

Title	Sphaerularia vespae sp. nov. (Nematoda, Tylenchomorpha, Sphaerularioidea), an Endoparasite of a Common Japanese Hornet, <i>Vespa simillima</i> Smith (Insecta, Hymenoptera, Vespidae)
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# *Sphaerularia vespae* sp. nov. (Nematoda, Tylenchomorpha, Sphaerularioidea), an Endoparasite of a Common Japanese Hornet, *Vespa simillima* Smith (Insecta, Hymenoptera, Vespidae)

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*Sphaerularia vespae* sp. nov., an endoparasite of a common Japanese hornet, *Vespa simillima* is described from Hokkaido, Japan, and its molecular sequence profiles are given. This newly discovered nematode appears to belong to the genus *Sphaerularia*, judging from its characteristic parasitic form, the uterus, which looks like a sausage with many verrucae on its surface. *Sphaerularia vespae* sp. nov. is distinguished from *S. bombi*, the only other nominal species of the genus, by the morphology of the male bursa, female tail, and anterior end of parasitic juveniles. SSU, ITS, and D2D3 (LSU) DNA sequences were determined for *S. vespae* sp. nov. and compared with those from related nematodes obtained from the GenBank database. The sequences from *S. vespae* sp. nov. were close to those of *S. bombi* and several other tylenchid entomoparasitic nematodes. *Sphaerularia vespae* sp. nov. parasitizes overwintering gynes of the hornet and practically sterilizes them, as *S. bombi* does for bumblebee gynes.

**Key words:** hornet, Japan, new species, *Sphaerularia*, taxonomy

## INTRODUCTION

The genus *Sphaerularia* was established by Dufour (1837), as an extraordinary and unusual parasite of bumblebees, and is currently a monospecific genus comprised of only *S. bombi* Dufour, 1837, after generic revisions (Siddiqi, 2000). However, 270 years after the first identification of sphaerularid nematodes (Réaumur, 1742), we have discovered another *Sphaerularia* that is an endoparasite of the overwintered gynes (potential queens) of the hornet, *Vespa simillima* Smith (Sayama *et al.*, 2007). The new species clearly belongs to the genus *Sphaerularia*, based on its characteristic morphology of the "uterus" female, the parasitic form, but is distinguished from *S. bombi* by several morphological features and its molecular profiles using SSU, ITS, and D2/D3 (LSU) rRNA gene sequences. The nema-

tode is described and figured herein as *Sphaerularia vespae* sp. nov. Preliminary observations on its biological features are also given.

## MATERIALS AND METHODS

### Collection of hornets

Overwintered gynes of *V. simillima* were caught using transparent plastic traps baited with clear liquor (distilled spirit) and orange juice from May to August 2005 at Hitsujigaoka, Sapporo, Hokkaido, Japan (E 141°23', N 42°59'). Most trapped hornets were fixed in Carnoy's solution (ethanol : chloroform : glacial acetic acid=6 : 3 : 1) and preserved in 80% ethanol, and were later dissected for examination of parasitism, though some individuals found alive in the traps were immediately dissected to obtain living nematodes.

### Rearing of the nematodes and morphological observation

Parasitic juveniles and uteria of the nematode were isolated from the haemocoel of a gyne of *V. simillima* caught on 21 July 2005. A water suspension containing the juvenile nematodes was pipetted onto autoclaved sawdust in a Petri dish (diameter 6.5 cm, height 9.0 cm). Infective adults of the nematode obtained from a 6-month-old culture and parasitic juveniles isolated from a gyne hor-

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net caught on 20 July 2005 were heat-relaxed at 65°C, fixed in TAF (triethanolamine+formalin), and processed through a glycerol-ethanol series by using Seinhorst's method (see Hooper, 1986). The glycerol specimens of the parasitic juveniles and infective adults were mounted in glycerin following the method of Maeseneer and d'Herde (see Hooper, 1986), and the uteria were stored in a small glass vial.

### Molecular profiles

The molecular profiles, *i.e.*, nearly full-length SSU, ITS (ITS 1, 5.8S, and ITS 2) and D2D3 (expansion segment of the LSU) rRNA gene sequences, of the new species were determined from a single

free-living female, obtained from the sawdust medium. We prepared a DNA sample following the methods described in Ye *et al.* (2007), and DNA fragments were amplified with the primer sets SSU\_F4 (5'- GCT TGT CTC AAA GAT TAA GCC -3') and SSU\_R81 (5'- TGA TCC WKC YGC AGG TTC AC -3') (SSU: provided at Dr. M. Blaxter's website, <http://www.nematodes.org/barcoding/sourhope/nemoprimer.html>), 18SF (5'- CGT AAC AAG GTA GCT GTA G -3') and 28SR (5'- TTT CAC TCG CCG TTA CTA AGG -3') (ITS: Ferris *et al.*, 1993; Vrain, 1993), and D2a (5'- ACA AGT ACC GTG AGG GAA AGT TG -3') and D3b (5'- TCG GAA GGA ACC AGC TAC TA -3') (D2D3: Nunn, 1992). The amplified fragments were sequenced by PCR direct sequencing by using a Big Dye

**Table 1.** Morphometric values of *Sphaerularia vespae* sp. nov. All measurements are in micrometers. The abbreviations are defined as: V=vulval position relative to body length (%); T=testis length relative to body length (%).

	Holotype (female)	Patatypes (females) <sup>a</sup>	Patatypes (males) <sup>b</sup>	Parasitic juveniles <sup>a</sup>
n (number of specimens)	–	12	2	10
L (body length)	1338	1392±61 (1300–1500)	1162, 905	959±54 (871–1024)
a (body length / maximum body width)	55.6	52.7±6.9 (39.0–59.6)	43.3, 39.1	26.8±3.4 (21.7–32.2)
c (body length / tail length)	8.2	8.2±0.4 (7.8–8.8)	21.5, 18.8	14.8±0.7 (13.9–15.5)
c' (tail length / anal body width)	5.0	5.2±0.5 (4.2–5.7)	3.3, 3.2	2.7±0.3 (2.3–3.1)
V or T	84.2	83.9±0.7 (82.4–85.0)	68.0, 57.2	–
M (conus length relative to stylet length (%))	25.6	27.9±1.6 (25.6–30.6)	30.0, 26.7	–
Lip width	10.5	10.5±0.4 (9.5–11.0)	9.0, 8.0	–
Lip height	4.5	4.5±0.4 (4.0–5.5)	4.0, 3.0	–
Stylet cone	4.5	4.5±0.2 (4.0–5.0)	4.0, 4.0	–
Stylet length	18.0	16.5±0.8 (15.5–18.0)	14.0, 14.0	6.5±0.6 (5.0–7.0)
Nerve ring	100	118±7.7 (100–126)	108, 94	101±3.8 (98–108)
Excretory pore	135	147±7.4 (135–158)	122, 107	114±4.2 (109–121)
Maximum body width	24.0	26.5±3.8 (24.0–34.5)	27.0, 23.0	36.0±3.4 (30.5–40.0)
Ovary+oviduct or testis length	158	165±20 (129–188)	790, 517	–
Spermatheca	198	412±141 (198–691)	–	–
Uterus+ quadricolumella	115	94±24 (38–122)	–	–
Post uterine sac	11.5	14±5.9 (7–28)	–	–
Testis reflection	–	–	0, 41.0	–
Vulva-anus length	136.0	147±17 (121–175)	–	–
Anal body width	15.0	15.0±1.8 (13.0–18.5)	16.5, 15.0	23.5±2.9 (21.0–28.5)
Tail length	76	79±4.8 (74–89)	54, 48	64±2.9 (58–67)
Spicule length	–	–	24.0 <sup>c</sup> , 27.0	–
Gubernaculum length	–	–	6.0 <sup>c</sup> , 8.5	–

<sup>a</sup>Average±SD (range).

<sup>b</sup>First and second individual, respectively.

<sup>c</sup>Subventral view.

Terminator Cycle Sequencing Kit v. 3.0<sup>®</sup> (PE Applied Biosystems, Foster City, CA).

The SSU and D2D3 sequences from a uterium were also determined and compared with those obtained from a single female to confirm species identification. A DNA sample was prepared from an ethanol-fixed uterium using a DNeasy Mini Kit<sup>®</sup> (Qiagen Japan, Tokyo) following the manufacturer's instructions. PCR amplification was conducted as above. To separate the nematode's amplicons from the host's amplicons, the PCR fragments were cloned with a pCR8/GW/TOPO TA cloning Kit<sup>®</sup> (Invitrogen, Tokyo), and sequenced using M13 sequencing primers 5'-GTA AAA CGA CGG CCA G -3' and 5'-GTA AAA CGA CGG CCA G -3', following the manufacturer's manual.

## TAXONOMY

### *Sphaerularia vespae* sp. nov.

(Figs. 1–6)

**Measurements.** See Table 1.

**Description.** *Female* (Figs. 1, 3). Body slender, annulated, relaxed or slightly ventrally bent when killed by heat. Lateral field with four conspicuous incisures, originating in the region of procorpus extending to close to the tail tip. Lip fused, smooth, slightly constricted, difficult to distinguish. Stylet conspicuous, conus and shaft separated. Stylet conus short, stout, triangular. Stylet shaft long, slightly curved, basal thickening absent. Procorpus tube-like, about two body diameters long. Two esophageal glands observed, but their openings very obscure. Esophageal glands approx. 5–6 body diameters long, constricted by a nerve ring at about anterior 1/4 position, posterior part overlapping intestine. Esophageal-intestinal junction very vague, not observed. Nerve ring conspicuous, about 120 µm behind anterior end. Excretory pore very obvious, about one body diameter behind nerve ring. Hemizonid just anterior to excretory pore. Deirids conspicuous, located in the middle of the lateral field, just posterior to the excretory pore. Ovary single, outstretched, sometimes tangled. Oocytes arranged in double or triple file. Oviduct long, cylindrical, comprising thick cells arranged in two rows. Spermatheca long, often filled with sperm mass. Quadricolumella obscure, cells not clearly distinguishable. Uterus elongated, cylindrical, consisting of large and thick cells. Vulva slit-like, vulval lip flat, not protuberant. Vagina at 90° to body wall, vaginal wall thick. Postuterine sac very short, less than a half body diameter long, semicircular. Rectum present, approx. one body diameter long, not muscular. Anus present, not protuberant, sometimes very difficult to observe. Rectum and anus may be nonfunctional. Tail long, anterior part tapered slightly, posterior part conical, sometimes possessing one constriction. Tail tip finger-like shape, mostly rounded but bluntly pointed in some individuals.

*Male* (Figs. 1, 3). Body slender, ventrally bent when killed by heat. Lateral field extends to level of spicules connecting to bursa. Anterior region similar to that of female. Testis single, outstretched or reflexed. Sperm cells, arranged in irregular multiple rows in anterior 1/4 part, develop into tiny spermatozoa in posterior part. Spicule paired, separated, arcuate, manubrium rounded, distinct. Gubernaculum present, straight, thorn-like. Caudal papillae absent. Bursa subterminal, starts just anterior to spicule. Tail conical, ventrally bent. Tail tip bluntly pointed.

*Parasitic juvenile* (Figs. 2, 4). Found in haemocoel of

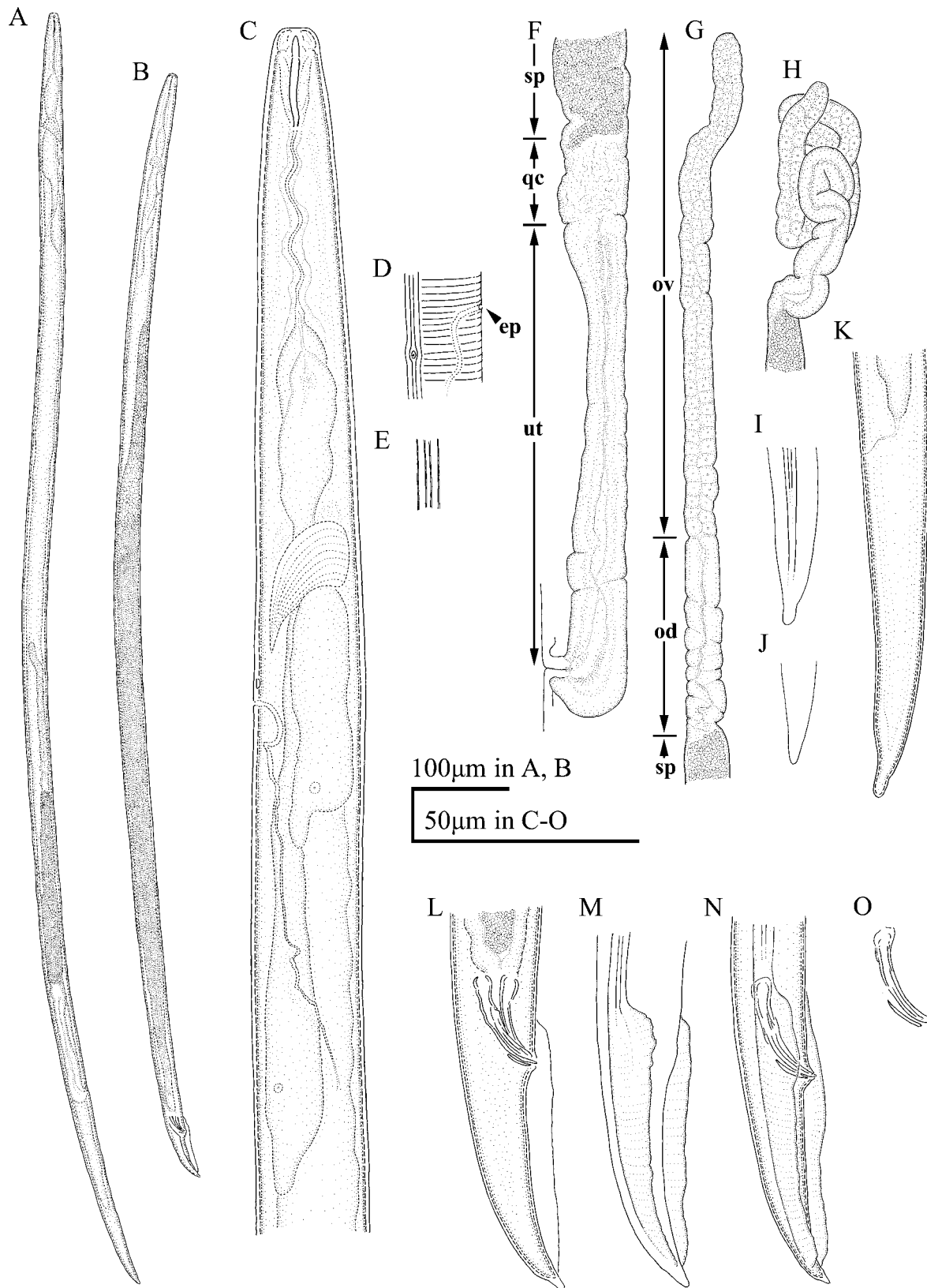
the host insect. Body stout, smoothly curved ventrally. Cuticle soft, thick, possessing annulation on its surface except for anterior and posterior end. Lateral field probably present, but not conspicuous. Lip fused, square in lateral view. Stylet short, fine, without obvious conus/shaft separation. Procorpus tube-like. Two esophageal glands observed, but openings very obscure. Esophageal glands approx. 4 body diameters long, constricted by nerve ring at about anterior 1/3 position, posterior part overlapping intestine. Esophageal-intestinal junction not observed. Nerve ring conspicuous, about 101 µm from anterior end. Excretory pore very obvious, about one body diameter behind nerve ring. Hemizonid just anterior to excretory pore. Rectum present but very obscure, approx. 1/2 body diameter long, not muscular. Anus present, not protuberant, sometimes very difficult to observe. Rectum and anus may be nonfunctional. Tail short, conical, possessing one to three constrictions. Tail tip has a rounded, finger-like shape.

*Uterium (mature parasitic female)* (Figs. 5, 6). Found in haemocoel of the host insect. Consists of the female reproductive organ. Anfractuous and sausage-shaped, sometime growing to more than 15 mm in length. Female body atrophied, sometimes found as an appendage of uterium, but usually not visible. Uterus everted from nematode body, enlarged, possessing many verrucae, which are derived from uterine cell nuclei, on its surface. The other parts of the reproductive organs invaginate the everted uterus. Quadricolumella and spermatheca sac-like, difficult to distinguish from each other. Oviduct containing well-developed eggs. Ovary well-developed, elongated, with many well-developed oocytes present.

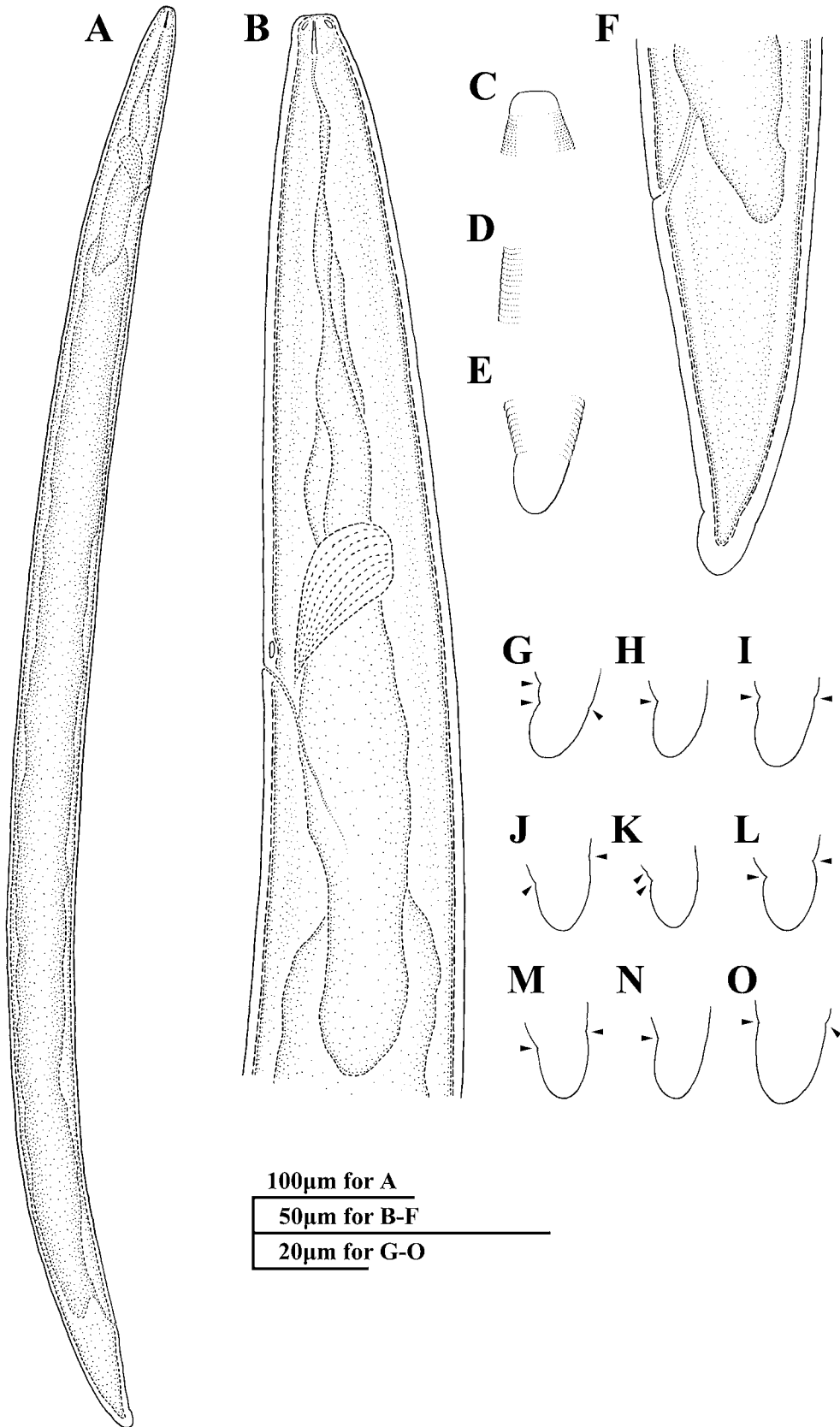
**Diagnosis and relationships.** *Sphaerularia vespae* sp. nov. is characterized by the rounded and finger-like tail tip of females and the conspicuous male bursa. Only one species, *S. bombi* Dufour was previously known in the genus *Sphaerularia*. The new species is distinguished from *S. bombi* by the length and shape of the female tail and male bursa, *i.e.*, *S. bombi* has a shorter (47–68 µm) tail with a bluntly pointed tail tip in females and an inconspicuous male bursa, while *S. vespae* sp. nov. has a longer (74–89 µm) tail, with a finger-like and mostly rounded tail tip in females and a large, conspicuous male bursa. The head shape of parasitic juveniles also distinguishes the new species from *S. bombi*. The anterior end of *S. vespae* sp. nov. is square in lateral view, while that of *S. bombi* is rounded and dome-like.

**Type host and locality.** The culture from which the type specimens were obtained was made from parasitic juveniles of *Sphaerularia vespae* sp. nov. isolated from the body cavity of an overwintered gyne of *Vespa simillima* Smith, caught with a bait trap at Hitsujigaoka, Sapporo, Hokkaido, Japan (42°59'N, 141°23'E), 21 July 2005.

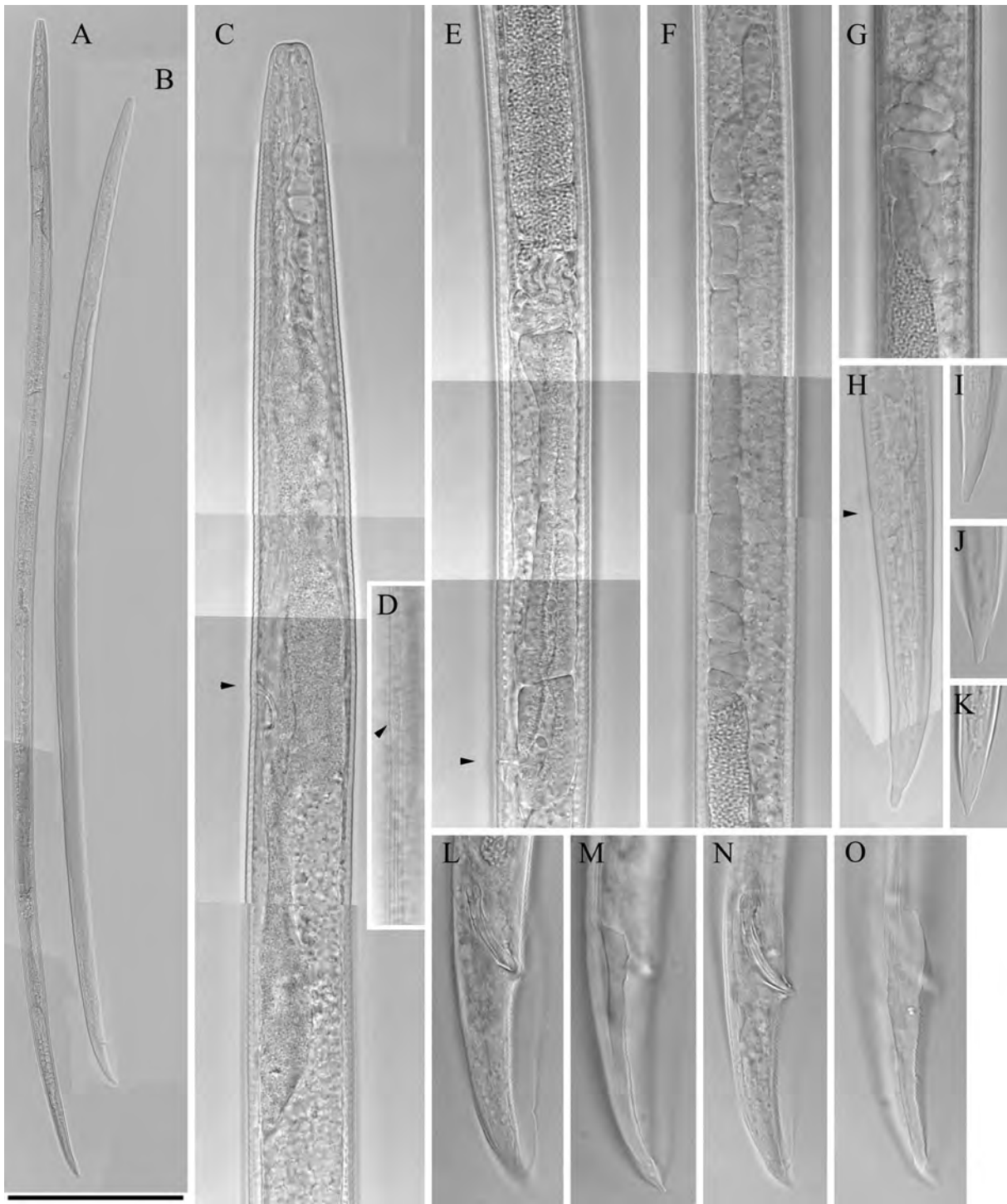
**Type material.** Type specimens were obtained from a 6-month-old culture on wet sawdust media on 10 February 2006. Holotype (female), slide number *Sphaerularia vespae* F-01 (USDANC Number: T-613t). Paratypes, slide numbers *Sphaerularia vespae* F-02-05 (USDANC Number: T-5635p-T-5638p) (four females); M-01 (USDANC Number: T-5639p) (one male) and *Sphaerularia vespae* P-01-04 (USDANC Number: T-5640p-T-5643p) (four parasitic juveniles) deposited in the USDA Nematode Collection, Beltsville, MD, USA;



**Fig. 1.** *Sphaerularia vespae* sp. nov. **(A)** Lateral view of female. **(B)** Lateral view of male. **(C)** Lateral view of female anterior region. **(D)** Body surface of female excretory pore region. **(E)** Lateral field at mid-body. **(F)** Female reproductive organ (vulva to spermatheca). **(G)** Female reproductive organ (spermatheca to outstretched ovary). **(H)** Female reproductive organ (spermatheca to out-tangled ovary). **(I–K)** Lateral view of female tail. **(L)** Subventral view of male tail (internal structure). **(M)** Subventral view of male tail (bursa and lateral field). **(N)** Lateral view of male tail. **(O)** Male spicule and gubernacula. Abbreviations: ep, excretory pore; ut, uterus; qc, quadricolumella; sp, spermatheca; od, oviduct; ov, ovary.



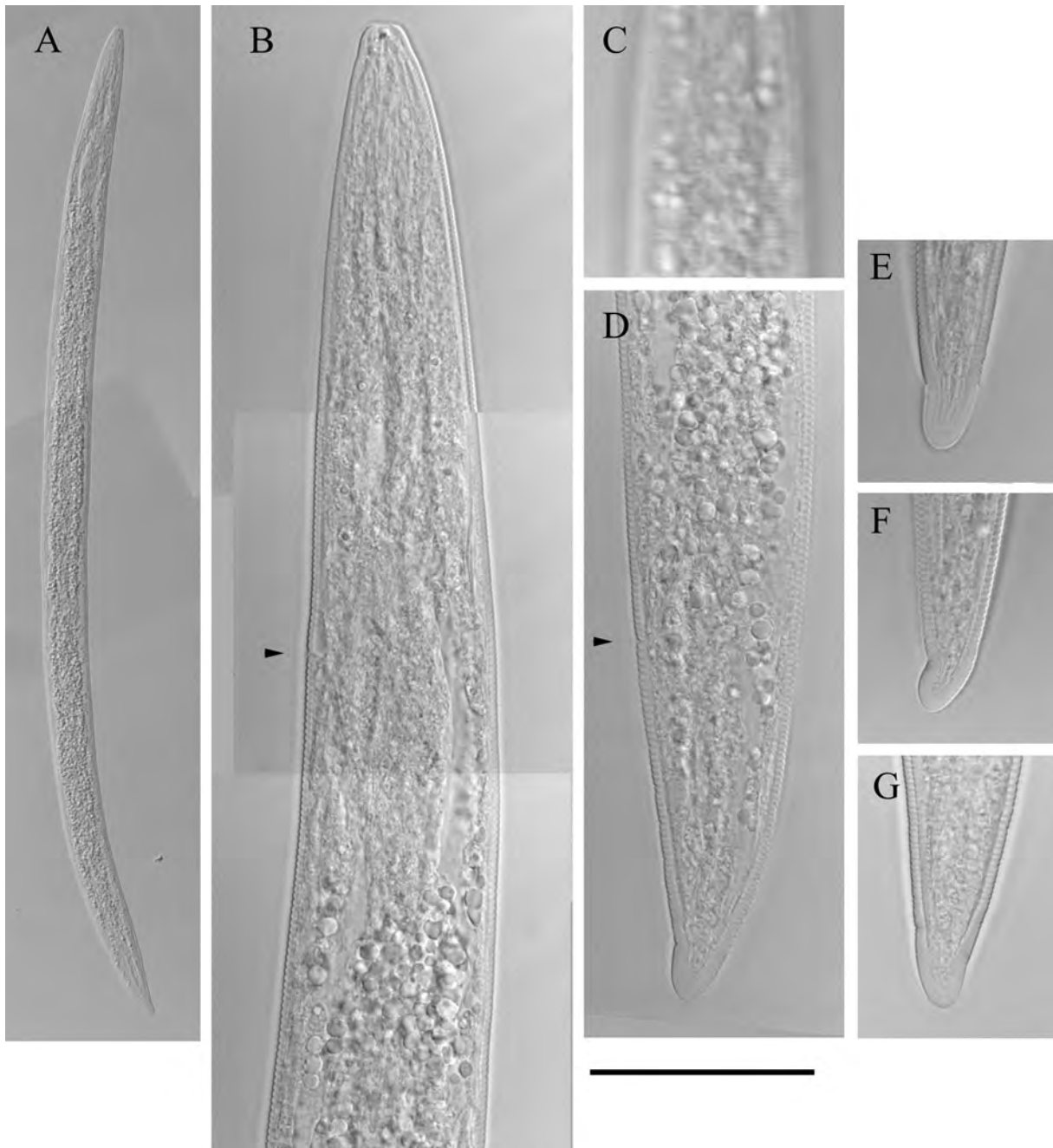
**Fig. 2.** Parasitic juvenile of *Sphaerularia vespae* sp. nov. **(A)** Lateral view. **(B)** Lateral view of anterior region. **(C)** Body annulation of anterior end. **(D)** Body annulation of mid-body. **(E)** Body annulation of posterior end. **(F)** Lateral view of tail. **(G–O)** Variety of tail tip (left lateral view). Arrowhead, constriction.



**Fig. 3.** *Sphaerularia vespae* sp. nov. (A) Lateral view of female. (B) Lateral view of male. (C) Lateral view of female anterior region (arrowhead, excretory pore). (D) Body surface and lateral field of female excretory pore region (arrowhead, deirid). (E) Female reproductive organ (vulva to spermatheca; arrowhead, vulva). (F) Female reproductive organ (spermatheca to outstretched ovary). (G) Female reproductive organ (spermatheca to out-tangled ovary). (H) Lateral view of female tail (arrowhead, anus). (I–K) variety of female tail tip. (L) Subventral view of male tail (bursa and lateral field). (M) Subventral view of male tail (bursa and lateral field). (N) Lateral view of male tail (inside structure). (O) Lateral view of male tail (bursa and lateral field). Bar=100  $\mu$ m in A and B; 40  $\mu$ m in C–O.

slide numbers *Sphaerularia vespae* F-06-10 (five females) and *Sphaerularia vespae* P-05-08 (four parasitic juveniles), deposited in The Herbarium and Insect Museum of the

National Institute of Agro-Environmental Science, Tsukuba, Ibaraki, Japan; slide number *Sphaerularia vespae* F-11-13 (three females), M-02 (one male) and P-09-10 (two parasitic



**Fig. 4.** Parasitic juvenile of *Sphaerularia vespae* sp. nov. **(A)** Lateral view. **(B)** Lateral view of anterior region (arrowhead, excretory pore). **(C)** Body annulation of mid-body. **(D)** Lateral view of tail (arrowhead, anus). **(E–G)** Variety of tail tip (left lateral view). Bar=100  $\mu$ m in A; 40  $\mu$ m in B–G.

juveniles), deposited in the Forest Pathology Laboratory Collection, Forestry and Forest Product Research Institute, Tsukuba, Japan.

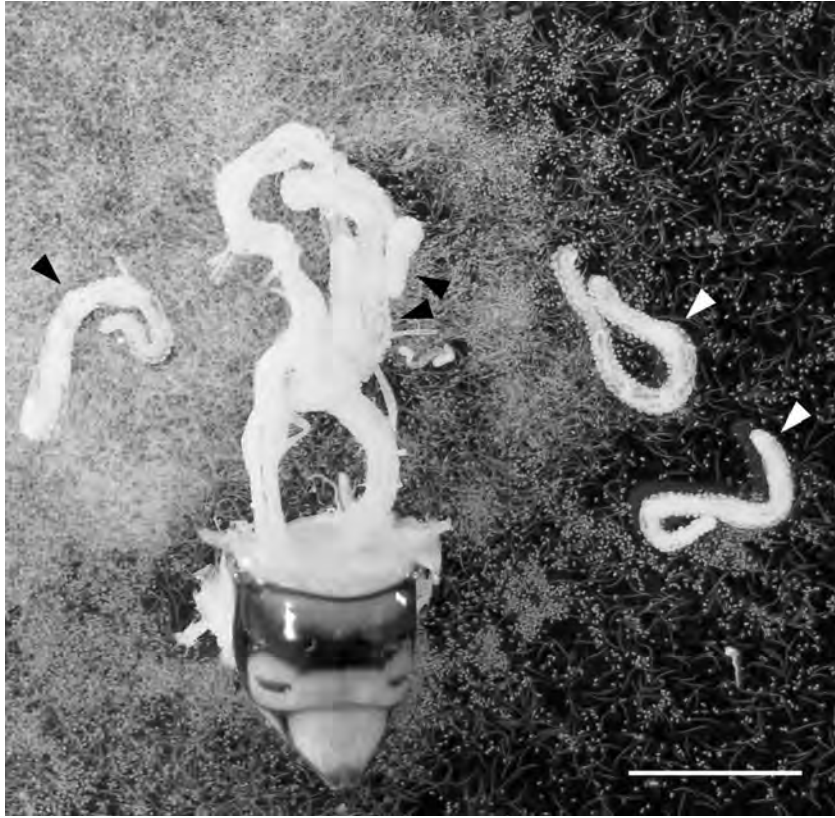
**Etymology.** Specific epithet derived from *Vespa*, the generic name of the host insect.

**Distribution.** Besides the type locality, *S. vespae* sp. nov. was found on Mt. Moiwa, Sapporo, Hokkaido, Japan; Shiraikawa, Sapporo, Hokkaido, Japan; and Nishi-Nopporo, Ebetsu, Hokkaido, Japan.

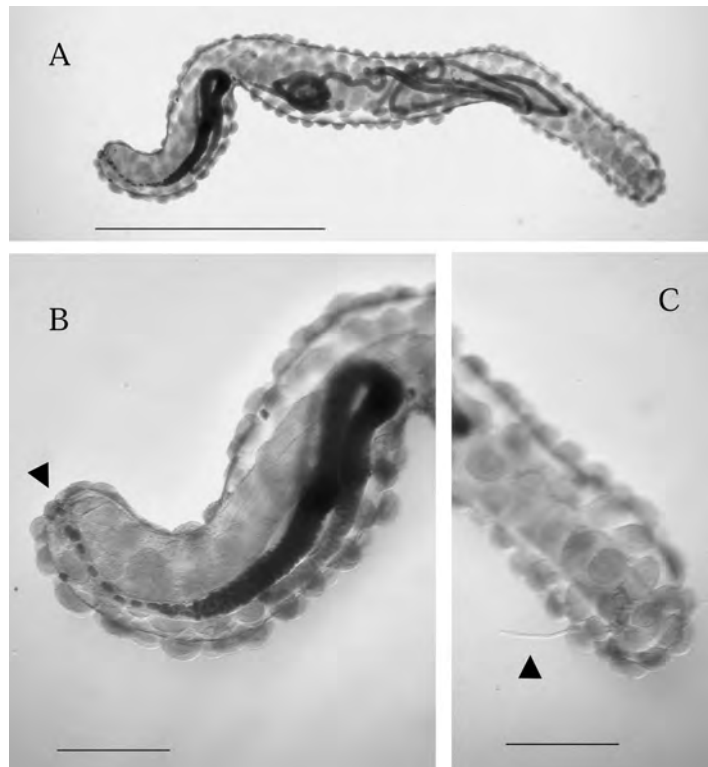
**Molecular profiles.** The profiles obtained from the uterium and those from a single infective female were identical

to each other. The partial SSU+ITS and D2/D3 sequences of *S. vespae* sp. nov., which were obtained from the single female, were deposited in the GenBank database under accession numbers AB300595 and AB300596, respectively. The closest sequences to these three molecular regions were from *S. bombi* (AB250213) for SSU, *Tylenchorhynchus leviterminalis* (EF030984) for ITS, and *S. bombi* (DQ328726) for D2/D3. The sequence similarities in the SSU and D2/D3 regions between *S. vespae* sp. nov. and *S. bombi* are 99% (1,709 identical bases per 1,727 bp: 4 substitutions and 14 insertions/deletions) and 90% (639 identical bases per 708





**Fig. 5.** Mature parasitic female (uterium) of *Sphaerularia vespae* sp. nov. (arrowhead) and eggs and juveniles (small graining and lines, respectively, sometimes appearing cloudy) found during dissection of abdomen of *Vespa simillima* gyne. Bar=5 mm.



**Fig. 6.** Glycerol-processed mature parasitic female (uterium) of *Sphaerularia vespae* sp. nov. **(A)** Entire uterium. **(B)** Genital pore (arrowhead) of uterium and eggs in the genital tract (close-up of left side A). **(C)** Body of *S. vespae* sp. nov. (arrowhead) attached to uterium (close-up of right side A). Bar=5 mm in A; 1 mm in B and C.

bp: 47 substitutions and 22 insertions/deletions), respectively.

## DISCUSSION

*Sphaerularia bombi*, the only known congener of *S. vespae* sp. nov., is an endoparasite of bumblebees of the genera *Bombus* and *Psithyrus* (Macfarlane *et al.*, 1995; McCorquodale *et al.*, 1998; Schmid-Hempel, 1998), and has also been reported from two species of *Vespula* wasps (Cobbold, 1888). These two *Vespula* wasps were referred to as *Vespa rufa* and *Vespa vulgaris* in Cobbold's original 1888 paper. Poinar (1975) and Siddiqi (2000) referred to *Vespa* as the host of *S. bombi*, without citing their source. We assume that these two authors were confused by the generic revision of the host.

The life cycle of *S. bombi* is summarized by Bedding (1984) and Poinar and van der Laan (1972) as follows. Mated females invade bumblebee gynes that overwinter underground. Parasitized gynes usually do not found colonies due to their undeveloped ovaries (but see Röseler, 2002) but repeatedly visit potential overwintering sites in spring and early summer, where they disperse numerous parasitic juveniles. These juveniles grow into adults and mate, and wait for new hosts that happen to enter the overwintering sites in autumn.

Although the details of the life cycle of *S. vespae* sp. nov. are not yet clear, they appear basically similar to those of *S. bombi*. Parasitized gynes of *V. simillima*, which carry no mature eggs, captured with bait traps in the spring, have enlarged uteria of *S. vespae* sp. nov. in the gaster (Sayama *et al.*, 2007). This strongly suggests that these gynes are incapable of establishing colonies, as is the case with parasitized bumblebees. Furthermore, parasitized gynes are frequently trapped in July and August, while unparasitized gynes are rarely captured in these months (Sayama *et al.*, 2007). This is consistent with the presumed behavior of parasitized gynes, which continue wandering outside in early summer, visiting potential overwintering sites and dispersing parasitic juveniles.

The prevalence of *S. vespae* sp. nov. in overwintered gynes of *V. simillima* is often very high: more than 60% of gynes collected in June and July were infested with the nematode in Sapporo, northern Japan (Sayama *et al.*, 2007). This implies that the nematode is potentially effective in controlling hornet populations. We do not know, however, whether the prevalence of *S. vespae* sp. nov. changes among populations of hornets or varies according to geographical region, or if it occurs in vespine species other than *V. simillima*. Future studies should reveal these points as well as the details of the life cycle of *S. vespae* sp. nov. in relation to its host.

## ACKNOWLEDGMENTS

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