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Subscriptions: Year 2023 (Volume 63): 450 €
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Previous volumes (2010-2021): 250 € / year (4 issues)
Acarologia, CBGP, CS 30016, 34988 MONTFERRIER-sur-LEZ Cedex, France
ISSN 0044-586X (print), ISSN 2107-7207 (electronic)

The digitalization of Acarologia papers prior to 2000 was supported by Agropolis Fondation under the reference ID 1500-024 through the « Investissements d’avenir » programme (Labex Agro: ANR-10-LABX-0001-01)

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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), eriophyid 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,
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 1 (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’-GCTGCGAGTGAACTGGAATCAAGCCT-3’) and 929R (5’-AGGTCACCACATTTTCGTTGC -3’) (Dowling and OConnor 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 2 min.
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 (Kimura2-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 *Gynaeceius* and *Phytoseiulus* examined in the study.

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

Neoselulus 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 (gd2, gd1, gd6, gd8, gd9) and 13 pairs of poroids (id1, id1a, id1b, idm2, idm3, idm4, idm5, idm6, isl, idl1, idl3, idl4); 328–347 long and 178–189 wide at s4 level; j1 17–21, j3 62–66, j4 63–65, j5 66–70, j6 74–77, J2 79–81, J5 7–9, z2 70–75, z4 69–76, z5 35–38, Z1 74–84, Z4 74–77, Z5 78–80, s4 69–83, S2 76–80, S4 56–59, S5 22–24, r3 64–69, R1 63–70.

Table 2 Character measurements of adult females of Neoselulus 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 et al. 2016) (n=2) | India (Gupta 1986) (n=2) | The Philippines & Taiwan (Liao et al. 2020) (n=10) | Korea (Lee and Ryu 1989) (n=a) | China (Xin et al. 1981) (n=1) |
|----------------|---------------------|----------------------|-----------------------|---------------------------------------------|--------------------------|--------------------------------------------------|-------------------------------|--------------------------------|
| DS-L           | 328.347             | 317.330              | 340.367               | 325.337                                      | 298.348                  | 341.3-499.8                                      | 301.1                         |                                |
| DS-W           | 178.189             | 165.179              | 182.193               | 183.195                                      | 180                      | 170.201                                          | 175.3                         |                                |
| j1             | 17.21               | 17.20                | 16.19                 | 16.20                                        | 16.20                    | 15.22                                            | 18.3                          |                                |
| j3             | 62.66               | 64.72                | 62.66                 | 63.65                                        | 50.60                    | 54.76                                            | 53.1                          |                                |
| j4             | 63.65               | 61.66                | 67.69                 | 55.60                                        | 54.62                    | 52.66                                            | 41.6-0.3                      |                                |
| j5             | 66.70               | 68.74                | 60.64                 | 73.75                                        | 67.72                    | 54.80                                            | 63                            |                                |
| j6             | 74.77               | 73.79                | 75.78                 | 70.75                                        | 72                       | 58.88                                            | 62.4-77.0                     | 67.9                           |
| j7             | 79.81               | 72.79                | 80.84                 | 78.80                                        | 71.81                    | 53.88                                            | 65.5-81.1                     | 72                             |
| j8             | 7.9                 | 7.8                  | 6.8                   | 6.7                                          | 7.10                     | 8.3-10.0                                         | 9.5                           |                                |
| z2             | 70.75               | 68.74                | 66.69                 | 66.70                                        | 69                       | 53.98                                            | 52.0-77.0                     | 62.1                           |
| z4             | 69.76               | 72.78                | 75.77                 | 73.75                                        | 70.76                    | 56.92                                            | 57.2-78.0                     | 70                             |
| z5             | 35.38               | 30.37                | 32.45                 | 30                                           | 31.36                    | 25.42                                            | 21.8-39.5                     | 29.3                           |
| Z1             | 74.84               | 76.80                | 80.85                 | 75.77                                        | 73.76                    | 64.95                                            | 66.8-81.1                     | 71.3                           |
| Z4             | 74.77               | 68.76                | 71.74                 | 71.72                                        | 63.69                    | 61.4-79.0                                        | 69.6                          |                                |
| ZS             | 78.80               | 79.84                | 80.85                 | 75.85                                        | 78.80                    | 64.94                                            | 77.0-91.5                     | 74                             |
| s4             | 69.83               | 79.84                | 82.86                 | 85.88                                        | 80.82                    | 71.103                                           | 69.7-85.3                     | 75.6                           |
| S2             | 76.80               | 69.77                | 74.77                 | 73.74                                        | 76.78                    | 66.97                                            | 62.4-79.0                     | 67.6                           |
| S4             | 56.59               | 62.66                | 60.65                 | 58.60                                        | 59.61                    | 56.74                                            | 49.9-64.5                     | 58.5                           |
| S5             | 22.24               | 21.26                | 20.24                 | 12.15                                        | 18                       | 12-24                                            | 33.3-51.0                     | 16.2                           |
| r3             | 64.69               | 57.69                | 62.65                 | 52.53                                        | 58                       | 52.70                                            | 44.7-64.5                     | 56.4                           |
| R1             | 63.70               | 56.64                | 62.66                 | 65.68                                        | 67                       | 45.78                                            | 47.8-70.7                     | 57.2                           |
| st2-st2        | 58.62               | 58.66                | 54.57                 | 60.61                                        | -                       | 56.64                                            | -                             | -                              |
| st1-st3        | 59.64               | 57.67                | 58.59                 | 54.55                                        | -                       | 57.63                                            | -                             | -                              |
| st5-st5        | 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                                            | -                             | -                              |
| J5             | 62.66               | 57.65                | 61.66                 | -                                            | 67                       | 56.92                                            | -                             | -                              |
| Sl/V           | 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                        | 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 sl–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) posteromesal JV2, gv3–gv3 16–28; JV3 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 7–5, 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, RI 41–46.

Venter. Stermogenital shield striated, sl–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) posteromesal 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 a 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 54–60, S2 48–53, S4 34–43, S5 10–13, r3 30–36, R1 29–34.

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

| 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 | 0-2/1-0/0-0 | 0-2/1-2/0-0 | 1-1/1-0/0-1 | 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 | 0-1-0-0 |
|                           | 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 | 1-1/0-1/0-1 | 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 | 1-1/0-1-0-1 | 1-2/1-2/0-1 | 1-1/2-0/0-1 | 1-1-1-1-1  | 1-1-1-1 |
Figure 2 Neoseiulus longispinosus (Evans). Deutonymph (female); A – dorsum; B – venter.

distinct pair of pores (gv₁) 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 (gv₁) 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.
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)
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 (id_1, idl_1, id_2, idm_2, idm_3, idm_4, idm_5, id_4, idm_6, isl, idl_4, idl_5, j1); 331–359 long and 175–203 wide at s4 level; j1 16–19, j3 52–57, j4 47–57, j5 55–59, j6 67–70, J2 74–81, J3 8–10, z2 52–66, z4 64–73, z5 31–35, Z1 73–76, Z4 73–77, Z5 84–90, s4 73–76, S2 68–77, S4 62–66, S5 45–51, r3 47–56, R1 50–55. All setae very finely serrated, except setae j1 and J3 short and smooth.

Venter (Fig. 5B). Sternal and genital shields striated, distance between setae st1–st3 55–60, st2–st2 59–63, st5–st5 52–59. Sternal shield with two pairs of poroids (iv_1, iv_2). Ventrianal shield striated, 99–121 long, 77–93 wide at level JV2 and 71–75 wide at the anus level; distance between setae JV2–JV2 42–56; pores (gv_1) posteromesad JV2, gv_3–gv_3 14–21; JV5 62–69.

Peritreme. Extending to bases of setae j3.

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

| Character | Taiwan (828) (n=10) | Japan (907) (n=10) | Japan (908) (n=10) | Australia (Schicha 1975) (n=5) | Taiwan & Japan (Liao et al. 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                              |
| j1        | 17-19                | 16-19              | 16-18              | 14±1                        | 14-24                                | 25                               |
| j3        | 64-67                | 52-57              | 60-65              | 58±7                        | 42-63                                | 56                               |
| j4        | 53-60                | 47-57              | 55-61              | 59±3                        | 42-60                                | 56                               |
| j5        | 64-67                | 55-59              | 63-66              | 63±5                        | 50-64                                | 60                               |
| j6        | 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                               |
| z2        | 65-70                | 52-66              | 60-65              | 69±6                        | 53-70                                | 62                               |
| z4        | 72-78                | 64-73              | 74-80              | 75±3                        | 56-81                                | 78                               |
| z5        | 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                               |
| s4        | 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                               |
| r3        | 59-63                | 47-56              | 52-59              | 59±5                        | 41-61                                | -                                |
| R1        | 60-64                | 50-55              | 58-62              | 68±5                        | 47-62                                | -                                |
| st2-st2   | 58-64                | 59-63              | 61-66              | -                           | 60-67                                | -                                |
| st1-st3   | 57-63                | 55-60              | 54-56              | -                           | 56-66                                | -                                |
| st5-st5   | 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                                | -                                |
| JV5       | 62-70                | 62-69              | 59-65              | -                           | 52-77                                | -                                |
| StIV      | 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.

Negm, M. W. et al. (2021), *Acarologia* 61(2): 432-452; DOI 10.24349/acarologia/20214440 442
Neoseiulus womersleyi (Schicha). Deutonymph (female); A – dorsum; B – venter.

Venter. Sternogenital shield striated; \( st1–st5 \) 98–101, \( st2–st2 \) 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 \( (gv_{j}) \) 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, \( S5 \) 35–39, \( S5 \) 22–26, \( r3 \) 22–27, \( R1 \) 24–29.

Venter. Sternogenital shield striated; \( st1–st5 \) 98–101, \( st2–st2 \) 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 \( (gv_{j}) \) 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.
Figure 7 Neoseiulus womersleyi (Schicha). Protonymph; A – dorsum; B – venter.

(gv₃) 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 (gv₃) 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 (gv3) present and setae ZVI, 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 interspecific 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 S3 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

| Species              | NL  | NW  | NC  | GL  | PP  |
|----------------------|-----|-----|-----|-----|-----|
| *N. longispinosus*   | 0.17|     |     |     |     |
| *N. womersleyi*      | 6.44| 0.07|     |     |     |
| *N. californicus*    | 7.80| 3.32| 0.00|     |     |
| *G. liturivorus*     | 9.33| 6.02| 6.78| 0.00|     |
| *P. persimilis*      | 7.80| 3.61| 3.62| 6.17| 0.00|
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.
Body color of females in various strains of *Neoseiulus longispinosus* (Nl) 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.

Acknowledgements

We are very grateful to Drs. Hidenari Kishimoto (NARO, Morioka), Hiroshi Oida (Hosei University), M. S. Ullah (Bangladesh Agricultural University) and Toshiyuki Tezuka (Agrisect Inc.) for providing phytoseiid mites. We also thank Drs. Suguru Ohno (Okinawa Prefectural Agricultural Research Center) and Yasuki Kitashima (Ibaraki University) for helping in mite collection.

Conflicts of Interest

The authors declare no conflicts of interest.

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