SPIXIANA	46	2	173–177	München, April 2024	ISSN 0341-8391

Molecular phylogeny confirms that "*Pseudolepeophtheirus*" *schmidti* Gusev, 1951 belongs in *Lepeophtheirus*

(Crustacea, Copepoda, Caligidae)

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Kakui, K. 2024. Molecular phylogeny confirms that "*Pseudolepeophtheirus*" *schmidti* Gusev, 1951 belongs in *Lepeophtheirus* (Crustacea, Copepoda, Caligidae). Spixiana 46 (2): 173–177.

Pseudolepeophtheirus schmidti was recently transferred to *Lepeophtheirus*, as *Pseudolepeophtheirus* was synonymized with *Lepeophtheirus*, but its phylogenetic position had not been tested molecularly. This study determined the 18S rRNA (18S) of "*Pseudolepeophtheirus*" *schmidti*, constructed an 18S-based phylogenetic tree of caligids, and revealed that "*P.*" *schmidti* nests within the *Lepeophtheirus* clade, confirming it belongs in *Lepeophtheirus*. This is the first record of this species from Japanese waters and the first from outside the Sea of Japan.

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Introduction

The caligid genus *Pseudolepeophtheirus* Markevich, 1940 contained two species, *Pseudolepeophtheirus longicauda* Markevich, 1940 (the type species) and *Pseudolepeophtheirus schmidti* Gusev, 1951. It had been distinguished from the closely related genus *Lepeophtheirus* von Nordmann, 1832 by having a reduced leg 4. However, because this character was not suitable for distinguishing between other closely related generic pairs in Caligidae, Dojiri & Ho (2013) synonymized *Pseudolepeophtheirus* with *Lepeophtheirus*. Homma et al. (2020) subsequently determined 18S rRNA (18S) and cytochrome *c* oxidase subunit I (COI) sequences from "*Pseudolepeophtheirus*" *longicauda* and found that this species nested within the *Lepeophtheirus* clade in an 18S tree. This provided molecular support for Dojiri & Ho's (2013) conclusion based on morphology that "*P.*" *longicauda* belongs in *Lepeophtheirus*.

"*Pseudolepeophtheirus*" *schmidti* was originally described from the pleuronectid flounder *Myzopsetta punctatissima* (Steindachner, 1879) collected from Nakhodka Bay in the Sea of Japan, and was later reported from *M. punctatissima* and *Pseudopleuronectes herzensteini* (Jordan & Snyder, 1901) collected from the Peter the Great Bay and Tatar Strait, Sea of Japan (Vinogradov 2012). Leg 4 was much smaller in "*P.*" *schmidti* (uniarticulate) than in *Lepeophtheirus longicauda* (biarticulate; exopod observed), raising the possibility that "*P.*" *schmidti* did not belong in *Lepeophtheirus*. Although Gusev (1951) and Vinogradov (2012) described the morphology of "*P.*" *schmidti* in detail, its phylogenetic position had not been investigated molecularly.

In this study, based on individuals collected from *M. punctatissima* landed at Nemuro, eastern Hokkaido, Japan (Fig. 1), I attempted to amplify the COI and 18S genes for "*P.*" *schmidti* (only 18S was successfully amplified) and constructed an 18S tree to determine its phylogenetic position within Caligidae.

Material and methods

A fresh individual of *M. punctatissima* (total length 29 cm; Fig. 2A, B) landed at Nemuro, Hokkaido, Japan, was bought at a supermarket in Sapporo, Hokkaido, on 19 July 2023. Twenty-eight caligids were collected from the inner surface of the left and right opercula of the

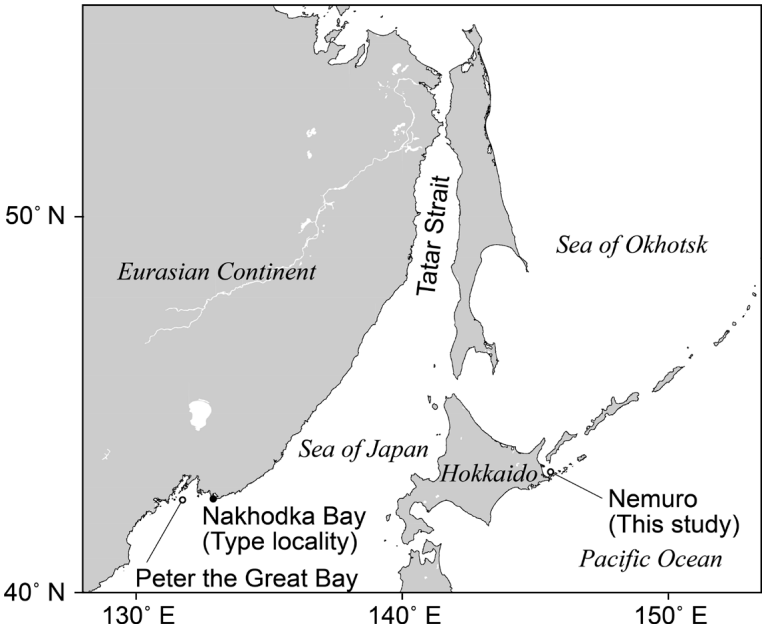

Fig. 1. Map showing collection localities for "*Pseudolepeophtheirus*" *schmidti*.

fish, fixed in 70 % ethanol, and preserved in 99 % ethanol. A piece of host muscle was fixed in 70 % ethanol and preserved in 99 % ethanol for DNA extraction; the remaining part of the fish was consumed by the author.

Total DNA was extracted from the egg sac of one copepod specimen and a piece of host muscle by using a NucleoSpin Tissue XS Kit (Macherey–Nagel, Germany). For the COI gene, PCR primers used for amplification and cycle sequencing were LCO1490 and HCO2198 (Folmer et al. 1994) for the copepod and COI_ff_F and COI_ff_R (Homma et al. 2020) for the host. Amplification primers for 18S were SR1 and SR12 (Nakayama et al. 1996); six primers (18S-b4F, 18S-b4R, 18S-a4R, 18S-b5F, 18S-b6F, and 18S-b8F; Kakui et al. 2011, 2021, Kakui & Hiruta 2022) were used in cycle sequencing. Conditions for PCR amplification of COI from the copepod with TaKaRa Ex taq DNA polymerase (TaKaRa Bio, Japan) were 94 °C for 1 min; 35 cycles of 98 °C for 10 s, 50 °C for 30 s, and 72 °C for 50 s; and 72 °C for 2 min. Those for the fish COI with KOD One PCR Master Mix (Toyobo, Japan) were 45 cycles of 98 °C for 10 s, 53 °C for 5 s, and 68 °C for 1 s. Conditions for the copepod 18S with KOD FX Neo polymerase (Toyobo, Japan) were 94 °C for 2 min; 45 cycles of 98 °C for 10 s, 65 °C for 30 s, and 68 °C for 1 min; and 68 °C for 2 min. Nucleotide sequences were determined by direct sequencing with a BigDye Terminator Kit ver. 3.1 and a 3130 or 3730 Genetic Analyzer (Life Technologies, USA), and fragments were concatenated by using MEGA7 (Kumar et al. 2016). Sequences obtained were deposited in the International Nucleotide Sequence Database (INSD) through the DNA Data Bank of Japan (DDBJ).

The copepod 18S dataset for phylogenetic analysis comprised the dataset from Homma et al. (2020) and the sequence I determined, representing 26 caligid species and three outgroup taxa from Dissonidae and Pandaridae. Sequences were aligned by eye according to the secondary structure of the honeybee 18S sequence (Gillespie et al. 2006) and the structure predicted with the RNAfold WebServer (Gruber et al. 2008, Lorenz et al. 2011); the aligned sequences were trimmed in MEGA7 to the shortest length among them. Alignment-ambiguous sites were then removed with Gblocks ver. 0.91b (Castresana 2000) in NGPhylogeny.fr (Lemoine et al. 2019) under the "relaxed" parameters described in Talavera & Castresana (2007). The aligned dataset contained 1616 positions. The optimal substitution model determined under the corrected AIC (Akaike information criterion) in ModelFinder (Kalyaanamoorthy et al. 2017) was GTR + F + R2. A maximum likelihood (ML) analysis was conducted in IQ-TREE ver. 2.1.2 (Minh et al. 2020); nodal support values were obtained from the Shimodaira–Hasegawa-like approximate likelihood-ratio test (SH-aLRT) with 10 000 replicates (Guindon et al. 2010) and an ultrafast bootstrap (UFBoot) analysis of 10 000 pseudoreplicates under the "bnni" option (Minh et al. 2013, Hoang et al. 2018). The ML tree was drawn with FigTree v1.4.4 (Rambaut 2023).

A map was generated by using GMT6 (Wessel et al. 2019) edited in Adobe Illustrator CS6. The copepod specimens were deposited in the Invertebrate Collection of the Hokkaido University Museum (ICHUM), Sapporo, Japan, under catalog numbers ICHUM8569 and ICHUM8570.

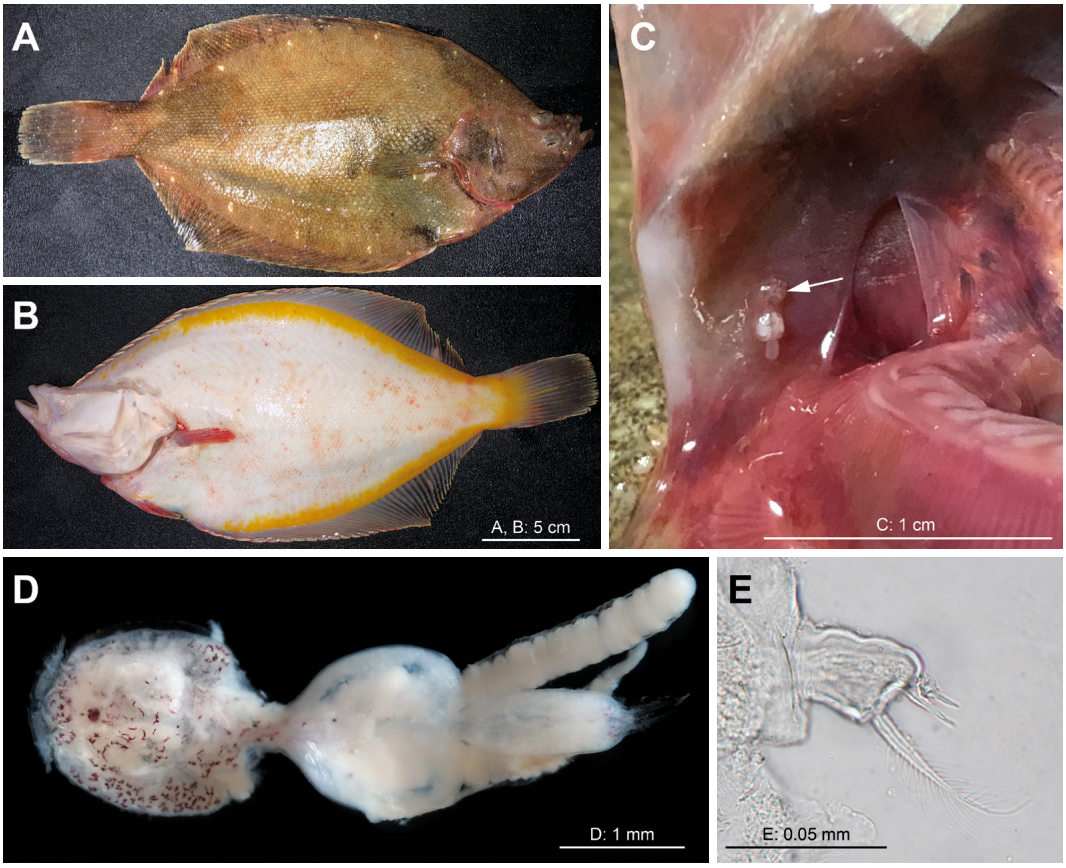

Fig. 2. "*Pseudolepeophtheirus*" *schmidti*, parasitic on the flounder *Myzopsetta punctatissima*. **A**, **B.** Fresh host fish, right and left views. **C.** Fresh "*P.*" *schmidti* (arrow) on inner surface of right operculum of host fish. **D.** Ethanol fixed "*P.*" *schmidti*, dorsal view. **E.** "*P.*" *schmidti*, right leg 4, ventral view.

Results and Discussion

The 18S (1761 bp; LC776724) sequence from the copepod and the COI sequence (619 bp, encoding 205 amino acids; LC776725) from the host fish were determined. My attempt to determine a COI sequence for the copepod was unsuccessful; instead, a contaminant bacterial sequence was determined (data not shown), likely due to primer mismatch. The sequence in the INSD determined by a BLAST search (Altschul et al. 1990) to be most similar to my copepod 18S sequence was from *Lepeophtheirus longicauda* (LC512444; identity score 99.77 %, query cover 100 %; Homma et al. 2020) and that to my fish COI sequence was from *Myzopsetta punctatissima* (MH032464; identity score and query cover, 100 %; Vinnikov et al. 2018). The ML tree (Fig. 3) shows "*P.*" *schmidti* to be nested in a *Lepeophtheirus* clade with high support (SH-aLRT = 98.4 %; UF-Boot = 99 %), confirming it belongs in *Lepeophtheirus*.

Among 126 valid species and two recognized subspecies in *Lepeophtheirus* (Homma et al. 2020, Kakui & Uyeno 2020, Hayes et al. 2021, Morales-Serna et al. 2023, this study), *Lepeophtheirus schmidti* most resembles *L. longicauda*, *L. longiventralis* Yü & Wu, 1932, *L. marcepes* C.B. Wilson, 1944, and *L. parvicruris* Fraser, 1920 in having a long abdomen, proportionally short cephalothorax, and the leg-4 exopod with two or fewer articles (for details, see Homma et al. 2020). The uniarticulate leg 4 (Fig. 2E) in *L. schmidti* is unique among congeners.

This is the first record of *L. schmidti* from Japanese waters and the first from outside the Sea of Japan. Further study in areas where host fishes occur may discover a broader distribution for this copepod species.

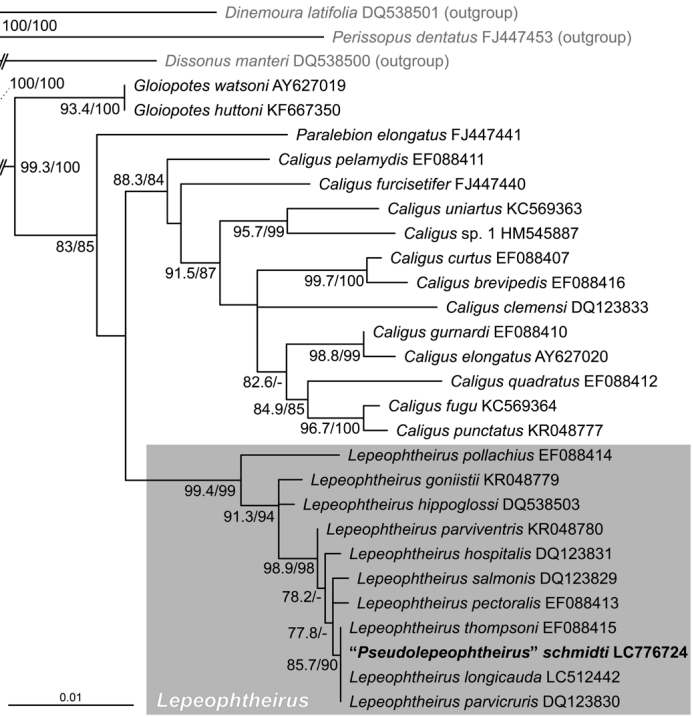

Fig. 3. ML tree for caligids based on 18S (1606 positions); numbers near nodes are SH-aLRT (left of slash) and ultrafast bootstrap (UFBoot; right of slash) values in percent; only values of SH-aLRT ≥75 % and UFBoot ≥80 % are shown. Scale at bottom indicates branch length in substitutions per site.

Acknowledgements

I thank Chizue Hiruta for help in copepod collection; Mizuho Munakata for help in molecular work; the World Register of Marine Species (WoRMS) and Flanders Marine Institute (VLIZ) for literature; and Matthew H. Dick for reviewing the manuscript and editing the English.

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