species by its hermaphroditic mode of reproduction. *P. taiwanensis* could be separated from the four other species by its shape of the stenostomatous right subventral ridge. The other three species are also potentially separated from each other, *e.g.*, the arrangement of v5-7 papillae and pd (Kanzaki et al. 2012a). However, these characters are very small and still show some variability within each species. In addition, morphometric values show large intraspecific variation as already noted for other *Pristionchus* species before (*e.g.* Herrmann et al. 2006a; Kanzaki et al. 2012a b, 2013a b c; Kanzaki and Giblin-Davis 2015; Ragsdale et al. 2015; Susoy

Table 2. Morphometrics of stenostomatous male and female specimens of *Pristionchus occultus* n. sp. RS5811 (temporary water mounts). All measurements made in μm and given in the form: mean ± SD (range)

Character	Stenostomatous male	Stenostomatous female
n	10	10
L	791 ± 93.3 (672-1008)	901 ± 110.5 (766-1110)
L’	673 ± 86.6 (565-875)	718 ± 84.6 (627-895)
a	16 ± 1.3 (14-19)	14 ± 1.1 (12-15)
b	5.6 ± 0.3 (5.2-6.1)	6.2 ± 0.5 (5.2-6.6)
c	6.7 ± 0.6 (5.7-7.6)	5 ± 0.4 (4.5-5.6)
c’	4 ± 0.6 (3.1-5.3)	6.6 ± 0.8 (5.5-8.2)
T or V	54 ± 2.7 (51-58)	47 ± 1.8 (44-50)
Maximum body diam.	50 ± 6.9 (42-66)	64 ± 8.8 (55-77)
Stoma length	10.2 ± 0.7 (9-11.1)	10.3 ± 1.9 (6.4-12.8)
Stoma diam.	4.4 ±0.5 (3.6-5.2)	5.6 ± 0.5 (4.7-6.5)
Pharynx length (head to base of pharynx)	131 ± 12.9 (112-158)	136 ± 12.3 (118-158)
Anterior pharynx (pro- + metacorpus)	76 ± 7.7 (66-91)	82 ± 6.4 (73-96)
Posterior pharynx (ithmus + basal bulb)	54 ± 5.8 (46-66)	54 ± 6.7 (44-66)
ant/total pharynx %	58 ± 1.5 (56-61)	60 ± 1.9 (57-63)
Median bulb diam.	24 ± 2.3 (21-29)	28 ± 2.8 (24-33)
Terminal bulb diam.	21 ± 3.8 (17-30)	24 ± 3.1 (19-29)
Testis length	431 ± 63.7 (363-586)	-
Ant. end to vulva	-	422 ± 45.3 (370-517)
Vulva to anus distance	-	296 ± 41.8 (256-378)
Cloacal or anal body diam.	30 ± 3 (26-35)	28 ± 4.2 (21-35)
Tail length	119 ± 12 (105-139)	183 ± 29.5 (139-231)
Spicule length (curve)	43 ± 3.2 (37-48)	-
Spicule length (chord)	34 ± 2.9 (30-39)	-
Gubernaculum length	17 ± 2 (15-22)	-

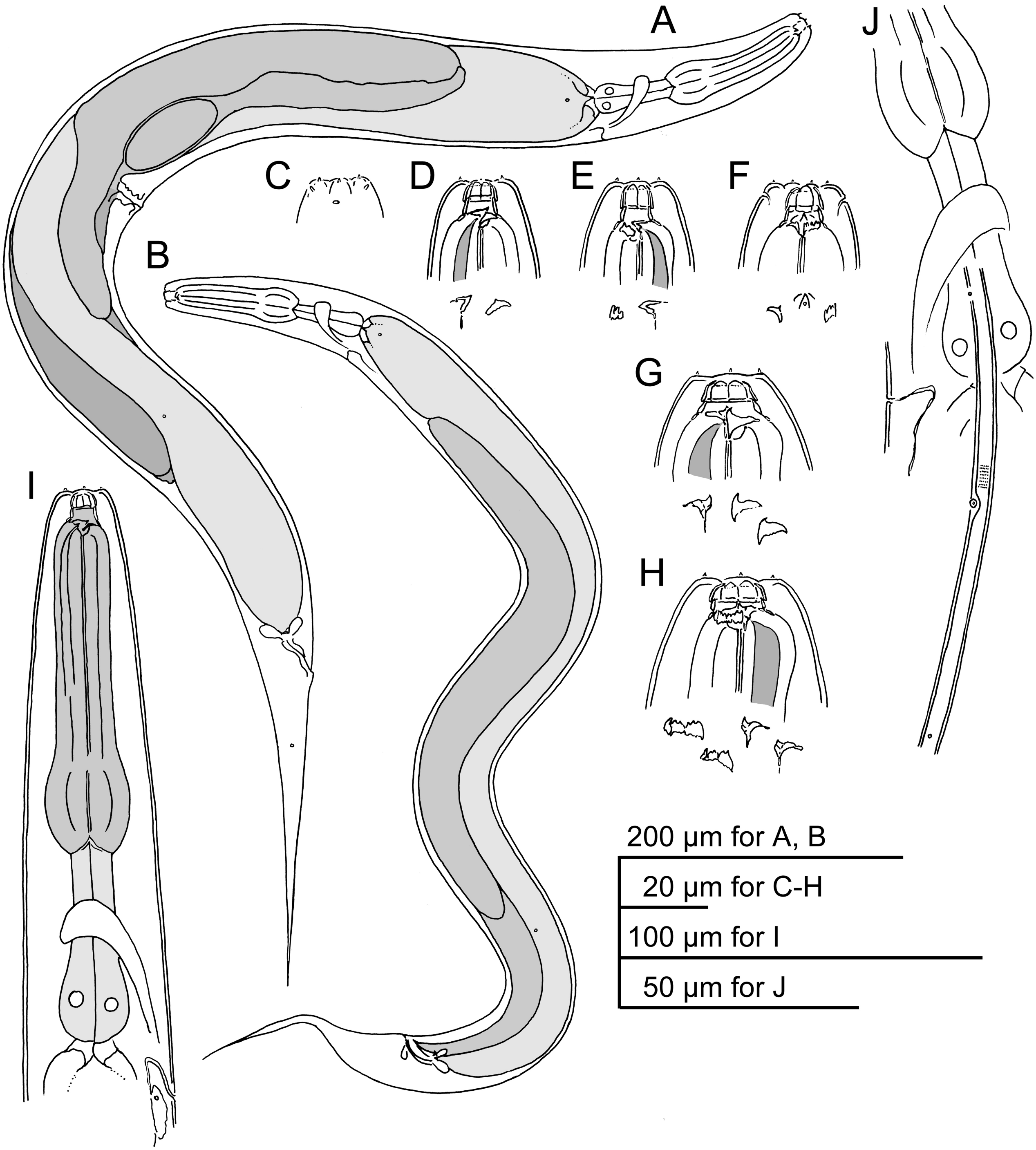

Fig. 4. *Pristionchus occultus* n. sp. Drawings are of live specimens (non-types) from temporary mounts. (A) Stenostomatous female, right lateral view. (B) Stenostomatous male, right lateral view. (C) Anterior end of stenostomatous male, right lateral view. (D) Stomatal region of stenostomatous female, right lateral view; below (from left to right) are dorsal tooth and right subventral denticle. (E) Stomatal region of stenostomatous female, left lateral view; below are left subventral ridge and dorsal tooth. (F) Stomatal region of stenostomatous female, ventral view; below are right subventral denticule, dorsal tooth and left subventral ridge. (G) Stomatal region of eurystomatous female, right lateral view; below are dorsal tooth and right subventral tooth. (H) Stomatal region of eurystomatous female, left lateral view; below are left subventral ridge and dorsal tooth. (I) Pharyngeal region of stenostomatous female, right lateral view. (J) Body surface showing longitudial striations and relative position of deirid and secretory pores.

et al. 2015). Therefore, biological and molecular characters are necessary to separate species.

DISCUSSION

The current study and description of two new *Pristionchus* species from Taiwan resulted from a field trip conducted in August 2015. The discovery of two new gonochoristic *Pristionchus* species that represent the closest known relatives of *P. exspectatus* and *P. arcanus*, respectively, is invaluable for the analysis of population genetics, macroevolution and speciation in the *P. pacificus* species-complex. However, given the fact that these two new species were recovered from only four gonochoristic isolates suggests that current sampling is far from saturation and that the real diversity of *Pristionchus* species in Taiwan might be much higher, demanding future sampling efforts.

Nonetheless, the work described in this study allows three major conclusions. First, our new findings expand the understanding of the

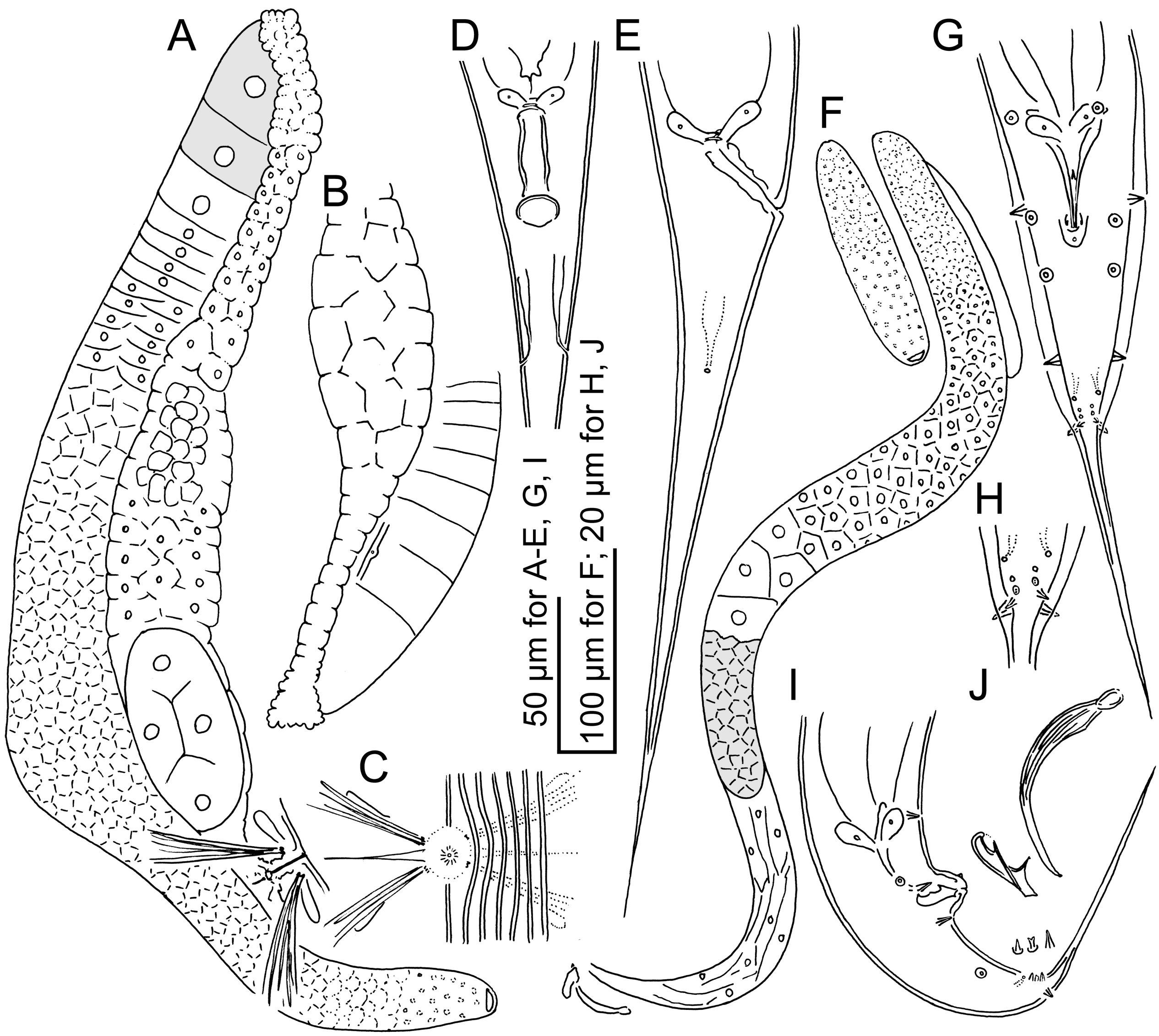

Fig. 5. *Pristionchus occultus* n. sp., (A) Anterior gonad of female, right lateral view. (B) relative position of female postdeirid. (C) Female vulva, ventral view. (D) Female anal region, ventral view. (E) Female tail, left lateral view. (F) Male gonad, left lateral view. (G) Male tail region, ventral view. (H) Male phasmid and genital papillae v5-7 and pd, ventral view. (I) Male tail region, right lateral view. (J) Spicule and gubernaculum, right lateral view.

biogeography of the *P. pacificus* species-complex. While *P. pacificus* has a cosmopolitan distribution and has so far been reported from all continents except Australia and Antarctica, *P. exspectatus*, *P. arcanus* and *P. japonicus* are presently only known from Japan (Kanzaki et al. 2012a, 2013a). These findings had provided first evidence for an East Asian origin of the species-complex. Our new findings provide additional evidence that the growing numbers of species in the *P. pacificus* species-complex are all found in Asia. However, our findings also indicate that these biogeo-graphic patterns have to be carefully evaluated. Specifically, a careful analysis of East Asian Pacific Islands is necessary to fully understand the history and biogeography of the *P. pacificus* species-complex: Samplings in more southern areas, such as the Phillipines and other territories nearby, will be necessary and might result in the identification of additional new species. Therefore, current sampling biases prevent a full understanding of the evolution of this species-rich nematode genus. Also, in cannot be ruled out that future samplings in other areas of the world will change the current perspective.

Nonetheless, current biogeographic patterns suggest a correlation between the broad geographic distribution and hermaphroditic reproduction in *P. pacificus*, which is not seen in any gonochoristic species of the *P. pacificus* species-complex. This observation may be explained by a more efficient dispersal of hermaphroditic species that require only a single individual to propagate (Herrmann et al. 2010; Kanzaki et al. 2012a). Additional support for this notion comes from the sampling described in this study, which in addition to the two newly described species revealed 26 new *P. pacificus* strains indicating the relative ease of dispersal of this hermaphroditic species.

Second, the identification of a species cluster with four gonochoristic species and the model organism *P. pacificus* has tremendous implications for the evolutionary biology of the genus. One research area that can substantially profit from such dense taxon sampling is the investigation of the evolutionary origin of hermaphroditism. With the addition of *P. taiwanensis* and *P. occultus*, the *P. pacificus* species-complex now consists of eight species. *P. pacificus* is the only species in the complex with a hermaphroditic mode of reproduction indicating that the mating type transition has evolved very recently. Studies to elucidate the genetic basis of hermaphroditism are feasible given the fact that *P. arcanus*, *P. exspectatus*, *P. taiwanensis*, *P. occultus* and *P. pacificus* sometimes form infertile hybrids. Therefore, detailed large-scale crossing experiments followed by backcrosses of the F1 hybrids with parental strains may represent a powerful tool to identify the underlying genetic and molecular mechanisms in future studies.

Third, our study on the morphology, taxonomy and systematics of the *P. pacificus* species-complex indicates the near absence of morphological characters available to diagnose them. It has been noted in recent years that morphology other than in the stoma and pharynx are relatively uniform across the family Diplogastridae (Kanzaki and Giblin-Davis 2015) and even more so in the genus *Pristionchus* (Ragsdale et al. 2015). Therefore, it was not surprising that *P. taiwanensis* and *P. occultus* are morphologically indistinguishable from the previously known species. Cryptic species are commonly recognized in many animal taxa in recent years (Pfenninger and Schwenk 2007). Therefore, integrative approaches based on reproductive isolation, molecular sequence divergence, and morphological characters will also bring the taxonomy of nematodes to its next level.

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Supplement Figures

Fig. 1S. Phylogenetic relationships of *Pristionchus* species inferred by maximum likelihood (ML) from a partial SSU rRNA fragment and 26 ribosomal protein-coding genes. Modified after Kanzaki et al. (2014). (download)

Fig. 2S. SSU sequence alignment and phylogenetic relationships of the newly described species and *P. pacificus*, *P. exspectatus* and *P. arcanus*. Alignment of SSU rRNA sequences for species of the *Pristionchus pacificus* species-complex. Sequences were aligned manually. Differences in sequences for new species described herein are considered diagnostic of those species. Sequences have been deposited in the GenBank database and ascribed accession numbers as described in text. (download)