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Morphological ontogeny and molecular analyses of geographic strains of two closely related *Neoseiulus* species (Acari: Phytoseiidae)

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Original research

ABSTRACT

The predatory mites, *Neoseiulus longispinosus* (Evans) and *N. womersleyi* (Schicha) (Phytoseiidae) are two morphologically close species. Although they can be mainly separated by the length of the dorsal seta *S5*, misidentifications may occur. The present study investigated various strains of the two closely related species collected from Japan, Taiwan and/or Thailand using a combination of morphological and molecular data. We described and illustrated the adults and immature stages. We also sequenced the 28S region of nuclear ribosomal DNA to determine whether these strains represent different species. The two species could be clearly separated based on the length of seta *S5* and the ratio of setae *Z5/S5*. In a 28S tree, the examined strains also formed separate clades with 100% bootstrap values. We conclude that a combination of different methods is often necessary to precisely distinguish these closely related taxa.

Keywords predator; phytoseiid mites; morphology; systematics; molecular; ribosomal DNA

Introduction


The Phytoseiidae Berlese is one of the most important families in the Acari that includes predatory mites used to control spider mites (Tetranychidae), eriophyoid mites (Eriophyoidea) and small pest insects (McMurtry and Croft 1997). *Neoseiulus* Hughes is one of the largest genera in the family Phytoseiidae, with about 415 species, including synonyms (Demite *et al.* 2020). Chant and McMurtry (2003) classified *Neoseiulus* species into 10 species groups mainly based on the presence or absence of setae *J1* and *ZV3*, seta *ST3* situated on or off the sternal shield, the shape of female ventrianal shield and spermatheca, and relative lengths of dorsocentral setae. *Neoseiulus longispinosus* (Evans, 1952) and *N. womersleyi* (Schicha,

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1975) belong to the *womersleyi* species subgroup of the *barkeri* species group. Because very few morphological features are known to separate *N. longispinosus* from *N. womersleyi*, and sterile female offspring were obtained from crosses between *N. longispinosus* females and *N. womersleyi* males (Ullah *et al.* 2017), we described all life stages to find out whether there might be new morphological characters that could be used to separate the species.

DNA sequences for the genes that encode proteins such as the cytochrome *c* oxidase subunit I (COI) and 12S rRNA of mitochondrial DNA (mtDNA), and the internal transcribed spacer (ITS) and 28S regions of nuclear ribosomal DNA (nrDNA) have been widely used to identify species and resolve phylogenetic relationships of phytoseiid mites (Okassa *et al.* 2009, 2020; Sonoda *et al.* 2012; Vicente dos Santos and Tixier 2017, 2018; Nguyen *et al.* 2019; Inak *et al.* 2020). We also examined whether the two closely related species (*N. longispinosus* and *N. womersleyi*) could be distinguished from each other as well as from other species using the 28S region of nrDNA.

Material and methods

Mite samples

Mite species and strains used in the morphological and DNA analyses are listed in Table 1. Laboratory stocks were separately reared on detached leaves of common bean, *Phaseolus vulgaris* L. (Fabaceae) placed on water-saturated polyurethane mats in plastic dishes (90-mm diameter, 20-mm depth) at 25±1 °C under a 16:8 h light:dark photoperiod. The two-spotted spider mite, *Tetranychus urticae* Koch (Tetranychidae) was provided as prey.

Morphological analyses

Adult females, males and immatures for certain strains were mounted on permanent slides using Hoyer's medium. The specimens were examined using a BX53[®] (Olympus, Tokyo, Japan) differential interference contrast microscope equipped with a DP72[®] digital camera (Olympus). Illustrations were done with Adobe Illustrator (Adobe Systems Incorporated, San Jose, CA, USA) and body parts and setae were measured using the imaging software Sensiv Measure1 ver. 2.6.0. All measurements are given in micrometers (µm) as minimum and maximum values from individuals examined. Body length measurements represent the distance between the anterior and posterior margins of dorsal shield. The setal nomenclature used for dorsal and ventral sides follows that of Lindquist and Evans (1965) as adapted by Rowell *et al.* (1978) and Chant and Yoshida-Shaul (1991), respectively. The notation for gland pores (solenostomes) or lyrifissures (poroids) follows Athias-Henriot (1975). The generic classification is according to the definitions given by Chant and McMurtry (2007). Voucher specimens of the redescribed species were deposited in the Laboratory of Applied Entomology and Zoology, Ibaraki University (AEZIU), Japan, under the serial voucher specimen numbers.

Molecular analyses

Adult females from each strain/species were arbitrarily selected and used for molecular analyses. Total DNA was extracted from the whole body of each female by crushing with a toothpick in 20 µl of 2x PCR Buffer for KOD FX Neo (Toyobo, Osaka, Japan). To amplify a fragment of the 28S region, the primers 43F (5'-GCTGCGAGTGAAGTGAATCAAGCCT-3') and 929R (5'-AGGTCACCATCTTTTCGGGTC-3') (Dowling and O'Connor 2010) were used. Polymerase chain reaction (PCR) was performed in a total of 20 µl of reaction solution containing 5 µl of DNA template, 0.4 µl of KOD FX Neo (1 U/µl, Toyobo), 0.6 µl of each primer (10 pmol/µl each), 4 µl of 2 mM dNTPs (Toyobo), 5 µl of 2x PCR Buffer for KOD FX Neo (Toyobo) and 4.4 µl of distilled water. PCR cycling conditions were 3 min at 94 °C, followed by 42 cycles of 10 sec at 98 °C, 30 sec at 52-58 °C and 1 min at 68 °C, and a final extension at 68 °C for

30 sec. PCR products were visualized by electrophoresis on an agarose gel. The PCR products showing a single band were purified using a Sephacryl S-300 HR column (GE Healthcare, Chicago, IL, USA) and directly sequenced. If the PCR products showed multiple bands, the bands of the expected size were gel purified using Wizard SV Gel and PCR Clean-Up System (Promega, Madison, WI, USA) and directly sequenced. The sequencing was carried out in both directions using the amplifying primers with BigDye Terminator Cycle Sequencing Kit v.3.1 (Applied Biosystems, Foster City, CA, USA) and on an ABI 3130xl Genetic Analyzer (Applied Biosystems).

All obtained sequence data were deposited in DDBJ/EMBL/GenBank International Nucleotide Sequence databases under the accession numbers LC591988–LC592035. The 28S sequences of outgroup taxon (*Hypoaspis miles* Berlese; Laelapidae; accession number: KU318163) were obtained from previously published data (Vicente dos Santos and Tixier 2017). Obtained sequences were aligned using CLUSTAL W and the numbers of parsimony informative sites were calculated in MEGAX (Kumar *et al.* 2018). Mean intra- and inter-specific genetic distances (*p*-distances; proportion (*p*) of nucleotide sites) were calculated by MEGAX. The maximum likelihood (ML) tree was constructed using the best-fit model (Kimura 2-Parameter model with gamma distribution) chosen by MEGAX. Branch robustness was tested by bootstrap analysis with 1,000 replications.

Table 1 Collection data and accession numbers of 17 strains of three *Neoseiulus* species and one species of each *Gynaeseius* and *Phytoseiulus* examined in the study.

Species	Locality	Geographic coordinates	Host plant	Collection date	Voucher specimen no. ^a (Experiment) ^b	Accession number
<i>N. longispinosus</i> (Evans)	Tarama, Okinawa, Japan	24°38' N – 124°43' E	<i>Symplocos kuroki</i> Nagam.	Nov. 21, 2019	897 (D)	LC591994-96
	Tarama, Okinawa, Japan	24°38' N – 124°43' E	<i>Bidens</i> sp.	Nov. 21, 2019	898 (D)	LC591997-99
	Tarama, Okinawa, Japan	24°40' N – 124°41' E	<i>Bidens</i> sp.	Nov. 21, 2019	900 (M, D)	LC592000-02
	Taitung, Taiwan	23°02' N – 121°09' E	<i>Fragaria</i> × <i>ananassa</i> Duchesne	Feb. 04, 2020	906 (M, D)	LC592006-08
	Bangkok, Thailand	13°45' N – 100°52' E	<i>Fragaria</i> × <i>ananassa</i> Duchesne	Oct. 01, 1999	725 (M)	-
<i>N. womerleyi</i> (Schicha)	Miyako, Okinawa, Japan	24°45' N – 125°17' E	Apiaceae	Nov. 22, 2019	903 (D)	LC592003-05
	Morioka, Iwate, Japan	39°76' N – 141°13' E	<i>Malus domestica</i> Borkh	Aug. 19, 2018	907 (M, D)	LC592009-11
	Taitung, Taiwan	22°53' N – 121°11' E	<i>Ipomoea nil</i> (L.) Roth	Nov. 09, 2010	828 (M, D)	LC591991-93
	Morioka, Iwate, Japan	39°76' N – 141°13' E	<i>M. domestica</i>	Oct. 19, 1999	724 (D)	LC591988-90
	Main Island, Okinawa, Japan	26°10' N – 127°43' E	<i>Luffa</i> sp.	April 26, 2016	908 (M, D)	LC592012-14
<i>N. californicus</i> (McGregor)	Ichihara, Chiba, Japan	35°43' N – 139°55' E	<i>Miscanthus sinensis</i> Andersson	Aug. 17, 1995	909 (D)	LC592015-17
	Spical [®]	Koppert Biological Systems	-	2007	910 (D)	LC592018-20
	Ami, Ibaraki, Japan	36°02' N – 140°13' E	<i>Pueraria montana</i> (Lour.) Merr.	June 07, 2019	911 (D)	LC592021-23
	Bangladesh	24°43' N – 90°26' E	<i>Rosa</i> sp.	Feb. 22, 2019	912 (D)	LC592024-26
	Miyako-Top [®]	Agrisect Inc.	-	2020	916 (D)	LC592033-35
<i>G. liturivorus</i> (Ehara)	Chiba, Japan	35°43' N – 139°55' E	<i>Allium fistulosum</i> L.	Nov. 2012	913 (D)	LC592027-29
<i>P. persimilis</i> Athias-Henriot	Chiri-Top [®]	Agrisect Inc.	-	2020	914 (D)	LC592030-32

^aVoucher specimens are preserved at the Laboratory of Applied Entomology and Zoology (Faculty of Agriculture, Ibaraki University) under the serial voucher specimen numbers.

^bEach strain of the five studied species was used for the morphological (M) and/or DNA (D) analyses.

Results

Taxonomy

***Neoseiulus longispinosus* (Evans, 1952)**

[Japanese name: Minami-kenaga-kaburidani]

(Figs. 1-4) (Table 2)

Description

Female (n=10)

Dorsum (Fig. 1A). Dorsal shield smooth, with few striae anterolaterally; with five pairs of solenostomes ($gd_2, gd_4, gd_6, gd_8, gd_9$) and 13 pairs of poroids ($id_1, idl_a, id_2, id_4, idm_2, idm_3, idm_4, idm_5, idm_6, isl, idl_1, idl_3, idl_4$); 328–347 long and 178–189 wide at s_4 level; j_1 17–21, j_3 62–66, j_4 63–65, j_5 66–70, j_6 74–77, J_2 79–81, J_5 7–9, z_2 70–75, z_4 69–76, z_5 35–38, Z_1 74–84, Z_4 74–77, Z_5 78–80, s_4 69–83, S_2 76–80, S_4 56–59, S_5 22–24, r_3 64–69, R_1 63–70.

Table 2 Character measurements of adult females of *Neoseiulus longispinosus* (Evans) strains collected in the present study comparing with those of previous studies (other than listed in Kreiter *et al.* 2020).

	Japan (900) (n=10)	Taiwan (906) (n=10)	Thailand (725) (n=10)	Dominican Rep. (Abo-Shnaf <i>et al.</i> 2016) (n=2)	India (Gupta 1986) (n/a)	The Philippines & Taiwan (Liao <i>et al.</i> 2020) (n=10)	Korea (Lee and Ryu 1989) (n=10)	China (Xin <i>et al.</i> 1981) (n=1)
DS-L	328-347	317-330	340-367	325, 337	325	298-348	341.5-499.8	301.1
DS-W	178-189	165-179	182-193	183, 195	180	170-201	216.5-391.5	175.3
<i>j</i> ₁	17-21	17-20	16-19	16, 20	16-20	15-22	16.6-23.2	18.3
<i>j</i> ₃	62-66	64-72	62-66	63, 65	50-60	54-76	45.8-68.6	53.1
<i>j</i> ₄	63-65	61-66	67-69	55, 60	54-62	52-66	41.6-60.3	54.2
<i>j</i> ₅	66-70	68-74	60-64	73, 75	67-72	54-80	49.9-67.6	63
<i>j</i> ₆	74-77	73-79	75-78	70, 75	72	58-88	62.4-77.0	67.9
<i>J</i> ₂	79-81	72-79	80-84	78, 80	71-81	53-88	65.5-81.1	72
<i>J</i> ₅	7-9	7-8	6-8	6, 7	7-10	7-17	8.3-10.0	9.5
<i>z</i> ₂	70-75	68-74	66-69	66, 70	69	53-98	52.0-77.0	62.1
<i>z</i> ₄	69-76	72-78	75-77	73, 75	70-76	56-92	57.2-78.0	70
<i>z</i> ₅	35-38	30-37	32-45	30	31-36	25-42	21.8-39.5	29.3
<i>Z</i> ₁	74-84	76-80	80-85	75, 77	73-76	64-95	66.6-81.1	71.3
<i>Z</i> ₄	74-77	68-76	71-74	71, 72	63-69	69-84	61.4-79.0	69.6
<i>Z</i> ₅	78-80	79-84	80-85	75, 85	78-80	64-94	77.0-91.5	74
<i>s</i> ₄	69-83	79-84	82-86	85, 88	80-82	71-103	69.7-85.3	75.6
<i>S</i> ₂	76-80	69-77	74-77	73, 74	76-78	66-97	62.4-79.0	67.6
<i>S</i> ₄	56-59	62-66	60-65	58, 60	59-61	56-74	49.9-64.5	58.5
<i>S</i> ₅	22-24	21-26	20-24	12, 15	18	12-24	33.3-51.0	16.2
<i>r</i> ₃	64-69	57-69	62-65	52, 53	58	52-70	44.7-64.5	56.4
<i>R</i> ₁	63-70	56-64	62-66	65, 68	67	45-78	47.8-70.7	57.2
<i>st</i> _{2-st} ₂	58-62	58-66	54-57	60, 61	-	56-64	-	-
<i>st</i> _{1-st} ₃	59-64	57-67	58-59	54, 55	-	57-63	-	-
<i>st</i> _{5-st} ₅	49-53	54-56	50-57	55, 58	-	51-70	-	-
VS-L	109-115	103-117	111-114	115, 120	107-116	111-122	-	-
VS-W/ZV2	90-99	90-96	91-96	82, 84	90-95	82-103	-	-
VS-W/anus	71-76	70-80	69-74	75, 80	-	68-84	-	-
<i>JV</i> ₅	62-66	57-65	61-66	-	67	56-92	-	57.6
<i>St</i> _{IV}	78-83	80-85	79-84	80, 85	60-70	71-90	-	73.6
SC-L	19-25	18-22	22-26	15, 20	-	15-24	-	-
SC-W	4-7	4-6	4-5	-	-	5-8	-	-
FD-L	25-28	22-27	22-25	-	-	23-26	-	-
MD-L	27-30	24-29	21-23	-	-	23-27	-	-

DS-dorsal shield; VS-ventrianal shield; SC-spermathecal calyx; FD-fixed digit; MD-movable digit; L-length; W-width.

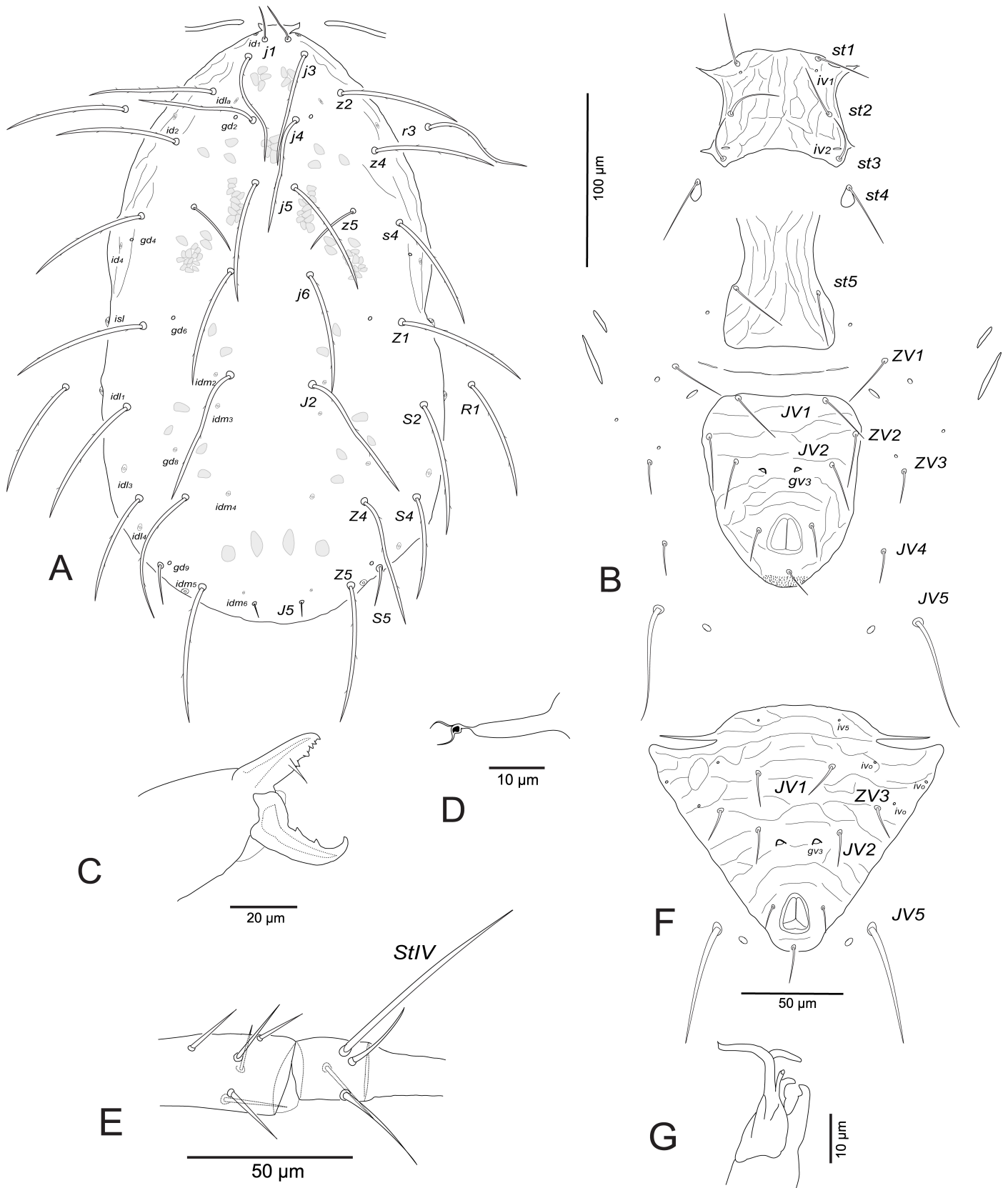


Figure 1 *Neoseiulus longispinosus* (Evans). Female; A – dorsum; B – venter; C – chelicera; D – spermatheca; E – leg IV; Male; F – ventrianal shield; G – spermatodactyl.

All setae very finely serrated, except setae *j1*, *J5* and *S5* short and smooth.

Venter (Fig. 1B). Sternal and genital shields striated, distance between setae *st1-st3* 59–64, *st2-st2* 58–62, *st5-st5* 49–53. Sternal shield with two pairs of poroids (*iv₁*, *iv₂*). Ventrianal shield striated, 109–115 long, 90–99 wide at level *ZV2* and 71–76 wide at the anus level; distance between setae *JV2-JV2* 46–58; one distinct pair of pores (*gv₃*) posteromesad *JV2*, *gv3-gv3* 16–28; *JV5* 62–66.

Peritreme. Extending to the level between setae *j1* and *j3*.

Chelicera (Fig. 1C). Movable cheliceral digit 27–30 long, with 2 teeth. Fixed cheliceral digit 25–28 long, with 4–5 teeth and a pilus dentilis.

Spermatheca (Fig. 1D). Calyx long, thin, flared distally, 19–25 long, 4–7 wide, constricted basally forming a short stalk at junction with a large semicircular atrium. Minor duct indistinct.

Legs. Leg IV with a long macroseta on basitarsus: *StIV* 78–83 (Fig. 1E). Chaetotactic formulae are shown in Table 3.

Male (n=7)

Dorsum. Dorsal shield smooth, with few striae anterolaterally; 253–261 long and 173–182 wide at *s4* level; *j1* 14–16, *j3* 57–60, *j4* 49–55, *j5* 57–63, *j6* 63–65, *J2* 66–72, *J5* 5–6, *z2* 56–60, *z4* 60–64, *z5* 30–33, *Z1* 55–59, *Z4* 58–61, *Z5* 60–64, *s4* 64–70, *S2* 58–63, *S4* 36–40, *S5* 14–16, *r3* 44–57, *R1* 41–46.

Venter. Sternogenital shield striated, *st1-st5* 105–109, *st2-st2* 51–55. Ventrianal shield entirely striated, wider than long, 104–122 long and 143–149 wide at the anterior corners level; with three pairs of pre-anal setae, one distinct pair of pores (*gv₃*) posteromesad *JV2*; *JV5* 50–59 (Fig. 1F).

Peritreme. Extending to level between setae *z2* and *z4*.

Spermatodactyl (Fig. 1G). Almost T-shaped, shaft 20–22 (transverse part). Movable cheliceral digit 18–20 long, fixed digit 16–17 long.

Legs. Leg IV with long setaceous macroseta on basitarsus: *StIV* 67–70. Chaetotactic formulae are shown in Table 3.

Deutonymph (female) (n=7)

Dorsum (Fig. 2A). Dorsal shield faint and smooth; 260–270 long and 170–179 wide at *s4* level; *j1* 15–17, *j3* 36–47, *j4* 40–51, *j5* 45–55, *j6* 57–63, *J2* 42–47, *J5* 7–8, *z2* 44–49, *z4* 49–61, *z5* 32–36, *Z1* 46–52, *Z4* 39–53, *Z5* 40–46, *s4* 55–65, *S2* 48–53, *S4* 34–43, *S5* 10–13, *r3* 30–36, *R1* 29–34.

Venter (Fig. 2B). Ventral shields indistinct, distance between setae *st1-st3* 68–71, *st2-st2* 50–56, *st5-st5* 35–37, *st1-st5* 120–126. Distances *JV1-JV1* 30–34, *JV1-JV5* 70–76; one

Table 3 Chaetotaxy of legs in different developmental stages of *Neoseiulus longispinosus* (Evans) and *N. womersleyi* (Schicha) strains collected in the present study.

Stage	Leg	Coxa	Trochanter	Femur	Genu	Tibia	Basitarsus
Larva	I	0-0/1-0/1-0	1-0/1-0/1-1	2-2/1-2/1-2	1-2/1-2/1-1	1-2/1-2/1-1	0-0-0-0
	II	0-0/1-0/1-0	1-0/1-0/1-1	1-2/1-2/0-1	1-2/0-2/0-1	1-1/1-2/1-1	1-1-1-1
	III	0-0/1-0/1-0	1-0/1-0/1-1	1-1/0-2/0-1	1-2/0-2/0-1	1-1/1-2/1-1	1-1-1-1
	IV	-	-	-	-	-	-
Protonymph	I	0-0/1-0/1-0	1-0/1-0/1-1	2-2/1-2/1-2	1-2/1-2/1-1	1-2/1-2/1-1	0-0-0-0
	II	0-0/1-0/1-0	1-0/1-0/1-1	1-2/1-2/0-1	1-2/0-2/0-1	1-1/1-2/1-1	1-1-1-1
	III	0-0/1-0/1-0	1-0/1-0/1-1	1-1/0-2/0-1	1-2/0-2/0-1	1-1/1-2/1-1	1-1-1-1
	IV	0-0/1-0/0-0	1-0/1-0/1-1	0-2/1-1/0-0	0-2/1-2/0-0	1-1/1-1/0-1	1-1-1-1
Deutonymph & adult	I	0-0/1-0/1-0	1-0/2-0/1-1	2-2/1-3/2-2	2-2/1-2/1-2	2-2/1-2/1-2	0-1-1-0
	II	0-0/1-0/1-0	1-0/2-0/1-1	1-2/1-2/2-2	1-2/0-2/0-2	1-1/1-2/1-1	1-1-1-1
	III	0-0/1-0/1-0	1-1/1-0/1-1	1-2/1-1/0-1	1-2/1-2/0-1	1-1/1-2/1-1	1-1-1-1
	IV	0-0/1-0/0-0	1-1/1-0/1-1	1-2/1-1/0-1	1-2/1-2/0-1	1-1/1-2/0-1	1-1-1-1

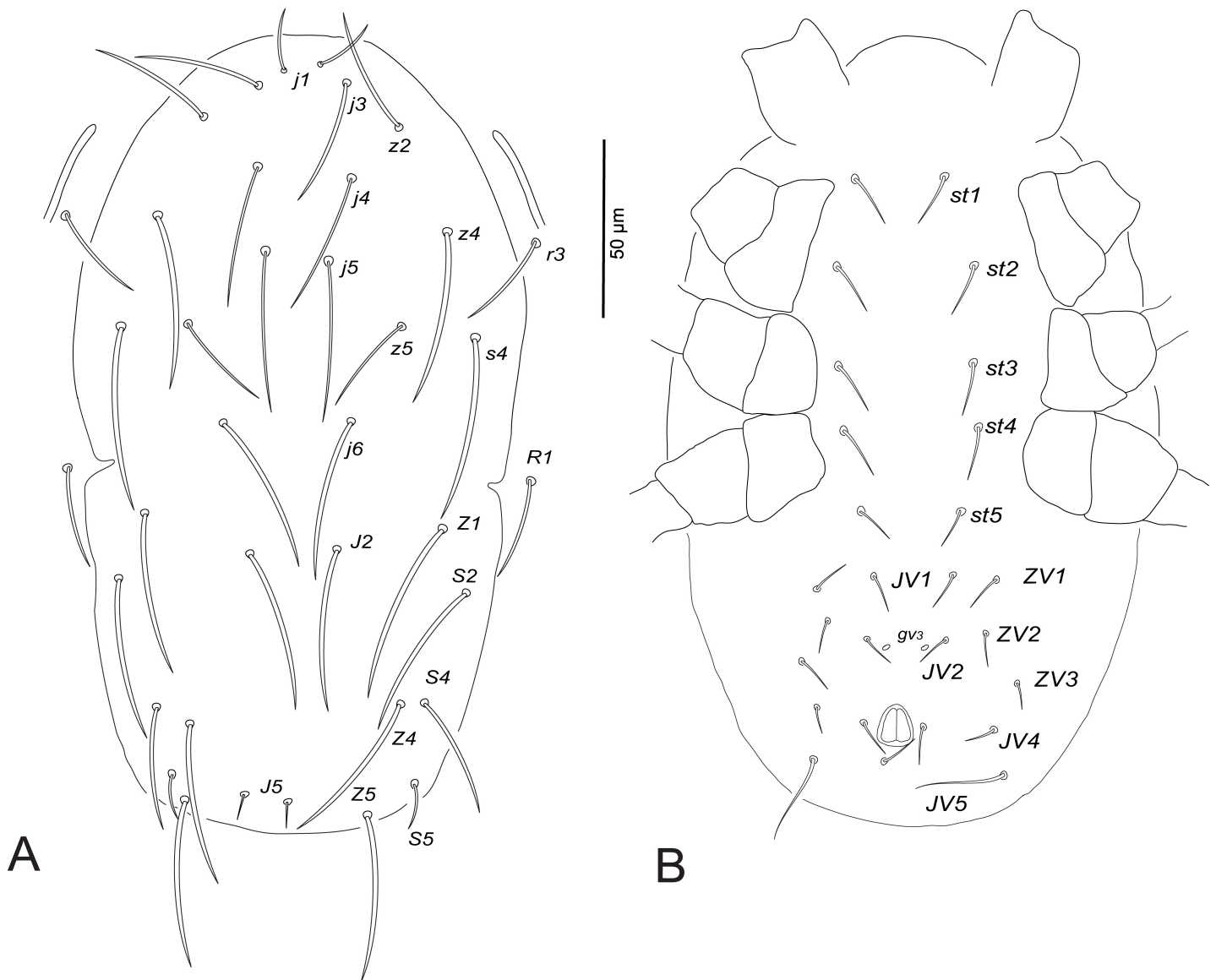


Figure 2 *Neoseiulus longispinosus* (Evans). Deutonymph (female); A – dorsum; B – venter.

distinct pair of pores (gv_3) posteromesad *JV2*; *JV5* 24–27.

Peritreme. Extending to the level between setae *z2* and *z4*, closer to *z2*.

Legs. Leg IV with one macroseta, *StIV* 65–70. Chaetotactic formulae are shown in Table 3.

Protonymph (n=6)

Dorsum (Fig. 3A). Dorsal shield indistinct; 238–245 long and 162–171 wide at *s4* level; *j1* 14–15, *j3* 33–43, *j4* 35–38, *j5* 38–40, *j6* 43–45, *J2* 31–37, *J5* 4, *z2* 40–42, *z4* 47–50, *z5* 25–28, *Z1* 27–34, *Z4* 33–37, *Z5* 33–40, *s4* 55–58, *S2* 33–36, *S4* 27–34, *S5* 8–10, *r3* 25–28, *R1* 23–29.

Venter (Fig. 3B). Sternal and genital shields indistinct, distance between setae *st1*–*st3* 65–67, *st2*–*st2* 51–55. Distances *JV1*–*JV1* 25–27, *JV1*–*JV5* 64–70; pores (gv_3) posteromesad *JV2*; *JV5* 18–21.

Peritreme. Very short, extending to level between setae *j6* and *s4*.

Legs. Leg IV with macroseta, *StIV* 61–67. Chaetotactic formulae are shown in Table 3.

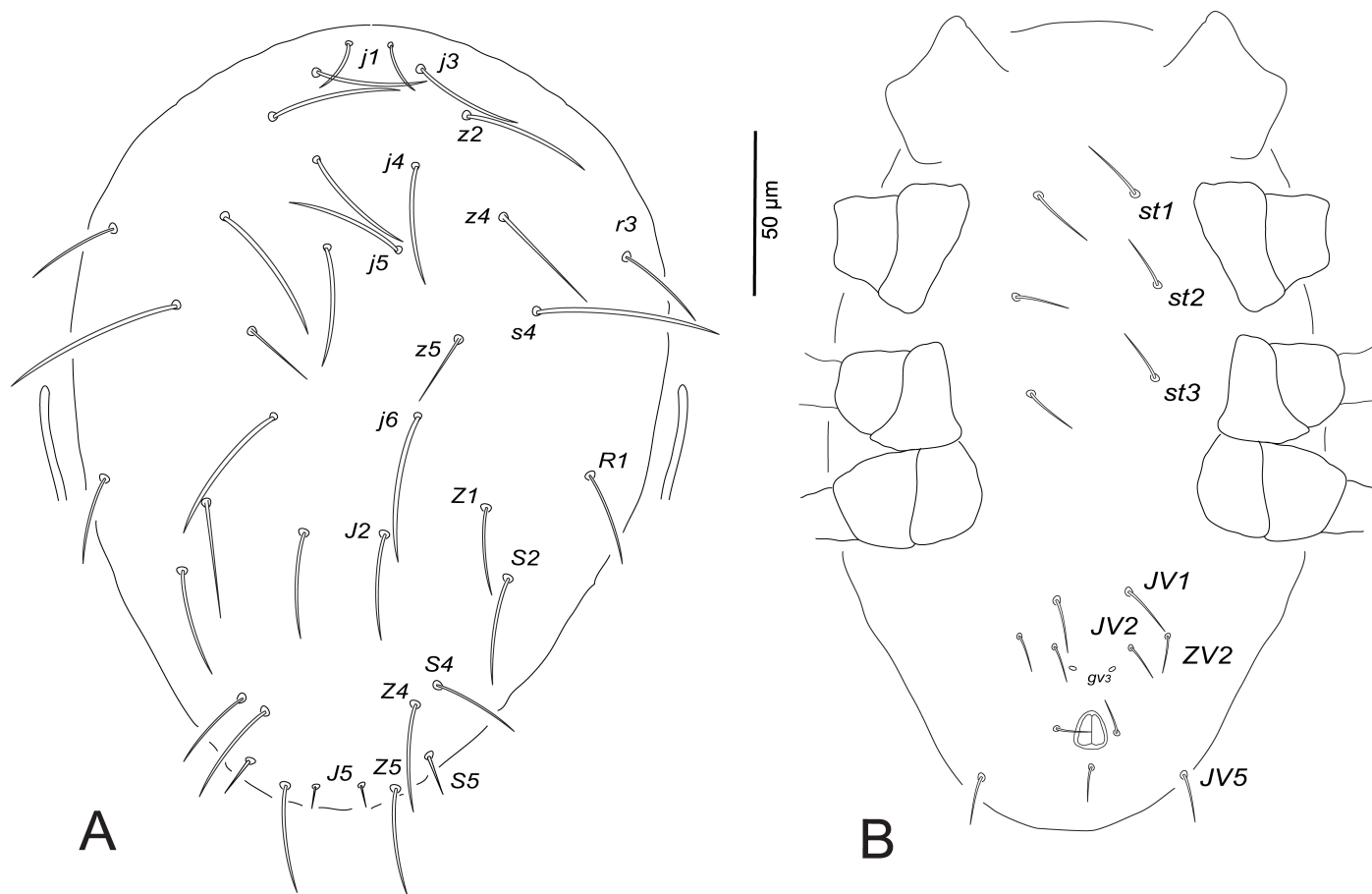


Figure 3 *Neoseiulus longispinosus* (Evans). Protonymph; A – dorsum; B – venter.

Larva (n=7)

Dorsum (Fig. 4A). Dorsal shield indistinct; 186–191 long and 133–137 wide at *s4* level; *j1* 18–23, *j3* 17–23, *j4* 10–13, *j5* 11–15, *j6* 53–57, *z2* 14–16, *z4* 17–23, *z5* 9–10, *Z1* 8–10, *Z4* 69–90 (whip-like), *s4* 54–61, *S2* 9–11.

Peritreme. Indistinct.

Venter (Fig. 4B). Distances between ventral setae *st1–st3* 62–66, *st2–st2* 54–57, *JV1–JV1* 19–22; pores (*gv3*) present and setae *ZV1*, *ZV3*, *JV4* absent. Dorsal seta *Z5* on posterior margin ventrally. Chaetotactic formulae of legs are shown in Table 3.

Material examined

10 females, 7 males, 7 deutonymphs, 6 protonymphs, 7 larvae (voucher specimen no. 900), Tarama, Okinawa, Japan (24°40' N – 124°41' E, T. Gotoh leg.), on *Bidens* sp. (Asteraceae); 10 females, 8 males, 10 deutonymphs, 8 protonymphs, 8 larvae (voucher specimen no. 906), Taitung, Taiwan (23°02' N – 121°09' E, T. Gotoh leg.), on *Fragaria* × *ananassa* Duchesne (Rosaceae); 10 females (voucher specimen no. 725), Bangkok, Thailand (13°45' N – 100°52' E, M. Kongchuensin leg.), on *F.* × *ananassa* (Table 1).

***Neoseiulus womersleyi* (Schicha)**

[Japanese name: Kenaga-kaburidani]
(Figs. 5-8) (Table 4)

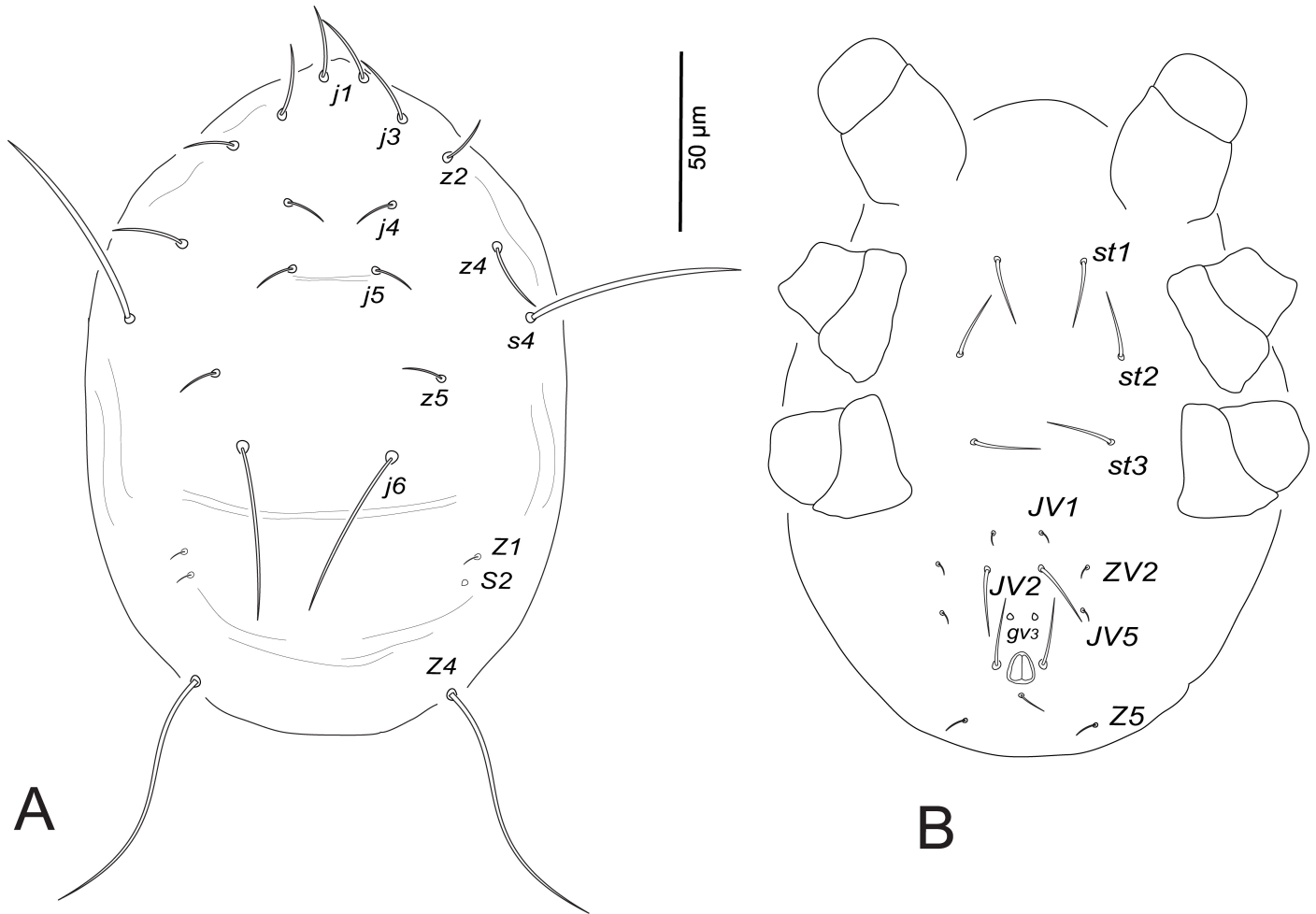


Figure 4 *Neoseiulus longispinosus* (Evans). Larva; A – dorsum; B – venter.

Description

Female (n=10)

Dorsum (Fig. 5A). Dorsal shield smooth, with few striae anterolaterally; with five pairs of solenostomes ($gd_2, gd_4, gd_6, gd_8, gd_9$) and 13 pairs of poroids ($idl_1, idl_a, id_2, id_4, idm_2, idm_3, idm_4, idm_5, idm_6, isl, idl_1, idl_3, idl_4$); 331–359 long and 175–203 wide at s_4 level; j_1 16–19, j_3 52–57, j_4 47–57, j_5 55–59, j_6 67–70, J_2 74–81, J_5 8–10, z_2 52–66, z_4 64–73, z_5 31–35, Z_1 73–76, Z_4 73–77, Z_5 84–90, s_4 73–76, S_2 68–77, S_4 62–66, S_5 45–51, r_3 47–56, R_1 50–55. All setae very finely serrated, except setae j_1 and J_5 short and smooth.

Venter (Fig. 5B). Sternal and genital shields striated, distance between setae st_1 – st_3 55–60, st_2 – st_2 59–63, st_5 – st_5 52–59. Sternal shield with two pairs of poroids (iv_1, iv_2). Ventrianal shield striated, 99–121 long, 77–93 wide at level ZV_2 and 71–75 wide at the anus level; distance between setae JV_2 – JV_2 42–56; pores (gv_3) posteromesad JV_2 , gv_3 – gv_3 14–21; JV_5 62–69.

Peritreme. Extending to bases of setae j_3 .

Chelicera (Fig. 5C). Movable cheliceral digit 24–29 long, with 2 teeth. Fixed cheliceral digit 22–27 long, with 4 teeth and a pilus dentilis.

Spermatheca (Fig. 5D). As in females of *N. longispinosus*. Calyx 12–21 long, 4–5 wide, slightly flared distally, constricted basally at junction with atrium. Atrium bulged and rounded, deeply forked at the junction with the major duct. Minor duct indistinct.

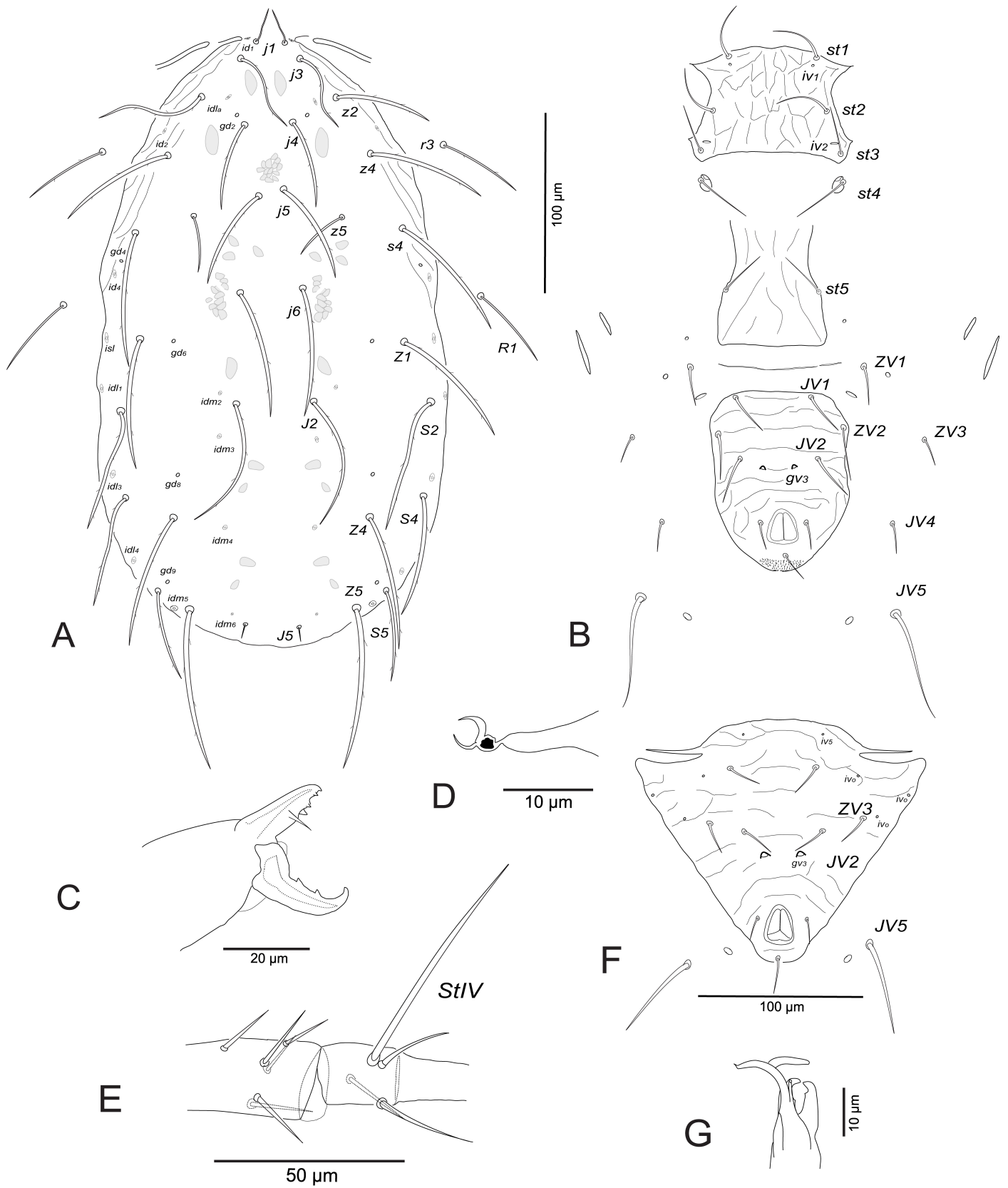


Figure 5 *Neoseiulus womersleyi* (Schicha). Female; A – dorsum; B – venter; C – chelicera; D – spermatheca; E – leg IV; Male; F – ventrianal shield; G – spermatodactyl.

Legs. Leg IV with long, smooth, pointed macroseta on basitarsus: *StIV* 69–80 (Fig. 5E). Chaetotactic formulae are shown in Table 3.

Male (n=8)

Dorsum. Dorsal shield smooth, with few striae anterolaterally; 243–254 long and 162–170 wide at *s4* level; *j1* 11–14, *j3* 33–36, *j4* 29–33, *j5* 31–33, *j6* 45–49, *J2* 47–51, *J5* 6–7, *z2* 37–40,

Table 4 Character measurements of adult females of *Neoseiulus womersleyi* (Schicha) strains collected in the present study comparing with those of previous studies.

	Taiwan (828) (n=10)	Japan (907) (n=10)	Japan (908) (n=10)	Australia (Schicha 1975) (n=5)	Taiwan & Japan (Liao <i>et al.</i> 2020) (n=9)	Australia (Womersley 1954) (n=2)
DS-L	324-331	331-359	314-352	348±12	325-362	357
DS-W	180-188	175-203	179-189	191±12	173-195	201
<i>j1</i>	17-19	16-19	16-18	14±1	14-24	25
<i>j3</i>	64-67	52-57	60-65	58±7	42-63	56
<i>j4</i>	53-60	47-57	55-61	59±3	42-60	56
<i>j5</i>	64-67	55-59	63-66	63±5	50-64	60
<i>j6</i>	69-74	67-70	70-75	77±5	56-78	70
J2	78-80	74-81	78-84	79±4	54-71	70
J5	7-9	8-10	8-9	13±2	8-12	14
<i>z2</i>	65-70	52-66	60-65	69±6	53-70	62
<i>z4</i>	72-78	64-73	74-80	75±3	56-81	78
<i>z5</i>	24-30	31-35	27-32	49±5	22-30	40
Z1	74-77	73-76	77-79	77±4	60-79	70
Z4	76-80	73-77	75-79	80±5	57-76	75
Z5	82-87	84-90	80-87	80±4	76-100	84
<i>s4</i>	83-87	73-76	80-85	84±4	68-86	84
S2	75-80	68-77	73-79	78±4	65-79	56
S4	64-68	62-66	61-67	59±9	55-67	75
S5	45-53	45-51	48-53	47±5	34-50	42
<i>r3</i>	59-63	47-56	52-59	59±5	41-61	-
R1	60-64	50-55	58-62	68±5	47-62	-
<i>st2-st2</i>	58-64	59-63	61-66	-	60-67	-
<i>st1-st3</i>	57-63	55-60	54-56	-	56-66	-
<i>st5-st5</i>	60-67	52-59	60-64	-	59-66	-
VS-L	100-110	99-121	104-109	-	112-134	-
VS-W/ZV2	85-90	77-93	88-91	-	85-96	-
VS-W/anus	72-75	71-75	65-70	-	70-79	-
<i>JV5</i>	62-70	62-69	59-65	-	52-77	-
<i>StIV</i>	72-76	69-80	73-79	-	54-84	-
SC-L	17-20	12-21	16-18	-	19-25	-
SC-W	5-6	4-5	4-6	-	4-6	-
FD-L	25-29	22-27	20-25	-	23-27	-
MD-L	21-26	24-29	20-24	-	24-28	-

DS-dorsal shield; VS-ventrianal shield; SC-spermathecal calyx; FD-fixed digit; MD-movable digit; L-length; W-width.

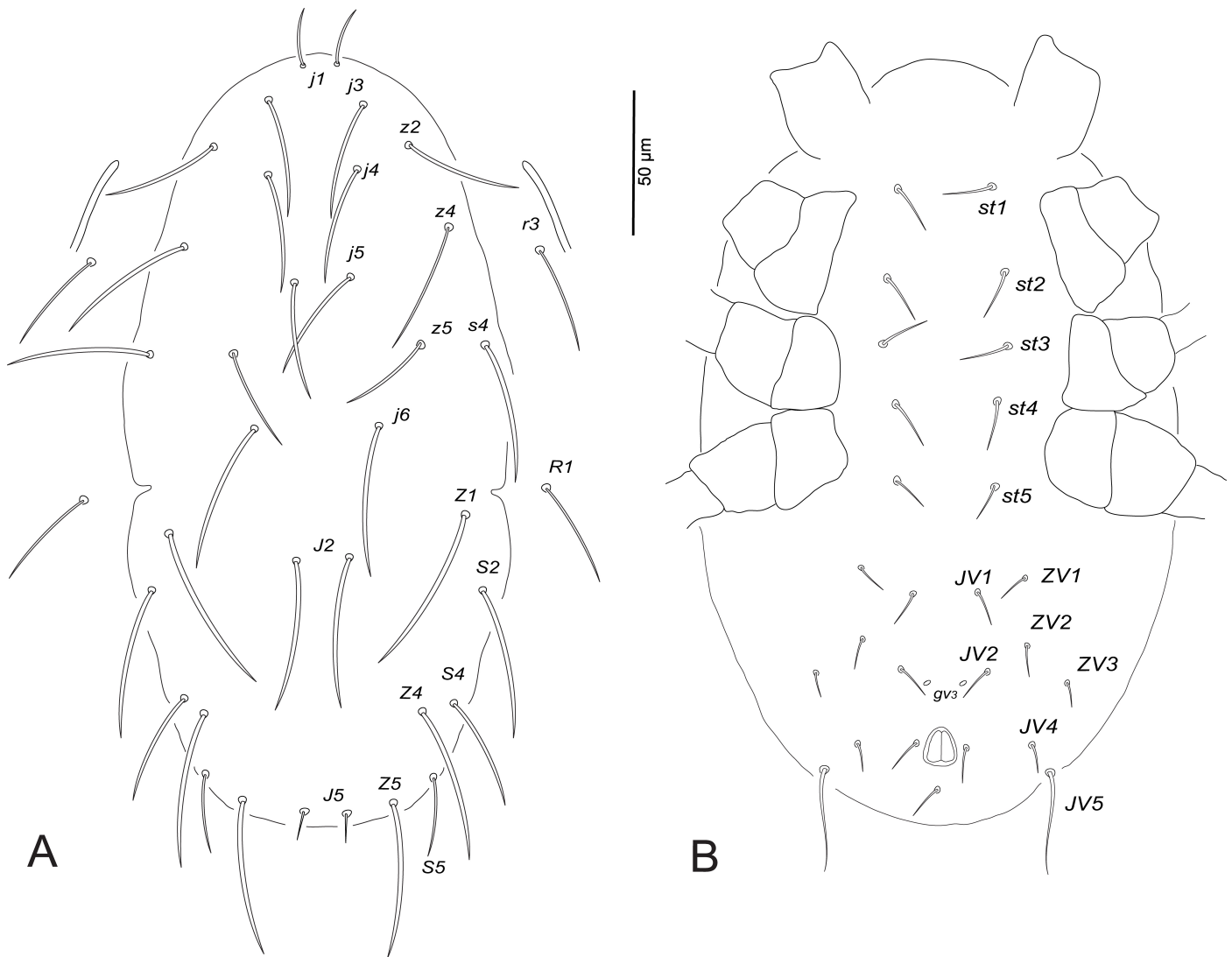


Figure 6 *Neoseiulus womersleyi* (Schicha). Deutonymph (female); A – dorsum; B – venter.

z4 43–46, *z5* 22–26, *Z1* 45–48, *Z4* 49–54, *Z5* 56–63, *s4* 50–56, *S2* 45–48, *S4* 32–34, *S5* 22–26, *r3* 22–27, *R1* 24–29.

Venter. Sternogenital shield striated; *st1–st5* 98–101, *st2–st5* 50–54. Ventrianal shield entirely striated, wider than long, 102–131 long and 139–157 wide at the anterior corners level; with three pairs of pre-anal setae; pores (*gv₃*) posteromesad *JV2*; *JV5* 41–51 (Fig. 5F).

Peritreme. Extending to level beyond setae *z2*.

Spermatodactyl (Fig. 5G). T-shaped, shaft 21–26 (transverse part). Movable cheliceral digit 16–19 long, fixed digit 17–19 long.

Legs. Leg IV with macroseta: *StIV* 57–63. Chaetotactic formulae are shown in Table 3.

Deutonymph (female) (n=7)

Dorsum (Fig. 6A). Dorsal shield smooth; 264–270 long and 145–151 wide at *s4* level; *j1* 13–20, *j3* 42–46, *j4* 36–37, *j5* 42–45, *j6* 52, *J2* 47–50, *J5* 5, *z2* 41–44, *z4* 46–56, *z5* 28–30, *Z1* 47–59, *Z4* 48–54, *Z5* 54–58, *s4* 49–60, *S2* 50–54, *S4* 35–39, *S5* 22–27, *r3* 27–38, *R1* 31–38.

Venter (Fig. 6B). Ventral shields indistinct, distance between setae *st1–st3* 65–69, *st2–st2* 51–54, *st5–st5* 39–41, *st1–st5* 119–124. Distances *JV1–JV1* 32–34, *JV1–JV5* 75–80; pores

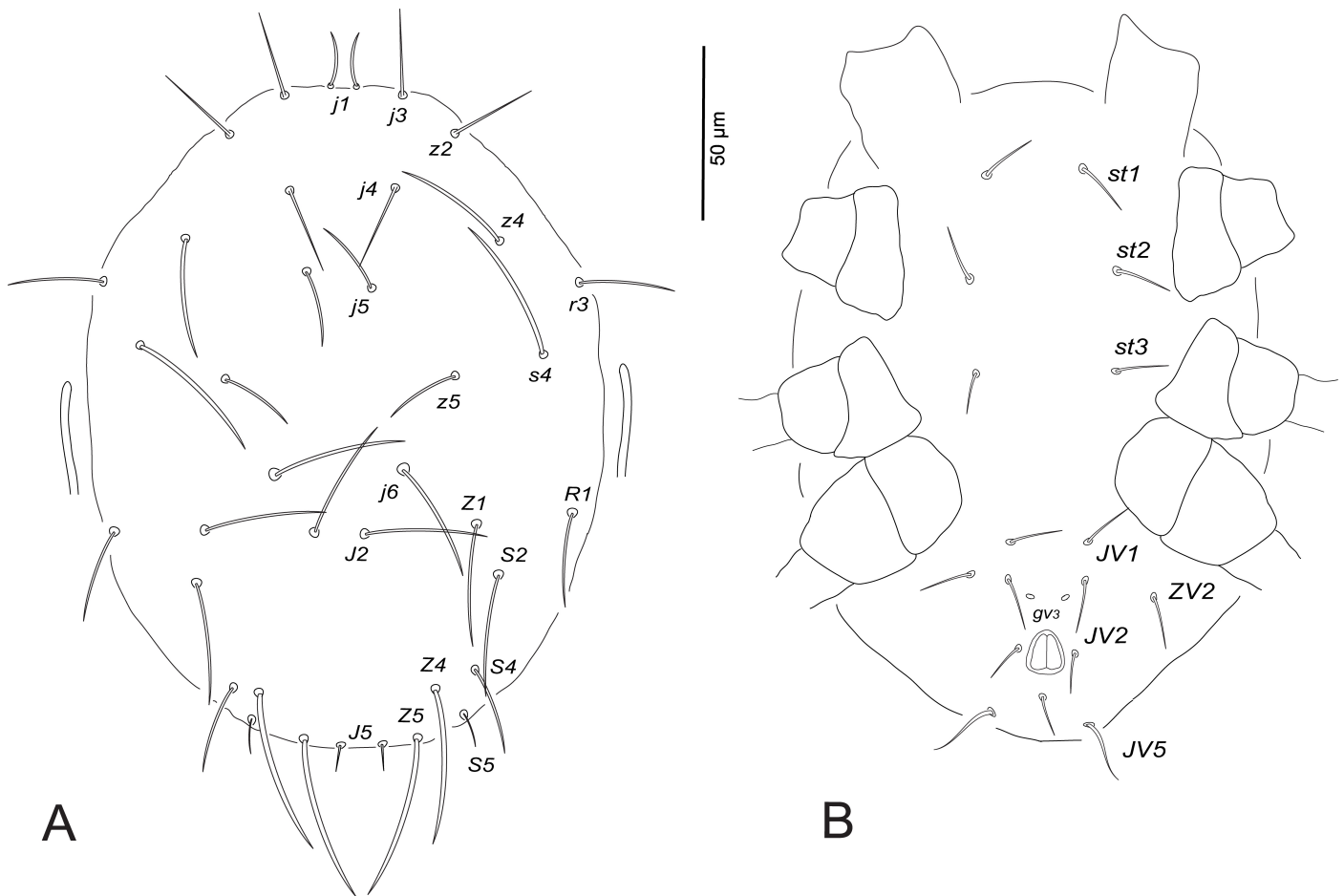


Figure 7 *Neoseiulus womersleyi* (Schicha). Protonymph; A – dorsum; B – venter.

(*gv*₃) distinct, posteromesad *JV2*; *JV5* 35–38.

Peritreme. Extending to the same level of setae *j4*.

Legs. Leg IV with macroseta: *StIV* 62–68. Chaetotactic formulae are shown in Table 3.

Protonymph (n=7)

Dorsum (Fig. 7A). Dorsal shield indistinct; 189–210 long and 140–164 wide at *s4* level; *j1* 14–15, *j3* 25–33, *j4* 25–31, *j5* 22–32, *j6* 32–39, *J2* 35–36, *J5* 5–6, *z2* 26–28, *z4* 35–37, *z5* 21–26, *Z1* 35–37, *Z4* 39–51, *Z5* 40–51, *s4* 43–53, *S2* 35–38, *S4* 22–26, *S5* 10–12, *r3* 18–28, *R1* 24–28.

Venter (Fig. 7B). Ventral shields indistinct, distance between setae *st1*–*st3* 60–64, *st2*–*st2* 48–52. Distances *JV1*–*JV1* 21–24, *JV1*–*JV5* 46–50; pores (*gv*₃) distinct, posteromesad *JV2*; *JV5* 17–18.

Peritreme. Very short, extending to beyond the level of setae *s4*.

Legs. Leg IV with macroseta: *StIV* 60–65. Chaetotactic formulae are shown in Table 3.

Larva (n=10)

Dorsum (Fig. 8A). Dorsal shield indistinct; 170–177 long and 139–144 wide at *s4* level; *j1* 17–19, *j3* 16–20, *j4* 10–14, *j5* 10–12, *j6* 48–54, *z2* 13–16, *z4* 16–18, *z5* 6–8, *Z1* 4–10, *Z4* 75–99 (whip-like), *s4* 57–60, *S2* 6–9.

Peritreme. Indistinct.

Venter (Fig. 8B). Distances between ventral setae *st1*–*st3* 63–67, *st2*–*st2* 56–58, *JV1*–*JV1*

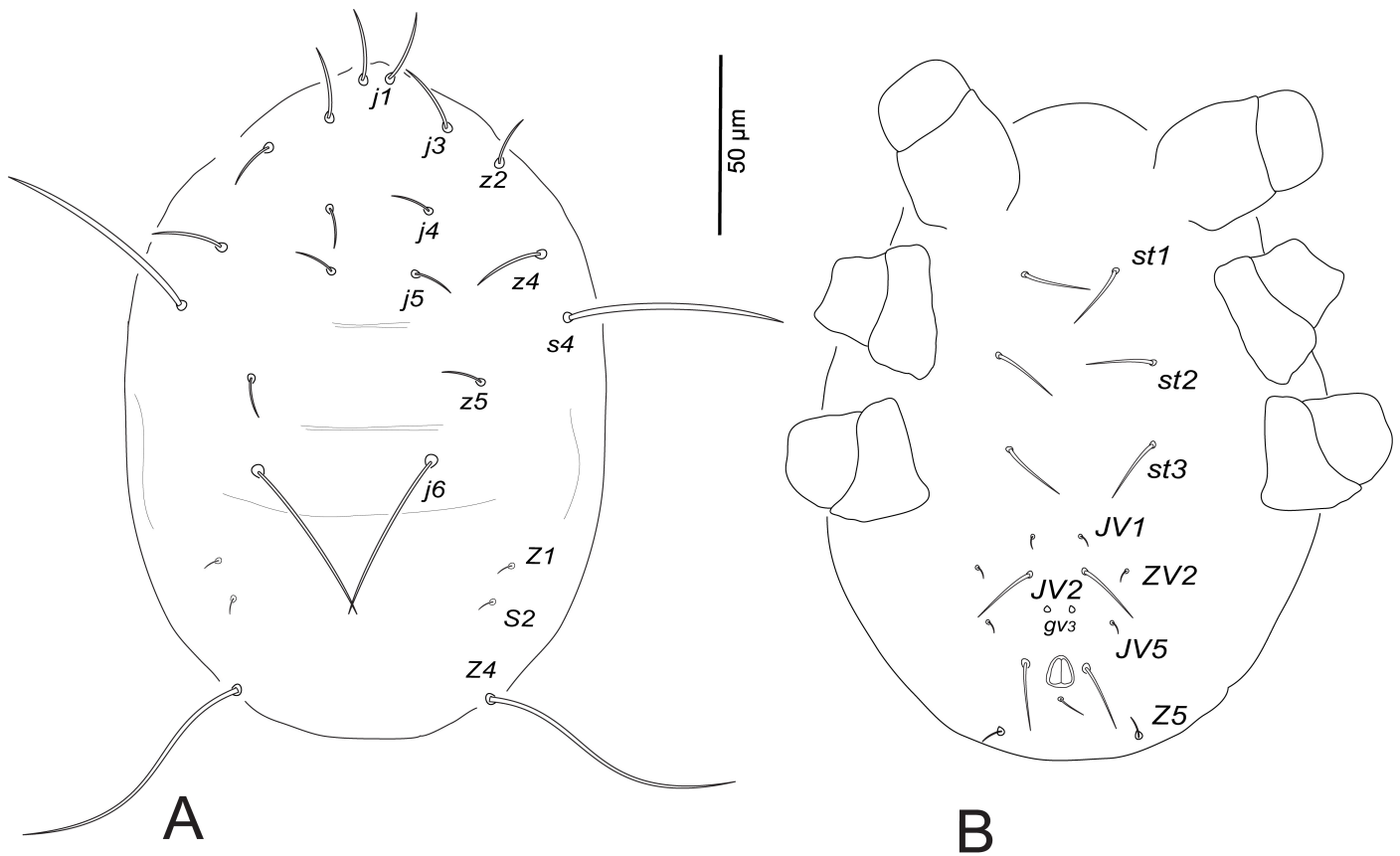


Figure 8 *Neoseiulus womersleyi* (Schicha). Larva; A – dorsum; B – venter.

20–23; pores (gv_3) present and setae *ZV1*, *ZV3*, *JV4* absent. Dorsal seta *Z5* on posterior margin ventrally. Chaetotactic formulae of legs are shown in Table 3.

Material examined

10 females, 8 males, 7 deutonymphs, 7 protonymphs, 10 larvae (voucher specimen no. 907), Morioka, Iwate, Japan (39°76' N – 141°13' E, H. Kishimoto leg.), on *Malus domestica* Borkh (Rosaceae); 10 females, 10 males, 8 deutonymphs, 8 protonymphs, 8 larvae (voucher specimen no. 828), Taitung, Taiwan (23°53' N – 121°11' E, T. Gotoh leg.), on *Ipomoea nil* (L.) Roth (Convolvulaceae); 10 females (voucher specimen no. 908), Main Island, Okinawa, Japan (26°10' N– 127°43' E, T. Gotoh leg.), on *Luffa* sp. (Cucurbitaceae) (Table 1).

Molecular analyses

After alignment, the 28S fragment had 666 nucleotide sites, of which 93 were parsimony informative. Comparison of the mean genetic distances of the 28S region showed an inter-specific divergence (3.32-9.33%) of phytoseiid mites (Table 5) and it was obviously higher than intraspecific divergence (0-0.17%). This result is consistent with our previous study of herbivorous spider mites (Tetranychidae, genus *Oligonychus*) that the genetic distances of the 28S region showed an intra- and interspecific divergence, 0-0.1% and 0.4-10.7%, respectively (Matsuda *et al.* 2012). A maximum likelihood tree of five species of phytoseiid mites based on the 28S nrDNA clearly separated the five species (Fig. 9). *N. longispinosus* and *N. womersleyi* can be distinguished from each other by the 28S region of nrDNA sequences as well as by morphological characters. In the ML tree, the genus *Neoseiulus* appeared to be polyphyletic.

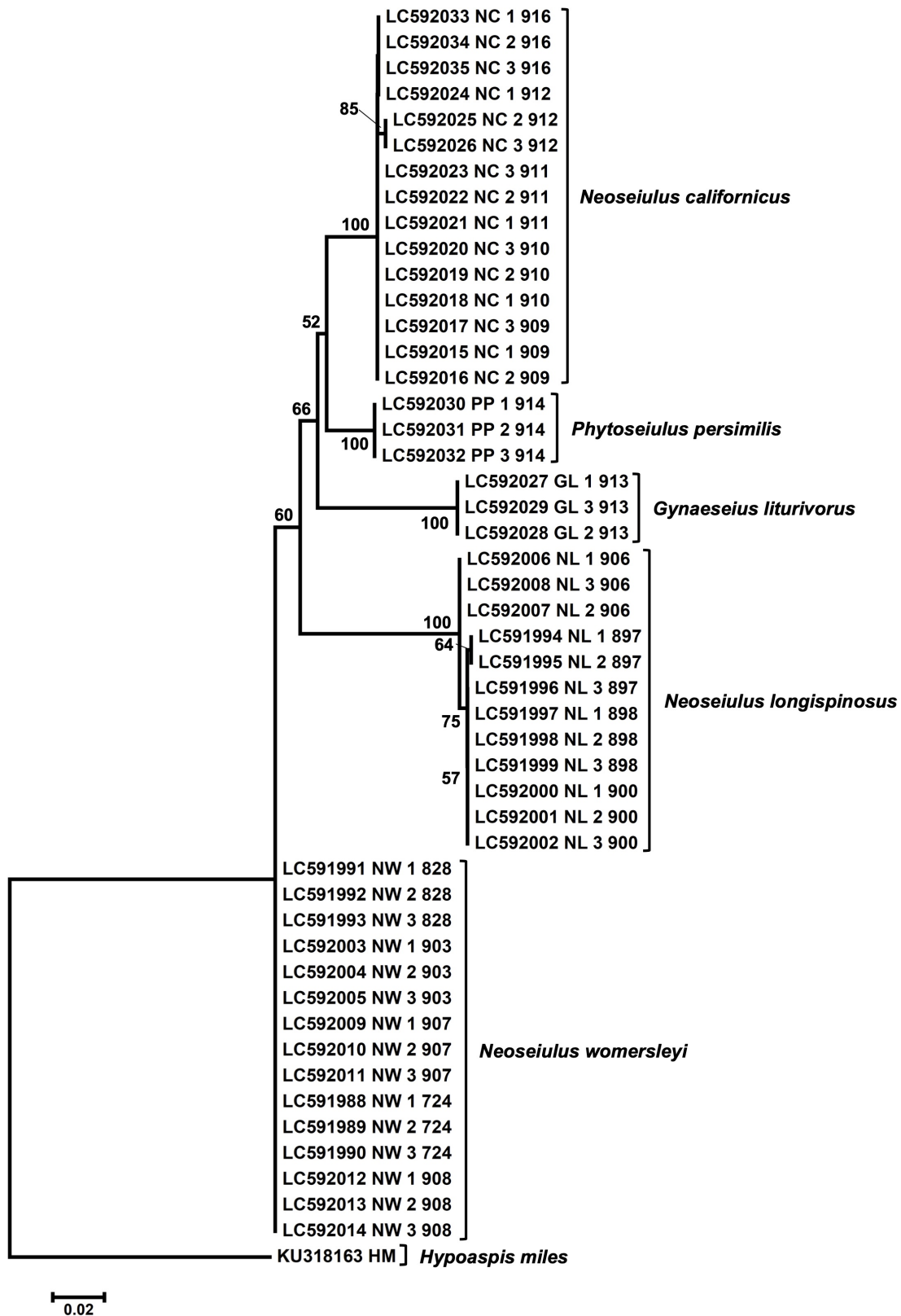


Figure 9 Maximum likelihood (ML) tree based on the 28S regions (666 bp) of nuclear ribosomal DNA (nrDNA) of phytoseiid mites using Kimura 2-Parameter model with gamma distribution. Bootstrap values based on 1,000 replications are indicated at the nodes. Only bootstrap values >50% are shown. Each operational taxonomic unit is indicated by accession number, abbreviation of species, individual identification number (three individuals for each strain/species) and voucher specimen number.

Discussion

The present study provides morphological and molecular information to support the species identification of *N. longispinosus* and *N. womersleyi*. Because females of these species are morphologically close, there has been confusion in species delimitation (Xin *et al.* 1981; Collyer 1982; Mallik and ChannaBasavanna 1983). Xin *et al.* (1981) misidentified *N. womersleyi* specimens and described them as new species, namely *N. pseudolongispinosus*. Collyer (1982) noted that the length of seta *S5* is highly variable and synonymized *N. longispinosus* with *N. womersleyi*. Some of Collyer’s *N. longispinosus* specimens seemed to be *N. womersleyi* (Minor 2020). In the present study, the length of seta *S5* and the ratio of setae *Z5/S5* in adult females greatly helped to separate the examined strains. Setae *S5* in *N. longispinosus* (20–26µm) are distinctly shorter than those of *N. womersleyi* (45–53µm); and setae *Z5* are about three to four times longer than *S5* in *N. longispinosus* ($Z5/S5 = 3.03-4.25$), but only around two times longer in *N. womersleyi* ($Z5/S5 = 1.56-2.08$; Tables 2 and 4). Furthermore, Schicha (1975) redescribed *N. longispinosus* from Australia and separated it from *N. womersleyi* based on the length of seta *S5* and the distance between the two ventrianal pores (*gv3*). He also indicated that the distance *gv3–gv3* was about twice as long in *N. womersleyi* as it was in *N. longispinosus*. However, we found this feature variable among individuals of the examined strains, considering it unreliable intra- and interspecific variation: *gv3–gv3* was 16-28µm for *N. longispinosus* and 14-21µm for *N. womersleyi*.

The measurements of *N. longispinosus* adult females in the examined strains are relatively similar to those published in the previous works (Table 2); however, the length of seta *S5* of the Korean specimens reported by Lee and Ryu (1989) (33.3-51.0µm) is questionable because it is noticeably longer than normal setal range. High similarity in *S5* measurements was also observed between the three strains of *N. longispinosus* examined in the present study (20-26µm) and those previously described from Taiwan (17µm; Tseng 1983), India (18µm; Gupta 1986) and Thailand (18-25µm; Oliveira *et al.* 2012); however, the length of *S5* is little shorter in the specimens described from Martinique (13-15µm; Kreiter *et al.* 2018), China (16.2µm; Xin *et al.* 1981) and the Dominican Republic (12, 15µm; Abo-Shnaf *et al.* 2016). Regarding *N. womersleyi*, the measurements of adult females collected in the present study were compared with those of previous studies (Table 4). To our knowledge, no descriptions have been done for *N. womersleyi* except for the main works of Womersley (1954), Schicha (1975), Ehara (1958), and most recently by Liao *et al.* (2020). Interestingly, Womersley (1954) redescribed *N. longispinosus* based on specimens collected from Australia, and although he noted that setae *S5* and *j1* are longer than that in the original description of *N. longispinosus*, he did not describe the Australian specimens as a new species but considered this variation as intraspecific. Conversely, Schicha (1975) emphasized the importance of this trait and described Womersley’s specimens along with other collected materials as new species, namely *N. womersleyi*.

Recently, there has been more interest in studying the ontogeny of mite groups to address the morphological variations among immatures of different species (Zhang 2018). Several studies have also described the immature stages of phytoseiid mites (MacGill 1939, Evans

Table 5 Mean intra- and interspecific genetic distance (% *p*-distances) between five species of phytoseiid mites in the 28S region.

Species	NL	NW	NC	GL	PP
<i>N. longispinosus</i> (NL)	0.17				
<i>N. womersleyi</i> (NW)	6.44	0.07			
<i>N. californicus</i> (NC)	7.80	3.32	0.00		
<i>G. liturivorus</i> (GL)	9.33	6.02	6.78	0.00	
<i>P. persimilis</i> (PP)	7.80	3.61	3.62	6.17	0.00

○ *N. womersleyi* ● *N. longispinosus*

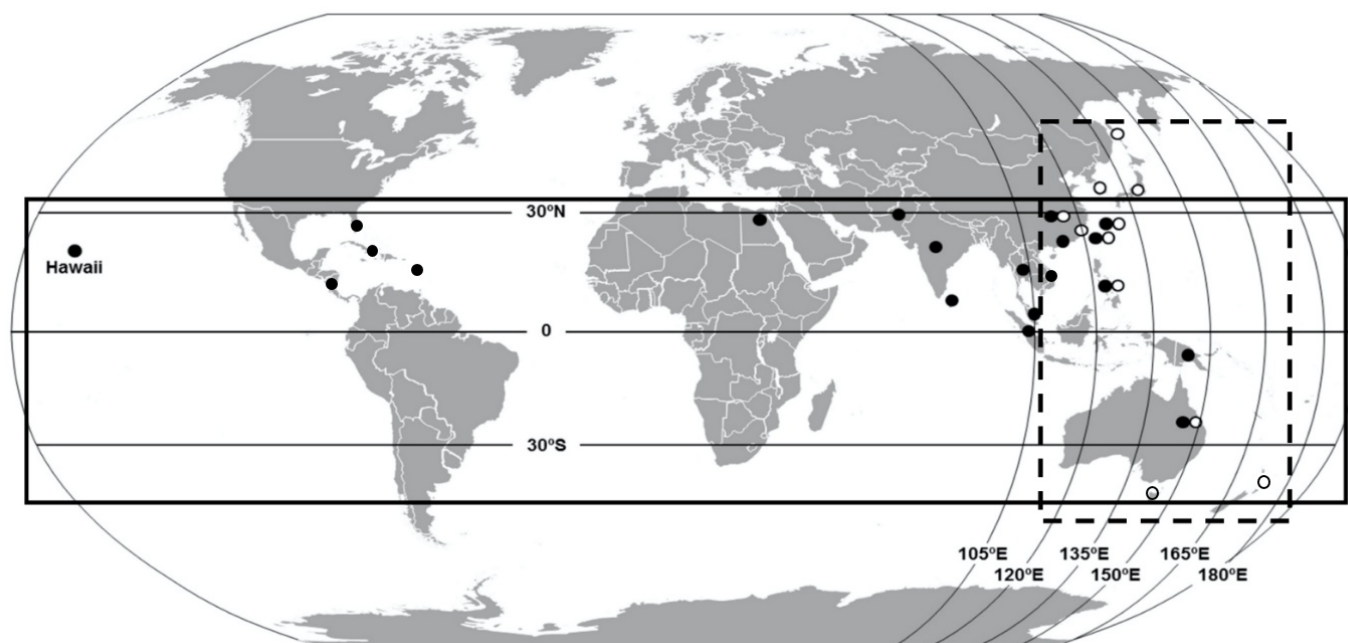


Figure 10 World distribution of *Neoseiulus womersleyi* (open circles; from Akimov and Kolodochka 1991; Ho *et al.* 1995, 2003; Ehara and Amano 2004; Moraes *et al.* 2004) and *N. longispinosus* (closed circles; from Ho *et al.* 1995; Lin *et al.* 2000; Ehara 2002; Moraes *et al.* 2004; Ohno *et al.* 2012).

1953, Chant 1958, Prasad 1973, Xin *et al.* 1981, El-Banhawy and Abou-Awad 1985; Aponte and McMurtry 1987; Li *et al.* 2020; Ma *et al.* 2020a, b). As for the immature stages of the present specimens of *N. longispinosus*, the dorsal chaetotaxy of the larvae has 12 setae (Fig. 4A), whereas Mallik and ChannaBasavanna (1983) described the larva collected from India with only nine pairs of dorsal setae, apparently overlooking setal pairs *z5*, *Z1* and *S2*. Also, the present specimens have eight pairs of ventral setae (excluding the para- and postanal setae) for the larva, but Mallik and ChannaBasavanna (1983) depicted only five pairs of ventral setae, missing the setae *ZV2*, *JV5* and *Z5*. For the protonymphs, the dorsal setae appear likewise in the adults (with 19 pairs), while the ventral side still lacks some setae (*ST4*, *ST5*, *ZV1*, *ZV3*, *JV4*). Subsequently, the deutonymph gets complete dorsal and ventral setations just as in the adult stages. It is worth mentioning that the deutonymphal stages reflect the adult differences (lengths of setae *S5* and *Z5*) in contrary to protonymphs, which have little variation in the length of setae *Z5* (37-40µm for *N. longispinosus* and 40-45µm for *N. womersleyi*). Moreover, the chaetotaxy of legs is also similar in the developmental stages of *N. longispinosus* and *N. womersleyi* strains examined in the present study (Table 3), with little setal variations from the redescribed species, *N. barkeri* Hughes, *N. californicus* (McGregor) and *N. kikuya* Ma, Fan & Zhang (Li *et al.* 2020; Ma *et al.* 2020a, b).

Concerning the geographical distribution of *N. longispinosus* and *N. womersleyi*, Evans (1952) first described *N. longispinosus* from Indonesia, while Schicha (1975) described *N. womersleyi* from Australia. Since then, both predatory species have been found in several countries (Ho *et al.* 1995; Ehara 2002). It seems that *N. longispinosus* has broader distribution than *N. womersleyi*. *N. longispinosus* is distributed latitudinally around the globe between 30°N and 30°S. On the other hand, *N. womersleyi* is distributed longitudinally from Siberia, Russia, to Australia along the Pacific coast region of East and Southeast Asia and Oceania

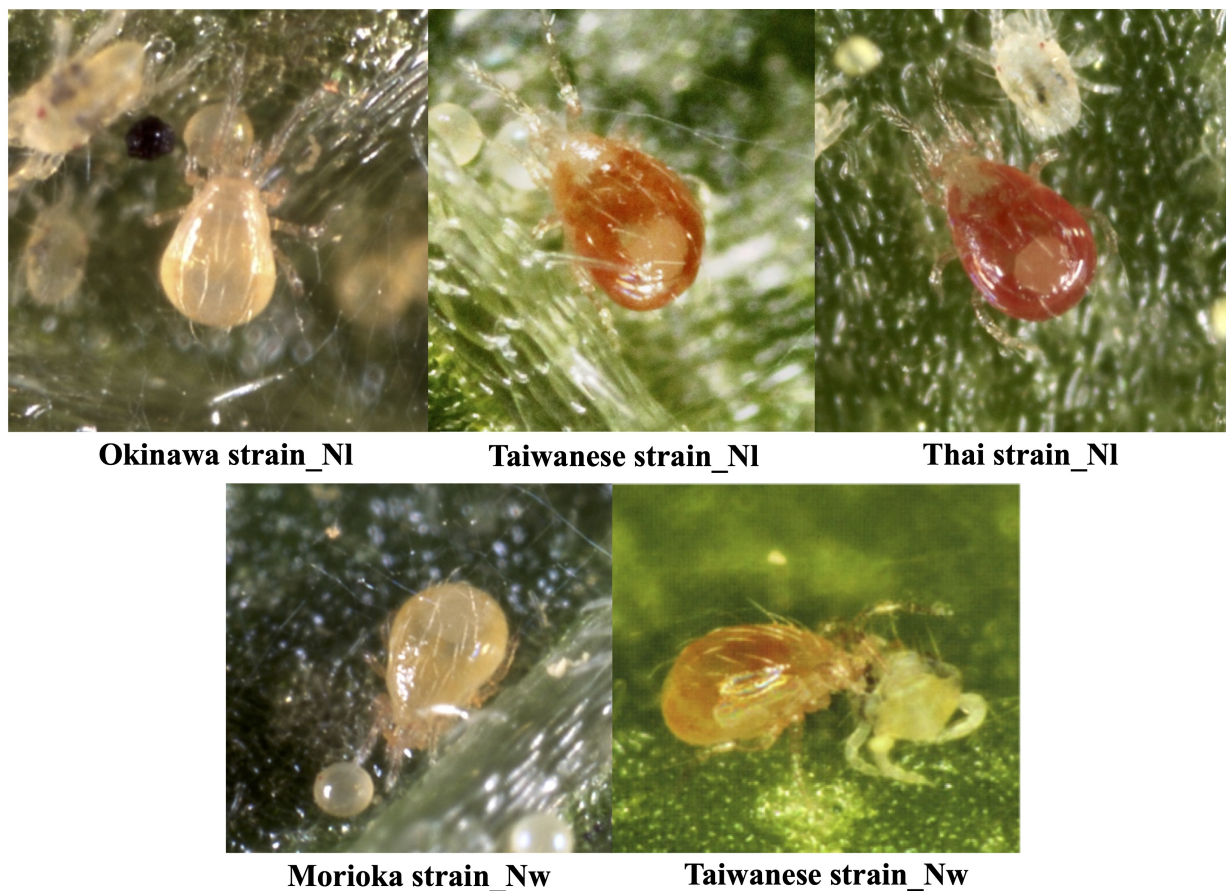


Figure 11 Body color of females in various strains of *Neoseiulus longispinosus* (NI) and *N. womersleyi* (Nw).

(Akimov and Kolodochka 1991; Ho *et al.* 1995, 2003; Lin 2000; Ehara 2002; Ehara and Amano 2004; Moraes *et al.* 2004; Ohno *et al.* 2012). However, some overlapping distributions exist between the two species (Fig. 10), which are correctly confirmed by literatures as well as specimens, considering that there may be some more misidentifications. For example, Akimov and Kolodochka (1991) mentioned that *N. longispinosus* is distributed in Siberia, but their drawing clearly shows that it is *N. womersleyi* due to having long *S5* ($Z5/S5 = 2.0$). In Japan, *N. longispinosus* is limited to Tarama Isl. and Irabu Isl. (ca. 24°N) of Okinawa Prefecture (Ohno *et al.* 2012), whereas *N. womersleyi* has been reported from more than 35 out of 47 prefectures from Hokkaido (ca. 44°N) to Okinawa (ca. 24°N) (Toyoshima *et al.* 2013). Although Ehara (1958) at first mistakenly redescribed *N. womersleyi* as *N. longispinosus* based on specimens collected from Fukushima Prefecture (Honshu; 37-38°N), but he subsequently corrected the species identity to *N. womersleyi* (Ehara *et al.* 1994). In Thailand, Kongchuensin *et al.* (2005) showed how the distributions of *N. longispinosus* strains were related to their host plant species. The body color of adult females differs in the current examined strains (Fig. 11). For example, *N. longispinosus* is reddish in the Taiwanese and Thai strains regardless of food types, but whitish in the Okinawa strain. However, *N. womersleyi* is whitish in all studied strains.

Crossbreeding and reproductive interference experiments carried out by Ho *et al.* (1995) and Ullah *et al.* (2017), respectively, confirmed that *N. longispinosus* and *N. womersleyi* represent distinct species. A significant genetic divergence was also reported between *N.*

longispinosus and *N. womersleyi* by using a random amplified polymorphic DNA (RAPD) analysis (Yeh *et al.* 2000). In the present study, the 28S nucleotide sequences showed that the two *Neoseiulus* species are different species as they formed separate clades. The ML tree also showed that the genus *Neoseiulus* appeared to be polyphyletic, which agrees with previous phylogenies based on the 12S rRNA genes of mtDNA (Tsolakis *et al.* 2012) and the ITS region of nrDNA (Inak *et al.* 2020). The current morphological and molecular analyses strengthen the taxonomic separation of the two species. Several case studies have used integrative taxonomy and employed the same methods to separate close species (Famah Sourassou *et al.* 2012; Arabuli *et al.* 2019). We conclude that a combination of different methods is required to distinguish and separate between closely related species.

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Conflicts of Interest

The authors declare no conflicts of interest.

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