

Two New Species of *Pristionchus* (Nematoda: Diplogastridae) from Taiwan and the Definition of the *pacificus* Species-Complex Sensu Stricto

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Abstract

Pristionchus pacificus Sommer, Carta, Kim, and Sternberg, 1996 is an important model organism in evolutionary biology that aims to integrate developmental biology and evo-devo with population genetics and ecology. Functional studies in *P. pacificus* are supported by a well-established phylogenetic framework of around 30 species of the genus *Pristionchus* that have been described in the last decade based on their entomophilic and necromenic association with scarab beetles. Biogeographically, East Asia has emerged as a hotspot of *Pristionchus* speciation and recent samplings have therefore focused on Islands and mainland settings in East Asia. Here, we describe in a series of three publications the results of our sampling efforts in Taiwan, Japan, and Hongkong in 2016 and 2017. We describe a total of nine new species that cover different phylogenetic species-complexes of the *Pristionchus* genus. In this first publication, we describe two new species, *Pristionchus sikae* sp. n. and *Pristionchus kurosawai* sp. n. that are closely related to *P. pacificus*. Together with five previously described species they form the “*pacificus* species-complex sensu stricto” that is characterized by all species forming viable, but sterile F1 hybrids indicating reproductive isolation. *P. sikae* sp. n. and *P. kurosawai* sp. n. have a gonochorist mode of reproduction and they are described using morphology, morphometrics, mating experiments, and genome-wide sequence analysis. We discuss the extreme diversification in the *pacificus* species-complex sensu stricto in East Asia and its potential power to study speciation processes.

Key words

Evolution, *Pristionchus pacificus*, Scarab beetles, Stoma, Taiwan

The nematode *Pristionchus pacificus* (Sommer et al., 1996) has been developed as a model system in evolutionary developmental biology (evo-devo), for comparison with *Caenorhabditis elegans* and, more recently, for integrative studies in evolutionary biology that aim to link developmental genetics and evo-devo with population genetics and ecology (Sommer, 2009, 2015). A suite of functional tools is available in *P. pacificus* that enable mechanistic insight building on a completely sequenced genome (Dieterich et al., 2008; Rödelsperger et al., 2017), DNA-mediated

transformation (Schlager et al., 2009) and forward and reverse genetics including CRISPR/Cas9-based engineering (Sommer et al., 1996; Tian et al., 2008; Witte et al., 2015). A well-established phylogenetic framework of the genus *Pristionchus* (Kreis, 1932) supports developmental and evolutionary studies in *P. pacificus*. Originally, field studies indicated that European *Pristionchus* species are often associated with scarab beetles (Herrmann et al., 2006), and *P. pacificus* was later found in association with the oriental beetle *Exomala orientalis* in Japan and the

United States (Herrmann et al., 2007). Subsequently, systematic beetle and insect samplings resulted in the isolation, characterization, and description of 30 *Pristionchus* species, all of which are available as living cultures. These species form four distinct species-complexes, most of which are biogeographically restricted (for review, see Ragsdale et al., 2015). For example, *P. pacificus* itself is part of the ‘*pacificus* species-complex’ that originated in East Asia and currently consists of *P. pacificus*, *Pristionchus exspectatus*, and *Pristionchus arcanus* (Kanzaki et al., 2012), the recently described *Pristionchus taiwanensis* and *Pristionchus occultus* (Herrmann et al., 2016) and the more distantly related *Pristionchus japonicus*, *Pristionchus maxplancki*, and *Pristionchus quartus-decimus* (Kanzaki et al., 2013).

In general, species designation in the genus *Pristionchus* requires morphology, morphometrics, mating experiments, and molecular sequence analysis. It is important to note though, that morphological and morphometric data alone are insufficient for species diagnosis because intraspecific variability often exceeds inter-species divergence (Kanzaki et al., 2012, 2013; Ragsdale et al., 2015). Remarkably, mating experiments and sequence analysis of a 1.6 kb fragment of the large subunit ribosomal ribonucleic acid (LSU) strongly correlate and characterize *Pristionchus* species in all species-complexes of the genus (Ragsdale et al., 2015). However, in contrast to *Pristionchus* species in other species-complexes of the genus, many members of the ‘*pacificus* species-complex’ form viable, but sterile F1 hybrids indicating their extreme close relationship. The formation of F1 sterile hybrids coincides with the inability of the LSU sequence to properly distinguish *P. pacificus*, *P. exspectatus*, *P. arcanus*, *P. taiwanensis*, and *P. occultus*. Therefore, the elucidation of the exact phylogenetic relationship of these species required whole genome sequencing and the use of 700,000 genome-wide variable sites that were genotyped in all species (Herrmann et al., 2016). Most recently, we established whole transcriptome sequencing combined with phylogenetic analysis of all cultivable *Pristionchus* species for the elucidation of their phylogenetic relationships (Rödelsperger et al., 2018).

Sampling efforts in East Asia between 2011 and 2015 have resulted in the isolation of the *Pristionchus* species mentioned above, but they also indicated that current samplings are far from saturation (Herrmann et al., 2016). Therefore, we have undertaken additional sampling trips to Japan, Taiwan, and Hongkong in 2016 and 2017. In a series of three publications we here describe the isolation, characterization, and description of nine new *Pristionchus* species. In this

publication, we describe the gonochoristic species *Pristionchus sikae* sp. n. and *Pristionchus kurosawai* sp. n. using morphology, morphometrics, mating experiments, and genome-wide sequence analysis. These species are similar to *P. pacificus*, *P. exspectatus*, *P. arcanus*, *P. taiwanensis*, and *P. occultus* in that they form viable but sterile F1 hybrids. Based on these findings, we define the ‘*pacificus* species-complex sensu stricto’ consisting of seven extremely closely related species that are only defined by their inability to form F2 hybrids and by using genome-wide molecular markers. We discuss the extreme diversification in the *pacificus* complex sensu stricto in East Asia and its potential power to study speciation processes.

Materials and methods

Beetle sampling and nematode isolation

Two field trips to Taiwan were conducted from August 10th to 17th, 2016 and May 15th to 28th, 2017. We sampled the areas of Huisun (2016), Lanyu Island, Taroko National Park and Dongyanshan National Forest (2017) and collected more than 600 specimens of scarab beetles belonging to 47 species in 22 genera. Nematodes were isolated using standard procedures as previously described (Herrmann et al., 2006a). In total, we isolated and characterized 25 strains of the Diplogastridae family with 15 of these 25 strains representing members of the genus *Pristionchus*. Eight of the *Pristionchus* strains are hermaphroditic and were shown by sequencing of the SSU and mating experiments (see below) to represent new isolates of *P. pacificus* and *Pristionchus fissidentatus*. Three strains could be identified as new strains of *P. occultus*. The new *P. pacificus*, *P. fissidentatus*, and *P. occultus* 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. Two gonochoristic isolates could not be attributed to a known species and are subject of this analysis. The field trips to Japan and Hongkong are described in detail in the accompanying publications.

Nematode cultivation

Pristionchus sikae sp. n. was isolated from an adult of *Dorcus titanus sika* (Kriesche, 1920) (Coleoptera: Lucanidae) collected at Huisun, Taiwan. The strain of *Pristionchus kurosawai* n. sp. was isolated from an adult of *Lucanus kurosawai* (Sakaino, 1995; Coleoptera: Lucanidae) collected at Songquangang, Taiwan.

Both 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 RS5901 (*P. sika* sp. n.) and RS5914 (*P. kurosawai* sp. n.).

Morphological observation and preparation of type material

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 (see Hooper, 1986).

Remark concerning morphometric measurements: We use a multi-dimensional approach for modern description of nematodes, combining molecular, morphological, biological, and ecological information to characterize new material as broadly as possible. This is based on repeated findings that measurements of certain nematode dimensions can change over time as a result to different culture conditions and that many *Pristionchus* species are morphologically cryptic (Kanzaki et al., 2012, 2013; Fonderie et al., 2013; Ragsdale et al., 2015; Herrmann et al., 2016).

Molecular characterization and phylogenetic analysis

A species phylogeny of the complete *Pristionchus* genus was reconstructed as described in Rödelsperger et al. (2018). In brief, transcriptome libraries were generated from mixed-stage worm cultures and sequenced on an Illumina HiSeq 3,000. Raw reads were assembled de novo and clustered into orthologous gene families. Protein sequences of more than 2,000 orthologous gene clusters without duplications were further aligned and a maximum likelihood tree was reconstructed based on the concatenated alignment (see Rödelsperger et al., 2018 for full details).

Mating experiments

To examine reproductive isolation of *P. sika* and *P. kurosawai* with related species we crossed them with all described gonochoristic species in the *pacificus* species-complex sensu stricto. Mating experiments were performed as previously described with some modifications (Herrmann et al., 2006b). Three virgin females of a strain were tested with six males of

another strain on a mating plate that contained a small amount of *E. coli* OP50 bacteria. All crosses were performed reciprocally and in quadruplicate. To test for the production of viable F1 hybrids, crosses were set up between all possible pairs and 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

Arthropod samplings in East Asia identify nine new *Pristionchus* species

Field trips to Taiwan, Japan, and Hongkong were conducted in 2016 and 2017 as described in detail in Materials and Methods section and in the accompanying publications (Herrmann et al., 2018; Kanzaki et al., 2018). Scarab beetles, but also other arthropods, including millipedes were the primary target for nematode isolation following protocols previously described (Herrmann et al., 2006; Ragsdale et al., 2015). Nematodes of the genus *Pristionchus* were identified based on their morphological characteristics using light microscopy. Fourteen *Pristionchus* strains were hermaphroditic and sequence analysis of the LSU rRNA gene revealed that they are new isolates of *P. pacificus*, *Pristionchus entomophagus* or *P. fissidentatus*. These strains are not considered further in this study.

From thousands of arthropod samples, we isolated a total of 17 gonochoristic *Pristionchus* strains, five strains from Taiwan, 10 strains from Japan and two strains from Hongkong. These strains were first subjected to LSU sequence analysis. Based on the LSU sequencing results mating experiments with reference strains of available *Pristionchus* species with identical or closely related LSU sequences were performed. While this strategy gave reliable results for most species to be described in the accompanying publications, some new isolates of species of the '*pacificus* species-complex' were not distinguishable using LSU sequences or morphological characters. Therefore, these strains were subjected to RNA sequencing analysis and mating experiments were performed with all closely related known species. In total, the 17 gonochoristic strains represent 13 *Pristionchus* species, nine of which are novel. Six strains represent new isolates of *P. arcanus*, *P. occultus*, *P. quartusdecimus* and *Pristionchus fukushimae*. In particular the new isolates of *P. arcanus*, *P. occultus* and *P. quartusdecimus* are invaluable for future studies as only single isolates of these species were previously known (Kanzaki et al., 2012, 2013; Herrmann et al., 2016). For example,

pairwise mating experiments between three new isolates of *P. occultus* and with the reference alleles RS5811 revealed perfect viability of F1 and F2 progeny.

Of the nine new species of *Pristionchus*, seven are more distantly related to the model organism *P. pacificus*. These species will be compared in the accompanying publications (Kanzaki et al., 2018; Herrmann et al., 2018). Below, we describe by morphology, morphometrics, and phylogenetic analysis the two species that belong to what now will be referred to as ‘*pacificus* species-complex sensu stricto’.

Mating experiments

We performed mating experiments between the two species and all described gonochoristic species in the *pacificus* species-complex sensu stricto (Fig. 1). Although almost all crosses produced viable F1 progeny, none of the crosses resulted in F2 progeny. In three species pairs, viable F1 progeny were only observed in one direction, whereas the reciprocal crosses had no offspring. The reduction of viable F1 progeny between *P. taiwanensis* and *P. sikae* sp. n. or *P. kurosawai* sp. n. is consistent with the phylogenetic relation among the species.

*Pristionchus sikae** sp. n. (Figs. 2–4, 7a–c; Table 1)

*The species name is derived from the type host (carrier) insect of the nematode species, *Dorcus titanus sika* (Coleoptera: Lucanidae).

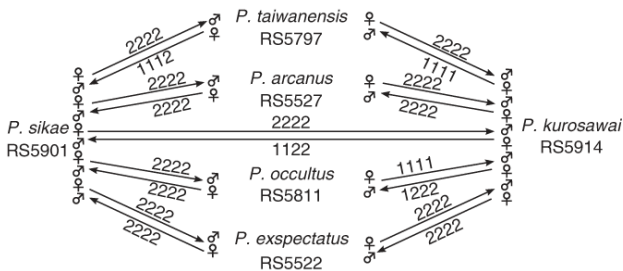


Figure 1: Results of reciprocal mating experiments between gonochoristic species of the *pacificus* species-complex sensu stricto. Numbers indicate the results of four replications of mating experiments. Production of no F1 progeny is indicated as ‘1’, whereas formation of viable sterile F1 progeny is indicated as ‘2’.

Type host (carrier) and locality

The culture from which the type specimens were obtained was originally isolated by N. Kanzaki from an adult *Dorcus titanus sika* collected at Huisun, Taiwan in June 2016.

*Pristionchus kurosawai** sp. n. (Figs. 5–7d,e; Table 1)

Type host (carrier) and locality

The culture from which the type specimens were obtained was originally isolated by K. Yoshida from an adult *Lucanus kurosawai* collected at Songquan-gang, Taiwan in June 2017.

Description

Two new *Pristionchus* species described here, *P. sikae* sp. n. and *P. kurosawai* sp. n. are typologically identical to each other in the key characters, e.g., stomatal morphology and male tail characters. Thus, to avoid redundancy, the typological characters are described as common character for those two species.

Adult

Body cylindrical, stout. Cuticle thick, with fine annulation and clear longitudinal striations. Lateral field consisting of two lines, only weakly distinguishable from body striation. Head 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 margin of cheilo- and gymnostom. Stomatal dimorphism present, with stenostomatous (narrow mouthed) and eurystomatous (wide mouthed) forms occurring in both males and females, but male eurystomatous form is not as common as females. Detailed stomatal morphology is described below. 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). Procorpus very muscular, stout, occupying half to two thirds of corresponding body diam. Metacarpus very muscular, forming well-developed median bulb. Isthmus narrow, not

*The species name is derived from the type host (carrier) insect of the nematode species, *Lucanus kurosawai* (Coleoptera: Lucanidae).

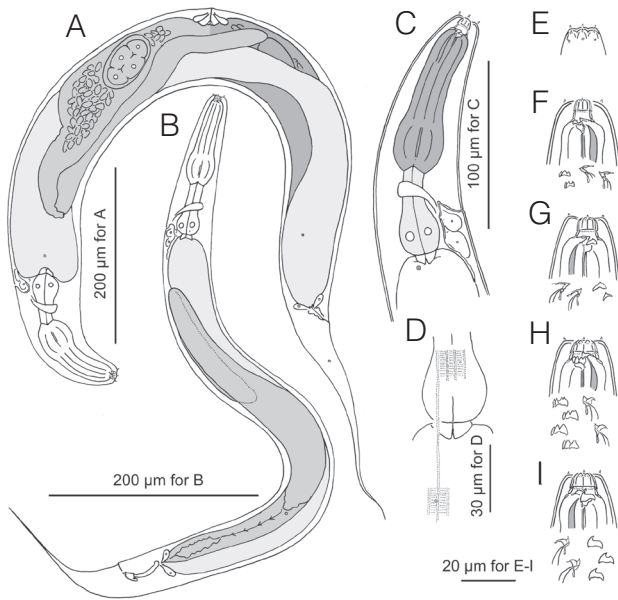


Figure 2: *Pristionchus sikae* sp. n. Drawings are of live specimens (non-types) from temporary mounts. A, Stenostomatous female in right lateral view. B, Stenostomatous male in left lateral view. C, Anterior region of stenostomatous female in left lateral view. D, Body surface showing longitudinal striations,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

diam. posterior to the junction. Hemizonid not clearly observed. Lateral glands, small pores connected to secretory cell, present and observed on the lateral body surface, with positions inconsistent among individuals, numbering 5 to 8 for males and 9 to 13 for females.

Stenostomatous form

Cheilostom consisting of six per- and interradi-al 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 form small flap. Gymnostom short, cuticular ring-like anterior end overlapping cheilostom internally. Dorsal gymnostomatal wall slightly thickened compared with ventral side. Stegostom separated into three subsections: pro-meso, meta, and telostegostom. Pro-meso stegostom forming weakly cuticularized ring surrounding the anterior edge of pharynx. Metastegostom bearing: conspicuous and movable triangular or flint-shaped dorsal tooth with strongly sclerotized surface giving an

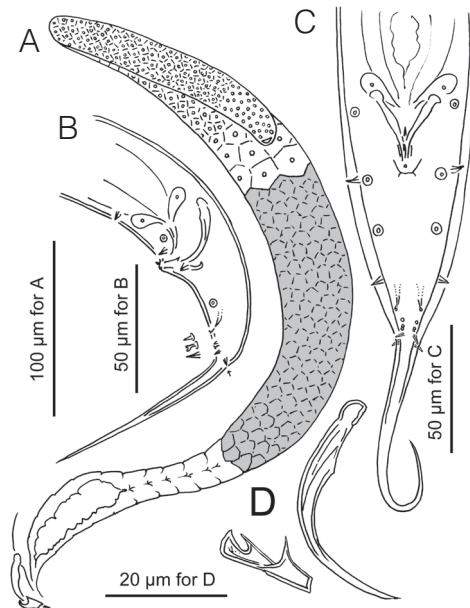


Figure 3: Adult male of *Pristionchus sikae* sp. n. Drawings are of live specimens (non-types) from temporary mounts. A, Gonadal system in right lateral view. B, Tail region in left lateral view with schematic drawings of distal ventral triplet papillae. C, Tail region in ventral view. D, Spicule and gubernaculum in right lateral view.

muscular. Basal bulb glandular. Pharyngo-intestinal junction clearly observed, well developed. Nerve ring usually surrounding middle region of isthmus. Excretory pore not conspicuous, ventrally located at level of isthmus to pharyngo-intestinal junction, excretory duct extending anterior and reflexed back to position of pore. Deirid observed laterally, located from slightly anterior to pharyngo-intestinal junction to a half body

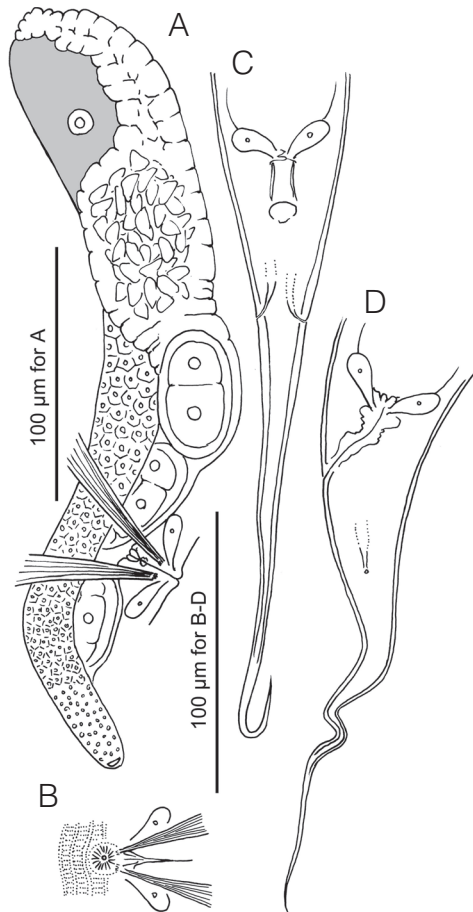


Figure 4: Adult female of *Pristionchus sikae* sp. n. Drawings are of live specimens (non-types) from temporary mounts. A, Gonadal system in right lateral view. B, Vulval region in ventral view. C, Tail region in ventral view. D, Tail region in left lateral view.

appearance as inverted V-shape in light microscopic observation in lateral view; pointed left subventral ridge with 3 min adventitious denticles on a plate; pointed right subventral ridge, often with distinct distal adventitious denticle. Telostegostom forming a weakly sclerotized cup-like cavity connecting stoma and pharynx.

Eurystomatous form

Cheilostom divided into six distinctive per- and inter-radial plates. Anterior end of each plate rounded and elongated to project from stomatal opening, forming small flap. Gymnostom with thick cuticle, forming a short, ring-like tube being thicker posteriorly. Anteri-

or end of gymnostom internally overlapping posterior end of cheilostomatal plates. Stegostom separated into three subsections: pro-meso, meta, and telostegostom. Pro-meso stegostom forming weakly cuticularized ring surrounding the anterior edge of pharynx. Metastegostom bearing: large claw-like dorsal tooth; large claw-like right subventral tooth; ridge of left subventral denticles or cusps of varying numbers and size, i.e., three large cusps, the tip of two lateral cusps sometimes split into two small tips. Dorsal and right subventral teeth movable. No movement observed for left subventral denticles. Telostegostom forming a weakly sclerotized cup-like cavity connecting stoma and pharynx.

Male

Ventrally arcuate, strongly ventrally curved at tail region when killed by heat. Testis single, ventrally located, anterior part reflexed to right side. Spermatogonia arranged in three to five rows in reflexed part, then well-developed spermatocytes arranged as three to four rows in anterior two-thirds of main branch, then mature amoeboid spermatids arranged in multiple rows in remaining, proximal part of gonad. *Vas deferens* not clearly separated from other parts of gonad. Three (two subventral and one dorsal) cloacal gland cells observed at distal end of testis and intestine. Spicules paired, separate. Spicules smoothly curved in ventral view, adjacent to each other for distal third of their length, each smoothly tapering to pointed distal end. Spicule in lateral view smoothly ventrally arcuate, giving spicule about 100° curvature, oval manubrium present at anterior end, lamina/calomus complex (blade) clearly expanded slightly posterior to manubrium (ca 1/4 of blade length from anterior), then smoothly tapering to pointed distal end. Gubernaculum conspicuous, about one-third of spicule length, broad anteriorly such that dorsal wall is slightly recurved and that dorsal and ventral walls separate at 50 to 60° angle at posterior end. Dorsal side of gubernaculum possessing single, membranous, anteriorly directed process, and lateral pair of more sclerotized, anteriorly, and obliquely ventrally directed processes. In lateral view, anterior half of gubernaculum with two serial curves separated by anteriorly and obliquely ventrally directed process, with anterior terminal curvature highly concave and almost closed, and with deep posterior curvature being one-third of gubernaculum length; posterior half forming tube-like process enveloping spicules. Thick cuticle around tail region, falsely appearing as a narrow leptoderan bursa in ventral view. Cloacal opening (CO) slit-like in ventral

Table 1. Morphometrics of *Pristionchus sikae* sp. n. and *Pristionchus kurosawai* sp. n.

Character	<i>P. kurosawai</i> RS5914 stenostomatous male	stenostomatous female	<i>P. sikae</i> RS5901 stenostomatous male	stenostomatous female
<i>n</i>	10	10	10	10
<i>L</i>	998±133 (828–1285)	1460±234 (1097–1831)	619±81.9 (533–813)	1003±70.6 (867–1121)
<i>L'</i>	861±127 (710–1128)	1186±175.2 (915–1454)	514±75.7 (434–708)	809±50.8 (699–895)
<i>A</i>	15±1.8 (12–17)	14±1.7 (12–17)	13±1.9 (10–15)	12±1 (11–14)
<i>B</i>	6.3±0.6 (5.1–7.0)	9±1.4 (7.5–11.7)	4.6±0.5 (3.8–5.4)	6±0.5 (5.2–6.6)
<i>C</i>	7.4±1.2 (4.9–8.9)	5.6±1.0 (3.5–7.1)	6.1±1 (4.6–7.7)	5.2±0.6 (4.4–6.2)
<i>c'</i>	3.4±0.9 (2.2–5.0)	6.1±2.3 (3.9–11.4)	3.7±0.5 (2.4–4.4)	6±0.7 (4.7–7.2)
<i>T</i> or <i>V</i>	60±5.2 (51–68)	46±3 (41–50)	51±5 (45–60)	46±2.6 (42–50)
Maximum body diam.	69±8.1 (54–79)	105±20.6 (76–134)	50±8.1 (40–64)	82±6.7 (72–93)
Stoma length	9.5±1.2 (8.1–12.1)	11.3±1.4 (9.8–13.6)	10±0.8 (9.1–11.6)	11.3±0.9 (10.3–13.1)
Stoma diam.	5.9±0.6 (5.3–7.0)	7.2±0.7 (6.0–8.6)	5.4±0.8 (4.5–6.8)	7.3±0.7 (6.3–8.2)
Pharynx length (head to base of pharynx)	149±12.4 (130–174)	154±14.1 (134–176)	126±10.6 (111–139)	157±7.8 (145–169)
Anterior pharynx (pro- + metacarpus)	88±7.2 (75–100)	92±9.4 (83–108)	78±6.4 (66–85)	98±5.6 (89–107)
Posterior pharynx (isthmus + basal bulb)	62±5.7 (55–74)	62±6.6 (47–68)	48±5.7 (37–56)	60±5.1 (55–70)
Ant/total pharynx %	59±1.2 (57–61)	60±2.5 (56–65)	62±2.5 (58–67)	62±2.3 (59–65)
Median bulb diam.	24±2.1 (21–28)	29±3.7 (24–35)	21±3.4 (17–26)	31±2.3 (28–35)
Terminal bulb diam.	26±3.5 (21–33)	29±3.9 (24–36)	21±3.4 (18–26)	30±2.2 (25–32)
Testis length	601±115.3 (450–799)	–	317±65.3 (250–485)	–
Ant. end to vulva	–	666±122 (542–795)	–	460±41.1 (372–510)
Vulva to anus distance	–	548±74.6 (433–683)	–	340±22.9 (309–372)
Cloacal or anal body diam.	42±10.9 (28–70)	46±8.9 (29–59)	29±4.6 (21–38)	32±2.4 (28–36)
Tail length	136±24.5 (111–186)	274±97.2 (182–525)	104±20.2 (78–142)	194±30.3 (155–237)
Spicule length (curve)	44±2.7 (40–49)	–	40±3.8 (33–44)	–
Spicule length (chord)	36±2.1 (33–39)	–	32±3.3 (27–37)	–
Gubernaculum length	16±1.6 (14–18)	–	13±1 (12–15)	–

view. One small, ventral, single genital papilla (vs) on anterior cloacal lip. Nine pairs of genital papillae (v1–v7, ad, pd) and a pair of phasmids (ph) present with an arrangement <v1, (v2d, v3) co, v4, ad, ph, (v5,

v6, v7), pd> in nomenclature of Sudhaus and Fürst von Lieven (2003), where v1 located at ca 1 cloacal body diam. (CBD) anterior to co; v2d and v3 close to each other and located at slightly anterior to co; v4 at

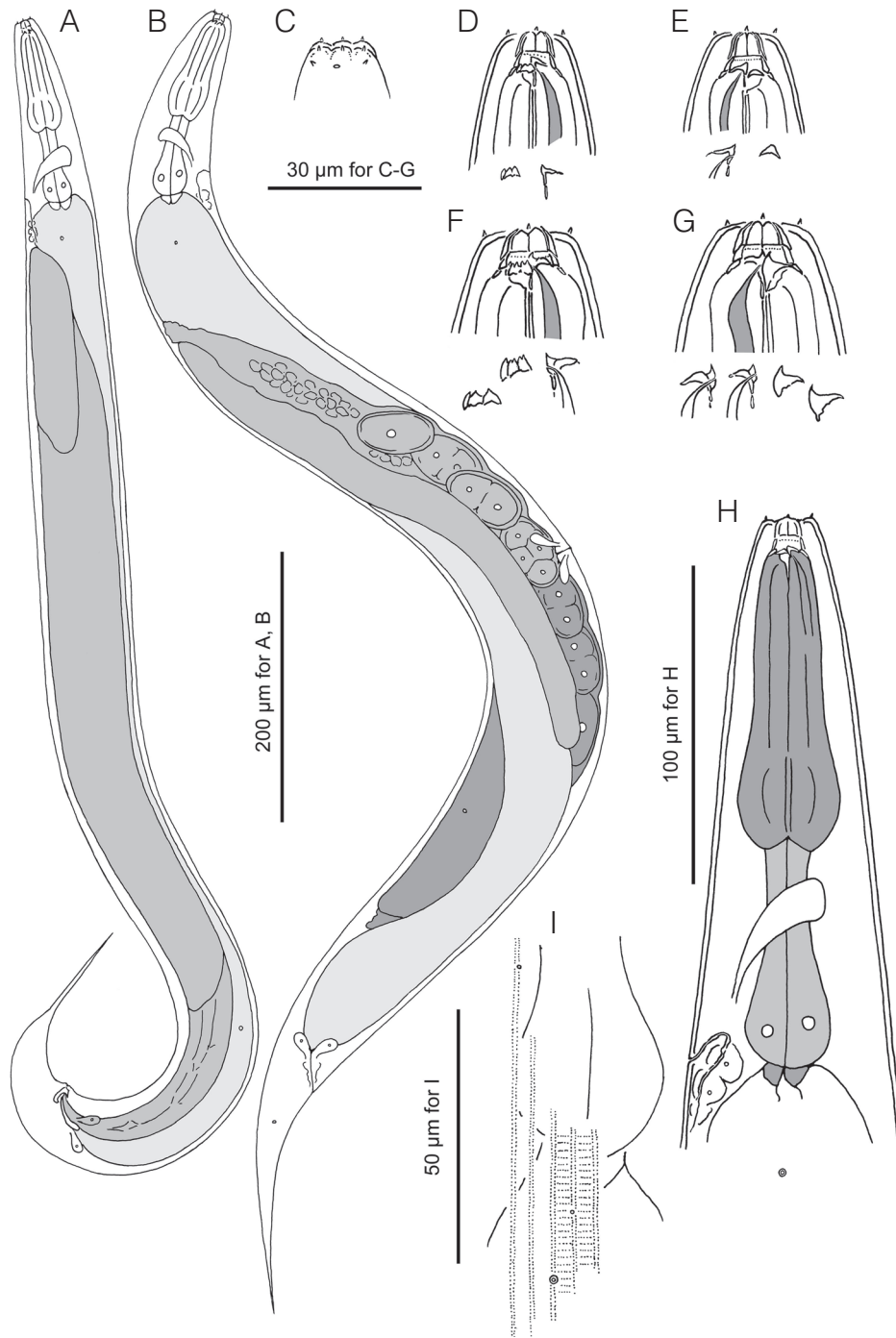


Figure 5: Male *Pristionchus kurosawai* sp. n. Drawings are of live specimens (non-types) from temporary mounts. A, Stenostomatous male in left lateral view. B, Stenostomatous female in right lateral view. C, Anterior end of stenostomatous male in left lateral view. D, Stomatal region of stenostomatous male in left lateral view; below (from left to right) are left subventral ridge and dorsal tooth. E, Stomatal region of stenostomatous male in right lateral view; below are dorsal tooth and right subventral denticle. F, Stomatal region of eurystomatous female in left lateral view; below are left subventral ridge and dorsal tooth. G, Stomatal region of eurystomatous female in right lateral view; below are dorsal tooth and right subventral tooth. H, Anterior region of stenostomatous female in left lateral view. I, Body surface showing longitudinal striations, annulations and relative position of deirid and lateral glands.

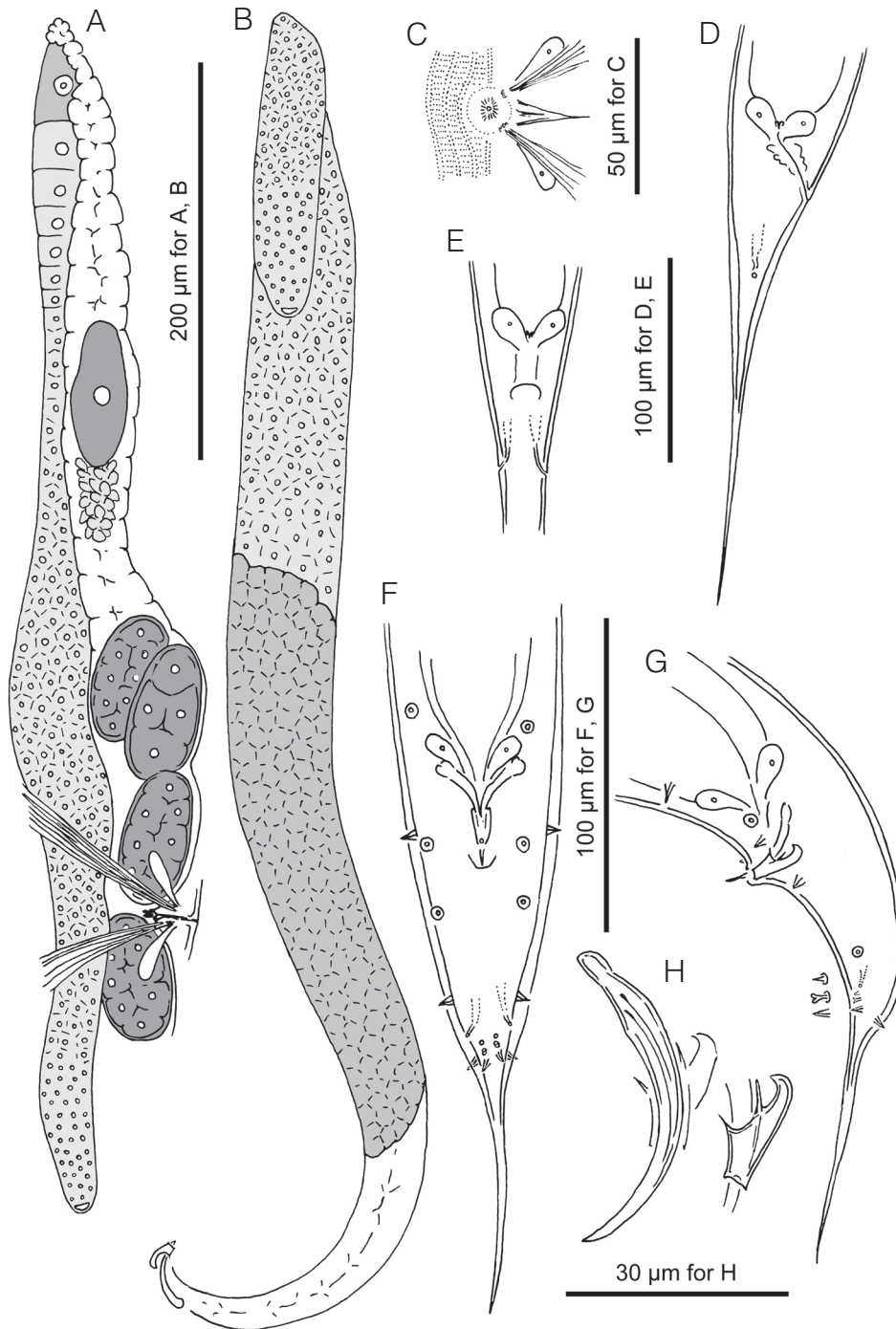


Figure 6: Female *Pristionchus kurosawai* sp. n. Drawings are of live specimens (non-types) from temporary mounts. A, Female gonadal system in right lateral view. B, Male gonadal system in left lateral view. C, Vulval region in ventral view. D, female tail region in left lateral view. E, Female tail region in ventral view. F, Male tail region in ventral view. G, Male tail region in left lateral view with schematic drawings of distal ventral triplet papillae. H, Spicule and gubernaculum in left lateral view.

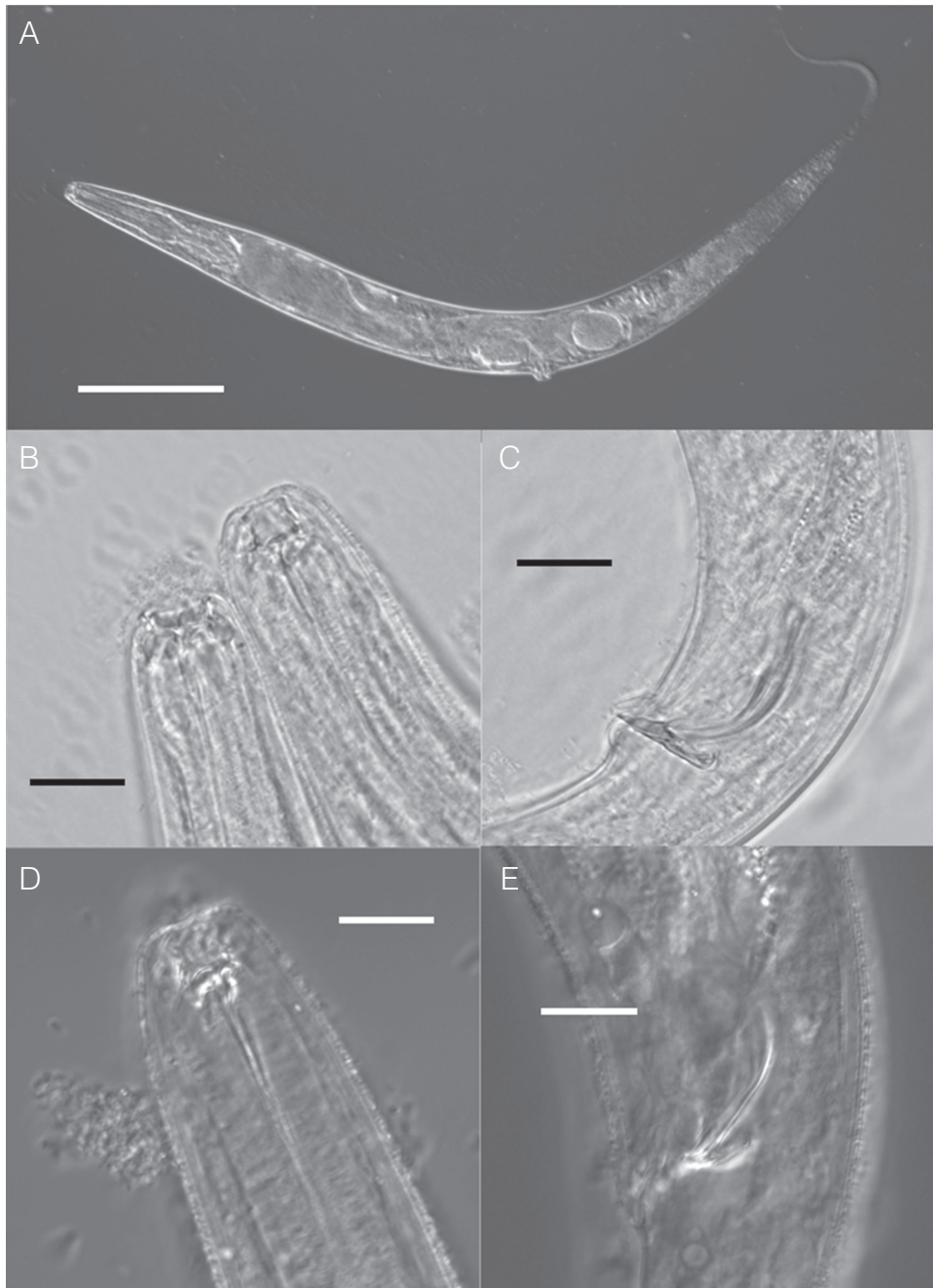


Figure 7: Nomarski Light Microscopy pictures of alive nematodes. A, Female *Pristionchus sikae* sp. n., total; B, Eurystomatous morph female, left & stenostomatous morph male, right, mouth region and C, male tail; D, *Pristionchus kurosawai* sp. n. mouth region, stenostomatous morph and E, male tail. Scale = 100 μ m in A, 10 μ m in B–E.

less than 1 CBD posterior to co; laterally located ad at the midway between co and the root of tail spike; subventrally located ph inbetween ad and v5; ventral v5-v7 forming triplet just anterior to the root of tail spike; and subdorsal pd between v7 and the root of tail spike. Anterior five pairs of papillae (v1, v2d, v3,

v4, and ad) almost equal in size, rather large and conspicuous, v7 and pd papillae obviously smaller than anterior five pairs, v5 and v6 very small, sometimes difficult to observe with light microscope. Anterior two pairs of the ventral triplet papillae (v5 and v6) papilliform and borne from socket-like base, v7 sim-

ple or typical thorn-like in shape. Tip of v5 papillae split into two small papilla-like projections. Tail conical, with long spike, about two to three cloacal body diam. 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 three to four rows in distal two-thirds of ovary and in double or single row in rest of ovary, distal tips of each ovary reaching oviduct of opposite gonad branch. Anterior end of oviduct (=junction tissue between ovary and oviduct) consists of rounded cells. Anterior part of oviduct consist of rounded cells and forming simple tube. Middle part of oviduct serving as spermatheca, consists of roundish and relatively large cells. Eggs in single to multiple-cell stage or even further developed at posterior part of oviduct (=uterus), which in young female being composed of squared or angular cells, long enough to contain one well developed oocyte. *Receptaculum seminis* not observed, i.e., the organ is not independent, and a part of oviduct/uterus works as the organ. 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 diam. long, intestine/rectum junction surrounded by well-developed sphincter muscle. Three anal glands (two subventral and one dorsal) present but not obvious. Anus in form of dome-shaped slit, posterior anal lip slightly protuberant. Phasmid about one to two anal body diam. posterior to anus. Tail long, smoothly tapered, distal end variable from filiform to long, and conical.

Type material, type strain, and nomenclatural registration

***Pristionchus sikae* sp. n.**

One slide holotype male (28512); two slides, each with paratypes one male and one female (28513&28514), deposited in the University of California Riverside Nematode Collection (UCRNC), CA. Two slides, each with with paratypes one male and one female (SMNHType-8995& 8996) deposited in the Swedish

Natural History Museum, Stockholm, Sweden. Two slides, each with paratypes one male and one female (SMNK-NEMA-T-0131&0132) deposited in the Natural History Museum Karlsruhe, Germany. Available in living culture and as frozen stock under culture code RS5901 in the Department of Evolutionary Biology, Max Planck Institute (MPI) for Developmental Biology, Tübingen, Germany, and can be provided to other researchers upon request. The new species binomial has been registered in the ZooBank database (zoobank.org) under the identifier. [urn:lsid:zoobank.org:act:0691C386-5A9C-4775-8BFD-3CCD9A1D545A].

***Pristionchus kurosawai* sp. n.**

One slide holotype male (28515); two slides, each with with paratypes one male and one female (28516&28517), deposited in the University of California Riverside Nematode Collection (UCRNC), CA. Two slides, each with with paratypes one male and one female (SMNHType-8997&8998) deposited in the Swedish Natural History Museum, Stockholm, Sweden. Two slides, each with paratypes one male and one female (SMNK-NEMA-T-0133&0134) deposited in the Natural History Museum Karlsruhe, Germany. Available in living culture and as frozen stock under culture code RS5914 in the Department of Evolutionary Biology, Max Planck Institute (MPI) for Developmental Biology, Tübingen, Germany, and can be provided to other researchers upon request. The new species binomial has been registered in the ZooBank database (zoobank.org) under the identifier. [urn:lsid:zoobank.org:act:22EC0C69-5BDE-4E92-B462-0E08DD504D74].

Diagnosis and relationship

Both new species are typologically identical, and characterized by the arrangement of male genital papillae, <v1, (v2d, v3) co, v4, ad, ph, (v5, v6, v7), pd>, and stomatal morphology of males and females, where stenostomatous form has conspicuous and movable triangular or flint-shaped dorsal tooth, pointed left subventral ridge with 3 min denticles and pointed right subventral ridge, and eurystomatous form has movable claw-like dorsal and right subventral teeth and left subventral denticles or cusps of varying numbers and size.

The new species and the previously known *pacificus* species-complex members *P. pacificus*, *P. exspectatus*, *P. arcanus*, *P. taiwanensis*, and *P. occultus*, form a cryptic species complex sharing the key characters, i.e., stomatal morphology and male tail characters (Kanzaki et al., 2012; Herrmann et al., 2016), and

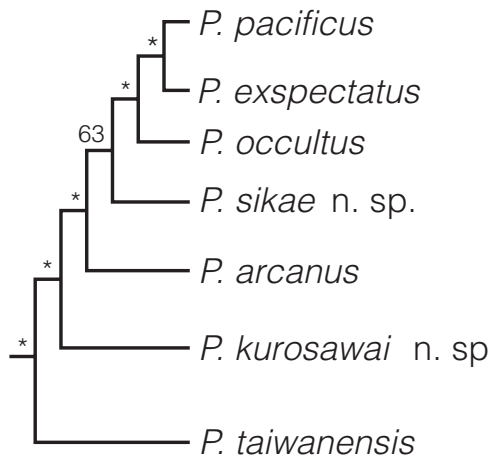


Figure 8: Phylogenetic relationship of the seven species of the '*pacificus* species-complex sensu stricto'. The schematic phylogeny shows a subtree of a phylogeny that was generated from transcriptome data of all cultivable *Pristionchus* species (Rödelsperger et al., 2018). Inner nodes are labeled with bootstrap support values and stars indicate full bootstrap support (100 replicates).

the morphometric values showing large intraspecific variation overlapping among species as demonstrated previously (Herrmann et al., 2006a, 2016; Kanzaki et al., 2012, 2013; Ragsdale et al., 2015). Therefore, these species are not distinguishable among each other solely by typological characters. However, both new species and previously described *P. pacificus* species complex can be distinguished from each other by the molecular phylogenetic characters, e.g., barcoding sequences, and biological characters, e.g., reproductive mode where *P. pacificus* and others are hermaphrodite and gonochoristic, respectively, and mating experiments.

Molecular characterization and phylogenetic analysis

The phylogenetic positions of the new species were determined by phylogenetic analysis of transcriptomes from all cultivable *Pristionchus* species (Rödelsperger et al., 2018). The resulting phylogeny (Fig. 8) shows a ladder-like structure and places *P. sikae* sp. n. and *P. kurosawai* sp. n. into a monophyletic subclade of *Pristionchus* species, all of which can produce F1 hybrids.

Discussion

The current study with the description of *P. sikae* sp. n. and *P. kurosawai* sp. n. from Taiwan further supports the notion of East Asia and particularly Taiwan as hotspot for speciation processes in the genus *Pristionchus*. This work allows two major conclusions. First, our new findings suggest that a group of at least seven extremely closely related *Pristionchus* species exists that is characterized by their inability to form F2 hybrids while viable F1 hybrids are readily observed. At the same time, LSU or other single molecular markers are unable to properly designate species in this group and only genome-wide sequence analysis allows the proper separation of these species. We therefore, designate this group of species as '*pacificus* species-complex sensu stricto' to clearly separate them from *P. japonicus*, *P. maxplancki*, and *P. quartusdecimus*, all of which can be diagnosed by the 1.6 kb fragment of the LSU or other molecular markers and none of which forms F1 hybrids. Future, more comprehensive studies in mainland East Asia including China will have to show how large the '*pacificus* species-complex sensu stricto' really is. The accompanying publication provides a first indication that this group of species is still incomplete and awaits future analysis (Herrmann et al., 2018). While technically and logistically challenging, we are optimistic that such samplings efforts will result in a comprehensive coverage of the northern part of East Asia in the years to come. We consider these sampling efforts a major contribution to obtain a systematic and comprehensive overview of the biodiversity of a single taxon, a challenging effort for most members of the meiofauna.

Second, the features characterizing members of the '*pacificus* species-complex sensu stricto' are unknown from species of other *Pristionchus* species-complexes and other nematodes. These observations suggest that East Asia and the '*pacificus* species-complex sensu stricto' represent a hotspot of speciation and diversification processes. Current and future sampling may therefore represent the basis for a molecular analysis of speciation processes, including the mating type transition from ancestral gonochorism to hermaphroditism as observed in *P. pacificus*. Consistent with both conclusions of this study, the additional seven *Pristionchus* species to be described in the accompanying publications do not show such extremely close phylogenetic relationships. All these species can readily be distinguished by their inability to hybridize and

utility to use single variable sequence markers for species designation. Together, these findings suggest a different tempo and mode of species processes in different *Pristionchus* species-complexes or alternatively but less likely, extremely different sampling biases in different groups of *Pristionchus* nematodes.

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