

Characterisation of, and entomopathogenic studies on, *Pristionchus aerivorus* (Cobb in Merrill & Ford, 1916) Chitwood, 1937 (Rhabditida: Diplogastridae) from North Carolina, USA

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Summary – During a survey of entomopathogenic nematodes in North Carolina, USA, a *Pristionchus* species was recovered using the *Galleria* bait method. Morphological studies with light microscopy and scanning electron microscopy, mating tests with reference strains, as well as molecular analyses of the near-full-length small subunit rRNA gene (18S) and D2-D3 expansion segments of the large subunit rRNA gene (28S) identified this isolate as *Pristionchus aerivorus*. Exposed *Galleria* larvae were killed within 48 h and high numbers of nematodes were recovered from the cadavers about 5 days later. Preliminary tests revealed that this nematode is capable of infecting at least two other insect species (*Helicoverpa zea* and *Tenebrio molitor*) under laboratory conditions. The status of the genus *Chroniodiplogaster* is discussed and confirmed as a junior synonym of *Pristionchus* based on morphological observation and molecular phylogenetic analysis.

Keywords – *Chroniodiplogaster*, description, DNA sequencing, molecular, morphology, phylogeny, SEM, taxonomy.

Pristionchus Kreis, 1932 belongs to the family Diplogastridae Micoletzky, 1922. These necromenic nematodes live in close association with beetles (Merrill & Ford, 1916; Herrmann *et al.*, 2006a, b, 2007, 2010; Kanzaki *et al.*, 2011, 2012a, b, 2013; Ragsdale *et al.*, 2015). At the beginning of this association, non-feeding dauers actively invade the beetle host and remain arrested in this stage until death of the host when they resume development within the cadaver. *Pristionchus* nematodes uses beetles as a refuge and as a means of transport, feeding on the fungi, bacteria and other nematodes growing in the body after the host has died (Dieterich *et al.*, 2008). In the last decade, a few species other than *Caenorhabditis elegans* have been developed as model systems for more detailed genetic and molecular studies. One such model species is *Pristionchus pacificus* Sommer, Carta, Kim & Sternberg, 1996 whose whole genome sequence was completed in

2008 (Dieterich *et al.*, 2008). Thus, the taxonomy of *Pristionchus* has received much attention. Study of the species in this genus from different geographic location expands the knowledge to the model species in terms of the morphology, biology and molecular phylogeny. In a review of the family Diplogastridae, Sudhaus & Fürst von Lieven (2003) recognised 27 valid species of *Pristionchus*, but the number has now increased to 49 nominal species (Ragsdale *et al.*, 2015).

Although most *Pristionchus* nematodes are considered as necromenic species phoretically associated with the insects, some are suspected to be weak pathogens or parasites of their insect host. Several reports have suggested that *P. aerivorus* (Cobb in Merrill & Ford, 1916) Chitwood, 1937 can cause lethal infections of insects. In 1916, Cobb (Cobb in Merrill & Ford, 1916) described *Diplogaster aerivora* (= *Pristionchus aerivorus*), which was

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found in the heads of dead termites (*Reticulitermes lucifugus* Rossi) from Kansas, USA, their death suspected to be caused by the high number of infecting nematodes. Davis (1919) found *P. aerivorus* in dead and dying larvae of a beetle (*Phyllophaga* sp.) and suggested that the nematodes were the cause of death. Banks & Snyder (1920) reported juveniles of *P. aerivorus* in the heads of active, normal-appearing *R. flavipes* Kollar whilst adult nematodes were found in sick and dead termites. *Pristionchus aerivorus* was responsible for the natural mortality of corn earworm larvae (*Heliothis obsoleta* Fab.) (Winburn & Painter, 1932) and the June beetle (*Phyllophaga anaxia* LeConte) (Lim *et al.*, 1981).

Poinar proposed a new genus, *Chroniodiplogaster* Poinar, 1990, and designated *P. aerivorus* as type. The dauer stages of this species occurred in the heads of worker termites of *R. tibialis* Banks in Colorado, USA (Poinar, 1990). The nematodes could occasionally enter the body cavity and kill the termites (Poinar, 1990). In a review paper by Sudhaus & Fürst von Lieven (2003), *Chroniodiplogaster* was considered as a junior synonym of *Pristionchus*.

Pristionchus aerivorus was isolated from June beetle larvae (*Phyllophaga* spp.) in southern Quebec, Canada (Lim *et al.*, 1981; Poprawski & Yule, 1991) and Steinkraus *et al.* (1993) found 1.25% of corn earworm pupae (Lepidoptera: *Helicoverpa zea* (Boddie)) were naturally infected with *P. aerivorus* in sandy soil in a corn field in Arkansas, USA. Wang *et al.* (2002) recovered this nematode from sluggish *R. lucifugus* Rossi from Mississippi, USA. In a survey of *Pristionchus* in the USA, *P. aerivorus* was found on scarab beetles in Massachusetts, New York, Ohio, Texas and Nebraska (Herrmann *et al.*, 2006b). So far, *P. aerivorus* has only been reported from the northern and southern states of the USA and Canada in association with insects, but it is considered as a weak parasite compared to entomopathogenic nematodes such as *Steinernema carpocapsae* and *Heterorhabditis bacteriophora* (6.25, 80 and 90% mean mortality, respectively) as judged from a laboratory bioassay (Steinkraus *et al.*, 1993). However, little is known about the occurrence of *P. aerivorus* in the south-eastern USA.

Within *Pristionchus*, many species cannot be identified by morphological methods, although this can be accomplished through DNA sequencing and by mating with reference strains to distinguish the different species (Herrmann *et al.*, 2006b; Kanzaki *et al.*, 2012b; Ragsdale *et al.*, 2013). In an effort to search for the presence of local entomopathogenic nematodes in North Carolina, high

numbers of a *Pristionchus* species were recovered using the insect bait method. In this study, we characterise this species based on morphological studies with light microscopy and scanning electron microscopy, molecular analyses of the near-full-length small subunit rRNA gene (18S) and D2-D3 expansion segments of the large subunit rRNA gene (28S), and mating studies with three reference species. In addition, its entomopathogenicity was tested under laboratory conditions.

Materials and methods

INSECT AND NEMATODE CULTURES

Insect- and nematode-rearing procedures followed previously published methodology (Torres-Barragan *et al.*, 2012). All insects were reared in the Arthropod Ecology Laboratory, Department of Entomology, North Carolina State University (NCSSU), using artificial or natural diets specific to each species (King & Leppla, 1984). The soil samples were collected from experimental plots under natural succession management at the Center for Environmental Farming Systems (CEFS, Goldsboro, NC); five *Galleria* 5th instars were exposed to 500 cm³ of soil (Meyling, 2007) collected from CEFS fields and brought into the laboratory for entomopathogen incidence assessment. Larval mortality was checked daily and dead larvae were surface-sterilised by immersing in 1% NaClO for 1 min, followed by two rinses in double-distilled water. Dead larvae were placed on White (1927) traps with an inverted 9 cm Petri dish lined with three layers of moistened filter paper (Whatman No. 1) in a 15 cm Petri dish filled with double-distilled water (Fig. 1A). Cadavers were maintained in darkness at room temperature and examined daily for nematode emergence. After a period of incubation, a *Pristionchus* species designated as strain S4B1 was recovered by dissection from one of several infected insects. The nematode has been subsequently maintained through periodic re-infections of *Tenebrio molitor* L. (Coleoptera: Tenebrionidae) to produce fresh nematodes for these experiments (Fig. 1A, B). Ten newly-moulted, unsclerotised, 6th instar *T. molitor* larvae were exposed to infection by 500 dauer juvenile nematodes. Cadavers of infected insects were then surface-sterilised by submersing in 1% NaClO for 1 min, followed by two rinses in double-distilled water. Insects were subsequently transferred to White traps (Glazer & Lewis, 2000) to facilitate nematode harvesting. Dauer juvenile nematodes were screened through a 500-mesh sieve, washed with 1%

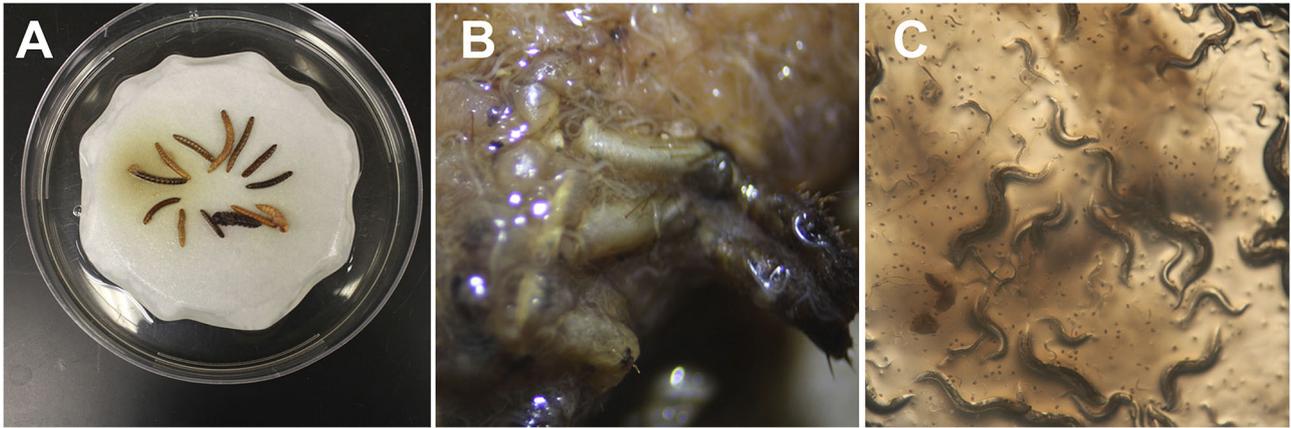


Fig. 1. Baiting and mating of *Pristionchus aerivorus*. A: *Tenebrio molitor* beetle larva cadaver in White trap for rearing nematodes; B: *Pristionchus aerivorus* multiplying in *T. molitor* larva cadaver; C: Progenies (females, males, juveniles and eggs) in a culture plate from a mating study of S4B1 from Raleigh, NC, USA, with RS5106 of *P. aerivorus* from Germany. This figure is published in colour in the online edition of this journal, which can be accessed via <http://booksandjournals.brillonline.com/content/journals/15685411>.

NaClO, suspended in double-distilled water and counted. Only newly-emerged dauer juvenile nematodes (<48 h) were used for entomopathogenicity assays. Nematodes recovered from *T. molitor* larvae were transferred to LB broth 1% agar in a 6 cm diam. Petri dish and incubated at room temperature (ca 20°C). The nematodes were sub-cultured approximately every 10 days.

MORPHOLOGICAL OBSERVATION

Nematodes were heat-killed and fixed in 4% formalin. The nematodes were processed to glycerin by a modification of a glycerin-ethanol series of Seinhorst's rapid method (1959) and permanently mounted on 25 × 75 mm microscope slides. Live nematodes were heat-killed in temporary water mounts for all measurements and microphotographs to assure quality and accuracy. Specimens were examined with a Leica DM2500 or Zeiss Imager A2 compound microscope with interference contrast at up to 1000× magnification. Drawings and measurements were made using a drawing tube. Stoma terminology by De Ley *et al.* (1995) and Sudhaus & Fitch (2001) was employed for description of the stoma parts. Spicules were measured from distal to proximal tip in a curved median line.

SCANNING ELECTRON MICROSCOPY

Nematodes in distilled water were transferred by pipette to modified BEEM capsules whose ends were cut open and fitted with 20 μm nylon mesh to hold the specimens through the entire preparation procedure (Bozzola

& Russell, 1999). The capped capsules were then transferred to a jar containing 3% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.2, at 4°C. Samples were washed in three changes of the same buffer, post-fixed in 2% osmium tetroxide in 0.1 M sodium cacodylate buffer, pH 7.2, for 16 h at 4°C in the dark in a refrigerator, washed in three more changes of the same buffer and then dehydrated through a graded ethanol series to 100%, 30 min per change, all on ice. Samples were allowed to equalise to room temperature and given two changes of 100% ethanol. Capsules containing the samples were then critical point-dried in liquid CO₂ (Samdri-795, Tousimis Research Corp.), mounted on aluminium stubs with double-stick tape, sputter-coated with 5 nm of gold-palladium (Hummer 6.2, Anatech USA) and viewed using a JEOL JSM-5900LV (JEOL USA) at 10 kV at the Center for Electron Microscopy, North Carolina State University.

MOLECULAR ANALYSES

Nematodes collected from *T. molitor* beetle larvae were used for the molecular study. Nematodes were picked into distilled water and their morphological identity was confirmed with light microscopy before being placed into 10 μl AE buffer (10 mM Tris-Cl, 0.5 mM EDTA; pH 9.0) on a glass microscope slide, macerated with a pipette tip and collected in final volume of 50 μl AE buffer. DNA samples were stored at -20°C until used as a PCR template. Primers for DNA amplification, PCR and DNA sequencing were the same as described in Ye *et al.* (2010).

Sequences were deposited in GenBank. DNA sequences were aligned by Clustal W (<http://workbench.sdsc.edu>, Bioinformatics and Computational Biology group, Department of Bioengineering, UC San Diego, San Diego, CA, USA). The molecular sequences of *P. aerivorus* were compared with those of the other nematode species available at the GenBank sequence database using the BLAST homology search program.

The model of base substitution was evaluated using Modeltest (Posada & Crandall, 1998; Huelsenbeck & Ronquist, 2001). The Akaike-supported model, the base frequencies, the proportion of invariable sites and the gamma distribution shape parameters and substitution rates were used in phylogenetic analyses. Bayesian analysis was performed to confirm the tree topology for each gene separately using MrBayes 3.1.0 (Huelsenbeck & Ronquist, 2001) running the chain for 1×10^6 generations and setting the 'burnin' at 1000. We used the Markov chain Monte Carlo (MCMC) method within a Bayesian framework to estimate the posterior probabilities of the phylogenetic trees (Larget & Simon, 1999) using 50% majority rule.

HYBRIDISATION TEST

Hybrid crosses were performed between S4B1 and three closely related species as informed by our phylogenetic analyses, namely *P. aerivorus* (RS5106), *P. pseudoaerivorus* Herrmann, Mayer & Sommer, 2006 (RS5139), and *P. americanus* Herrmann, Mayer & Sommer, 2006 (RS5140), strains of which were provided by the Max Planck Institute for Developmental Biology, Department of Evolutionary Biology, Germany. Juveniles of each strain were individually transferred to LB broth 1% agar in a 6 cm diam. Petri dish and incubated at room temperature (*ca* 20°C) until the nematodes moulted to the adult stage. Each cross consisted of one female of one strain and one male of another strain. Crosses were performed reciprocally and in at least five replicates for each direction. As a control, conspecific crosses were performed between one male and one female placed under the same conditions as for interspecies crosses. Crosses were checked daily and the F1 generation was subcultured and the presence of F2 generation was confirmed. Then, to confirm reproductive compatibility, the successfully hybridised strain was kept as laboratory culture for at least 2 months, *i.e.*, through at least the F2 generation (Fig. 1C).

NEMATODE ENTOMOPATHOGENICITY

Entomopathogenicity assays were performed following methodology described in Torres-Barragan *et al.* (2012). Nematode entomopathogenicity was evaluated using 4th instar *H. zea* and 6th instar *T. molitor*. Insect species and stages used for this study were selected based on their availability and ease of manipulation for experimental procedures.

Petri dishes lined with two layers of filter paper (Whatman No. 1) containing the test insects were used as experimental arenas and each insect species was infected separately. *Helicoverpa zea* larvae were placed individually in 6 cm diam. Petri dishes because of their cannibalistic nature. Five *T. molitor* larvae were exposed collectively in 10 cm diam. Petri dishes. Suspensions of IJ were prepared (Glazer & Lewis, 2000) and experimental units were inoculated with 500 IJ per larva, dispensed in 0.5 ml into small single insect arenas and 1 ml into large collective insect arenas. Insects were kept at 25°C in total darkness and mortality and time to death was recorded daily. The experiment was run in five trials (different dates) of five insects each for a total of 20 insects per species. After the insects died, larvae were rinsed with double-distilled sterile water and transferred to White traps, time to nematode emergence being recorded for each insect.

Results

***Pristionchus aerivorus* (Cobb in Merrill & Ford, 1916) Chitwood, 1937** (Figs 2-4)

MEASUREMENTS

See Table 1.

DESCRIPTION

General

Body ventrally arcuate or straight when heat-killed. Tail region strongly recurved ventrally in male. Cuticle smooth, finely annulated transversely. Cuticle traversed by *ca* 24 clear longitudinal striae, lateral lines indistinguishable even in SEM. Six separate lips, head not set off, continuous with the body contour, six papilliform labial sensilla, one on each lip, four papilliform cephalic sensilla

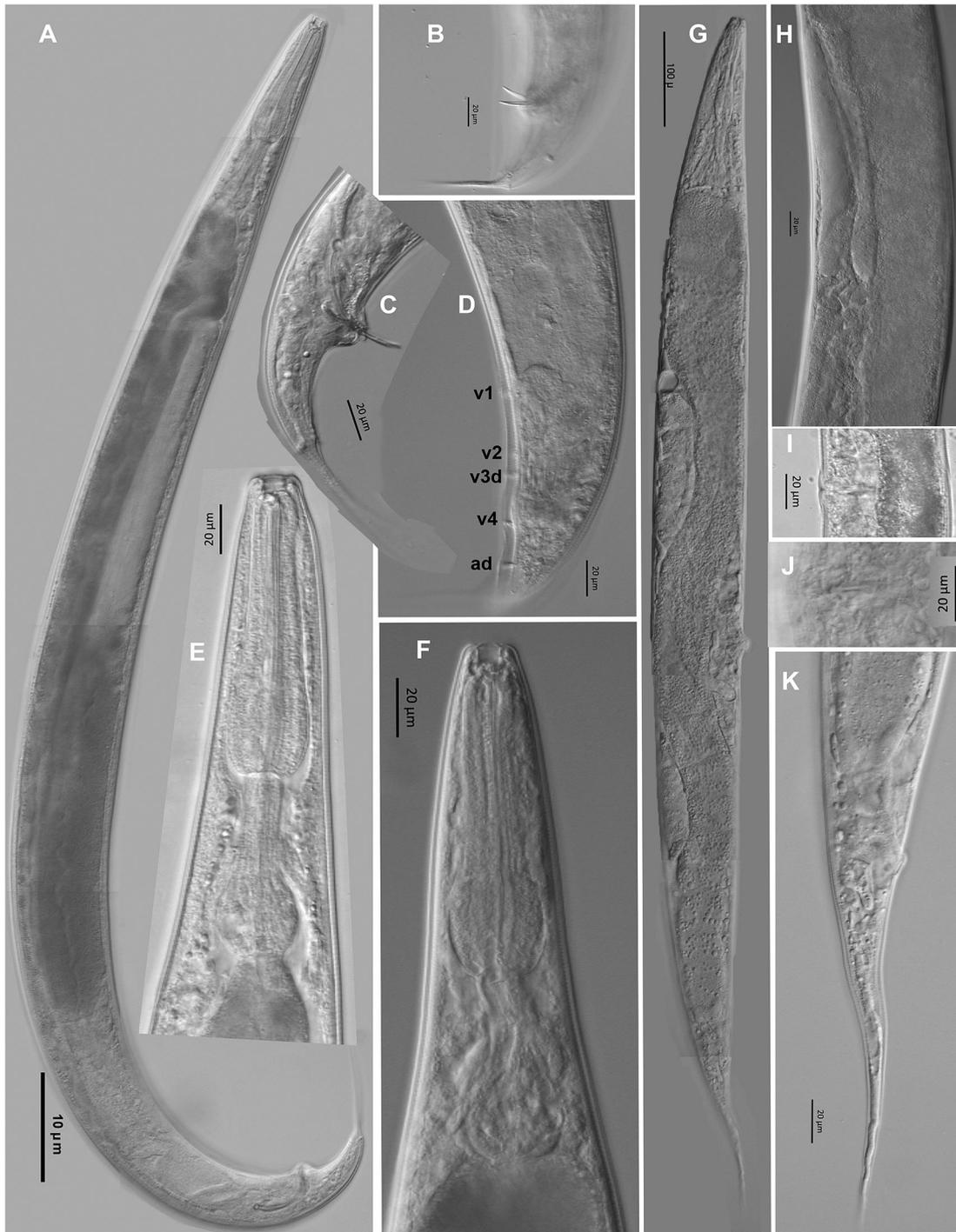


Fig. 2. Photographs of stenostomatous form of *Pristionchus aerivorus* (A-E: Male; F-K: Female). A: Entire body; B: Paired spicules; C: Lateral view of tail region; D: Lateral view of tail region showing genital papillae and bursa-like thickened cuticle; E, F: Pharyngeal region; G: Entire body; H: Reflexion of ovary; I: Lateral view of vulva; J: Ventral view of vulva; K: Tail region. Abbreviations: ad = anterior dorsal genital papilla; v + number = ventral genital papillae; v + number + d = ventro-lateral papillae.

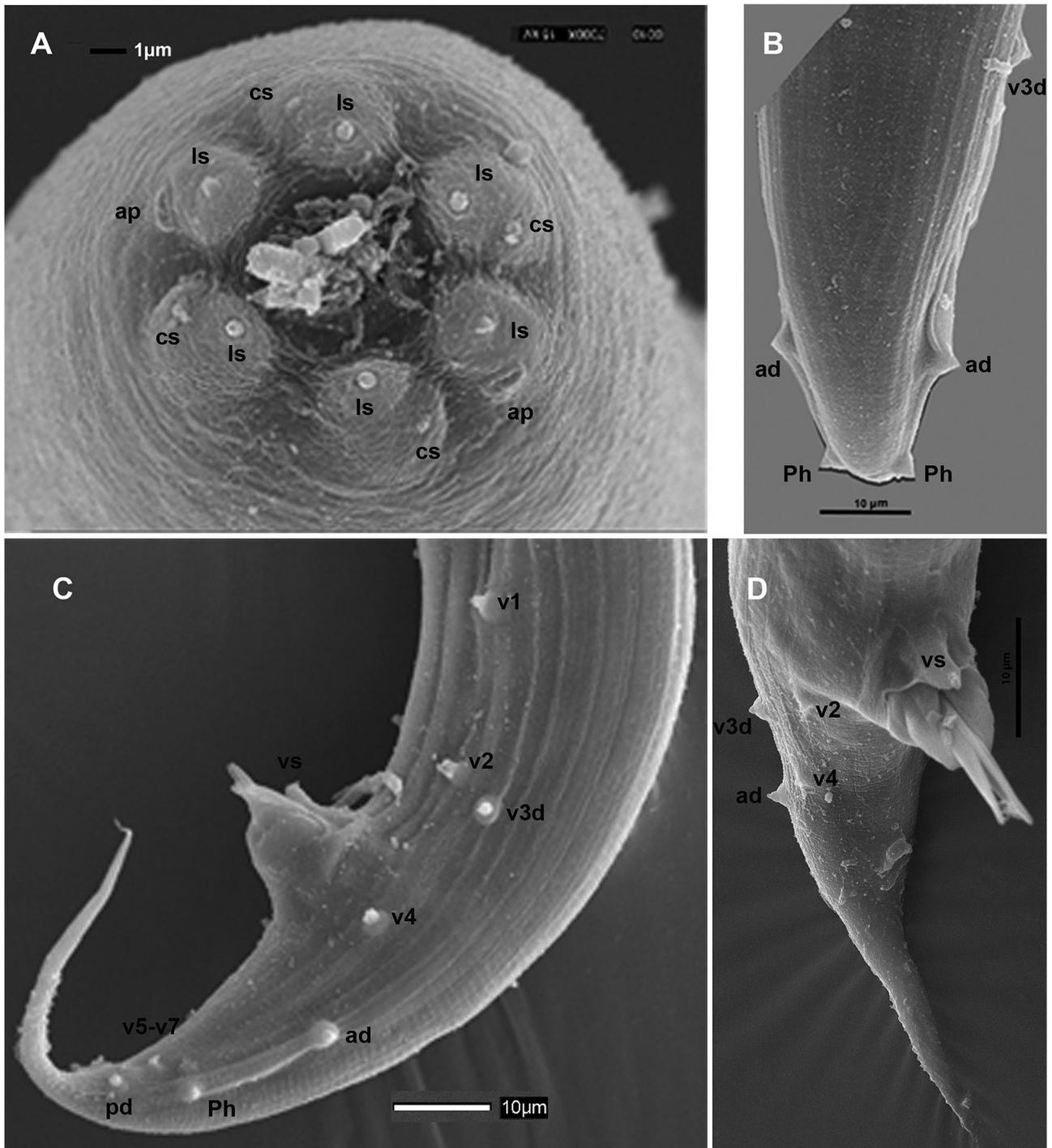


Fig. 3. Scanning electron microscope photographs of stenostomatous form of *Pristionchus aerivorus* male. A: Lip region *en face* view showing two amphidial apertures (ap), six labial sensilla (ls) and four cephalic sensilla (cs); B: Dorsal view of tail region; C: Ventral-lateral view of tail region; D: Ventral view of tail region showing paired spicules and genital papillae. Abbreviations: ad = anterior dorsal genital papilla; pd = posterior dorsal genital papilla; Ph = phasmid, v + number = ventral genital papillae, v + number + d = ventro-lateral papillae, vs = ventral single genital papilla.

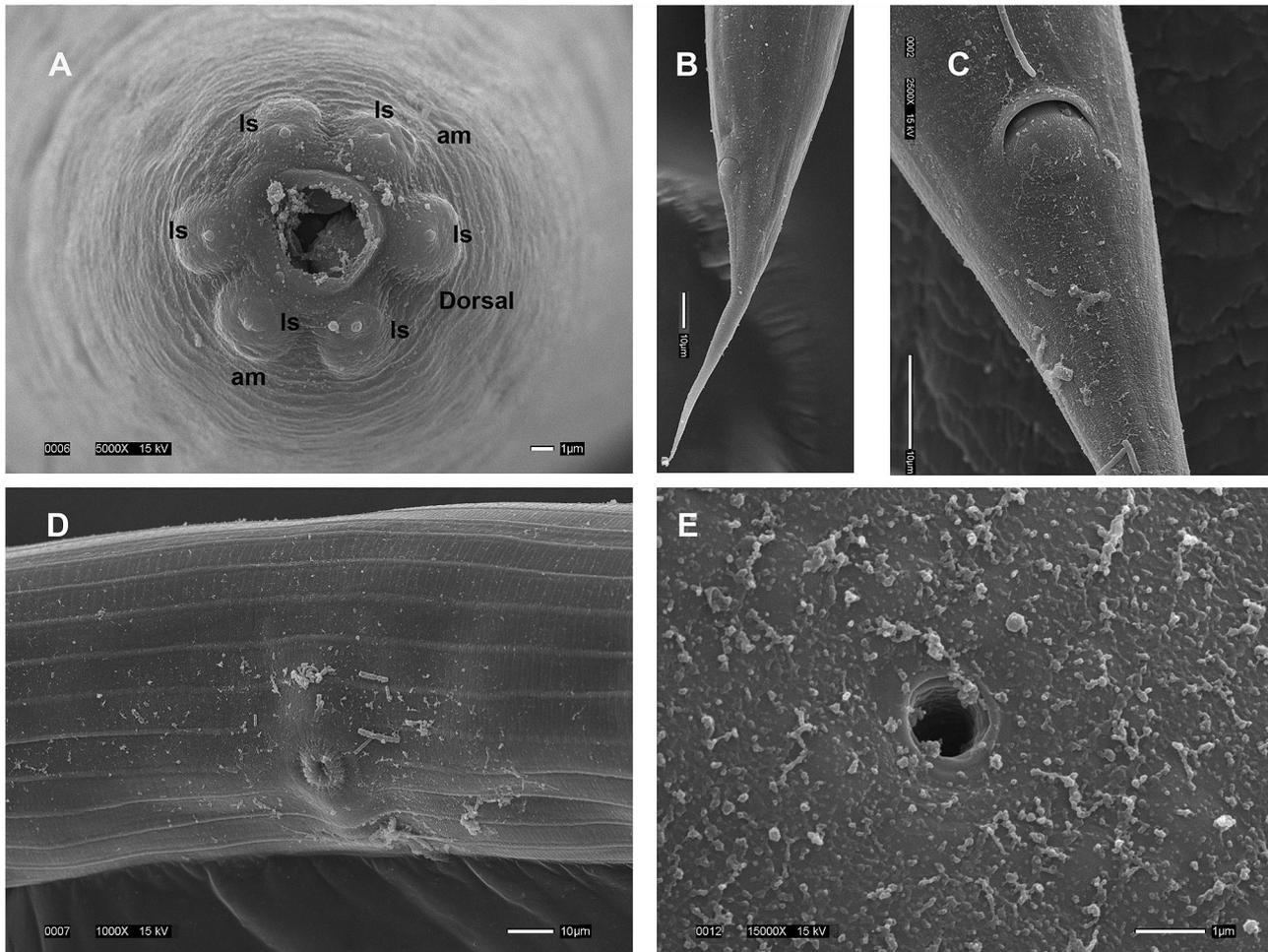


Fig. 4. Scanning electron microscope photographs of stenostomatous form of *Pristionchus aerivorus* female. A: Lip region *en face* view showing two amphidial apertures (am) and six labial sensilla (ls); B: Tail lateral view; C: Tail ventral view showing anus; D: Vulva region showing vulva and fine longitudinal striae; E: Excretory pore.

arranged as one on each sublateral lip only in male, no cephalic sensilla visible in female. Amphidial apertures elliptical, on lateral lips, located just posterior to anterior lip and next to labial sensillum. Stomatal morphology typical for genus. Dorsal metarhabdion with large protruding movable tooth, right subventral metarhabdion and left subventral metarhabdion with smaller tooth. Anterior part of pharynx (pro- and metacarpus) *ca* 1.5 times as long as posterior part (isthmus and basal bulb). Procorpus very muscular, stout, occupying two-thirds of corresponding body diam. Metacarpus very muscular, forming well developed median bulb. Isthmus narrow, not muscular. Basal bulb glandular. Nerve ring surrounding middle region of isthmus. Excretory pore not conspicuous under

light microscope. Deirids slightly posterior to pharyngo-intestinal junction. A 'post-deirid' (or 'pre-phasmid') associated with a gland-like cell observed at about midpoint between vulva and anus in female and about one-fifth body length from tail tip in male. Several other pores located along body and either slightly left or right of lateral between body striations, their positions inconsistent among individuals. Observation by LM could confirm 3-6 pores for males and 7-8 for females. In new (4-5 days old) cultures, almost all individuals of stenostomatous form, eurystomatous form occurring in old (more than 1 week old) cultures. Body size generally smaller in eurystomatous form than stenostomatous form, possibly as a result of starvation.

Table 1. Morphometrics of *Pristionchus aerivorus*. All measurements are in μm and in the form: mean \pm s.d. (range).

Character	Male (stenostomatous)	Female (stenostomatous)	Male (eurystomatous)	Female (eurystomatous)
n	9	11	12	12
L	1470 \pm 131.3 (1320-1700)	1680 \pm 200.9 (1200-1950)	781 \pm 164.3 (525-1030)	681 \pm 101.3 (575-875)
a	17.3 \pm 1.9 (13.6-19.4)	15.1 \pm 1.3 (13.4-17.3)	16.8 \pm 2.7 (11.9-21.6)	17.2 \pm 4.5 (12.0-29.2)
b	8.8 \pm 0.7 (7.9-10.4)	9.8 \pm 0.7 (7.7-10.6)	5.4 \pm 0.6 (4.2-6.3)	4.8 \pm 0.7 (3.2-5.9)
c	11.5 \pm 1.4 (9.6-13.5)	11.0 \pm 1.5 (7.7-14.1)	7.0 \pm 1.3 (5.0-9.4)	5.1 \pm 0.5 (4.2-6.2)
c'	3.1 \pm 0.4 (2.6-3.7)	3.8 \pm 0.5 (2.8-4.4)	3.8 \pm 0.9 (2.9-5.6)	5.6 \pm 0.7 (4.4-6.7)
T or V	72.5 \pm 5.0 (64.3-80.7)	50.0 \pm 1.4 (47.8-51.9)	63.8 \pm 8.2 (43.5-74.2)	49.4 \pm 4.0 (43.1-56.1)
Pharynx length	168 \pm 14.7 (150-200)	172 \pm 12.9 (154-190)	143 \pm 18.4 (120-182)	146 \pm 31.8 (120-245)
Mid-body diam.	86 \pm 14.4 (68-118)	112 \pm 18.0 (80-135)	47 \pm 9.6 (34-60)	41 \pm 8.6 (30-59)
Tail	130 \pm 15.2 (100-160)	157 \pm 21.1 (106-180)	119 \pm 11.7 (75-120)	133 \pm 15.4 (115-168)
Cloacal or anal body diam.	42 \pm 3.8 (36-48)	41 \pm 5.0 (35-50)	30.9 \pm 7.9 (20-42)	24.5 \pm 4.7 (20-38)
Stoma length	9.4 \pm 1.1 (8-11)	8.9 \pm 1.0 (7-10)	6.8 \pm 0.8 (5-8)	7.7 \pm 1.5 (5-10)
Stoma diam. (widest part)	8.3 \pm 0.9 (7-10)	9.3 \pm 1.0 (7-10)	9.5 \pm 2.1 (7-14)	12.0 \pm 3.3 (8-20)
Corpus length	105 \pm 12.0 (90-130)	107 \pm 6.6 (96-118)	93 \pm 11.8 (80-120)	99 \pm 19.9 (80-160)
Anterior end to excretory pore	–	–	137 \pm 11.9 (122-150)	–
Testis length	1041 \pm 155.1 (825-1330)	–	505 \pm 116.8 (250-640)	–
Testis reflexion	190 \pm 38.7 (108-250)	–	151 \pm 14.7 (130-170)	–
Spicule length	69 \pm 8.4 (60-84)	–	55 \pm 5.2 (44-64)	–
Gubernaculum length	21.8 \pm 3.6 (18-27)	–	17.6 \pm 2.8 (14-22)	–
Stoma length/stoma diam.	1.1 \pm 0.1 (1.0-1.4)	1.0 \pm 0.1 (0.8-1.3)	0.7 \pm 0.2 (0.5-1.1)	0.6 \pm 0.1 (0.35-0.9)

Stenostomatous form

Cheilostom consisting of six per- and interradial plates. Incisions between plates not easily distinguished by LM. Anterior end of each plate rounded and elongated so as to protrude from stomatal opening forming a small flap, but not conspicuous. Gymnostom short, cuticular ring-like anterior end overlapping cheilostom internally.

Dorsal gymnostomatal wall slightly thickened compared with ventral side. Stegostom bearing a large, conspicuous, flint-shaped (or inverted V-shaped) dorsal tooth, there is a half-circular or blunt triangular ridge in right subventral sector and a crescent-shaped left subventral ridge with 2-3 small blunt peaks, often with apparent cleavage between two main peaks. Dorsal tooth sclerotised at surface.

Eurystomatous form

Cheilostom divided into six distinct per- and interradial plates. Anterior end of each plate rounded and elongated to protrude from stomatal opening forming a small flap. Gymnostom with thick cuticle, forming a short, ring-like tube. Anterior end of gymnostom internally underlapping posterior end of cheilostomatal plates. Stegostom bearing a large claw-like dorsal tooth, with large base of varying size, a large claw-like right subventral tooth, and in left subventral sector a row of 4-6 small- to medium-sized triangular subventral peaks projecting from a common cuticular plate apparently split mid-way along its length. Dorsal and right subventral teeth movable, left subventral denticles immovable.

Male

Testis single, anterior end reflexed ventrally to right side; spermatocytes arranged in multiple rows. Posterior part of testis forming *vas deferens*. Distal end of *vas deferens* fused with distal end of intestine forming a simple cloacal tube. Three cloacal (anal) glands (two subventral and one dorsal) surrounding cloacal tube. Spicules paired, separate, ventrally arcuate, shaft slender, tips sharply pointed. Gubernaculum conspicuous, *ca* one third of spicule length. One small, ventral, single genital sensillum on anterior cloacal lip. Nine pairs of genital papillae and a pair of phasmids present, arranged as (v1, v2, v3d, v4, ad, Ph, (v5, v6, v7), pd) with the terminologies proposed by Sudhaus & Fürst von Lieven (2003). Three precloacal papillae (v1 and v2 ventrolateral, v3d lateral) spaced unevenly, v1 located much anterior, v4 ventrolateral just posterior to anus, lateral ad located around middle of tail, v5-v7 forming a ventral triplet, lateral phasmids and ventrolateral pd close to each other a little anterior to base of tail spike. Bursa or bursal flap absent, but thick cuticle around tail region falsely appearing as a narrow leptoederan bursa in ventral view. Tail conical with a spike occupying about half of tail length.

Female

Reproductive system didelphic, amphidelphic. Each gonad arranged, from vulva and vagina, as uterus, oviduct and ovary. Anterior and posterior gonads on right and left of intestine, respectively. Anterior gonad is described from distal part herein. Ovary reflexed anteriorly to left of intestine (antidromous reflexion). Oocytes mostly arranged in multiple rows, sometimes more than five rows in distal two-thirds of ovary, and in single row in remaining third of ovary, distal tip of each ovary sometimes reflexed to reach oviduct of opposite gonad

branch. Middle part of oviduct serving as spermatheca, sperm observed in distal part of oviduct, close to ovary. A few to many (sometimes >20) eggs in single- to multiple cell stage or even further developed in proximal part of oviduct (= uterus). *Receptaculum seminis* not observed. Dorsal wall of uterus at level of vulva thickened and appearing dark in LM observation. Four vaginal glands present but obscure. Vagina perpendicular to body surface, surrounded by sclerotised tissue. Vulva slightly protuberant in lateral view, pore-like in ventral view. Rectum long, *ca* one anal body diam. long. Three anal glands (two subventral and one dorsal) observed around intestine-rectal junction. Anus in form of dome-shaped slit. Tail elongate, slender, tapering gradually, *ca* four anal body diam. long.

MOLECULAR PHYLOGENETIC RELATIONSHIPS

For molecular analysis, the near-full-length 18S and the D2-D3 expansion segment of 28S rRNA region were sequenced for the four strains. The DNA sequences of these *Pristionchus* are available in the GenBank database under the accession numbers provided in Figures 5 and 6. Other *Pristionchus* species from GenBank with the highest matches after a BLASTN search were selected for phylogenetic analysis.

A BLASTN search of *P. aerivorus* (S4B1) with the 1670 bp 18S region revealed the highest match with 99% identity as *P. aerivorus* (GenBank accession number FJ040440, 2 bp differences), *P. maupasi* (Potts, 1910) Paramonov, 1952 (FJ040443, 3 bp differences), *P. pseudoaerivorus* (FJ040447, 6 bp differences), and *P. americanus* (FJ040445, 13 bp differences, 1 gap). S4B1 is also 99% identical with three reference isolates *P. aerivorus* (RS5106, 2 bp differences), *P. americanus* (RS5140, 4 bp differences) and *P. pseudoaerivorus* (RS5139, 5 bp differences). S4B1 is more distant from *P. lheritieri* (Maupas, 1919) Paramonov, 1952 (FJ040439, identities = 1620/1670 (97%), gaps = 1/1670 (0%)), *P. entomophagus* (Steiner, 1929) Sudhaus & Fürst von Lieven, 2003 (FJ040441, identities = 1617/1670 (96%), gaps = 1/1670 (0%)), and *P. uniformis* Fedorko & Stanuszek, 1971 (FJ040444, identities = 1614/1670 (96%), gaps = 1/1670 (0%)).

A BLASTN search of *P. aerivorus* (S4B1) with the 727 bp 28S D2-D3 region revealed the highest match as *P. aerivorus* (AB477071, identities = 315/319 (98%)), *P. maupasi* (DQ059065, identities = 421/430 (97%)), *P. pacificus* (EU195982, identities = 677/723 (93%), gaps = 5/723 (0%)), *P. cf. pacificus* (AB597247, identities =

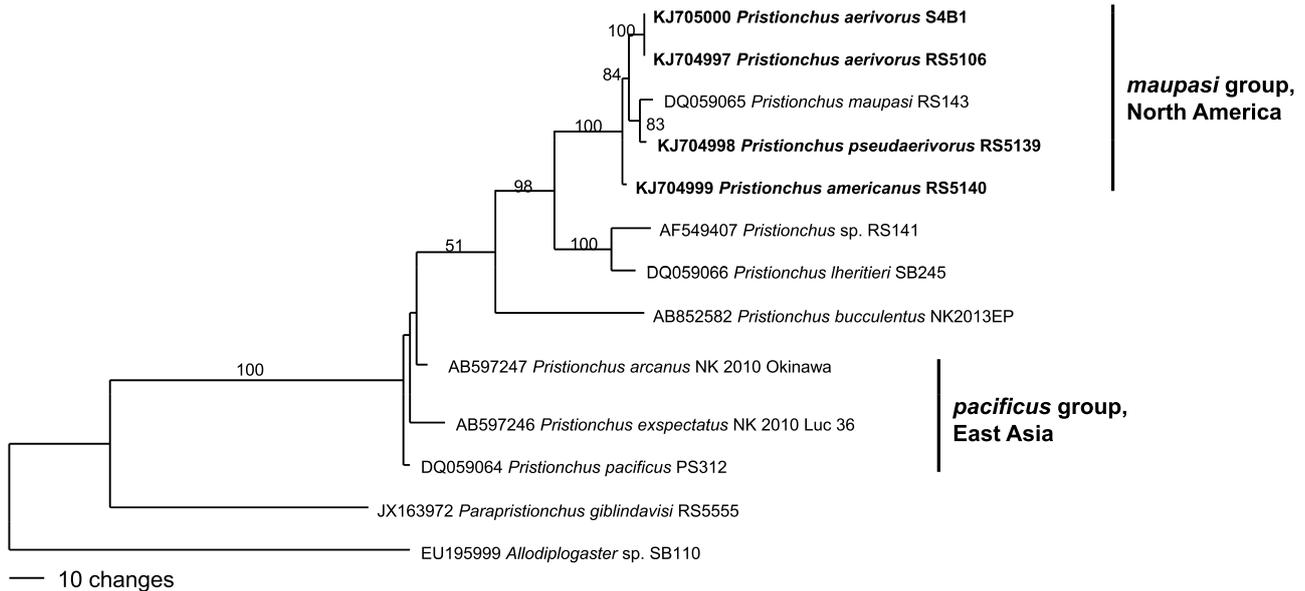


Fig. 6. The 10 001st Bayesian likelihood tree inferred from 28S D2-D3 under the GTR + I + G model ($-\ln L = 3029.1047$; AIC = 6076.2095; freqA = 0.2128; freqC = 0.2114; freqG = 0.3239; freqT = 0.2518; R(a) = 0.364; R(b) = 1.2095; R(c) = 0.3822; R(d) = 0.2694; R(e) = 3.6856; R(f) = 1; Pinvar = 0; Shape = 0.391). Posterior probability values exceeding 50% are given on appropriate clades.

650/700 (92%), gaps = 6/700 (0%)), *P. cf. pacificus* (AB597246, identities = 650/700 (92%), gaps = 5/700 (0%)), *P. pacificus* (DQ059064, identities = 388/430 (90%), gaps = 5/430 (1%)) and *P. lheritieri* (DQ059066, identities = 387/430 (90%), gaps = 2/430 (0%)). S4B1 is identical with the reference isolate *P. aerivorus* (RS5106) in D2-D3 sequence, but different from two reference isolates: *P. americanus* (RS5140, 7 bp differences) and *P. pseudoaerivorus* (RS5139, 9 bp differences).

Figure 5 presents a phylogenetic tree based on the near full-length 18S rRNA from a multiple alignment of 1698 total characters. This dataset has 1318 constant characters (77.6%) and includes some *Pristionchus* sequences with 471-559 bp as shown in Figure 5. Using *Allodiplogaster* sp. as an outgroup taxon, this tree inferred a 100% supported monophyletic clade which includes two groups: the *maupasi* group from North America and the *pacificus* group from East Asia. The study strain of *P. aerivorus* (S4B1) and three reference strains *P. aerivorus* (RS5106), *P. americanus* (RS5140) and *P. pseudoaerivorus* (RS5139) are in the North American *maupasi* group. This group also comprised *P. maupasi*, *P. mariannae* Herrmann, Mayer & Sommer, 2006 and *P. pauli* Herrmann, Mayer & Sommer, 2006. This tree generally agrees with the tree from a partial 18S rRNA and 26 ribosomal protein-

coding genes by Kanzaki *et al.* (2014). Figure 6 presents a phylogenetic tree based on rRNA 28S D2-D3 sequences from a multiple alignment of 800 total characters. This dataset has 496 constant characters (62.0%), but only few sequences are available to compare from GenBank. Using *Allodiplogaster* sp. as an outgroup taxon, this tree inferred species from *maupasi* group from North America as a highly supported monophyletic clade, *pacificus* group from East Asia are in basal position. Similar to the 18S tree, the study strain *P. aerivorus* (S4B1) and three reference strains *P. aerivorus* (RS5106), *P. americanus* (RS5140) and *P. pseudoaerivorus* (RS5139) were in the North American *maupasi* group.

HYBRIDISATION TEST

Hybrid crosses were only successful between S4B1 and *P. aerivorus* (RS5106) with viable offspring produced for at least 2 months. Eggs and juveniles were observed between S4B1 and *P. pseudoaerivorus* (RS5139) and between S4B1 and *P. americanus* (RS5140), but with no production of viable offspring from the F1 generation. Conspecific crosses of these four strains were all successful between one male and one female for interspecies crosses.

NEMATODE ENTOMOPATHOGENICITY

Our *Pristionchus* isolate was amenable to laboratory colony maintenance in microbiological media, as well as through periodical inoculation onto live insect hosts. Interestingly, both *T. molitor* and *H. zea* were readily infected by our *P. aerivorus* strain, with infections resulting in 100% mortality of both hosts. However, time to host death was significantly ($F_{1,38} = 33.515$, $P < 0.001$) longer for *H. zea* compared with *T. molitor*, with *H. zea* taking approximately twice as long to die after inoculation than *T. molitor* (Fig. 7). On the other hand, time to nematode emergence after host death was longer for *T. molitor* (71 ± 8.95 h) compared with *H. zea* (49 ± 6.84 h), although this difference was only statistically significant at $P = 0.01$ ($F_{1,38} = 3.67$, $P < 0.0630$).

Species in *Pristionchus* are generally regarded as insect associates. The resulting mortalities of the two insect species after being challenged with the *P. aerivorus* in this study suggested its potential use as a biological control agent. This species can be easily mass-cultured in LB broth agar by feeding on the bacteria associated with this nematode. Further study is necessary to include more insect species and field studies. The roles of the associated bacteria that the nematode carried deserve characterisation.

REMARKS ON THE STATUS OF *CHRONIODIPLOGASTER*

Poinar (1990) proposed *Chroniodiplogaster* and the combination *C. aerivorus* for *P. aerivorus*. A second species in the genus, *C. formosiana*, was later described and *P. uniformis* was transferred to this genus (Poinar et al., 2006). According to Poinar et al. (2006), *Chroniodiplogaster* can be separated from most diplogastrid

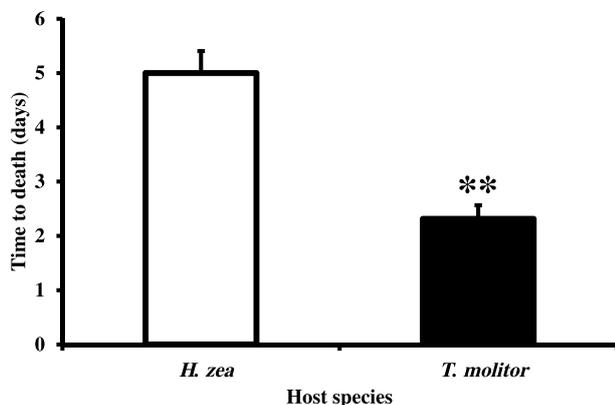


Fig. 7. Time to host death (h) after inoculation of two hosts with 250 *Pristionchus aerivorus* IJ under laboratory conditions.

genera “by possessing a gubernaculum in which the distal portion forms an enclosed sheath (or tube) that surrounds the spicule shafts, the number and position of genital megapapillae and micropapillae, separate bursae associated with the megapapillae and micropapillae, and an elongate to filamentous tail”. However, those generic characters overlap with those of *Pristionchus*, e.g., the tube-like process of the gubernaculum has been found in most of the species, and the so-called leptoderan bursae are considered to be the result of a ventral view of thickened cuticle (see Ragsdale et al., 2015). Further, no molecular data was employed to support the genus status in their studies. Thereafter, Sudhaus & Fürst von Lieven (2003) considered *Chroniodiplogaster* as a junior synonym of *Pristionchus*. *Chroniodiplogaster formosiana* was later transferred to *Pristionchus* by Fürst von Lieven & Sudhaus (2008) and confirmed to be a junior synonym of *P. pacificus* (Ragsdale et al., 2015). Based on the near-full-length 18S rRNA sequencing data of this study, *P. aerivorus* has only a few nucleotide differences from many other *Pristionchus* species and falls into a monophyletic clade in the *maupasi* group which is a sister to *pacificus* group (Fig. 5). Because *P. aerivorus*, *P. pacificus* (= *C. formosiana*) and *P. uniformis* were, based upon 18S rRNA sequences, inferred to be monophyletic with other *Pristionchus* species, the generic status of *Chroniodiplogaster* is not supported and it is therefore confirmed as a junior synonym of *Pristionchus* following the opinion of Sudhaus & Fürst von Lieven (2003) and Ragsdale et al. (2015).

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