Research Article

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Morphological and molecular studies of *Eudiplozoon nipponicum* (Goto, 1891) and *Eudiplozoon kamegaii* sp. n. (Monogenea; Diplozoidae)

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Abstract: *Eudiplozoon nipponicum* (Goto, 1891) Khotenovsky, 1985 (Monogenea: Diplozoidae), is known to parasitise *Cyprinus carpio* Linnaeus and species of *Carassius*. In this study, we conducted a taxonomic re-examination of *E. nipponicum* using genetic analysis and morphological comparisons from different host species from a single water system. rDNA nucleotide sequences of the internal transcription spacer 2 (ITS-2) region (645 bp) showed interspecific-level genetic differences among diplozoids from species of *Carassius* and *C. carpio* (p-distance: 3.1–4.0%) but no difference among those from different species of *Carassius* (0–0.4%) or between those from *C. carpio* collected in Asia and Europe (0–1.1%). Large variation was observed among 346 bp cytochrome *c* oxidase subunit I (COI) sequences (0.3–16.0 %); the topology of the phylogenetic tree showed no relationship to host genera or geographical regions of origin. Morphological observation showed that average clamp size of diplozoids from *C. carpio* was larger than those from *Carassius* spp. The number of folds on the hindbody was 10–25 for diplozoids from *C. carpio* and 12–19 for those from *Carassius* spp. Thus, our ITS-2 sequence and morphological comparison results indicate that diplozoids from *C. carpio* and species of *Carassius* belong to different species. The scientific name *E. nipponicum* should be applied to the species infected to the type host, *Carassius* sp. of Nakabo (2013) (Japanese name gimbuna). The diplozoid infecting *C. carpio* (Eurasian type) should be established as a new species: *Eudiplozoon kamegaii* sp. n. A neotype of *E. nipponicum* is designated in this report because the original *E. nipponicum* specimens are thought to have been lost.

Keywords: genus *Eudiplozoon*, neotype, new species, host specificity, molecular phylogeny, morphology, monogenea

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Diplozoon nipponicum Goto, 1891 was described by Goto (1891) (Monogenea: Diplozoidae) based on specimens found on *Carassius vulgaris* (Nordmann) from Japan, but Goto (1891) designated neither the type specimens nor type locality. Khotenovsky (1981) later assigned this species to a new genus, *Sindiplozoon* Khotenovsky, 1981, followed by another new genus, *Eudiplozoon* Khotenovsky, 1985. *Eudiplozoon nipponicum* (Goto, 1891) is now the only species in the genus *Eudiplozoon*.

The type host *C. vulgaris* is now considered a synonym of *Carassius carassius* (Linnaeus) (Kottelat 1997). However, *C. carassius* is not distributed in Japan. Matejusová et al. (2001) misinterpreted the type host designated by Goto (1891) as *C. carassius*. *Carassius vulgaris*, as described by Goto (1891), may have been either *Carassius* sp. (Japanese name gimbuna) (Shimazu et al. 2015) or *Carassius buergeri* subsp. 2 (Japanese name kinbuna) (Nagasawa 2016).

In Japan, five species or subspecies of cyprinid fishes have been reported as natural hosts of *E. nipponicum* (sci-

entific names follow Nakabo 2013): *Cyprinus carpio* Linnaeus (Japanese name koi) (Kamegai et al. 1966, Kamegai 1968, 1976, Ogawa 2010–2011), *Carassius* sp. (Japanese name gimbuna) (Kitahara et al. 1986, Hirose et al. 1987, Mizuo et al. 1999, Sicard et al. 2003), *C. buergeri* subsp. 2 (Japanese name kinbuna) (Kitahara et al. 1986, Sicard et al. 2003), *Carassius buergeri grandoculis* Temminck et Schlegel (Japanese name nigorobuna) (Kamegai 1975b, Ogawa 2010–2011), and *Carassius cuvieri* Temminck et Schlegel (Japanese name gengorobuna) (Kamegai 1977, Nagasawa 2016).

In other countries, the following fish species have been also recorded as hosts of *E. nipponicum*: *Carassius auratus gibelio* (Bloch) and *Channa argus* (Cantor) (Channidae) from China (Jiang et al. 1989, Jiang 2000, Gao et al. 2007), *Cyprinus carpio rubrofasciatus* Lacépède, *Abramis brama* (Linnaeus) (Cyprinidae), *Capoeta capoeta heratensis* (Keyserling) (Cyprinidae) and *Luciobarbus capito conocephalus* (Kessler) (Cyprinidae) from Russia (Pugachev et

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Fig. 1. Sampling localities of host fish. **A** – Shiga prefecture; **B** – out of Shiga. Black circle, localities where *Eudiplozoon* were collected; open circle, localities where *Eudiplozoon* were absent.

al. 2010), *Leuciscus vorax* (Heckel) (Cyprinidae) and *Mesopotamichthys sharpeyi* (Günther) (Cyprinidae) from Iraq (Al-Saadi et al. 2010, Mhaisen and Abdul-Ameer 2014).

Sicard et al. (2003) compared *E. nipponicum* obtained from *C. carpio* in France and *Eudiplozoon* sp. found on *Carassius auratus* (Linnaeus) (in fact *Carassius* sp. – Japanese name ginbuna; T. Shimazu personal communication) from Japan using the internal transcribed spacer 2 (ITS-2) rDNA region and found a genetic distance of 0.028 (Kimura's two-parameter model) between *E. nipponicum* and *Eudiplozoon* sp. This value was lower than the average interspecific distance (0.094) for the genus *Paradiplozoon* (Diplozoidae) (Sicard et al. 2003).

These authors suggested that the genetic distance between *E. nipponicum* from *C. carpio* in Europe and *Eudiplozoon* sp. from *Carassius auratus* in Japan supports the view that *E. nipponicum* should be divided into two species or subspecies. However, they did not analyse diplozoids from *C. carpio* in Japan and therefore could not determine whether this genetic difference was derived from host segregation or geographical distances (there are no records of *E. nipponicum* from *Carassius* in Europe). Sequence data for the diplozoids analysed by Sicard et al. (2003) have not been published.

Zhang et al. (2018) subsequently sequenced the complete mitogenome and ITS-2 region of *Eudiplozoon* sp. obtained from a *C. auratus* complex from Tangxun Lake, Hubei Province, China, and found a significant difference from *E. nipponicum* found on *C. carpio* from France (AY009163) in ITS-2 sequences (94–95% of similarity) and COI sequence (83% of similarity). Their mitogenome

sequence (MG458326) is the only sequence of *Eudiplozoon* from *Carassius* registered with the International Nucleotide Sequence Database. Zhang et al. (2018) suspected the existence of cryptic species of *Eudiplozoon* and discussed the need of taxonomic revision of the genus.

To resolve the problem of species composition of *Eudiplozoon* is necessary to collect diplozoids from both *C. carpio* and sympatrically distributed species of *Carassius* and compare their morphology and DNA sequences. It is thought that all *E. nipponicum* specimens collected by Goto (1891) have already been lost (Shimazu and Araki 2006). Therefore, it is necessary to designate a neotype of *E. nipponicum*.

In this study, we compared the molecular and morphological characteristics of diplozoids found on *C. carpio* and species of *Carassius* captured in a single water system in Japan to clarify genetic and/or morphological divergences in diplozoids on different host genera that were not geologically isolated. We redescribed *E. nipponicum* with the designation of neotype and also described one new species.

MATERIALS AND METHODS

Collection and identification of the host fishes

We collected species of *Carassius* and *Cyprinus carpio* from 8 locations in Shiga Prefecture, Japan (1–8 in Table 1 and Fig. 1A). Specimens of *Eudiplozoon* were obtained from 4 localities of them: Harie (1), 20 pairs of adult diplozoids (40 individuals) and 1 diporpa from *Carassius buergeri grandoculis*; Ohta (2): 7 pairs of adult diplozoids (16 individuals) from *C. buergeri grandoculis* and 5 pairs of adult diplozoids (8 individuals) from *Carassius*

Table 1. Sampling locality, sampling period and host species of diplozoids examined.

Locality	Sampling period	Fish species examined	n	range of the standard length (cm)	No. of fish infected	No. of paired diplozoids	No. of diporpaes
(1) Harie, Shin-asahi Town, Takashima City, Shiga Prefecture (35°22'17"N; 136°02'47"E)	May–August 2016, May–November 2017 and June 2019	<i>Carassius buergeri grandoculis</i> (nigoro-buna)	68	11.7–32.9	10	20	1
		<i>Carassius</i> sp. (gin-buna)	71	7.0–31.5	0	0	0
		<i>Carassius</i> spp. (not identified)	7	2.4–32.7	0	0	0
		<i>Cyprinus carpio</i>	8	11.1–59.3	0	0	0
(2) paddy field pond of Ohta biotope, Shin-asahi Town, Takashima City, Shiga Prefecture (35°20'24"N; 136°04'13"E)	May–November 2018 and June 2019	<i>Carassius buergeri grandoculis</i>	50	5.5–33.7	5	7	0
		<i>Carassius</i> sp.	38	9.6–35.8	4	5	0
(3) Kitafunaki, Adogawa Town, Takashima City, Shiga Prefecture (35°19'59"N; 136°04'03"E)	May 2016	<i>Carassius buergeri grandoculis</i>	9	8.3–14.9	0	0	0
		<i>Carassius</i> sp.	6	8.8–24.8	0	0	0
		<i>Carassius</i> spp. (not identified)	1	11.6	0	0	0
		<i>Carassius buergeri grandoculis</i>	2	7.9–14.1	0	0	0
(4) shore net (<i>eri</i>) off the shore of Lake Biwa at Hachiyado, Otsu City, Shiga Prefecture (35°10'53"N; 135°55'10"E) in February 2017	June–August 2018	<i>Carassius</i> sp.	5	8.3–13.6	0	0	0
(5) Wani fisheries port, Otsu City, Shiga Prefecture (35°09'55"N; 135°55'26"E)	February 2017	<i>Cyprinus carpio</i>	1	34.4	1	2	0
(6) biotope on the coast of Lake Biwa at Shinhamu, Kusatsu City, Shiga Prefecture (34°59'52"N; 135°54'30"E)	November 2017 and August–November 2018	<i>Carassius</i> sp.	6	10.8–32.7	0	0	0
		<i>Cyprinus carpio</i>	10	9.0–17.9	5	8	2
(7) Jinjo pond, Satsuma, Hikone City, Shiga Prefecture (35°13'44"N; 136°09'37"E)	August 2018	<i>Carassius buergeri grandoculis</i>	1	5.1	0	0	0
		<i>Carassius</i> sp.	4	6.4–8.0	0	0	0
		<i>Carassius cuvieri</i> (gengoro-buna)	2	11.1–14.7	0	0	0
(8) Kaideima Bridge, Kaideima, Hikone City, Shiga Prefecture (35°15'23"N; 136°13'39"E)	June 2018	<i>Carassius buergeri grandoculis</i>	1	21.3	0	0	0
(9) Chikuma River, Senbonyanagi, Chikuma City, Nagano Prefecture (36°29'59"N; 138°08'01"E)	May 2016	<i>Carassius</i> sp.	10	7.1–12.8	0	0	0
(10) Tanjo River, Hirohata Town, Yonezawa City, Yamagata Prefecture (37°56'47"N; 140°04'13"E)	August 2016	<i>Carassius buergeri</i> subsp. 2 (kin-buna)	17	3.8–9.8	0	0	0
(11) Ichinomiya, Tama City, Tokyo Metropolitan area (35°39'16"N; 139°26'21"E)	September 2017	<i>Carassius</i> sp.	10	2.8–6.6	0	0	0

sp.; Wani (5): 2 pairs of adult diplozoids (four individuals) from *Cyprinus carpio*; and Shinhamu (6): 8 pairs of adult diplozoids (16 individuals) from *C. carpio*. We also examined fishes from 3 sites out of Shiga Prefecture, but none of them was infected with diplozoids (9–11 in Table 1 and Fig. 1B).

Collected fish were brought to the laboratory and kept in a water tank until dissection. Fish that were dead or weak at the time of collection were brought back to the laboratory on ice and dissected immediately. Their gills were excised and placed in Petri dishes with 0.4 % saline, and diplozoids on the gills were identified under a stereoscopic microscope. Detected diplozoids were placed on glass slides, and a small piece of the forebody was cut for DNA analyses. These pieces were stored at -18°C until DNA analyses. Remaining worm bodies were fixed and stained for morphological study (see below).

Fish samples were photographed for morphological identification and standard length (SL) was measured before dissection. Among *Carassius* species, body height ratio (SL/body height), number of branched soft rays on the dorsal fin and number of gill rakers were measured for species identification (Miyaji et al. 1976, Nakabo 2013). When the sample fish could not be identified by morphology, the blood cell diameter were measured to distinguish *Carassius* sp. (triploids) from other diploid species (Fujiwara and Usuki 1994, Kohashi 1994). However, this method

was not applied in the early period of the study and some fish remained unidentified to the species level.

Two types of *C. carpio* are distributed in Japan: the Eurasian type, derived from individuals introduced from the Eurasian continent, and the native type, which was naturally distributed in prehistoric times (Mabuchi et al. 2005, Miyamoto and Nakajima 2006, Mabuchi et al. 2008, 2010). When diplozoids were detected from *C. carpio*, the host fish type was distinguished using a simplified molecular identification technique (Mabuchi and Nishida 2006). Fish DNA was extracted from a piece of muscle using the Wizard SV Genomic DNA Purification System (Promega, Madison, WI). A partial sequence of the cytochrome-b (Cyt-b) region of the mitochondrial DNA (mtDNA) was amplified via polymerase chain reaction (PCR), following the protocol by Mabuchi and Nishida (2006). PCR products were subjected to electrophoresis with 10 x Midori Green Direct (Nippon Genetics Co., Ltd., Tokyo, Japan), and amplified DNA fragment lengths were verified (ca. 400 and 280 bp for the Eurasian and native types, respectively; Mabuchi and Nishida 2006).

Molecular analysis

Genomic DNA was extracted from frozen tissue samples using a Wizard SV Genomic DNA Purification System (Promega, Madison, Wisconsin, USA). In all newly obtained samples, partial regions of the ITS-2 from rDNA were amplified by PCR;

Table 2. P-distance of the sequences of ITS-2 (645 bp) and COI (346 bp) between samples of *Eudiplozoon* Khotenovsky, 1985 obtained in the present study and data from DNA Data Bank of Japan, and *Inustiatius* spp. and *Sindiplozoon ctenopharyngodoni* (outgroups). The maximum, minimum and the average (in parentheses) values are shown.

species	host	n	<i>E. nipponicum</i>	<i>E. nipponicum</i>	<i>Eudiplozoon</i> sp.*	<i>E. kamegaili</i> sp. n.	<i>E. nipponicum</i> * (= <i>E. kamegaili</i>)
			<i>Carassius buergeri</i> <i>grandoculis</i>	<i>Carassius</i> sp.	<i>Carassius auratus</i>	<i>Cyprinus carpio</i> Eurasian type	<i>Cyprinus carpio</i>
ITS-2							
<i>Eudiplozoon japonicum</i>	<i>Carassius buergeri</i> <i>grandoculis</i>	15	0.0–0.5 (0.1)				
<i>Eudiplozoon japonicum</i>	<i>Carassius</i> sp.	2	0.0–0.4 (0.1)	0,0			
<i>Eudiplozoon kamegaili</i> n.sp.	<i>Cyprinus carpio</i> Eurasian type	11	3.1–4.0 (3.2)	3.1–3.6 (3.2)		0.0–0.7 (0.3)	
<i>Eudiplozoon nipponicum</i> * (= <i>E. kamegaili</i>) (outgroups)	<i>Cyprinus carpio</i>	6	3.1–3.8 (3.2)	3.1–3.4 (3.2)		0.0–1.1 (0.3)	0.0–0.4 (0.2)
<i>Sindiplozoon ctenopharyngodoni</i> *	<i>Ctenopharyngodon idella</i>	1	23.2–23.6 (23.4)	23.4		23.9–24.5 (24.1)	23.9–24.3 (24.0)
<i>Inustiatius inustiatius</i> , <i>I. aristichthysi</i> *	<i>Hypophthalmichthys molitrix</i> , <i>Aristichthys nobilis</i>	2	26.1–26.4 (26.2)	26.3		27.2–27.5 (27.2)	27.2–27.5 (27.3)
COI							
<i>Eudiplozoon japonicum</i>	<i>Carassius buergeri</i> <i>grandoculis</i>	3	0.6–14.9 (9.9)				
<i>Eudiplozoon</i> sp.*	<i>Carassius auratus</i>	1	10.5–16.0 (14.2)				
<i>Eudiplozoon kamegaili</i> sp.n.	<i>Cyprinus carpio</i> Eurasian type	2	0.3–15.2 (10.3)		9.3–10.8 (10.1)	2.6	
<i>Eudiplozoon nipponicum</i> * (= <i>E. kamegaili</i>) (outgroup)	<i>Cyprinus carpio</i>	1	2.3–13.7 (6.1)		16.0	13.7–14.0 (13.8)	
<i>Sindiplozoon</i> sp.*	<i>Mylopharyngodon piceus</i> , <i>Parabramis pekinensis</i> , <i>Spinibarbus hollandi</i>	1	19.2–20.1 (19.7)		18.1	19.2–19.5 (19.4)	19.2

* Sequences obtained from DDBJ

in a subset of samples, partial regions of cytochrome *c* oxidase subunit I gene (COI) from mtDNA were also amplified. PCR was performed in 20 µl reaction mixture containing 13.8 µl pure water, 2.0 µl 10×Ex Taq Buffer (Takara Bio Inc., Shiga, Japan), 1.6 µl dNTP Mixture (Takara Bio Inc.), 1.0 µl each of forward and reverse primers, 0.1 µl Ex Taq (Takara Bio Inc.) and 0.5 µl template DNA.

To amplify the ITS-2 region, we used the 3S (5′–GGT ACC GGT GGA TCA CTC GGC TCG–3′) and BD2 (5′–TAT GCT TAA RTT CAG CGG GT–3′) primer set (Bowles et al. 1995). To amplify the COI regions, we used the EuDiploCOI-longF (5′–GGT TTT GTT TAT TTA YTT ATG GG–3′) (originally designed) and ASmit2 (5′–TAA AGA AAG AAC ATA ATG AAA ATG–3′) (Littlewood et al. 1997) primers. We also used the EuDiploCOI-F (5′–ATG TTT CAG CAT ATG TTT TGR TTT–3′) and EuDiploCOI-R2 (5′–AAA YCA AAA CAT ATG CTG AAA CAT–3′) internal primers when necessary.

The thermocycling profile was as follows: 94°C at 30 s, 40 cycles of 94°C for 10 s, 50°C for 30 s, 72°C for 60 s. Then DNA fragments were extended at 72°C for 7 min, and stored at 4°C. PCR was performed using a 2720 Thermal Cycler (Applied Biosystems, Life Technologies Co., Carlsbad, California, USA).

PCR products were cleaned using Wizard SV Gel and PCR Clean-up System (Promega) and sequenced directly by FAS-MAC Co. (Kanagawa, Japan). Then, analysed sequences were registered in the DNA Data Bank of Japan (DDBJ; accession nos.

LC496174–LC496190 and LC517162–LC517172 for ITS-2 and LC517173–LC517177 for COI).

Sequences were aligned with those of *Eudiplozoon* registered in DDBJ (Supplementary Table 1) using the ClustalW with the default parameters. A total of 645 positions were included for ITS-2 analysis and 346 positions for COI analysis. A maximum likelihood (ML) tree was constructed using the MEGA7.0.20 (Kumar et al. 2016). Probabilities were tested via bootstrap analyses of 1,000 replicates. Prior to ML analyses, a DNA substitution model was selected using neighbor-joining trees; the NT+G model was selected for ITS-2 and applied for phylogenetic analyses with a default gamma parameter of 5. The HKY + I model was selected for COI analyses. *Sindiplozoon ctenopharyngodoni* (Ling, 1973) (DQ098898), *Inustiatius inustiatius* (Nagibina, 1965) (DQ098893) and *I. aristichthysi* (Ling, 1973) (DQ098894) were used as ITS-2 outgroups, and *Sindiplozoon* sp. (MG458326) was used as an outgroup for COI tree construction. We also calculated p-distances between samples for each of ITS-2 and COI.

Morphological study

Each worm body was slightly pressed between a glass slide and cover slip, fixed in alcohol formal acetic acid (AFA), stained with Heidenhain's iron hematoxylin following the standard protocol, serially dehydrated in ethanol, cleared in creosote, followed by xylene, and mounted in Canada balsam.

Fig. 2. Maximum-likelihood phylogenetic tree of diplozoids based on partial sequences of the ITS-2 (645 bp).

The mounted specimens were observed under a biological microscope and drawings were made with the aid of a camera lucida. Body length was measured from photographs of mounted specimens, using the Image J (v. 1.51j8) software. Inner organs were measured using a digital camera at the microscope setting, with the WraySpect (Ver. 2.) software. The number of folds on the hindbody was also counted under a microscope. These measurement sites followed those of Khotenovsky (1985). Posterior to anterior clamps on haptor were numbered as I–IV, following Hirose et al. (1987). Measurements of juveniles (paired immature individuals) and diporpa were excluded from the statistical analyses. A portion of specimens used in the present study were deposited at the National Museum of Nature and Science, Tokyo, Japan (NMNS) and Institute of Parasitology, Biology Centre of the Czech Academy of Science, České Budějovice, Czech Republic (IPCAS).

We also measured many *Eudiplozoon* specimens deposited in Meguro Parasitological Museum, Tokyo (MPM), prepared by Satoru Kamegai and his collaborators from 1965 to 1980 (Supplementary Table 2). Fourteen pairs of *Eudiplozoon* specimens from *Carassius* sp., 42 pairs from *C. buergeri* subsp. 2, and 15 pairs from *Cyprinus carpio* (type unknown) were measured for clamp

size, and 25 pairs from *Carassius* sp., 35 pairs from *C. buergeri* subsp. 2, 5 pairs from unidentified *Carassius* spp. and 49 pairs from *C. carpio* (type unknown) were measured for the number of folds on the hindbody, to confirm whether they share morphological characteristics with new specimens obtained from host fishes of the same genera (*Carassius* or *Cyprinus*).

RESULTS

Diplozoid prevalence and intensity in collected fish

We collected 150 specimens of *Carassius* sp. (6.4–35.8 cm SL), 131 *C. buergeri grandoculis* (5.5–33.7 cm SL), 17 *C. buergeri* subsp. 2 (3.8–9.8 cm SL), 2 *C. cuvieri* (11.1–14.7 cm SL), 8 unidentified *Carassius* spp. (2.4–32.7 cm SL) and 19 *Cyprinus carpio* (9.0–59.3 cm SL) (Table 1). A total of 42 paired diplozoids and 3 diporpa were obtained: 5 pairs from *Carassius* sp., 27 pairs and 1 diporpa from *C. buergeri grandoculis*, 10 pairs and 2 diporpa from *C. carpio*. The prevalence of diplozoid infection around Lake Biwa was 3.1% (4/130) among *Carassius* sp., 11.5% (15/130) among *C. buergeri grandoculis*, and 31.6% (6/19)

Fig. 3. Maximum-likelihood phylogenetic tree of diplozoids based on partial sequences of the COI (346 bp).

Fig. 4. *Eudiplozoon nipponicum* (Goto, 1891) Khotenovsky, 1985, neotype (NSMT-Pl 6408). **A** – entire paired adults; **B** – anterior part of body, ventral view; **C** – posterior part of body, ventral view; **D** – reproductive organs; **E** – clamp I of a reference specimen (Urabe, personal collection); **F** – central hook; **G** – central hook of a reference specimen (NSMT-Pl 6410) (F and G bar 20 μm). **Abbreviations:** bc – buccal cavity; bs – buccal sucker; c – clamp; ch – central hook; cvd – common vitelline duct; e – egg; ed – egg deformed; f – fold; gic – genitointestinal canal; han – handle; hap – haptor; i – intestine; m – mouth; mg – musculo-glandular organs; od – oviduct; oe – oesophagus; ot – ootype; ov – ovary; ovd – ovovitelline duct; p – pharynx; s – shaft of hook; t – testis; tnc – transverse nerve commissure; u – uterus; uo – uterus opening; vt – vitelline follicles; w – wing of hook.

among *C. carpio*. No diplozoids were detected from *C. cuvieri* or unidentified species of *Carassius*. The mean intensities were 2.5 in *Carassius* sp. (paired diplozoids were counted as 2 individuals), 3.9 in *C. buergeri grandoculis*, and 3.7 in *C. carpio*.

Molecular analysis

We successfully sequenced 28 samples (10 pairs and 1 diporpa from *Cyprinus carpio*, 2 pairs from *Carassius*

sp., 14 pairs and 1 diporpa from *C. buergeri grandoculis*). Following the simplified molecular identification, all six *C. carpio* harbouring diplozoids were classified as the Eurasian type.

Diplozoids from *C. carpio*, including sequences obtained in the present study and from DDBJ, and those from species of *Carassius* were separated into different clades in the ML tree based on the 645 bp ITS-2 region (Fig. 2). The p-distance between diplozoids from differ-

Fig. 5. *Eudiplozoon kamegaii* sp. n. **A** – holotype (NSMT-PL 6405); **B** – anterior part of body, ventral view; **C** – posterior part of body, ventral view; **D** – reproductive organs; **E** – clamp II; **F** – central hook. **Abbreviations:** bc – buccal cavity; bs – buccal sucker; c – clamp; cvd – common vitelline duct; f – fold; gic – genitointestinal canal; mg – musculo-glandular organs; han – handle; hap – haptor; i – intestine; m – mouth; oe – oesophagus; ot – ootype; ov – ovary; ovd – ovovitelline duct; p – pharynx; s – shaft of hook; t – testis; tnc – transverse nerve commissure; u – uterus; uo – uterus opening; vt – vitelline follicles.

ent host genera were 3.1–4.0% (Table 2). Eight of eleven diplozoid sequences newly obtained from *C. carpio* (Eurasian type) were coincided with those of *Eudiplozoon nipponicum* from *C. carpio* in the Czech Republic and China (AJ300710 and DQ098897, respectively). Among the remaining three samples, 1–4 bases were substituted. The p-distances among diplozoids from *Carassius* species, including *Carassius* sp. and *C. buergeri grandoculis*, were 0–0.5% (Table 2), and the same sequences were shared between diplozoids from *Carassius* sp. and some diplozoids from *C. buergeri grandoculis*.

The ML tree based on COI (346 bp) showed large variation among sequences, with neither samples from the same host species nor from the same locality forming a single clade (Fig. 3). The p-distances of COI sequences between samples of *Eudiplozoon* were relatively large (reaching 16.0%) and there was large variation even between samples from *C. buergeri grandoculis* obtained from the Lake Biwa water system (Table 2). Two of three sequences obtained from diplozoids from *C. buergeri grandoculis* were similar and formed one clade with *E. nipponicum* from *C. carpio* in France. However, the remaining sequence was similar to those of diplozoids from *C. carpio* (Eurasian type) in the Lake Biwa water system, and forming one clade with *Eudiplozoon* sp. from China (Fig. 3).

Morphological comparison between specimens of *Eudiplozoon* from different host species

In all specimens obtained in this study, musculo-glandular organs were present anterior to buccal suckers and prominent folds were present on the hindbody. In addition, an egg filament was attached on the egg surface at the opposite end of the uterus opening among all seven gravid specimens. Therefore, all specimens were identified as belonging to the genus *Eudiplozoon* (Figs. 4, 5).

We focused on the number of folds on the hindbody and the clamp size as key characters for the distinction of diplozoids from *C. carpio* and species of *Carassius*. Diplozoids from *Carassius* sp. have 16–18 folds (median 17; $n = 2$). Diplozoids from *C. buergeri grandoculis* have 14–17 folds (15.5; $n = 8$). Diplozoids from *C. carpio* have 16–25 folds (21; $n = 17$). MPM specimens from species of *Carassius* (including *Carassius* sp., *C. buergeri* subsp. 2 and unidentified fishes) have 12–19 folds (15; $n = 102$), and those from *C. carpio* have 10–23 folds (18; $n = 80$).

Figures 4E and 5E show the clamp morphology of diplozoids from *C. buergeri grandoculis* and from *C. carpio*. Diplozoids from both host genera have clamps with a small projection at the anterior end of median sclerite, followed by a V-shaped sclerite, connecting with anterior clamp jaws. Additional thin projection, a ligament-like

Fig. 6. Box-plot of clamp width of *Eudiplozoon* spp. from different host species/locality. The bold line indicate the median; box indicates upper and lower quartiles; line indicates the maximum and minimum values within 1.5 times of the upper and lower quartiles. Left three box-plots indicating the measurements of *Eudiplozoon* specimens in Meguro Parasitological Museum (MPM) and right three box-plots indicating those of *Eudiplozoon* specimens obtained from Shiga Prefecture in the present study.

structure rather than a sclerite, at posterior end of median sclerite connecting with posterior clamp jaws.

No morphological differences were detected in the anterior joining sclerites of diplozoids from different host genera.

Figure 6 shows the clamp width of diplozoids from *Carassius* sp., *C. buergeri grandoculis* and *C. carpio* (Eurasian type), with the measurements of the specimens in MPM. In the Lake Biwa water system, clamp width ranges of diplozoids from different host genera (*Carassius* or *Cyprinus*) did not overlap for all four clamps. Among MPM specimens, clamp width overlapped between diplozoids from different host genera but, most individuals could be distinguished from worms obtained from the other host genera by the width of clamps II and III. Clamp II widths were < 120 μm in 97% of diplozoids from *Carassius* (*Carassius* sp. and *C. buergeri* subsp. 2), and ≥ 120 μm in 95%

of those from *C. carpio*. Clamp III widths were < 120 μm in 94% of diplozoids from *Carassius*, and ≥ 120 μm in 95% of those from *C. carpio* (Fig. 6). Clamp width is correlated to the host standard length in diplozoids from *C. buergeri grandoculis*, but is not in diplozoids from *C. carpio* (Fig. 7; an example of clamp III). Clamp width little overlapped between diplozoids from *Carassius* and those from *C. carpio* even if their host sizes are almost same.

Morphological descriptions of *Eudiplozoon nipponicum* and *Eudiplozoon kamegaii* sp. n.

Based on these molecular and morphological results, we concluded that specimens of *Eudiplozoon* from species of *Carassius* spp. and from *Cyprinus carpio* (Eurasian type) are separate species. We therefore redescribe *E. nipponicum* and designate its neotype, and describe a new species from *C. carpio*.

Fig. 7. Relationship between host standard length and clamp III width. Based on seven pairs of *Eudiplozoon* from 5 *Cyprinus carpio* (closed circle), six pairs from five *Carassius buergeri grandoculis* (open circle) and two pairs from *Carassius* sp. (open triangle). Linear regression lines are shown for *Eudiplozoon* from *C. carpio* and from *C. buergeri grandoculis*.

Class Monogenea van Beneden, 1858

Family Diplozoidae Palombi, 1949

Genus *Eudiplozoon* Khotenovsky, 1985

Eudiplozoon nipponicum (Goto, 1891) Khotenovsky, 1985

Fig. 4

Syns. and records: *Diplozoon nipponicum* Goto, 1891: Goto (1891), pp. 151–190, pls XXI–XXIII; Yin and Sproston (1948), pp. 82–85, table VI; Reichenbach-Klinke (1961), pp. 541–557, fig. 5e (after Goto 1891), Bychowsky (1962), p. 381 (in part), fig. 844 (species uncertain); Yamaguti (1963), p. 233 (only citation of Goto 1891); Kamegai (1968), pp. 1–8 (in part), figs. 2–5 (oncomiracidia, species uncertain); Osmanov (1971), p. 138 (in part); Kamegai (1976), pp. 1–9 (host record only); Kawatsu (1978), pp. 1315–1319 (host record only); Kimura (1977), pp. 5–14 (in part), figs. 3–5 (species uncertain); Kitahara et al. (1986), pp. 1307–1312 (host record only); Mizuo et al. (1999), pp. 46–50 (host record only); Anonymous (2002), pp. 206–218 (host record only);

Diplozoon sp.: Strelkov (1971), p. 71, No. 134 (host record only);

Sindiplozoon nipponicum (Goto, 1891) Khotenovsky, 1981 (in part): Khotenovsky (1981), pp. 166–178 (in part);

Eudiplozoon nipponicum (Goto, 1891) Khotenovsky, 1985 (in part): Khotenovsky (1985), pp. 214–218 (figs. 152–156 show *E. kamegaii*); Bauer et al. (1985), pp. 379–380 (in part), figs. 603, 604 (after Khotenovsky 1985, showing *E. kamegaii*); Jiang et al. (1989), pp. 259–269 (in part); Nagasawa et al. (1989), p. 9 (in part) (host record only); Jiang (1991), pp. 193–194 (in part) (fig. 197 and pls 1, 2 perhaps show *E. kamegaii*); Ogawa (1994), p. 62 (in part) (host record only); Lü et al. (1996), p. 49 (in part) (host record only); Jiang (2000), pp. 672–674 (in part) (fig. 597A after Jiang 1991, 597B after Chen 1973, both showing *E. kamegaii*), plate II–8, 9 (species uncertain); Molodozh-

nikova and Zhokhov (2006), p. 346 (in part) (host record only); Pugachev et al. (2010), pp. 508–509 (in part), figs. 703–704 (after Khotenovsky 1985, showing *E. kamegaii*); Ogawa (2011), pp. 1463, 1464 (in part) (host record only); *Eudiplozoon* sp.: Sicard et al. (2003), pp. 199–211; Zhang et al. (2018), additional file 8a–d.

Although there are a lot of abstracts on *Diplozoon nipponicum* by Satoru Kamegai, most of them record only host species and include no taxonomical information (see Nagasawa 2016 for these abstracts). There are also many books referring to *D. nipponicum* or *E. nipponicum* without any original data, but we do not list most of these references in this paper.

Neotype (one pair): NSMT-PI 6408 (neotype), National Museum of Nature and Science, Tokyo (Fig. 4A–D)

Host of neotype: *Carassius buergeri grandoculis* (Cyprinidae) (Japanese name nigorobuna).

Site of infection: Gills.

Collection site of neotype: A canal near Lake Biwa in Harie, Shin-asahi Town, Takashima City, Shiga Prefecture, Japan.

Collection date: 27 June 2016.

Reference specimens: (1) NSMT-PI 6409 (host: *C. buergeri grandoculis* collected at Harie on 13 July 2017), NSMT-PI 6410 (host: *C. buergeri grandoculis* collected at Ohta on 9 June 2019), NSMT-PI 6411 (host: *Carassius* sp. collected at Ohta on 9 June 2019), Institute of Parasitology M-725 (host: *C. buergeri grandoculis* collected at Ohta on June 19, 2018), three adult pairs found on *Carassius* sp. collected from the Ohta biotope collected on 4 and 19 June 2018 and 9 June 2019 in M. Urabe's personal collection, and 24 pairs found on *C. buergeri grandoculis* from the Harie and Ohta biotope in May–August 2016, May–July 2017, May–June 2018, and 9 June 2019, at M. Urabe's personal collection. (2) 39 pairs of adults collected from *Carassius* sp. (ginbuna), 69 from *C. buergeri* subsp. 2 and 1 from *Carassius* sp. (species unknown) deposited in the Meguro Parasitological Museum during 1965 to 1980 (see Supplementary Table 2).

Description of neotype. Measurement of paired worms (2 individuals) in micrometres (μm) unless otherwise stated. A pair of individuals fusing together to form X-shaped body (Fig. 4A). Body constructed of two parts: forebody filled with vitelline follicles and hindbody with haptors including four pairs of clamps and one pair of central hooks for host attachment. In holotype, one haptor missing two clamps and both haptors missing one hook. Body 4.94–5.20 mm long. Forebody 2.85–3.19 mm long. Hindbody 0.97–1.06 mm long, with 15–16 folds, terminated as blunt haptor. Posterior third of hindbody except haptor dilated, with 5–6 wide folds (Fig. 4C). Anterior end of median sclerite of clamps having small projection connected to arcuate sclerite. Arcuate sclerite connecting median sclerite with clamp jaws. Anterior and posterior clamp jaws having cross striation on their inner side (Fig. 4E). Clamp I (posterior), 32 long ($n = 1$) and 67–77 wide ($n = 2$); clamp II, 38 long ($n = 1$) and 76 wide ($n = 2$); clamp III, 40–44 long ($n = 2$) and 76–81 wide ($n = 2$); clamp IV (anterior), 44–69 long ($n = 2$) and 77–81 wide ($n = 2$). Central hooks medial to clamps IV, having handle 50 long ($n = 2$) and shaft 21–22 long ($n = 2$) (Fig. 4F).

Mouth opens anteroventral, almost closing, leading to large buccal cavity (Fig. 4B). Buccal suckers 106–127 long and 76–93 wide ($n = 4$). Musculo-glandular organs (“sticky gland” of Goto 1891) anterior to buccal sucker, 37–52 long and 31–40 wide ($n = 4$). Pharynx elliptic, 68–77 long and 31–40 wide. Transverse nerve commissure dorsal to oesophagus. Lateral diverticula of intestine in forebody unclear. Intestine in hindbody tubulate, ending blindly (Fig. 4C). Intestine loop in hindbody described by Goto (1891) unclear.

Testis one, with several shallow-notched lobules, posteromedial to ovary, in base of hindbody, 432–508 long and 159 wide ($n = 2$) (Fig. 4D). Ovary pretesticular, kidney-shape with one elongated projection, 635–752 long and 317–336 wide ($n = 2$). Oviduct arising from nearly distal end of ovary, receiving genitointestinal canal and common vitelline duct forming short ovovitelline duct, posterior to ovary. Distal part of genitointestinal canal not clearly observed. Ovovitelline duct leading to ootype. Ootype large, elliptic, sinistral to ovary.

Uterus running along anterior edge of ovary of another individual. Uterus opening at body fusion part, without any visible structure. Eggs spindle-shaped, with single long filament on opposite side of uterus opening. Egg in uterus 290 long (not including filament) and 89 wide. Egg filament in uterus coiling, unmeasured.

Measurements and supplemental description of reference specimens

Based on 32 specimens obtained in the present study, including neotype. Body 3.11–10.24 mm long ($n = 25$). Forebody 1.34–6.34 mm long ($n = 26$). Hindbody 0.79–3.12 mm long ($n = 26$). Third section of quarters of hindbody broader, with 3–4 thick folds. Based on 32 specimens obtained in the present study, including neotype. Body 3.11–10.24 mm long ($n = 25$). Forebody 1.34–6.34 mm long ($n = 26$). Hindbody 0.79–3.12 mm long ($n = 26$).

Third section of quarters of hindbody broader, with 3–4 thick folds. Clamp I, 43–90 wide ($n = 22$); clamp II 74–101 wide ($n = 21$); clamp III 72–103 wide ($n = 21$); clamp IV, 58–102 wide ($n = 22$). Central hook 40–56 long ($n = 22$), sometimes with wing (Fig. 4F). Musculo-glandular organs 27–52 long and 44–49 wide ($n = 18$). Buccal sucker 72–150 long and 63–147 wide ($n = 36$). Pharynx 20–77 μm long and 19–53 wide ($n = 11$). Intestine in forebody having lateral diverticula. Intestine loop in hindbody described by Goto (1891) unclear. Egg in uterus 250–438 long (not including filament) and 81–219 wide ($n = 7$). Hindbody with 14–18 folds (median 16; $n = 10$).

Remarks. These diplozoids are identified as *E. nipponicum*, according to the original description of this species by Goto (1891). Goto (1891) described the type host of *D. nipponicum* as *Carassius vulgaris*, which is probably *Carassius* sp. (ginbuna) or *C. buergeri* subsp. 2 (kinbuna) (Shimazu et al. 2015, Nagasawa 2016). Therefore, we agree with Shimazu et al. (2015) that the scientific name *E. nipponicum* should be applied to the species from *Carassius* spp., not to the species from *Cyprinus carpio*. The present study reveals *C. buergeri grandoculis* is another host of *E. nipponicum*.

There is no record of the type locality in the original description by Goto (1891). Fujita (1937) remarked the type locality as “Senju, Tokyo”, which is now Adachi Ward, Tokyo. Kamegai et al. (1966) and Kamegai (1968) remarked the type locality as “a pond in the Shimura district, a suburb of Tokyo”, which probably refers to Shimura, Itabashi Ward, Tokyo (Shimazu et al. 2015, Nagasawa 2016). Therefore, it is likely that Goto (1891) collected type host from a pond in northern Tokyo, although the exact location remains unknown. We attempted to collect *Carassius* fish from some localities near Tokyo, but we could obtain only 10 fish, which harboured no diplozoids, from one locality (Ichinomiya). Goto (1891) also reported that *D. nipponicum* was found in various places in Japan.

Geographical distribution: Japan (Hokkaido, Honshu), China, Russia (Amur and Volga regions)

Hosts described in the literature: *Carassius vulgaris* (now ginbuna, *Carassius* sp. or kinbuna *C. buergeri* subsp. 2 – Goto 1891, Shimazu et al. 2015, Nagasawa 2016): goldfish *Carassius carassius* (misidentification of *C. auratus*?) (Yin and Sproston, 1948), *C. cuvieri* (?) (no morphological description) (Kamegai 1977); *Carassius auratus* (ginbuna, now *Carassius* sp.) (Sicard et al. 2003, Shimazu et al. 2015)

Eudiplozoon kamegaii sp. n.

Fig. 5

ZooBank number for species:

[url:https://zoobank.org/act:35BC1E5F-A9EA-4382-9B84-124FD997F98A](https://zoobank.org/act:35BC1E5F-A9EA-4382-9B84-124FD997F98A)

Syns. and records: *Diplozoon nipponicum* Goto, 1891: Bychowsky (1962), p. 391 (in part), fig. 844 (species uncertain); Kamegai et al. (1965), pp. 607, 608; Kamegai et al. (1966), pp. 2–9, figs. 1–6; Kamegai (1968), pp. 1–8 (in part), figs. 2–5 (oncomiracidia, species uncertain); Kovalev and Pashkevishute (1969), pp. 232, 233 (host record)

only); Kamegai (1970a), pp. 21–25, figs. 1, 2, 4; Kamegai (1970b), p. 20, fig. (not numbered); Osmanov (1971), p. 138 (in part); Kamegai (1973), p. 98; Chen (1973), p. 154, plate XCIV, figs. 234–238; Kamegai (1976), pp. 1–9 (host record only); Kimura (1977), pp. 5–14 (in part), figs. 3–5 (species uncertain); Lambert and Denis (1982), pp. 533–542, figs. 1–3 (oncomiracidia); Denis et al. (1983), pp. 128–134 (host record only); Le Brun et al. (1985), pp. 105–109, photos 1A,B; Rhee (1985), pp. 331–333, figs. 1–4; Kamegai (1986), p. 35 (host record only); Hirose et al. (1987), Figs. 1 and 4 (oncomiracidium); Izyumova (1987), p. 137, 206 (host record only); Le Brun et al. (1988), pp. 395–400; Samman (1989), pp. 45–50 (host record only); Jovelín and Justine (2001), pp. 393–401; Al-Nasiri (2003), pp. 95–99, fig. 1;

Diplozoon sp.: Strelkov (1971), p. 71, No. 135 (host record only);

Sindiplozoon nipponicum (Goto, 1891) Khotenovsky, 1981 (in part); Khotenovsky (1981), pp. 166–178;

Eudiplozoon nipponicum (Goto, 1891) Khotenovsky, 1985 (in part); Kornakova (1983), pp. 102–105, figs. 7–10 (*nomen nudum*, without a description or definition that are purported to differentiate the taxon ICZN: 13.1.1); Khotenovsky (1985), pp. 214–218, figs. 152–156; Bauer et al. (1985), pp. 379–380 (in part), figs. 603, 604 (after Khotenovsky 1985); Jiang et al. (1989), pp. 259–269 (in part); Nagasawa et al. (1989), p. 9 (in part) (host record only); Svobodová et al. (1990), pp. 3–13 (host record only); Jiang (1991), pp. 193, 194 (in part) (fig. 197 and pls 1,2 perhaps show *E. kamegaii*); Nedeva (1991), pp. 35–37, fig. 6; Pojmańska and Chabros (1993), pp. 101–108 (host record only); Lü et al. (1996), p. 49 (in part) (host record only); Ogawa (1994), p. 62 (in part) (host record only); Jažić (1997), pp. 82, 83, fig. 1 (after Khotenovsky 1985); Székely and Molnár (1998), pp. 45–54 (host record only); Jiang (2000), pp. 672–674 (in part), fig. 597A (after Jiang 1991), 597B (after Chen 1973), plate II–8, 9; Littlewood and Olson (2001), pp. 262–278 (18S sequence, accession no. AJ287510); Matejusová et al. (2001), pp. 465–474, fig. 1; Sicard et al. (2001), pp. 709–717; Zurawski et al. (2001), pp. 783–792, figs. 1–4; Olson and Littlewood (2002), pp. 233–244; Sicard et al. (2002), pp. 85–87; Cojocar (2003), pp. 73–74 (host record only); Sicard et al. (2003), pp. 199–211; Zurawski et al. (2003a), pp. 198–200, figs 1,2; Zurawski et al. (2003b), pp. 349–357, figs. 1–4 (diporae); Matejusová et al. (2004), pp. 817–822, figs. 2H; Oganessyan (2004), pp. 88–93 (host record only); Schabussova et al. (2004), pp. 63–68, fig. 1; Gao et al. (2007), pp. 695–703; Molodozhnikova and Zhokhov (2006), p. 346 (in part) (host record only); Stojanovski et al. (2008), pp. 389–398, figs. 14–15; Oganessyan (2009), pp. 32–37, fig. 1 (copy from Khotenovsky 1985, showing *E. kamegaii*); Pugachev et al. (2010), pp. 508–509 (in part), figs. 703, 704 (copy from Khotenovsky 1985, showing *E. kamegaii*); Hodová and Matejusová (2010), pp. 702–709, figs. 1–5; Košková et al. (2010), pp. 909–914, fig. 1; Hodová et al. (2011), pp. 2–4, figs. 1–5 (after Hodová and Matejusová 2010 and Valigurová et al. 2011); Ogawa (2011), pp. 1463–1464 (in part) (host record only); Valigurová et al. (2011), pp. 383–

394, figs. 1–6; Rohlenová et al. (2011), p. 120 (host record only); Ondračková et al. (2012), pp. 1487–1493 (host record only); Zhang et al. (2012), pp. 576–580 (host record only); Šimková et al. (2013), Tavakol et al. (2013), p. 92 (host record only); Mhaisen and Abdul-Ameer (2014), p. 98, fig. (2)–2 (after Khotenovsky 1985); Mhaisen et al. (2015), p. 54 (host record only); Konstanzová et al. (2016), pp. 1493–1500, figs. 2A, B, 3a–d; Šmiga et al. (2016), pp. 169–172 (host record only); Jedličková et al. (2016), pp. 494–506; Mhaisen and Al-Rubae (2016), p. 8 (host record only); Ilgová et al. (2017), pp. 1–12 (host record only); Konstanzová (2017) p. 30 (host record only); Jedličková et al. (2018), pp. 1–17; Mhaisen et al. (2018), pp. 195–196 (host record only); Roudnický et al. (2018), pp. 1–13; Caña-Bozada et al. (2019), pp. 1–11; Fedorovych and Guttyj (2019), pp. 146–151 (host record only); Ilgová et al. (2019), pp. 1–4 (host record only); Jedličková et al. (2019), pp. 337–346, fig. 8; Mhaisen (2019), p. 159 (host record only); Chmúřčáková et al. (2020), pp. 1–8 (host record only).

Type host: *Cyprinus carpio* Linnaeus (Cyprinidae) (Eurasian type; Japanese name koi)

Site of Infection: Gills.

Type locality: Shinhami (near Lake Biwa), Kusatsu City, Shiga Prefecture, Japan.

Date: 10 November 2017.

Holotype (one pair): NSMT-PI 6405, National Museum of Nature and Science, Tokyo

Paratypes (three pairs): NSMT-pl 6406 (collected at Hachiyado on February 25, 2017), NSMT-pl 6407 (collected at Shinhami on September 25, 2018), Institute of Parasitology M-726 (collected at Shinhami on November 10, 2017)

Reference specimens: Six pairs of adults collected at Shinhami and Hachiyado during 2017–2018 in M. Urabe's personal collection; 76 pairs of adults in the Meguro Parasitological Museum (see Supplementary Table 2).

Description of holotype. A pair of individuals fusing together to form an X-shaped body (Fig. 5). Body consisting of forebody filled with vitelline follicles and hindbody with haptors including four pairs of clamps and one pair of central hooks, but only one hook remaining in holotype. Body 6.86–7.51 mm long. Forebody 3.59–3.97 mm long. Hindbody 2.23–2.47 mm long, having 23–25 folds. Third section of quarters of hindbody dilating, with 4–5 thick folds. Hapter occupying posterior quarters of hindbody, having 4 pairs of clamps (Fig. 5C). Haptor missing 3 of 4 central hooks. Anterior end of median sclerite of clamps having small projection connected to arcuate sclerite (Fig. 5E). Arcuate sclerite connecting median sclerite with clamp jaws. Weak cross striation present both on posterior clamp jaw, lateral sclerite of posterior jaw, and upper part of anterior clamp jaw. Clamp I, 69–79 long and 120–130 wide (n = 4); clamp II, 83–100 long and 148–161 wide (n = 4); clamp III, 86–96 long and 149–171 wide (n = 4); clamp IV, 90–101 long (n = 3) and 146–159 wide (n = 4). Central hook submedian, medial to clamp II, having handle 38 long and shaft 22 long (n = 1).

Mouth anterovental, leading to large buccal cavity (Fig. 5B). Buccal sucker paired, elliptic, 161–180 long and 128–146 wide ($n = 4$). Musculo-glandular organs anterior to oral sucker, subterminal, paired, globular, smaller than buccal sucker and pharynx, 57.9–68.9 long and 49–58 wide ($n = 4$). Pharynx elliptic, 88–89 long and 56–67 wide ($n = 2$). Transverse nerve commissure dorsal to oesophagus. Lateral diverticula of intestine in forebody unclear. Intestine in hindbody with weak lateral diverticula, ending blindly anterior to clamps (Fig. 5C).

Testis one, with several shallow-notched lobules, in second section of quarters of hindbody, 444 long and 254–368 wide ($n=2$) (Fig. 5D). Ovary pretesticular, anterior margin obvious, 527–603 long and 317 wide ($n = 2$). Oviduct arising from distal or proximal end of ovary, receiving genitointestinal canal. Distal part of genitointestinal canal not clearly observed. Oviduct receiving common vitelline duct near ootype, forming short ovovitelline duct. Ootype large, elliptic, sinistral to ovary. Uterus running forward gonads of another individual. Uterus opening at body fusion part, without any visible structure. No eggs observed in uterus.

Measurements and supplemental description of paratypes and reference specimens

Based on 10 pairs (including holotype) obtained in this study. Body 3.79–7.53 mm long ($n = 20$). Forebody 2.13–3.97 mm long ($n = 20$). Hindbody 1.12–2.50 mm long ($n = 20$), with 16–25 folds (median 21; $n=17$). Clamp I distal, 89–128 wide ($n = 18$); clamp II 103–170 wide ($n = 19$); clamp III 108–174 wide ($n = 18$); clamp IV proximal, 103–160 wide ($n = 18$). Central hook 38–53 long ($n = 24$). Musculo-glandular organs 24–69 long and 27–61 wide ($n = 22$). Buccal sucker 87–180 long and 74–146 wide ($n = 34$). Pharynx globular or oval, 62–106 long and 42–87 wide ($n = 18$). Intestine in hindbody tubulate with small projections. Ovary and testis in hindbody, sometimes with ambiguous outline. No eggs found in our specimens.

Etymology: This new species is named in honour of Satoru Kamegai (1909–2002), founder of the Meguro Parasitological Museum, Tokyo, Japan.

Geographical distribution: China, Japan (Hokkaido, Honshu), Russia (native Amur region?), South Korea.

Introduced countries: Armenia, Belarus, Bosnia-Herzegovina, Bulgaria (?), Czech Republic, France, Hungary, Iran (?), Iraq (?), Kazakhstan, Macedonia (?), Moldova, Poland, Romania (?), Slovakia (?), Ukraine.

Hosts described in the literature: *Cyprinus carpio* sensu lato (Kamegai 1973, Le Brun et al. 1985, Matejusková et al. 2001, 2004, Sicard et al. 2001, 2002, Gao et al. 2007, Hodová and Matejusková 2010, Pugachev et al. 2010 – in part, Wan and Yue 2015).

Remarks. Clamp width in this species was generally larger than that of *Eudiplozoon nipponicum* sensu stricto in all clamps (Fig. 6). The hindbody often has more than 19 folds in this species, whereas the hindbody of *E. nipponicum* never exceeds 19 folds. Although their ranges partly overlap, generally, these two species can be morphologically distinguished by these characteristics.

Genetic variation in the ITS-2 region of this species is small in relation to its wide geographic distribution (Table 2). Among the 14 individuals analysed in this study, 8 had identical sequences to those of *E. nipponicum* from *C. carpio* obtained in the Czech Republic (AJ300710) and China (DQ098897). The distribution and phylogeny of wild carp remain controversial because they have been transplanted by humans for many years (Kohlmann and Kersten 2013, Dong et al. 2015, Xu et al. 2019). It is very plausible to assume that *E. kamegaii* has also spread rapidly with its hosts, as suggested by Khotenovsky (1981). In Europe, *E. kamegaii* (reported as *E. nipponicum*) is considered to be an introduced species (Lambert and Denis 1982, Jedličková et al. 2018).

There are many records of *E. nipponicum* in *C. carpio* from Japan and many Eurasian countries. These can be identified as *E. kamegaii* when their descriptions include DNA information, clamp measurements, or number of folds on hindbody (Kamegai et al. 1965, 1966, Osmanov 1971, Chen 1973, Kamegai 1973, Le Brun et al. 1985, Rhee 1985, Nedeva 1991, Jovelín and Justine 2001, Matejusková et al. 2001, 2004, Sicard et al. 2001, 2002, 2003, Olson and Littlewood 2002, Gao et al. 2007, Hodová and Matejusková 2010, Pugachev et al. 2010, Valigurová et al. 2011, Wan and Yue 2015). There are many papers that reported *E. nipponicum* from *C. carpio* but provided neither sufficient morphological description nor genetic analysis (e.g., Kamegai 1968, 1970a,b, Lambert and Denis 1982, Denis et al. 1983, Hirose et al. 1986, Lambert and Le Brun 1988, Le Brun et al. 1988, Zurawski et al. 2001, 2003a,b, Al-Nasiri 2003, Stojanovski et al. 2008, Konstanžová et al. 2016, Jedličková et al. 2016). It is probably that these papers also reported *E. kamegaii*, but further confirmation is needed.

DISCUSSION

Specimens of *Eudiplozoon* from species of *Carassius* and *Cyprinus carpio* collected from the same water system were classified into different clusters in the phylogenetic tree based on the ITS-2 region (Figs. 2 and 3). The substitution rates of bases between different host genera were 3.1–4.0% among ITS-2 sequences, significantly higher than those within the clusters, and comparable to those between monogenean species (6.9–24.5%) reported in a previous study (Matejusková et al. 2001).

There was large variation among COI haplotypes in *Eudiplozoon* spp.; neither individuals from the same host genus nor those from the same locality formed a single clade. Incongruence is frequently observed between phylogenetic trees calculated based on mitochondrial or nuclear DNA in invertebrates, including monogeneans, for several reasons including hybridisation (e.g., Ziętara et al. 2010, Lumme et al. 2017). Further data are clearly needed for COI phylogenetic tree of *Eudiplozoon* spp.

There are no significant differences in clamp sclerite morphology, which is a key characteristic often used to distinguish diplozoid species (Matejusková et al. 2001, Pugachev et al. 2010). We could not describe the exact morphology of intestine branching, which is also a key characteristic in diplozoids (Pugachev et al. 2010). It was

hard to observe this characteristic in our specimens, so further study is needed using some newly collected, living samples. However, they were generally distinguishable using clamp width or the number of folds on the hindbody (Fig. 6). Clamp width correlated with host standard length in specimens of *Eudiplozoon* from *Carassius buergeri grandoculis*. In specimens from *C. carpio*, clamp width did not correlate with host standard length, but the plots in Fig. 7 suggest that it has a positive correlation with host standard length in the cases of small hosts. Anyway, when one compares individuals from *Carassius* and those from *C. carpio* with almost same size, their clamp width was clearly different from each other, as shown in Fig. 7. These morphological differences support the view that these specimens from different host genera belong to different species. We therefore concluded that *Eudiplozoon* from species of *Carassius* and those from *C. carpio* belong to different species.

Eudiplozoon nipponicum from species of *Carassius* has been recorded only from Japan (Goto 1891, Kamegai 1968, 1974a,b, 1975a,b, 1976, 1977, Kawatsu 1978, Ichihara et al. 1980, Nagasawa et al. 1989, Mizuo et al. 1999, Anonymous 2002, Sicard et al. 2003), China (Yin and Sproston 1948, Jiang 1991, Zhang et al. 2018), and Russia (Amur River) (Bychowsky 1962, Strelkov 1971), not considering a questionable record from Uzbekistan, which lists only common host names in Russian (Osmanov 1971). All records of *E. nipponicum* from Europe are from *C. carpio*.

Therefore, it is likely that *E. nipponicum* sensu stricto is distributed only in East Asia, although it should be taxonomically reinvestigated, especially based on nuclear DNA sequence. There are also many studies on *E. nipponicum* from Japan and Eurasian countries, which regarded diplozooids from *C. carpio* and species of *Carassius* spp.

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- as a single species, often without supporting morphological or host information (e.g., Kamegai 1968, 1976, Kimura 1977, Nagasawa et al. 1989, Ogawa 1994, Šimková et al. 2013, Nagasawa 2016, Jedličková et al. 2018). Further taxonomical study is needed to clarify these specimens.
- There are some records of *Eudiplozoon* spp. from fishes which are not investigated in this study as mentioned in the Introduction (*Carassius auratus gibelio*, *Cyprinus carpio rubrofasciatus*, *Abramis brama*, *Capoeta capoeta heratensis*, *Luciobarbus capito conocephalus*, *Leuciscus vorax*, *Mesopotamichthys sharpeyi*, *Channa argus*). None of them are described morphologically nor studied for genetic information, and we cannot estimate whether they are *E. nipponicum*, *Eudiplozoon kamegaii*, or unknown species.
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