



A new species of *Ceratophysella* (Collembola: Hypogastruridae) from Japan, with notes on its DNA barcode and a key to Japanese species in the genus

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Abstract

Ceratophysella comosa **sp. nov.** was collected from ascomata of *Ciborinia camelliae* in Japan and the morphological and molecular characteristics of the species are described here. The species has 3 + 3 cephalic spines as in *Ceratophysella loricata* and *Ceratophysella pilosa*, but a plurichaetosis intermediate between *C. loricata* (absent) and *C. pilosa* (strong). The new species can be distinguished from these two species also by the number of setae on the first thorax segment and ventral tube. Partial DNA sequences of the mitochondrial cytochrome *c* oxidase subunit 1 (*COI*) gene were used as DNA barcodes to distinguish species. Interspecific genetic distances of the gene were higher than the intraspecific distances between *Ceratophysella* species for which sequence data are available. An identification key of Japanese *Ceratophysella* is provided.

Key words: *Ceratophysella comosa* **sp. nov.**, chaetotaxy, fungus feeding, plurichaetosis

Introduction

The genus *Ceratophysella* Börner (Hypogastruridae) comprises about 142 species (Bellinger *et al.* 1996–2012), of which 15 are known from Japan (Furuno *et al.* 2000), following revisions by Skarżyński and Christiansen (2008) and Bellinger *et al.* (1996–2012). Plurichaetosis is seen in some species and is well developed in *Ceratophysella horrida* (Yosii, 1960) and *Ceratophysella pilosa* (Yosii) (Babenko *et al.* 1994; Yosii, 1960). We collected a new species with moderate plurichaetosis from the ascomata of *Ciborinia camellia* L. M. Kohn in Japan.

The new species, *Ceratophysella comosa* **sp. nov.**, is described morphologically below together with partial DNA sequence of its mitochondrial *cytochrome c oxidase subunit 1* (*COI*) gene, a DNA barcode now commonly used for species characterisation (Hebert *et al.* 2003). The sequence was compared to those of congeneric species, including *C. pilosa*. Some Japanese species were not included in the keys for *Ceratophysella* in Babenko *et al.* (1994) and Thibaud *et al.* (2004) so an updated key is included here.

Materials and Methods

Specimens of *C. comosa* **sp. nov.** were collected from ascomata of *Ciborinia camelliae* on 9 April 2011 at Nagasaki (32°46'N, 129°53'E; 360 m above sea level) and were fixed in 100% ethanol. In order to study a same specimen both morphologically and molecularly, one of antennae was dissected in 100% ethanol for DNA extraction. For morphological studies, the antennal-dissected specimens were cleared in 10% KOH aqueous solution for a few minutes, mounted between cover slides with Andre and Hoyer's fluid and examined morphologically under an optical microscope. The terminology for the morphological description follows that in Yosii (1960), Babenko *et al.* (1994), Fjellberg (1998) and Thibaud *et al.* (2004). Seven specimens were examined

To distinguish between *C. comosa* **sp. nov.** and the closely related species *C. loricata* (Yosii, 1960) (described from the USA), a holotype and seven paratypes of *C. loricata* were examined. Eight specimens were labelled as

paratypes of *C. loricata*, but one did not belong to this species; several other specimens were labelled "Japan (Kyoto): Yase, on Fungus, 23 XI 1970, and leg. R. Yoshii; *Hypogastrura (Cyclograna) loricata* Ys., det. Yoshii" and "Japan (Nagano): Mt Kurobegoro, 19 VII 1938, leg. R. Yoshii; *Hypogastrura (Cyclograna) loricata* Ys., det. Yoshii", but these were not *C. loricata* but instead *Ceratophysella denisana* (Yosii) or another *Ceratophysella* species.

For comparison of interspecific and intraspecific genetic divergences between species of the genus, specimens of *C. pilosa* were collected on 10 April 2011 in Otsu (34°56'N, 135°53'E; 130 m above sea level). Of these, four specimens were treated as described above. The specimens are preserved in the Natural History Museum and Institute, Chiba (Nos. CBM-ZI 146424–146427).

Genomic DNA was extracted from a dissected antenna using a QIAGEN DNeasy Mini Kit (Qiagen), according to the manufacturer's instructions. The DNA sequence of a 658-bp region of the mitochondrial *COI* gene was obtained following Protocol 1. In cases where there was insufficient polymerase chain reaction (PCR) product for sequencing, Protocol 2 was used (of 96 specimens of *Ceratophysella*, *Hypogastrura*, and *Schaefferia* tested, 83 specimens yielded amplicons with the Protocol 1, 7 specimens required Protocol 2, and 6 specimens did not yield amplicons with either protocol; these latter 6 samples were collected eight years before and the rest within 3 years of the DNA extraction).

Protocol 1: A partial region of the mitochondrial *COI* gene was amplified by PCR from genomic DNA with the primer pair M13-LCOdent (5'-TGTAACACGACGGCCAGT-TCAACAAATCRYAARGAYATYGG-3', where R = A or G and Y = C or T) and M13R-HCOdent (5'-CAGGAAACAGCTAT-GACCACTTCTGGRTGNCCRAARAATCA-3', where N = A, C, G, or T) (modified from LCO1490 and HCO2198, respectively; Folmer *et al.* 1994); the M13 universal primer sequence was added for sequencing (Ivanova *et al.* 2007). Each PCR mixture contained 2 µl of DNA template, 6.25 µl of 2×PCR buffer, 2.5 µM of each primer, 0.4 mM of dNTPs, and 0.25 units of *Taq* DNA polymerase (KOD FX Neo; Toyobo). Water was added to reach a total reaction volume of 12.5 µl. The PCR conditions consisted of 94°C for 2 min; 45 cycles at 98°C for 10 s, 52°C for 30 s, and 68°C for 1 min, with a final 5-min extension at 68°C. The PCR products were loaded onto 1.5% agarose gels for electrophoresis, dissected out from the gels, cleaned using a QIAquick Gel Extraction kit (Qiagen), and sequenced directly from both directions with primers M13 and M13R on an ABI3730 sequencer using a BigDye 3.1 sequencing kit (Applied Biosystems), according to the manufacturer's instructions.

Protocol 2: The procedure was same as in Protocol 1 except that nested PCR was carried out in Protocol 2. First, 25 PCR cycles were carried out with the primer pair (LCOdent-out: GGTCACAAATCRYAARGA; HCOdent-out: TAAACTTCTGGR-TGNCCRAA). The 1/100-diluted amplicon was used as the template for 35 nested PCR cycles with the primer pair M13-LCOdent and M13R-HCOdent.

All sequences were verified as being derived from Collembola using the BLASTn algorithm of the basic local alignment search tool against GenBank. The resulting sequences were edited and aligned using ClustalW. The sequences were submitted to GenBank (Accession No. AB740020 for the holotype of *C. comosa* **sp. nov.**; Nos. AB740021–AB740026 for the paratypes of *C. comosa* **sp. nov.**; AB740027–AB740030 for *C. pilosa*).

Pairwise nucleotide divergences within and between *C. comosa* **sp. nov.** and other congeneric species (Table 1) were calculated using corrected Kimura-2 parameter distances. The distance tree was inferred using the Neighbour-Joining method (Saitou and Nei 1987) from 1,000 bootstrap. In addition to the *C. pilosa* sequence data, those for the following *Ceratophysella* species were obtained from GenBank: *C. denticulata* (Bagnall) (HQ732030–HQ732038) and *C. gibbosa* (Bagnall) (HQ732039, HQ732040; Greenslade *et al.* 2011). The sequence data were analysed using MEGA (ver. 5; Tamura *et al.* 2011).

TABLE 1. *Ceratophysella* species used for nucleotide divergences of the mitochondrial *COI* gene.

Species	GenBank Accession No.	Country	Locality	References
<i>C. comosa</i> sp. nov.	AB740020–AB740026	Japan	Nagasaki	This study
<i>C. denticulata</i>	HQ732030–HQ732034	Australia	Macquarie Island	Greenslade <i>et al.</i> (2011)
	HQ732035	Chile	Parque Nacional Chilo	
	HQ732036	New Zealand	Mt. Stokes	
	HQ732037, HQ732038		Poor Knights Islands	
<i>C. gibbosa</i>	HQ732039	France	Paris	Greenslade <i>et al.</i> (2011)
	HQ732040	Argentina	Parque Nacional Lanin	
<i>C. pilosa</i>	AB740027–AB740030	Japan	Otsu	This study

Ceratophysella comosa sp. nov.

Figs. 1–9

Description.

Body length 0.8–1.3 mm. Colour dark brown to dark grey. Tegumentary granulation coarse on part of the dorsal side of thoracic terga II–abdominal terga VI. Abdominal tergum V with 12–15 tegumentary granules between setae p1. Antennal segment I with seven setae; antennal segment II with 12 setae. Antennal III organ with two sensilla guarded by raised cuticle and two guard sensilla (Fig. 1). Antennal segment III microsensilla present. Antennal segment IV with a simple apical antennal bulb and 5–7 poorly differentiated blunt sensilla (Fig. 1); ventral file with about 40 peg-like setae surrounding a central seta (Fig. 2). Head with 3 + 3 cephalic spines in positions d2, d5, and sd5 (Fig. 5). Eyes 8 + 8. Postantennal organ with four lobes, the posterior two lobes surrounding an accessory boss (Fig. 3). Ocelli area with 3 + 3 setae. Labrum with 5, 5, and 4 setae; labral margin with four tubercles. Maxillary outer lobe with two sublobal hairs. Basolateral field of ventral labium with 4 + 4 setae. Basomedian field of ventral labium with 5 + 5 setae (Fig. 6). Claw with an internal tooth and two lateral teeth (Fig. 8). Empodium with a lamella and a filament reaching the internal tooth of the claw. Ventral tube laterally with 5 + 5 setae (3 + 3 setae located distally and anteriorly to the other 2 + 2 setae; Fig. 4). Tenaculum with 4 + 4 teeth (Fig. 7). Dens with seven setae on posterior side (Fig. 9). Mucro curved, with lateral lamella. Dens/mucro ratio = ca. 2. Anal spines long, yellow to brown in colour, situated on basal papillae. Anal spines/inner edge of hind claws ratio = ca. 1.3.

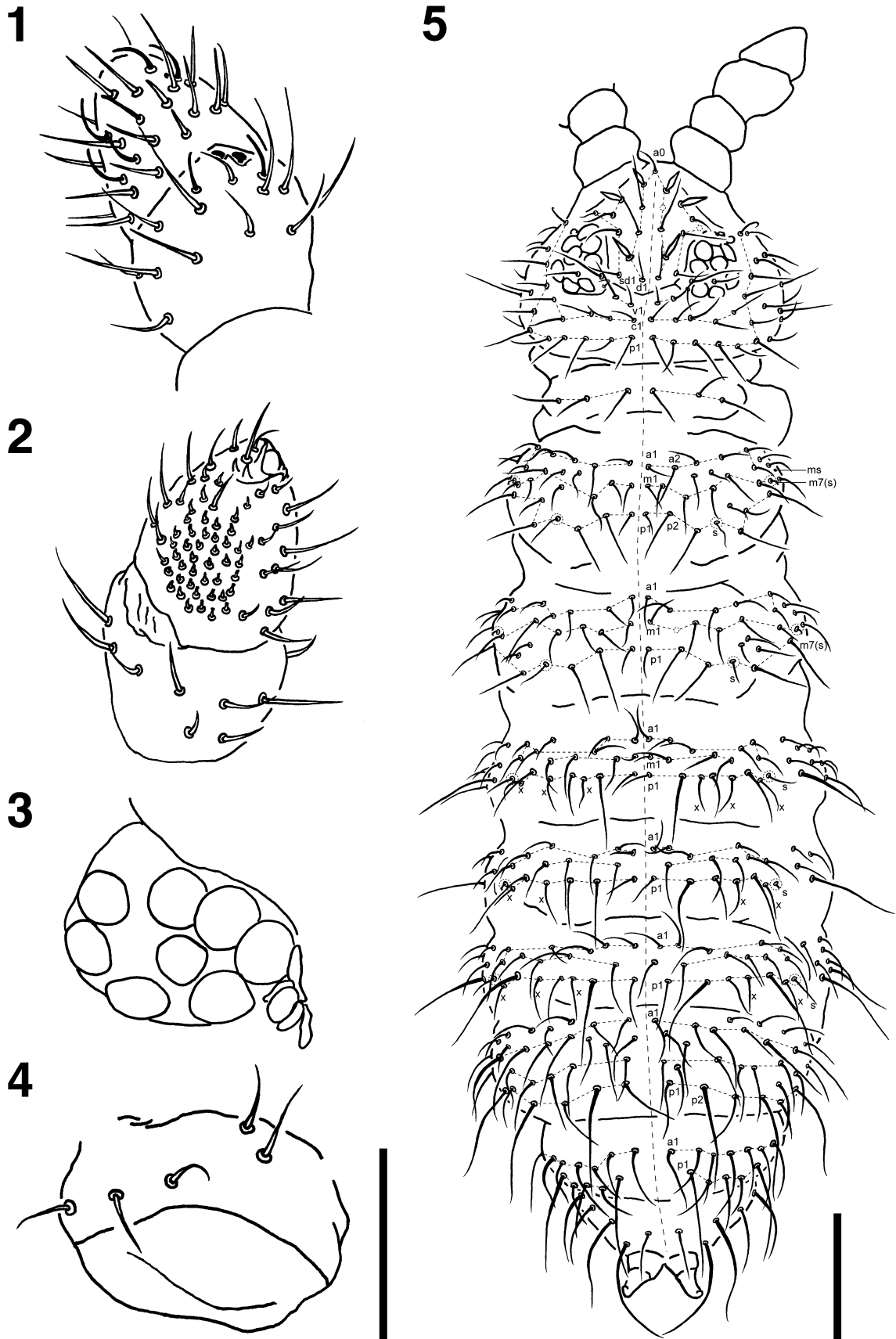
Body setae slender, smooth or slightly serrated and pointed at the apex (Fig. 5). Sensory setae long and weakly differentiated from body setae; those on thoracic tergum II (m7), abdominal tergum II (p7–8), and the lateral aspect of thoracic tergum III (m7) very short and curved. One pair of lateral microsensilla present on thoracic tergum II. Plurichaetosis absent on head thoracic tergum I, and abdominal tergum VI, weak on thoracic terga II–III and abdominal terga I–III and V, more distinct on abdominal tergum IV. Body setae, especially supernumerary setae, often asymmetric in position and number. Head with setae v2 about twice as long as setae v1 or less. Thoracic tergum I with 3 + 3 setae. Thoracic tergum II with setae a2 about twice as long as setae a1 or less, and setae p2 about 1.5 times as long as setae p1. Thoracic terga II–III with sensory setae at the location of p4. Abdominal terga I–III with sensory setae at the location of p7–8. Abdominal tergum I with setae m1. Abdominal tergum IV with setae p2 about 1.5 times as long as setae p1 or less and sensory setae at the location of p5. Abdominal segment II normally with 4 + 4 ventral setae (Fig. 7). Abdominal segment III with 10 + 10 ventral setae. Trochanters I–III with 7, 7, 6 (6–8) setae. Femurs I–III with 14, 13, 12 setae. Tibiotarsi I–III with 19, 19, 18 setae (Fig. 8).

Remarks

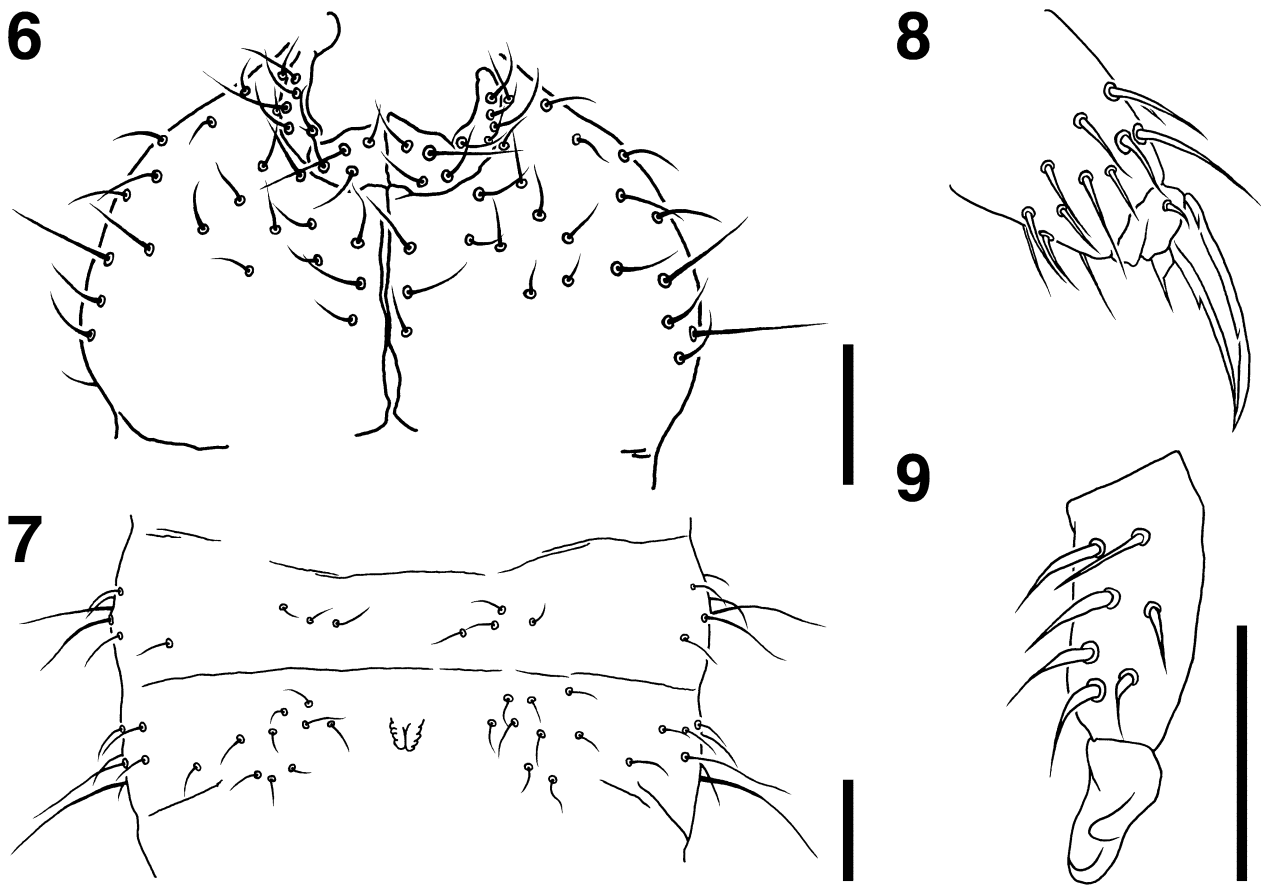
Within the genus *Ceratophysella*, the presence of 3 + 3 cephalic spines and the shape of postantennal organ (the posterior two lobes surrounding an accessory boss) place *C. comosa* sp. nov. near *C. loricata*, and *C. pilosa*. The morphological features of the new species generally fit the original description of *C. loricata*. Very short sensory setae on thoracic tergum II, abdominal tergum II, and the lateral aspect of thoracic tergum III are also present in types of *C. loricata*. However, *C. comosa* sp. nov. has only weak plurichaetosis, whereas plurichaetosis is absent in *C. loricata*. As a result the apparent locations of sensory setae on abdominal terga I–III differ between these species (p7–8 in *C. comosa* sp. nov.; p5 in *C. loricata*). Furthermore, differentiation of macro- and microsetae is weaker in *C. comosa* sp. nov. than in *C. loricata* (in types of *C. loricata*, head with setae v2 about four times as long as v1 or more; thoracic tergum II with setae a2 about three times as long as setae a1 or more, and setae p2 about four times as long as setae p1 or more; abdominal tergum IV with setae p2 about twice as long as setae p1). In addition, tegumentary granules are slightly finer in *C. comosa* sp. nov. than in *C. loricata* (the numbers of tegumentary granules between setae p1 on abdominal tergum V are 12–15 in *C. comosa* sp. nov. and 11–13 in types of *C. loricata*). As strong plurichaetosis is seen in *C. pilosa*, the new species can be easily distinguished from it in having 3 + 3 setae on thoracic tergum I (more setae in *C. pilosa*) and from *C. loricata* by having 5 + 5 setae on ventral tube (4 + 4 in *C. loricata*).

Etymology

The species name *comosa* is a Latin word meaning hairy, in reference to the plurichaetosis in the long setae on abdominal tergum IV of the new species.



FIGURES 1–5. *Ceratophysella comosa* sp. nov.: **1**, dorsal view of antennal segments III–IV; **2**, ventral view of antennal segments III–IV; **3**, ocelli and postantennal organ, right side; **4**, lateral view of ventral tube; **5**, dorsal chaetotaxy. ms indicate microsensilla; s indicates sensory setae; x indicates supernumerary setae in posterior rows on abdominal terga I–III. Bar 50 μm (Figs. 1–4), 100 μm (Fig. 5).



FIGURES 6–9. *Ceratophysella comosa* **sp. nov.**: **6**, ventral view of head; **7**, ventral view of abdominal segments II and III; **8**, inner view of claw and tibiotarsus III; **9**, dens and mucro. Bar 50 μ m.

Type materials

A holotype male and six paratypes (one male and five females), collected in Nagasaki, Japan (32°46'N, 129°53'E; 360 m above sea level) on 9 April 2011 from ascomata of *Ciborinia camellia*, have been deposited in the Natural History Museum and Institute, Chiba (the holotype: No. CBM-ZI 146417; the paratypes: Nos. CBM-ZI 146418–146423). Sequence data for the mitochondrial *COI* gene for the type specimens were registered in GenBank (the holotype: Accession No. AB740020; the paratypes: Nos. AB740021–AB740026).

DNA sequences

The interspecies sequence divergences of the mitochondrial *COI* gene were larger than intraspecies divergences between *C. comosa* **sp. nov.**, *C. denticulata*, *C. gibbosa*, and *C. pilosa* individuals examined (Table 2; Fig. 10). These results suggest that sequencing the mitochondrial *COI* gene is useful for discrimination of *C. comosa* **sp. nov.** from congeneric species, and supports the applicability of this region for species discrimination as indicated by previous studies on DNA barcoding of Collembola (Hogg and Hebert 2004; Porco *et al.* 2010). However, a recent study with broader sampling documented cryptic diversity of the mitochondrial *COI* gene in *C. denticulata* and distinguished three lineages (Porco *et al.* 2012). DNA barcoding data are available for only a few of the 142 *Ceratophysella* species. Further investigations are needed for DNA barcoding of this genus.

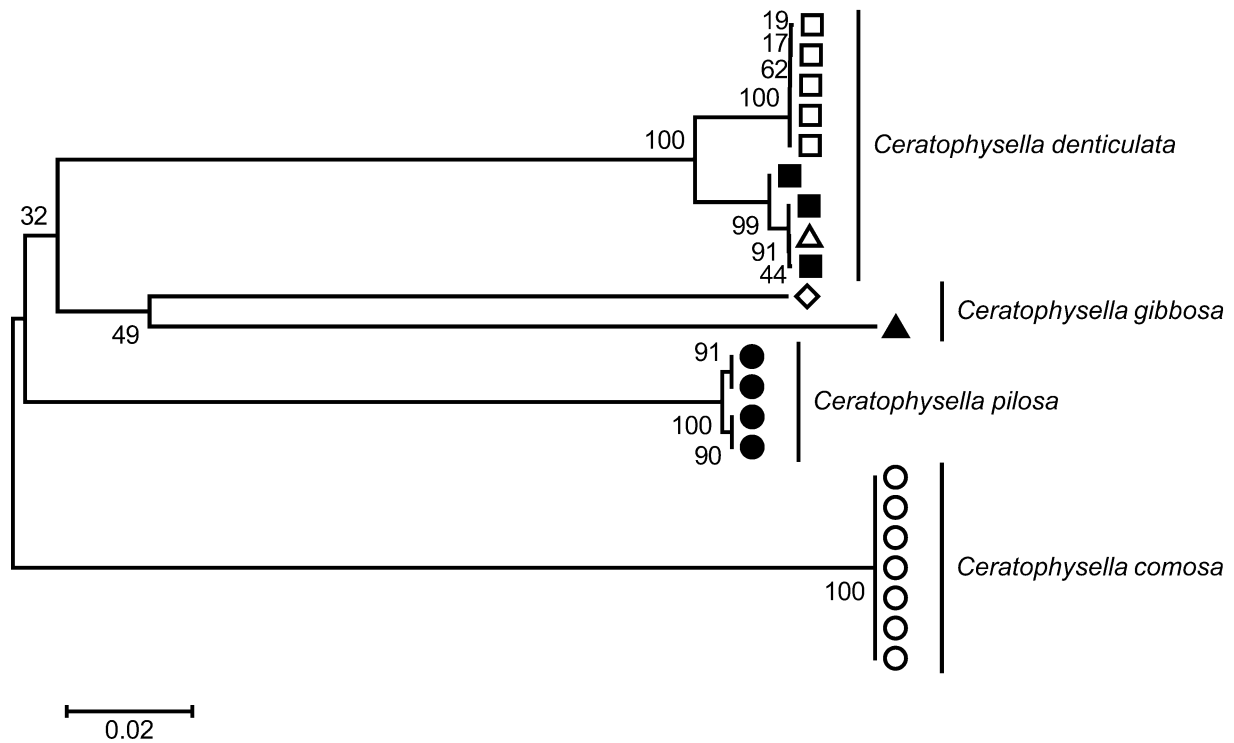


FIGURE 10. Genetic distance tree of mitochondrial *COI* gene in *Ceratophysella* inferred using the Neighbour-Joining method. The bootstrap consensus tree was inferred from 1,000 replicates. The percentages of replicate trees in the bootstrap test are shown next to the branches. The distances were computed using the Kimura 2-parameter method. Symbols indicate localities from which the specimens were collected: open circle, Japan (Otsu); closed circle, Japan (Nagasaki); open square, Australia; closed square, New Zealand; open triangle, Chile; closed triangle, Argentina; open diamond, France. See Table 1 for sources of sequence data.

TABLE 2. Inter- and intraspecific sequence divergences of mitochondrial *COI* gene in *Ceratophysella* (Kimura-2 parameter pairwise distances).

Species	1	2	3	4
1 <i>C. comosa</i> sp. nov. (<i>n</i> = 7)	0			
2 <i>C. denticulata</i> (<i>n</i> = 9)	0.255–0.267	0–0.029		
3 <i>C. gibbosa</i> (<i>n</i> = 2)	0.244–0.283	0.231–0.249	0.217	
4 <i>C. pilosa</i> (<i>n</i> = 4)	0.251–0.253	0.228–0.237	0.233–0.263	0–0.003

See Table 1 for sources of sequence data.

Key to the species of Japanese *Ceratophysella*

- 1 Postantennal organ with posterior lobes encircling an accessory boss 2
- Postantennal organ never with posterior lobes encircling an accessory boss 4
- 2 Head with 2 + 2 spines *C. horrida* (Yosii)
- Head with 3 + 3 spines 3
- 3 Thoracic tergum I with 3 + 3 setae *C. comosa* sp. nov.
- Thoracic tergum I with more than 3 + 3 setae *C. pilosa* (Yosii)
- 4 Abdominal tergum IV with setae p1 subequal to setae p2 in length *C. yakushimana* (Yosii)
- Abdominal tergum IV with setae p1 longer or shorter than setae p2 5
- 5 Abdominal tergum IV with setae p1 longer than setae p2 6
- Abdominal tergum IV with setae p1 shorter than setae p2 13
- 6 Abdominal tergum IV with setae p3 subequal to setae p2 7
- Abdominal tergum IV with setae p3 longer than setae p2 9
- 7 Tibiotarsi I–III with three clavate tenent hairs *C. fujisana* Itoh
- Tibiotarsi I–III with at most one clavate tenent hair 8

8	Thoracic tergum I with 3 + 3 setae. Antennal segment I with seven setae. Claws without internal teeth . . .	<i>C. sakayori</i> Tamura
-	Thoracic tergum I with 2 + 2 setae. Antennal segment I with six setae. Claws with one internal tooth	<i>C. ateruui</i> Tamura
9	Abdominal tergum V with medial spine-like integumentary projection between setae p1	<i>C. tergilobata</i> Cassagnau
-	Abdominal tergum V without this projection	10
10	Abdominal tergum V with setae p1 modified into a spine	<i>C. duplicispinosa</i> Yosii
-	Abdominal tergum V with setae p1 normal, not modified into a spine	11
11	Head with setae oc2 and v2 modified into a spine	<i>C. wrayia</i> (Uchida & Tamura)
-	Head with setae oc2 and v2 hair-like, not modified into a spine	12
12	The tip of mucro pointed	<i>C. ainu</i> Yosii
-	Mucro blunt-tipped	<i>C. denisana</i> Yosii
13	Dens with six setae	<i>C. proserpinae</i> (Yosii)
-	Dens with seven setae	14
14	Body granulation on abdominal tergum V fine: abdominal tergum V with more than 15 granules between setae p1	<i>C. communis</i> (Folsom)
-	Body granulation on abdominal tergum V fine: abdominal tergum V with fewer than 15 granules between setae p1	15
15	Body colour white (alive) or straw-yellow (in alcohol)	<i>C. troglodites</i> Yosii
-	Body colour dark	<i>C. denticulata</i> Bagnall

Remarks: The key is based partly on Babenko *et al.* (1994) and Thibaud *et al.* (2004) with modifications for identifying Japanese species.

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