Bull. Natl. Mus. Nat. Sci., Ser. A, 41(3), pp. 137–154, August 21, 2015

Paradiplozoon skrjabini (Monogenea, Diplozoidae), an Ectoparasite on the Gills of Freshwater Fishes (Cyprinidae, Leuciscinae) of Japan and Primorsky Region, Russia: a Morphological and Molecular Study

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(Received 8 May 2015; accepted 24 June 2015)

Abstract Monogenean specimens of *Paradiplozoon* Akhmerov, 1974 (Diplozoidae) were found on the gills of *Tribolodon hakonensis* (Günther, 1877), *Tribolodon sachalinensis* (Nikolskii, 1889), *Phoxinus steindachneri* Sauvage, 1883 and *Phoxinus oxycephalus* (Sauvage and Dabry de Thiersant, 1874) (Cyprinidae, Leuciscinae) from Japan; and *Leuciscus waleckii* (Dybowski, 1869) and *Rhynchocypris lagowskii* (Dybowski, 1869) (Leuciscinae) from Primorsky Region, Russia. The second internal transcribed spacer (ITS2) region of the ribosomal DNA (rDNA) was sequenced for many of them. The ITS2 (624 bp) and 5.8S-ITS2-28S (720 bp) sequences obtained were phylogenetically compared with some previously published ITS2 sequences of diplozoids by the neighbor joining (NJ), maximum likelihood (ML) and maximum persimony (MP) methods. All the present specimens are identified as *Paradiplozoon skrjabini* Akhmerov, 1974 from the present morphological and molecular studies. Some institutional specimens from Hokkaido are also identified as *P. skrjabini*, and *Tribolodon brandtii* (Dybowski, 1872) from Hokkaido is added as the host. *Paradiplozoon skrjabini* unusually has low host specificity (on seven species of four genera of leuciscine fishes) and a wide range of geographical distribution (in eastern Eurasia: the Amur River system of Russia and China; Primorsky Region and Sakhalin Island, Russia; and Hokkaido and Honshu, Japan). In adult morphology and molecular phylogeny, *P. skrjabini* in eastern Eurasia is most closely related to *Paradiplozoon homoion homoion* (Bychowsky and Nagibina, 1959) in western Eurasia. Adult morphology is described. Molecular data are given.

Key words: Monogenea, Diplozoidae, *Paradiplozoon skrjabini*, morphology, ITS2 sequences, leuciscine cyprinids, Japan, Russia.

Introduction

Monogeneans of the Diplozoidae Palombi, 1949 are ectoparasitic on the gills of mainly freshwater cyprinid fishes in Europe, Asia and Africa (Khotenovsky, 1985a, b; Jiang, 2000; Pugachev *et al.*, 2010). Two adult individuals are found fused together in pairs in permanent copula on the host gills (*e.g.*, Goto, 1891; Khotenovsky, 1985a), and this body fusion is unique compared with the other monogenean families.

In Japan, it has been well known that *Diplozoon nipponicum* Goto, 1891 (now in *Eudiplozoon* Khotenovsky, 1985) is a parasite on the

gills of *Carassius carassius* [most likely referring to *Carassius auratus langsdorfii* Temminck and Schlegel, 1846 at that time, Japanese name: Gin-buna] and *Cyprinus carpio* Linnaeus, 1758 (Cyprinidae, Cyprininae) (*e.g.*, Kamegai, 1968; Ogawa, 1994) since Goto (1891) described this species from *Carassius vulgaris* [*sic*, most likely referring to Gin-buna] in Japan (locality not specified). Sicard *et al.* (2003) used a sample of *Eudiplozoon* sp. found on the gills of *C. auratus* [Gin-buna] from the Chikuma River [at Chikaraishi, Kamiyamada Town then, Nagano Prefecture, on 15 September 2000], Japan for their molecular study of diplozoids. We consider that this *Eudiplozoon* sp. refers to *E. nipponicum* (Goto, 1891).

One (T. S.) of us collected a large number of specimens of *Paradiplozoon* Achmerov, 1974 from leuciscine cyprinids in various places in Hokkaido and Honshu, Japan, from 1984 to 2000. Sicard *et al.* (2003) used only a small part of them as *Paradiplozoon* sp. for their molecular study, but the rest of them were lost.

We collected some specimens of *Paradiplozoon* once more from *Tribolodon hakonensis* (Günther, 1877), *Tribolodon sachalinensis* (Nikolskii, 1889), *Phoxinus steindachneri* Sauvage, 1883 and *Phoxinus oxycephalus* (Sauvage and Dabry de Thiersant, 1874) (Cyprinidae, Leuciscinae) in Japan for the present study. Since Akhmerov (1974) found *Diplozoon* (*Paradiplozoon*) *skrjabini* Akhmerov, 1974 (now in *Paradiplozoon*) on the gills of *Leuciscus waleckii* (Dybowski, 1869) (Leuciscinae) and *Diplozoon* (*Paradiplozoon*) sp. 3 of Akhmerov, 1974 on the gills of *Phoxinus lagowskii* Dybowski, 1869 (now in *Rhynchocypris*) (Leuciscinae) both from the Amur River basin, Russia, we also used some specimens found on the gills of *L. waleckii* and *R. lagowskii* from Primorsky Region, Russia, for the present study. The second internal transcribed spacer (ITS2) region of the ribosomal DNA (rDNA) was sequenced for many of the present specimens.

In the present paper, we describe adult morphology and molecular data, identify all the present specimens from Japan and Russia as *P. skrjabini*, and briefly discuss morphological and molecular phylogenetic relationships between *P. skrjabini* in eastern Eurasia and *Paradiplozoon homoion* (Bychowsky and Nagibina, 1959) in western Eurasia.

Specimens and Methods

Morphological study

Material examined. (1) 9 specimens (pairs) (NSMT-Pl 5916), adult, fixed in 70% ethanol *in situ* in fish, whole-mounted, on gills of *Tribolodon sachalinensis*, Lake Abashiri, Abashiri City, Hokkaido, Japan, 29 August 2013 (10 ethanol-fixed specimens 10670–10672 and 10674–10680 sequenced, Table 1). (2) 4 (NSMT-Pl 5917), adult, flattened, whole-mounted, on gills of *T. hakonensis*, Kotoni-hassamu River, Nishi-ku, Sapporo City, Hokkaido, Japan, 7 April 2011. (3) 2 (NSMT-Pl 5918), adult, slightly flattened, whole-mounted, on gills of *T. hakonensis*, Tama River, Fuchu City, Tokyo, Honshu, Japan, 13 August 2011. (4) 7 (MPM Coll. No. 20971), adult, slightly flattened, whole-mounted, on gills of *T. hakonensis*, Ueda City, Nagano Prefecture, Honshu, Japan, 30 October 2013 (2 ethanol-fixed specimens 12748–12749 sequenced, Table 1). (5) 16 (NSMT-Pl 5919), adult, strongly flattened, whole-mounted, on gills of *T. hakonensis*, Metoba River, Chuo-oote, Matsumoto City, Nagano Prefecture, Japan, 13 August 2011. (6) 96 (60, NSMT-Pl 5920; 36, MPM Coll. Nos. 20972 and 21104), adult, heat-killed, slightly flattened, whole-mounted, serially-sectioned, on gills of *T. hakonensis*, Sai River, Akashina-Nakagawate, Azumino City, Nagano Prefecture, Japan, 31 July 2012, 10 January 2013, 3 June 2013 (4 ethanol-fixed specimens 10556–10559 sequenced, Table 1). (7) 2 (MPM Coll. No. 21105), adult, slightly flattened, whole-mounted, on gills of *T. hakonensis*, Lake Kizaki, Oomachi City, Nagano Prefecture, Japan, 2 December 2011. (8) 2 (MPM Coll. No. 21106), adult, slightly flattened, whole-mounted, on gills of *T. hakonensis*, Tenryu River, Ina City, Nagano

Prefecture, Japan, 7 May 2014 (2 ethanol-fixed specimens 12746–12747 sequenced, Table 1). (9) 3 (MPM Coll. No. 21107), adult, fixed in 10% formalin *in situ* in fish, whole-mounted, on gills of *T. hakonensis*, Oota River, Higashino, Asaminami-ku, Hiroshima City, Hiroshima Prefecture, Honshu, Japan, 11 October 2014. (10) 3 (MPM Coll. No. 21108), adult, fixed in 10% formalin *in situ* in fish, whole-mounted, on gills of *T. hakonensis*, Takiyama River, Inoshiyama, Akioota Town, Hiroshima Prefecture, Japan, 17 October 2014. (11) 56 (MPM Coll. Nos. 20973–20974 and 21109–21113), adult, heat-killed, slightly flattened, whole-mounted, serially-sectioned, on gills of *Phoxinus steindachneri*, Metoba River, Asahi, Matsumoto City, Nagano Prefecture, Japan, 29 June 2011, 25 and 30 October 2012, 1 May 2013, 12 November 2013 (4 ethanol-fixed specimens 10560–10563 sequenced, Table 1). (12) 4 (MPM Coll. No. 21114), adult, heat-killed, on gills of *Phoxinus oxycephalus*, Takami River, Kotsugawa, Higashiyoshino Village, Nara Prefecture, Honshu, Japan, 6 August 2013 (4 ethanol-fixed specimens 10665–10668 sequenced, Table 1). (13) 16 (NSMT-Pl 5914), adult, fixed in 95% ethanol without flattening, whole-mounted, on gills of *Leuciscus waleckii*, Bolshaya Ussurka River (45°57′N, 133°53′E), Primorsky Region, Russia, 2 June 2014 (4 ethanol-fixed specimens 12738–12741 sequenced, Table 1). (14) 7 (NSMT-Pl 5915), adult, fixed in 95% ethanol without flattening, whole-mounted, on gills of *Rhynchocypris lagowskii*, Narva River (43°03′N, 131°22′E), Primorsky Region, 30 August 2014 (4 ethanol-fixed specimens 12742–12745 sequenced, Table 1).

Methods. Adult worms were made into permanent preparations mounted in Canada balsam by several methods: (1) killed in hot 0.6% saline, fixed with 10% neutral buffered formalin, stained with Ehrlich's hematoxylin and mounted; (2) slightly flattened, fixed with either 10% neutral buffered formalin or AFA, stained with either Ehrlich's hematoxylin or Heidenhain's iron hematoxylin or Grenacher's alum carmine and mounted; (3) fixed in 95% ethanol without flattening, stained with Grenacher's alum carmine and mounted; (4) fixed and stained with a mixture of saturated solution of ammonium picrate and glycerin (1 : 1) (Malmberg, 1957) and mounted; (6) obtained from the gills of host fish fixed in either 70% ethanol or 10% formalin, stained with Grenacher's alum carmine and mounted; or (7) fixed in hot 10% neutral buffered formalin, serially sectioned (10 μm thick) and stained with hematoxylin and eosin.

The body and some external and internal organs were measured on whole-mounted specimens. Besides eggs in whole-mounted specimens, laid eggs fixed in 10% formalin were also measured and figured. The terminology of the sclerites of the cramps and central hooks followed that of Pugachev *et al.* (2010). Measurements (length by width) are given in micrometers unless otherwise stated. Drawings were made with the aid of a camera lucida. Representatives of the specimens studied have been deposited in the National Museum of Nature and Science, Tsukuba, Japan (collection name code NSMT-Pl); and in the Meguro Parasitological Museum, Tokyo, Japan (collection name code MPM Coll. No.).

Molecular study

Specimens sequenced. Table 1 lists specimens used for sequencing the ITS2 region in the present study, with their host species, localities, dates, genotypes and GenBank accession numbers of their sequences. Most of them were fixed in 95% ethanol immediately after collection, and some were obtained from fish fixed in 70% ethanol (see *Material examined*).

DNA analyses. Total genomic DNA was extracted from ethanol-preserved tissues of the anterior part of the body of the present specimens and purified using the DNeasy Blood & Tissue Kit (QIAGEN, Hilden), according to the manufacturers' instructions. Total DNA was used to amplify DNA fragments (complete sequence of the ITS2 region contained partial sequences of the 5.8S and 28S regions) by polymerase chain reaction (PCR) with sets of universal D primer

Table 1. Specimens of *Paradiplozoon skrjabini* used for sequencing the ITS2 region, with their host species, sampling localities, dates, genotypes and GenBank accession numbers.

Specimen	n*	Host species	Sampling locality and date	Genotype	GenBank accession number
12748–12749	2	*Tribolodon hakonensis*	Ueda City, Nagano Prefecture, Honshu, Japan, 30 October 2013	Ps-G1	LC050521
10556–10559	4	*Tribolodon hakonensis*	Sai River, Akashina-Nakagawate, Azumino City, Nagano Prefecture, Honshu, Japan, 10 January 2013	Ps-G1	LC050522
12746–12747	2	*Tribolodon hakonensis*	Tenryu River, Ina City, Nagano Prefecture, Honshu, Japan, 7 May 2014	Ps-G1	LC050523
10560–10563	4	*Phoxinus steindachneri*	Metoba River, Asahi, Matsumoto City, Nagano Prefecture, Honshu, Japan, 1 May 2013	Ps-G1	LC050524
10665–10668	4	*Phoxinus oxycephalus*	Takami River, Kotsugawa, Higashiyoshino Village, Nara Prefecture, Honshu, Japan, 6 August 2013	Ps-G1	LC050525
10670–10672 10674–10680	10	*Tribolodon sachalinensis*	Lake Abashiri, Abashiri City, Hokkaido, Japan, 29 August 2013	Ps-G2	LC050526
10669	1	*Tribolodon hakonensis*	Lake Abashiri, Abashiri City, Hokkaido, Japan, 29 August 2013	Ps-G2	LC050527
12738–12741	4	*Leuciscus waleckii*	Bolshaya Ussurka River, Primorsky Region, Russia, 2 June 2014	Ps-G3	LC050528
12742–12745	4	*Rhynchocypris lagowskii*	Narva River, Primorsky Region, Russia, 30 August 2014	Ps-G4	LC050529

* Number of the specimens sequenced.

and the B1 primer (Bachellerie and Qu, 1993). Typically, 1.0 μl of templete was used in a 20.0 μl scaled PCR using the following conditions: 95℃ for 10 min; 30 cycles of 95℃ for 30 sec, 55℃ for 30 sec, 72℃ for 75 sec; 72℃ for 5 min, followed by storage at 4℃. PCR products were purified using the ExoSAP-IT or illustra ExoProStar (GE Healthcare, Buckinghamshire). Purified DNA fragments were sequenced directly by an automated method using the BigDye Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems, California) on an automated DNA Sequencer (ABI 3130 or 3130xl DNA Analyzer; Perkin Elmer/Applied Biosystems, California). In the sequencing reaction by the BigDye Terminator, the same primer set used at the time of the PCR amplification of the fragments of the ITS2 region. Typically, 1.0 μl of templete was used in a 10.0 μl scaled PCR using the following conditions: 96℃ for 30 sec; 25 cycles of 96℃ for 30 sec, 50℃ for 5 sec, 60℃ for 4 min; followed by storage at 4℃.

All the sequence data have been submitted to the DNA data bank of Japan (DDBJ database), and the accession numbers are given in Table 1. Sequence alignment and editing were performed for each gene separately using MEGA 5.02 (Tamura *et al.*, 2011), CLUSTAL W (Thompson *et al.*, 1994) and CLC Workbench software (CLC bio, Aarhus).

Phylogenetic analyses. Phylogenetic analyses were performed with some previously known ITS2 sequences of diplozoids (Table 2) by the neighbor joining (NJ) method (Saitou and Nei, 1987), maximum likelihood (ML) method (Felsenstein, 1981) using MEGA 5.02 (Tamura *et al.*, 2011) with 1,000 bootstrap replications, and maximum persimony (MP) method (Fitch, 1971). *Eudiplozoon nipponicum* (AJ300710) and *Inustiatus inustiatus* (DQ098893) were used as the outgroup.

Abbreviations used in the figures. aasa, anterior additional sclerite of anterior end of median sclerite; aasp, anterior additional sclerite of posterior end of median sclerite; acj, anterior clamp jaw; apb, anterior part of body; asa, additional sclerite of anterior clamp jaw; bc, buccal cavity;

Table 2. List of diplozoid species used for genetic comparison with *Paradiplozoon skrjabini* in the ITS2 sequences, with their host species, localities, GenBank accession numbers and sequence authors.

Parasite species	Host species	Locality	GenBank accession number	Author
Paradiplozoon bingolense Civáňová *et al.*, 2013*	*Garra rufa* Heckel, 1843	Göynük Stream (Murat River system), Turkey	HE653910	Civáňová *et al.* (2013)
P. bliccae (Reichenbach-Klinke, 1961)	*Blicca bjoerkna* (Linnaeus, 1758)	Morava River, Czech Republic	AJ300712	Matejusová *et al.* (2001)
P. homoion (Bychowsky and Nagibina, 1959)	*Phoxinus phoxinus* (Linnaeus, 1758) *Rutilus rutilus* (Linnaeus, 1758)	Morava River, Czech Republic	AJ300715	Matejusová *et al.* (2001)
P. ichthyoxanthon Avenant-Oldewage in Avenant-Oldewage *et al.*, 2014*	*Labeobarbus aeneus* (Burchell, 1822)	Vaal River, South Africa	HF566124	Avenant-Oldewage *et al.* (2014)
P. megan (Bychowsky and Nagibina, 1959)	*Leuciscus cephalus* (Linnaeus, 1758)	Morava River, Czech Republic	AJ300711	Matejusová *et al.* (2001)
P. nagibinae (Gläser, 1965)	*Abramis ballerus* (Linnaeus, 1758)	Kyjovka River, Czech Republic	AJ563371	Matejusová *et al.* (2004)
P. pavlovskii (Bychowsky and Nagibina, 1959)	*Aspius aspius* (Linnaeus, 1758)	Morava River, Czech Republic	AJ300714	Matejusová *et al.* (2001)
P. sapae (Reichenbach-Klinke, 1961)	*Abramis sapa* (Pallas, 1814)	Morava River, Czech Republic	AJ300713	Mattjusová *et al.* (2001)
P. vaalense Dos Santos *et al.*, 2015*	*Labeo umbratus* (Smith, 1841)	Vaal River system, South Africa	HG423142	Dos Santos *et al.* (2015)
Diplozoon paradoxum Nordmann, 1832	*Abramis brama* (Linnaeus, 1758)	Kyjovka River, Czech Republic	AJ563372	Matejusová *et al.* (2004)
Eudiplozoon nipponicum (Goto, 1891)	*Cyprinus carpio* Linnaeus, 1758	Morava River, Czech Republic	AJ300710	Matejusová *et al.* (2001)
Inustiatus inustiatus (Nagibina, 1965)	*Hypophthalmichthys molitrix* (Valenciennes, 1844)	Lake Tangxun, China	DQ098893	Gao *et al.* (2007)
Sindiplozoon ctenopharyngodoni (Ling, 1973)	*Ctenopharyngodon idella* (Valenciennes, 1844)	Lake Tangxun, China	DQ098898	Gao *et al.* (2007)

* See the Discussion of the present paper.

bfp, body fusion part; bs, buccal sucker; c, clamp; ch, central hook; cvd, common vitelline duct; e, esophagus; gic, genitointestinal canal; h, haptor; i, intestine; lid, lateral intestinal diverticulum; m, mouth; Mg, Mehlis' gland; ms, median sclerite; o, ovary; od, oviduct; ot, ootype; ovd, ovovitelline duct; p, pharynx; pasa, posterior additional sclerite of anterior end of median sclerite; pasp, posterior additional sclerite of posterior end of median sclerite; pcj, posterior clamp jaw; ppb, posterior part of body; sd, sperm duct; t, testis; tnc, transverse nerve commissure; to, trapezoid outgrowth; u, uterus; vf, vitelline follicles.

Results

Class Monogenea Carus, 1863

Family Diplozoidae Palombi, 1949

Genus *Paradiplozoon* Akhmerov, 1974

Paradiplozoon skrjabini Akhmerov, 1974

(Figs. 1–10)

Diplozoon (*Paradiplozoon*) *skrjabini* Akhmerov, 1974: 11–12, fig. 5, 1 text table.

Diplozoon (*Paradiplozoon*) sp. 3: Akhmerov, 1974: 18, fig. 10.

Paradiplozoon skrjabini: Khotenovsky, 1985a: 147, 150, figs. 104–105, table 11; Khotenovsky, 1985b: 371, fig. 590B; Jiang, 2000: 660, fig. 586; Pugachev *et al.*, 2010: 492, figs. 683–684.

Diplozoon sp.: Ogawa, 1994: 64.

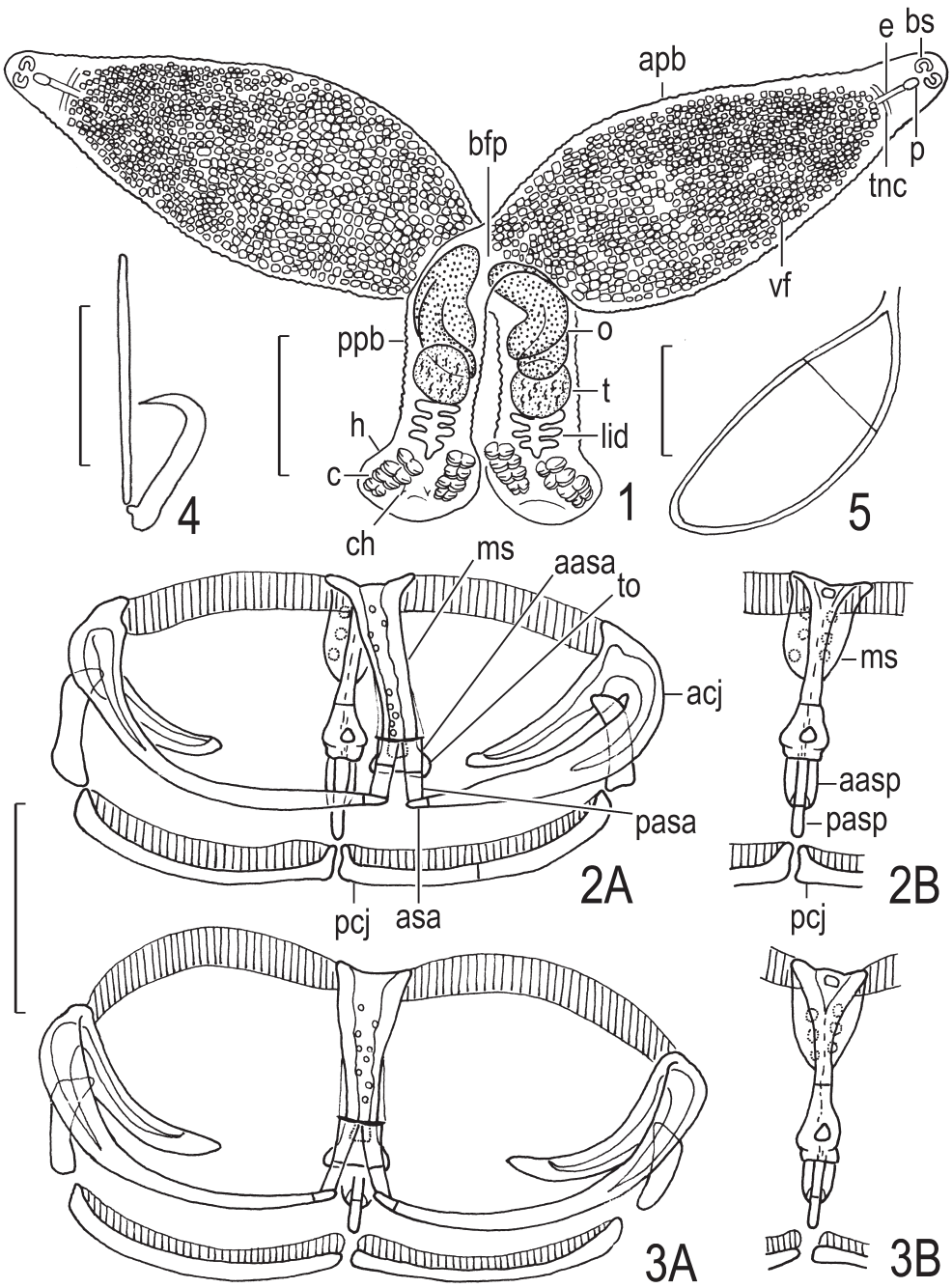
Paradiplozoon sp.: Sicard *et al.*, 2003: table 1.

Morphological study

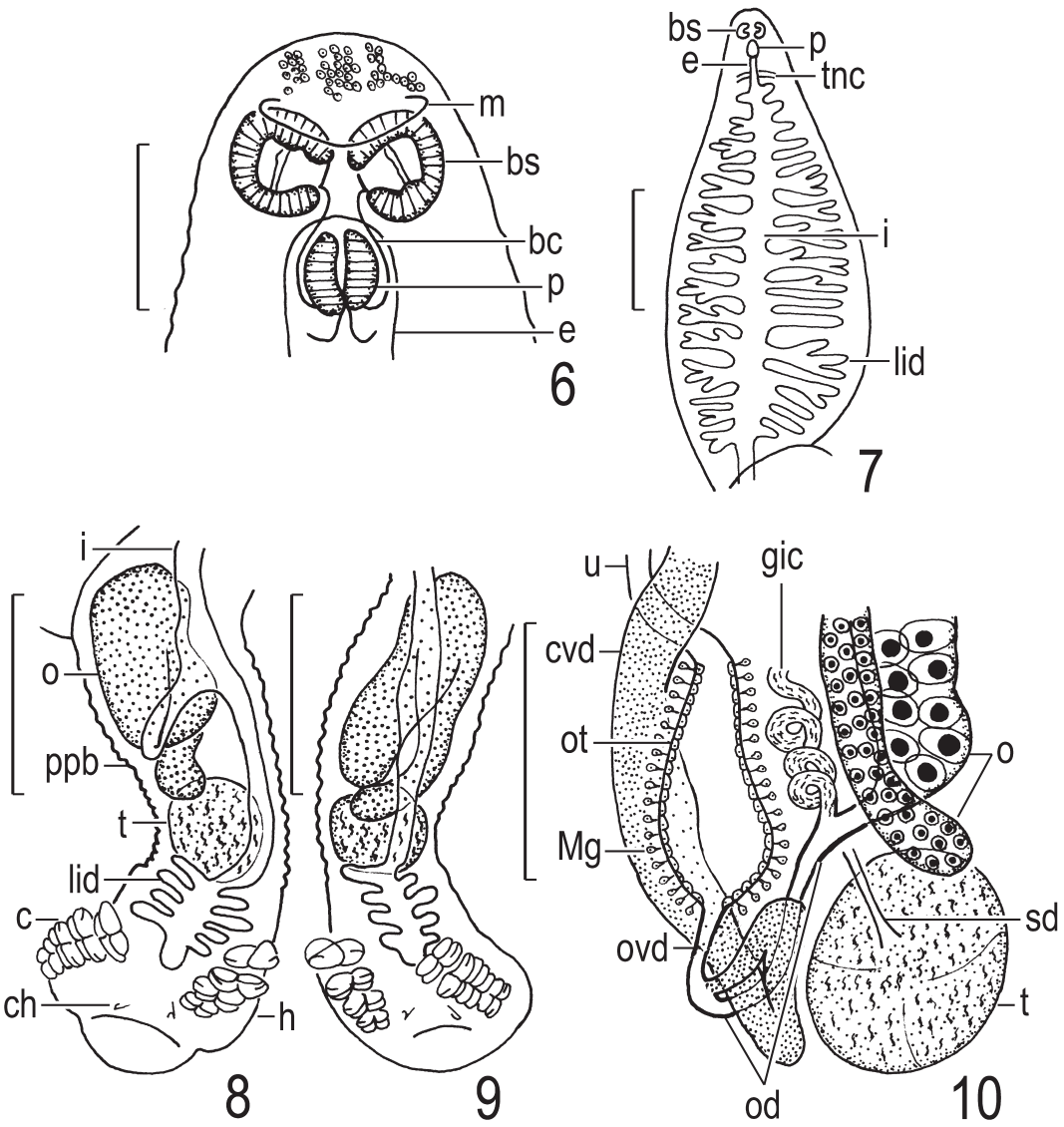
Description. 1) Based on many whole-mounted and serially sectioned adult specimens (pairs) found on *Tribolodon hakonensis* from Sai River and *Phoxinus steindachneri* from Metoba River (Figs. 1, 2, 4–8 and 10); 15 (NSMT-Pl 5920–5921, heat-killed) found on *T. hakonensis* measured. Two adult individuals partially fused together in X-shape. Body 2.09–3.89 mm long; anterior part of body dorsoventrally flat, ovate, tapering anteriorly, 1.33–2.54 mm by 0.52–1.09 mm from anterior extremity of body to body fusion part; posterior part of body cylindrical, 0.71–1.32 mm by 0.22–0.52 mm from body fusion part to posterior extremity of body, slender in anterior and middle thirds, expanded as haptor in posterior third. Tegumental ridges present in whole body except in haptor, annular, arranged regularly, much more prominent in middle third of posterior part of body. Sticky glands (terminology of Goto, 1891) anterior to buccal suckers absent. About six longitudinal small masses of cells (possibly ducts of cephalic glands) present in anterior tip of body. Transverse nerve commissure dorsal to esophagus, immediately anterior to vitelline follicles.

Haptor rectangular, 0.21–0.35 mm by 0.33–0.51 mm, thickened in posterolateral and posterodorsal margins and in posteroventral margin like lip, having four pairs of clamps and one pair of central hooks. Clamps set close together in longitudinal row along ventrolateral margins of haptor. One pair of anterior and posterior additional sclerites (our terminology; Fig. 2A, aasa, pasa) attached to anterior end of median sclerite, ventral to trapezoid outgrowth, thin, flat, looking like tendons of vertebrates rather than chitinous sclerites; posterior additional sclerites connecting to respective additional sclerites of anterior clamp jaws. Additional sclerites attached to trapezoid outgrowth absent. Anterior additional sclerite of posterior end of median sclerite having bulges; posterior additional sclerite round at tip. Longitudinal row of four clamps 188–288 long; clamp I (posterior) 47–88 by 91–116, II 47–85 by 109–151, III 50–85 by 103–147, IV (anterior) 50–88 by 103–150. Central hooks dorsal, between posterior clamps I, handle 32–46 long, anchor 16–26 long.

Mouth anteroventral, large, leading to funnel-shaped buccal cavity. Buccal suckers two, 50–85 by 47–75, opening into buccal cavity. Pharynx elliptical, 47–69 by 41–56, protruding into buccal cavity. Esophagus between pharynx and first diverticulum of intestine (slightly posterior to anterior vitelline follicles), fairly thick, swelling ventrally to pharynx, 0.19–0.20 mm long. Intestine median, ending blindly slightly anteriorly to haptor, with lateral diverticula on either side of body; diverticula long, about 15–17 on either side of anterior part of body, sometimes further diverticulated in parts, not forming anastomosis; one long lateral diverticulum ventral to ovary, four to six short lateral diverticula posterior to testis on either side of posterior part of body. Testis single, globular, large, may be partitioned into several lobules like lymph node, 135–266 by 150–251, dorsal, median or dextrally submedian, in middle third of posterior part of body. Sperm duct running forward, terminal part (or ejaculatory duct) not observed clearly. Ovary single, narrow ovate, folded once in inverted U-shape, ventral, pretesticular, dextrally submedian, in body fusion portion and anterior third of posterior part of body, sometimes slightly extending into anterior part of body; proximal half of ovary dorsal, thinner and longer than distal half, 314–659 by 84–141; distal half ventral, 235–628 by 84–282. Oviduct arising from posterior end of distal half of ovary, posterior to ovary, giving off genitointestinal canal, then receiving common vitelline duct. Genitointestinal canal convoluted, including sperm, distal part not clearly observed. Ovovitelline duct short, leading to ootype. Ootype elliptical, large, directed anteriorly, dorsal, sinistrally submedian, lined with layer of small cells, surrounded by scattered Mehlis' gland. Uterus running forward, almost straight, short; metraterm thick, ciliated, opening on sinistral margin of anterior part of body immediately anterior to body fusion part (not illustrated). Genital atrium not seen. Eggs in body 216–273



Figs. 1–5. *Paradiplozoon skrjabini*, adult specimens (pairs) found on gills of freshwater leuciscine fishes. — 1, entire body, NSMT-Pl 5921, *Tribolodon hakonensis*, Sai River, Honshu, Japan; 2, entire clamp (2A), posterior part of median sclerite (2B), MPM Coll. No. 21112, *Phoxinus steindachneri*, Metoba River, Honshu, ventral view; 3, entire clamp (3A), posterior part of median sclerite (3B), NSMT-Pl 5914, *Leuciscus waleckii*, Bolshaya Ussurka River, Primorsky Region, Russia, ventral view; 4, central hook, MPM Coll. No. 21112, *Ph. steindachneri*, Metoba River; 5, egg, laid by adults on *Ph. steindachneri*, Metoba River. Scale bars: 0.5 mm in Fig. 1; 100 μm in Fig. 5; 50 μm in Figs. 2–3; 30 μm in Fig. 4.



Figs. 6–10. *Paradiplozoon skrjabini*, adult specimens (pairs) found on gills of freshwater leuciscine fishes. — 6, anterior part of body, NSMT-Pl 5920, *Tribolodon hakonensis*, Sai River, Honshu, Japan; 7, intestine and its lateral diverticula (semischematic), NSMT-Pl 5920, ventral view; 8, posterior part of body, NSMT-Pl 5919, *T. hakonensis*, Metoba River, Honshu, ventral view; 9, posterior part of body, NSMT-Pl 5914, *Leuciscus waleckii*, Bolshaya Ussurka River, Primorsky Region, Russia, ventral view; 10, reproductive organs (semischematic), MPM Coll. No. 21112, *Phoxinus steindachneri*, Metoba River, dorsal view. Scale bars: 0.5 mm in Figs. 7–9; 100 μm in Figs. 6 and 10.

by 78–116, sometimes caved in on one side. Vitelline follicles numerous, small, filling up ventral and dorsal parenchyma of anterior part of body, entering median parenchyma between intestinal diverticula, absent anterior to transverse nerve commissure; common vitelline duct (vitelline reservoir) running posteriorly, convoluted sinistrally lateral to testis. Large mass of cells of unknown nature present posterior to testis. Excretory system not worked out.

Eggs. Laid eggs elliptical, slightly asymmetrical laterally, bright reddish brown, unembryonated,

220–267 by 72–116; operculum present at about border of first and second thirds from filamented pole; filament slender, very long (Fig. 5).

Measurements. 1) Based on 10 slightly flattened and ammonium picrate-fixed specimens (MPM Coll. No. 21112) found on *Phoxinus steindachneri*. Body 2.62–3.89 mm long; anterior part of body 1.74–2.54 mm by 0.54–1.19 mm; posterior part of body 0.68–1.27 mm long: longitudinal row of 4 clamps 198–283 long (abnormally 5 clamps per row in one specimen); clamp I 47–88 by 75–116, II 47–78 by 85–138, III 53–78 by 88–144, IV 53–84 by 94–154; handle of central hooks 36–48 long; anchor 17–22 long: buccal suckers 50–78 by 47–66; pharynx 53–69 by 44–60; testis 141–330 by 175–251; ovary 251–785 by 63–282 in proximal half, 251–659 by 110–314 in distal half: eggs in body 243–286 by 84–130; and laid eggs 229–304 by 88–104.

2) Based on 16 whole-mounted specimens (NSMT-Pl 5914) found on *Leuciscus waleckii* (Figs. 3 and 9). Specimens poorly prepared, difficult to observe external and internal organs closely. Body 3.39–4.92 mm long; anterior part of body 2.22–3.33 mm by 0.79–1.27 mm; posterior part of body 0.95–1.59 mm long. Longitudinal row of four clamps 266–314 long; clamps very similar in structure to those of specimens found on *T. hakonensis*; clamp I 63–78 by 116–132, II 72–78 by 132–173, III 72–94 by 141–179, IV 75–94 by 144–188. Handle of central hooks 33–48 long, anchor 17–25 long. Buccal suckers 78–94 by 63–94. Pharynx 63–94 by 56–85. Testis globular, large, 220–235 by 188–226. Ovary inverted U-shaped, large, 220–597 by 109–110 in proximal half, 377–486 by 157–173 in distal half. Eggs in body 243–286 by 103–130.

3) Based on 7 poorly prepared whole-mounted specimens (NSMT-Pl 5915) found on *Rhynchocypris lagowskii*. Body 1.35–2.94 mm long; anterior part of body 0.82–1.98 mm by 0.36–0.68 mm; posterior part of body 0.40–0.87 mm long; longitudinal row of 4 clamps 179–283 long; clamps very similar in structure to those of specimens found on *T. hakonensis*; clamp I 63–66 by 94–107, II 63–66 by 119–125, III 56–63 by 119–135, IV 056–88 by 138–150; handle of central hooks 38–40 long, anchor 19–22 long; buccal suckers 50–72 by 41–60; pharynx 53–60 by 41–56; and eggs in body 222–270 by 100–135.

Molecular analyses

Phylogenetic analyses. Complete sequences of the ITS2 region and partial sequences of the 5.8S and 28S regions were obtained for all of the specimens sequenced (Table 1). The ITS2 (624 bp) sequences were identical between the specimens 10556–10563, 10665–10668 and 12746–12749 from Honshu, Japan (genotype Ps-G1 in Table 1), between the specimens 10669–10672 and 10674–10680 from Hokkaido, Japan (Ps-G2), between the specimens 12738–12741 from Russia (Ps-G3), and between the specimens 12742–12745 from Russia (Ps-G4), respectively.

With respect to the sequenced data of the ITS2 region between the genotypes Ps-G1 to Ps-G4 and the closely related *Paradiplozoon homoion* from the Czech Republic, all of the site numbers of substitutions observed and respective nucleotide information are shown in Table 3. Differences identified in the nucleotide sequences of the ITS2 region between the genotypes Ps-G1 to Ps-G4 are found only at three sites (site numbers 139, 156 and 523 in Table 3) of the total 624 nucleotide bases (*i.e.*, substitution rate = 0.48%). Even including the closely related species *P. homoion* from the Czech Republic, substitutions were observed only at 17 sites (*i.e.*, substitution rate = 2.7%). Seven sites of these substitutions were identified as case of "indel (gap)" (*i.e.*, insertion or deletion). In other words, the ITS2 sequences between the genotypes Ps-G1 to Ps-G4 showed an extremely high degree of homology.

The results of the molecular phylogenetic analyses of *Paradiplozoon* spp., based on this genetic information, are shown in Figures 11–12. These analyses were performed applying the NJ, ML and MP methods to the sequences of the ITS2 (624 bp) and 5.8S-ITS2-28S (720 bp) regions of the four genotypes Ps-G1 to Ps-G4,

Table 3. Comparison of the nucleotide sequences (624 bp) of the ITS2 region between the genotypes Ps-G1 to Ps-G4 of Japanese and Russian *Paradiplozoon skrjabini* (Table 1) and Czech *P. homoion* (Table 2). Numbering refers to the position of nucleotide substitutions, the remaining unnumbered parts of the sequences being identical between the five sequences. n: multiple nucleotides, i.e., a heterozygotic site; -: alignment gap.

Species, genotype and sampling locality	ITS2 sequence
P. skrjabini, Ps-G1, Honshu, Japan	GC**G**AAATTTCTGCCGGTAG**n**CTGTTGGCG------AC
P. skrjabini, Ps-G2, Hokkaido, Japan	GC**G**AAATTTCTGCCGGTAG**C**CTGTTGGCG------AC
P. skrjabini, Ps-G3, Russia	GC**G**AAATTTCTGCCGGTAG**C**CTGTTGGCG------AC
P. skrjabini, Ps-G4, Russia	GC**A**AAATTTCTGCCGGTAG**C**CTGTTGGCG------AC
P. homoion, AJ300715, Czech Republic	GC**G**AAATTTCTGCCGGTAG**C**CTGTTGGCG**TTGGCG**AC
	↑ 139 ↑ 156 ↑ 166

Species, genotype and sampling locality	ITS2 sequence
P. skrjabini, Ps-G1, Honshu, Japan	TA**A**TTGTTGTCACTGCTACTTGCATATGTGCGAGTG
P. skrjabini, Ps-G2, Hokkaido, Japan	TA**A**TTGTTGTCACTGCTACTTGCATATGTGCGAGTG
P. skrjabini, Ps-G3, Russia	TA**A**TTGTTGTCACTGCTACTTGCATATGTGCGAGTG
P. skrjabini, Ps-G4, Russia	TA**A**TTGTTGTCACTGCTACTTGCATATGTGCGAGTG
P. homoion, AJ300715, Czech Republic	TA**T**TTGTTGTCACTGCTACTTGCATATGTGCGAGTG
	↑ 456

Species, genotype and sampling locality	ITS2 sequence
P. skrjabini, Ps-G1, Honshu, Japan	TG**-G**CCCGG**G**ATGAGATT**A**TGTGCCCATGTCATT**C**T
P. skrjabini, Ps-G2, Hokkaido, Japan	TG**-G**CCCGG**G**ATGAGATT**A**TGTGCCCATGTCATT**C**T
P. skrjabini, Ps-G3, Russia	TG**-G**CCCGG**G**ATGAGATT**A**TGTGCCCATGTCAT**G**CT
P. skrjabini, Ps-G4, Russia	TG**-G**CCCGG**G**ATGAGATT**A**TGTGCCCATGTCAT**G**CT
P. homoion, AJ300715, Czech Republic	TG**TA**CCCGG**A**ATGAGATT**T**TGTGCCCATGTCAT**G**CT
	↑ 492 ↑ 499 ↑ 508 ↑ 523

Species, genotype and sampling locality	ITS2 sequence
P. skrjabini, Ps-G1, Honshu, Japan	GT**A**GGTGTCACCAGTA**T**T**C**TGCTGTATTGTGTGTGCA
P. skrjabini, Ps-G2, Hokkaido, Japan	GT**A**GGTGTCACCAGTA**T**T**C**TGCTGTATTGTGTGTGCA
P. skrjabini, Ps-G3, Russia	GT**A**GGTGTCACCAGTA**T**T**C**TGCTGTATTGTGTGTGCA
P. skrjabini, Ps-G4, Russia	GT**A**GGTGTCACCAGTA**T**T**C**TGCTGTATTGTGTGTGCA
P. homoion, AJ300715, Czech Republic	GT**C**GGTGTCACCAGTA**C**T**T**TGCTGTATTGTGTGTGCA
	↑ 565 ↑ 579

and also utilizing data from some previously reported sequences of related diplozoids species (Table 2). As a result, the present specimens from both Japan and Russia have been evaluated as constituting a monophyletic group (Figs. 11–12). In addition, *P. homoion* was positioned as a sister group of this group (Figs. 11–12). Moreover, the monophyly of the present species and *P. homoion* was evaluated as being supported with an extremely high degree of confidence. The bootstrap probabilities (BP) of the ML, MP and NJ analyses of the ITS2 region (624 bp) being each 99, 99 and 100%, respectively (Fig. 11), and also for the BP of the ML, MP and NJ analyses for the 5.8S-ITS2-28S region (720 bp) the probabilities each being 99, 100 and 100%, respectively (Fig. 12).

Discussion

In adult morphology, the present specimens are keyed to *Paradiplozoon* Akhmerov, 1974

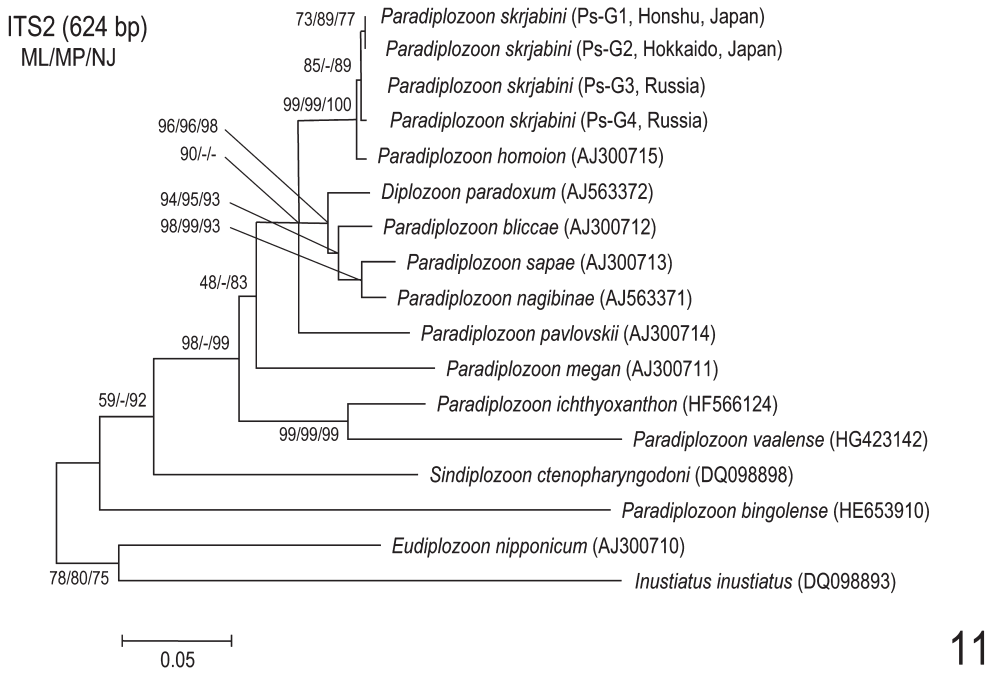


Fig. 11. Genetic relationships within the *Paradiplozoon* species, based on the 624 bp sequences of the ITS2 region. The ML dendrogram was constructed using *Sindiplozoon ctenopharyngodoni*, *Eudiplozoon nipponicum* and *Inustiatus inustiatus* as outgroups. The scale bar indicates substitutions per site. The topologies presented by the ML, MP and NJ trees are essentially identical to those presented by the ML tree. ML, MP and NJ bootstrap probabilities are specified only the main nodes. Ps-G1 to Ps-G4 of OTUs are the genotypes, referring to Table 1 for details regarding the corresponding specimens, host species, sampling localities, and GenBank accession numbers.

(Diplozoidae) *sensu* Khotenovsky (1981), because four paired clamps were present on the haptor, the sticky glands were absent anterior to the buccal suckers, and the middle part of the posterior part of the body was slender (Fig. 1). Akhmerov (1974) originally described *Diplozoon* (*Paradiplozoon*) *skrjabini* as a new subgenus and species in *Diplozoon* Nordmann, 1832 (Diplozoinae). Later, Khotenovsky (1981) raised this subgenus to the genus rank with *Paradiplozoon megan* (Bychowsky and Nagibina, 1959) as the type species.

The chitinous sclerite structure and measurements of the clamps (the third pair in particular) and central hooks are of taxonomic importance (Khotenovsky, 1985a, b; Pugachev *et al.*, 2010), though Matejusová *et al.* (2001) stated that the size of the attachment organ of monogenean parasites [the clamps and central hooks in diplozoids] can show extensive variation with different hosts, localities or temperatures and may not be a suitable indicator of species. Although the present specimens slightly differ in measurements including those of the clamps from material to material, the sclerite structure of the clamps was identical between them. Moreover, the measurements of the central hooks are almost the same. Therefore, we consider that all the present specimens belong to a single species of *Paradiplozoon* in adult morphology.

In eastern Russia, the following species of *Paradiplozoon* have previously been known (Akhmerov, 1974; Khotenovsky, 1985a, b; Pugachev *et al.*, 2010): *Paradiplozoon parabramisi* (Ling, 1973) [syn. *Diplozoon* (*Paradiplozoon*) *parabramidis* Akhmerov, 1974] found on

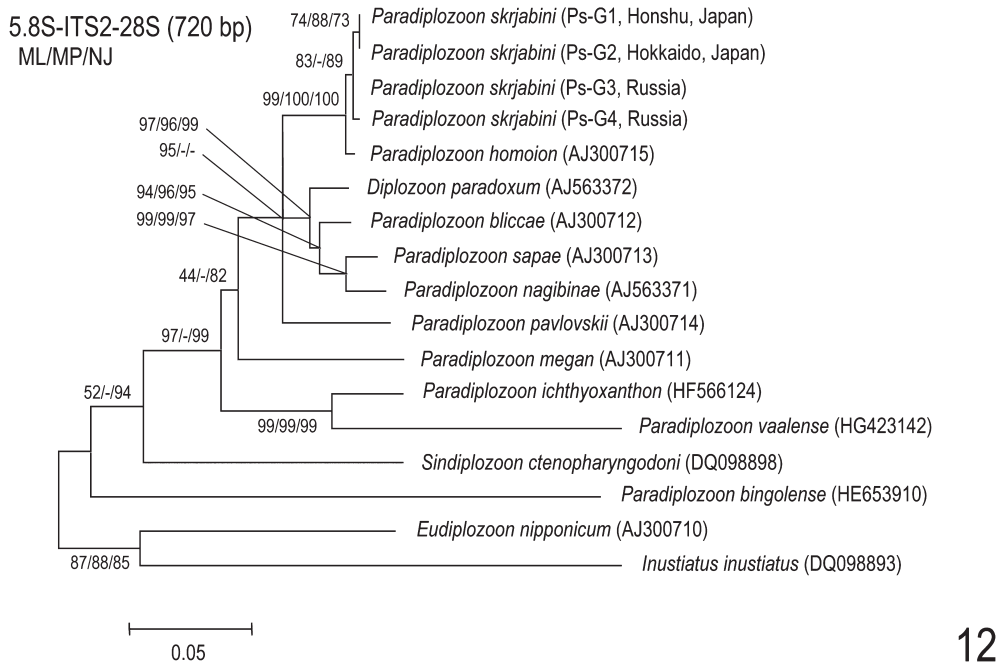


Fig. 12. Genetic relationships within the *Paradiplozoon* species, based on the 720 bp sequences of the 5.8S-ITS2-28S region. The ML dendrogram was constructed using *Sindiplozoon ctenopharyngodoni*, *Eudiplozoon nipponicum* and *Inustiatus inustiatus* as outgroups. The scale bar indicates substitutions per site. The topologies presented by the ML, MP and NJ trees are essentially identical to those presented by the ML tree. ML, MP and NJ bootstrap probabilities are specified only the main nodes. Ps-G1 to Ps-G4 of OTUs are the genotypes, referring to Table 1 for details regarding the corresponding specimens, host species, sampling localities, and GenBank accession numbers.

Parabramis pekinensis (Basilewsky, 1855) *etc*.; *Paradiplozoon marinae* Akhmerov, 1974 found on *Ctenopharyngodon idella* (Valenciennes, 1844) *etc*.; *Paradiplozoon cyprini* Khotenovsky, 1982 found on *Cyprinus carpio rubrofuscus* Lacepède, 1803; *Paradiplozoon megalobramae* Khotenovsky, 1982 [syn. *Diplozoon* (*Paradiplozoon*) sp. 2 of Akhmerov, 1974] found on *Megalobrama skolkovii* Dybowski, 1872; *Paradiplozoon hemiculteri* (Ling, 1973) found on *Hemiculter leucisculus* Basilewsky, 1855 *etc*.; *Paradiplozoon amurense* Akhmerov, 1974 found on *Pseudaspius leptocephalus* (Pallas, 1776); *Paradiplozoon skrjabini* Akhmerov, 1974 found on *Leuciscus waleckii*; and *Paradiplozoon erythroculteris* Akhmerov, 1974, *species inquirenda*, found on *Chanodichthys mongolicus* (Basilewsky, 1855). The present species most closely resembles *P. skrjabini* in morphology among them, because the posterior part of the body has tegumental ridges (folds in Pugachev *et al.*, 2010), the posterior clamp jaw lacks a cross striation, the anterior clamp jaw has a small additional sclerite on the tip, the median sclerite has a trapezoid outgrowth on the anterior end, the buccal suckers are slightly larger than the pharynx, the intestine has diverticula in the posterior part of the body, and the testis is entire and globular.

The present species morphologically differs from *P. skrjabini* as described by Akhmerov (1974), Khotenovsky (1985a, b) and Pugachev *et al.* (2010) as follows: (1) the testis is large (Figs. 1 and 8–9) instead of small; (2) the ovary is inverted U-shaped and large (Figs. 1 and 8–9) instead of slightly curved and small; (3) the anterior and posterior additional sclerites are attached

to the anterior end of the median sclerite (Fig. 2A, aasa, pasa) (not described by them); (4) the sclerites attached to the trapezoid outgrowth are absent instead of present; (5) the posterior additional sclerite of the posterior end of the median sclerite is round at the tip (Figs. 2–3) instead of bifurcated; and (6) the anterior clamp jaws are thin and slender instead of widened in the middle part. Accordingly, the specimens of *P. skrjabini* described by Akhmerov (1974), Khotenovsky (1985a, b) and Pugachev *et al.* (2010) need reexamining to compare them with the present specimens in adult morphology. We could not borrow any of them from Russia for reexamination, and so we asked Drs. Sergey G. Sokolov and Pavel I. Gerasev in Russia to reexamine them for us.

Akhmerov's (1974) seven original specimens (pairs) of *Diplozoon* (*Paradiplozoon*) *skrjabini* found on the gills of *Leuciscus waleckii* from the Amur River basin have been deposited in the Museum of the Center of Parasitology, Severtsov Institute of Ecology and Evolution, Russian Academy of Sciences, Moscow, since his death. (1) They have been dealt with as syntypes in the Museum, because none of them is labeled holotype, though Akhmerov (1974) designated it. (2) They are whole-mounted, two in Canada balsam and five in gelatin. (3) None of them is well preserved for morphological observation (S. G. Sokolov, personal communication, 12 September 2013). According to him (personal communication, 9 June 2013), it appeared that the testis was globular and large, and the ovary was inverted U-shaped (388 by 188 μm [in the proximal half] and 325 by 213 μm [in the distal half]) in one syntype (slide #432, Devyatka River near Kondon Village, 25 August 1958). Khotenovsky (1985a) described specimens of *P. skrjabini* found on the gills of *L. waleckii* from the Amur River basin and Sakhalin Island, Russia; but he did not use any of Akhmerov's type specimens at that time. Jiang (2000) reproduced the description and figures by Khotenovsky (1985a), with the almost same measurements, for *P. skrjabini* on *L. waleckii* from northeastern China. After examining 30 slides of *P. skrjabini* found on *L. waleckii* and 10 slides of *P. skrjabini* found on *Phoxinus phoxinus* from Khasan District, Primorsky Region, both in the collection of the Laboratory of Parasitic Worms, Zoological Institute of the Russian Academy of Sciences, Sankt Petersburg, P. I. Gerasev (personal communication, 13 February 2015) told us that Khotenovsky (1985a) described adult morphology of *P. skrjabini* correctly.

The measurements of the clamps and central hooks of the present specimens agree, with no significant differences, with those of *P. skrjabini* given by Akhmerov (1974), Khotenovsky (1985a, b) and Pugachev *et al.* (2010). Moreover, one of the present materials was found on the gills of *L. waleckii* (the type host of *P. skrjabini*) from the Amur River system (the type locality of *P. skrjabini*). It seems unlikely from the present study that two different species of *Paradiplozoon* parasitizing the same species fish in the same river system. Consequently, we conclude that the present species is identified as *P. skrjabini*. Accordingly, *P. skrjabini* unusually has low host specificity (on six species of four genera of leuciscine fishes) and a wide range of geographical distribution (in eastern Eurasia: the Amur River system of Russia and China; Primorsky Region and Sakhalin Island, Russia; and Hokkaido and Honshu, Japan). This is the first record of *P. skrjabini* from Japan. The above-mentioned morphological discrepancies between the present specimens and those deposited in the Laboratory of Parasitic Worms have remained to be explained. The Russian museum specimens require reexamining more closely.

The phylogenetic analyses of diplozoids in the present study have focused on molecular markers within the ITS2 region, for the purpose of comparison with sequence data published in several previous studies (Matejusová *et al.*, 2001; Sicard *et al.*, 2001; Matejusová *et al.*, 2002; Sicard *et al.*, 2003; Matejusová *et al.*, 2004; Gao *et al.*, 2007; Civáňová *et al.*, 2013; Avenant-Oldewage *et al.*, 2014; Dos Santos *et al.*, 2015). As shown in Table 3, the homology of the sequenced data within the ITS2 region of the present specimens

from Japan and Russia was extremely highly supported, *i.e.*, 99.5% support for homology. In addition, the results of the phylogenetic analyses that were conducted using two data sets (624 bp within the ITS2 region, and 720 bp within the 5.8S-ITS2-28S region) each having different analysis ranges indicated substantially homologous topologies (Figs. 11–12). As a result, the monophyly of the present specimens has been basically supported (Figs. 11–12). The molecular data also support the above-mentioned conclusion that the present species refers to *P. skrjabini*. Some of the present specimens lack their molecular data, but they are also assigned to *P. skrjabini* from morphology and measurements of the clamps and central hooks.

In addition, the monophyly of *P. skjrabini* on leuciscine cyprinids in eastern Eurasia and *P. homoion* [most likely referring to *P. homoion homoion* (Bychowsky and Nagibina, 1959) today] in western Eurasia was also strongly supported (Figs. 11–12) (see also Sicard *et al.*, 2003). Matejusová *et al.* (2001) sequenced the two samples of *P. homoion* found on *Phoxinus phoxinus* (Linnaeus, 1758) and *Rutilus rutilus* (Linnaeus, 1758) (Leuciscinae) (Table 2). Although the observed degree of homology between these two species was very high (97.3%), yet it is still considered that the 17 substitution sites observed, including the 7 indel sites as being significant (Table 3). At this stage, from these results, it is considered to be reasonable to treat both species as independent, as they would normally be treated according to conventional morphological taxonomy. It is desirable that evolutional relationship between *P. skrjabini* in eastern Eurasia and *P. homoion* in western Eurasia should be further studied. In addition, it is strongly suggested that these two species (*P. skjrabini* and *P. homoion*) are largely genetically differentiated from other *Paradiplozoon* species (Figs. 11–12).

Akhmerov (1974) described *Diplozoon* (*Paradiplozoon*) sp. 1 found on *Gobio* sp. and *Diplozoon* (*Paradiplozoon*) sp. 3 found on *Rhynchocypris lagowskii* (syn. *Phoxinus lagowskii*) from the Amur River basin. The former differs from *P. skrjabini* in that the buccal suckers (32–36 μm) are smaller than the pharynx (50–56 by 42 μm). The original description of the latter is too brief. We consider that *Diplozoon* (*Paradiplozoon*) sp. 3 is synonymous with *P. skryabini*, because one of the present materials was found on the gills of *R. lagowskii* from the Amur River system, and *P. skrjabini* had been identified from *Phoxinus phoxinus* in Khasan District as mentioned above. Sicard *et al.* (2003) sequenced the ITS2 region of a sample of *Paradiplozoon* sp. found on the gills of *Tribolodon hakonensis* (syn. *Tribolodon hakuensis* (Günther, 1877)), from the Tenryu River [at Ina City, Nagano Prefecture, on 9 September 2000], Japan; but they did not register the sequence yielded. The present specimens 12746–12747 were obtained from the same species fish in the same locality (Table 1). *Paradiplozoon* sp. of Sicard *et al.* (2003) is evidently synonymous with *P. skrjabini*.

With regard to the sclerites of the clamps, it has previously been said that one pair of the anterior joining sclerites was attached to the trapeze spur [trapezoid outgrowth in the present paper, Figs. 2–3] and connect with the respective additional sclerites of the anterior clamp jaws in many species of diplozoids (Khotenovsky, 1985a, b; Jiang, 2000; Matejusová *et al.*, 2001; Matejusová *et al.*, 2002; Matejusová *et al.*, 2004; Pugachev *et al.*, 2010). Matejusová *et al.* (2004) demonstrated that the combination of the shape and comparison of length of the trapeze spur and anterior joining sclerites could lead to accurate identification of diplozoid species. However, such anterior joining sclerites were absent in the present specimens. Instead, one pair of the anterior and posterior additional sclerites existed, attaching to the anterior end of the median sclerite; and the posterior ones of them connected with the respective additional sclerites of the anterior clamp jaws (Fig. 2A, aasa, pasa). Similar sclerites (but one sclerite each) were described by Gläser and Gläser (1964) in *Diplozoon homoion* Bychowsky and Nagibina, 1959 and by Khotenovsky (1985a) in *Paradiplozoon homoion*

homoion (Bychowsky and Nagibina, 1959). We examined three specimens (pairs) of *P. homoion* deposited in the Helminthological Collection, Institute of Parasitology, Czech Republic: No. Coll. M-305/1, *Paradiplozoon homoion gracile* (Reichenbach-Klinke), on gills of *Rutius rutius* (2157-245), Latorica River, Czechoslovakia then, 7 July 1964; No. Coll. M-306/1, *Paradiplozoon homoion homoion*, on gills of *Rutilus rutilus*, Kamenice River, Czechoslovakia then, 7 November 1979; and No. Coll. M-306/2, *Paradiplozoon homoion homoion*, on gills of *Carassius carassius* (2197-26), Szolnok, Hungary, 8 July 1964. They had one pair of the anterior and posterior additional sclerites but no anterior joining sclerites as in *P. skrjabini*. The handle of the central hook was 33–44 μm long, and the anchor was 14–20 μm long. The testis was globular to elliptical and large instead of lobed (Khotenovsky, 1985a). The ovary was inverted U-shaped and large. Accordingly, *P. skrjabini* is similar to *P. homoion* in adult morphology, though it is distinct from the latter in the ITS2 sequences as mentioned above (Table 3).

Besides the ITS2 sequences of the nine species of *Paradiplozoon* (Table 2), Gao *et al.* (2007) reported the ITS2 sequences of six species of diplozoids from China: *Paradiplozoon hemiculteri* (Ling, 1973) (*e.g.*, DQ098887), *Paradiplozoon jiangxiense* (Jiang, Wu and Wang, 1985) (DQ098885) (the original spelling *jiangxiensis* changed as mentioned below), *Paradiplozoon opsariichthydis* (Jiang, Wu and Wang, 1984) (*e.g.*, DQ098890), *Paradiplozoon parabramisi* (Ling, 1973) (*e.g.*, DQ098883), *Paradiplozoon parapeleci* (Jiang, Wu and Wang, 1984) (DQ098882) and *Eudiplozoon nipponicum* (*e.g.*, DQ098895). The five species of *Paradiplozoon* formed a clade that was distinctly separated from the clade of *P. skrjabini* and those of other diplozoid species in our unpublished preliminary analyses as seen in Gao *et al.* (2007), Civáňová *et al.* (2013) and Avenant-Oldewage *et al.* (2014). We used the sequence of *E. nipponicum* from Europe (Table 2) and excluded the sequences of the five species of *Paradiplozoon* from China in the present analyses.

In the present molecular phylogenetic trees (Figs. 11–12), *Paradiplozoon bliccae*, *P. nagibinae* and *P. sapae* were clustered together with *Diplozoon paradoxum* in a clade but separately with the other species of *Paradiplozoon* as seen in Matejusová *et al.* (2004), Gao *et al.* (2007) and Civáňová *et al.* (2013), suggesting that *Paradiplozoon* is polyphyletic as pointed out by Gao *et al.* (2007). *Paradiplozoon* requires revision.

We here discuss nomenclatural problems of the four species of *Paradiplozoon* (see also Table 2). (1) The original spellings *jiangxiensis* and *bingolensis* (either masculine or feminine) of *P. jiangxiensis* and *P. bingolensis* are changed to *jiangxiense* and *bingolense* (neuter), respectively, because the generic name *Paradiplozoon* is neuter. (2) The species name, author and date of *P. ichthyoxanthon* first appeared as "*Paradiplozoon ichthyoxanthon* Avenant-Oldewage, 2013" in a paper accessible electronically (doi:10.1017/S0022149X12000879) by Avenant-Oldewage, le Roux, Mashego and Jansen van Vuuren in *Journal of Helminthology* in 2013. "*Paradiplozoon ichthyoxanthon* n. sp." and "A new species, *Paradiplozoon ichthyoxanthon*" are used in the title and abstract of it, respectively. No paper by Avenant-Oldewage (2013) is cited in the References of it. We consider that Avenant-Oldewage individually described this new species in it, though this is clearly indicated nowhere in it. Because it has not been registered in the *Official Register of Zoological Nomenclature* (ZooBank) and contains no evidence in it itself that such registration has occurred, it is not considered published according to Article 8.5.3 of the *International Code of Zoological Nomenclature*, hereafter the Code (International Commission on Zoological Nomenclature, 2012). It is regarded as a preliminary version of a work issued and distributed electronically in advance of publication (Articles 9.9 and 21.8.3 of the Code). Such a paper does not constitute a published work (Article 9.9 of the Code). The final version was printed in the same journal in 2014 (see the References of the present paper). Therefore, we here

cite the author and date of *P. ichthyoxanthon* as Avenant-Oldewage in Avenant-Oldewage, le Roux, Mashego and Jansen van Vuuren, 2014. (3) Similarly, the date of "*Paradiplozoon vaalense* n. sp." is regarded as 2015. The preliminary version of a paper accessible electronically (doi:10.1017/S0022149X1300059X) by Dos Santos, Jansen van Vuuren and Avenant-Oldewage appeared in the same journal in 2013, and the final version was printed in 2015 (see the References of the present paper).

Ogawa (1994) briefly mentioned specimens of *Diplozoon* sp. found on the gills of *T. hakonensis* from the Horobetsu River, Teshio River at Iwaonai Dam, a tributary of the Teshio River, Mena River, Chitose River and Lake Toro; *T. sachalinensis* (syn. *Tribolodon ezoe* Okada and Ikeda, 1937) from the Teshio River at Iwaonai Dam and Lake Toro; and *Tribolodon brandtii* (Dybowski, 1872) from Lake Toro, all in Hokkaido. We reexamined part of the specimens: (1) 6 specimens (pairs) (MPM Coll. No. 19627), whole-mounted, on gills of *T. hakonensis*, Mena River, 20 August 1981; (2) 23 (MPM Coll. No. 19628), whole-mounted, on gills of *T. hakonensis*, Chitose River at Ebetsu, 28 August 1982; (3) 11 (MPM Coll. Nos. 19236 and 19629), whole-mounted, on gills of *T. hakonensis*, Lake Toro, 10 May 1977, 17 June 1984; (4) 8 (MPM Coll. Nos. 19237 and 19239), whole-mounted, on gills of *T. sachalinensis*, Lake Toro, 11 May 1977; (5) 12 (MPM Coll. No. 19235), whole-mounted, on gills of *T. brandtii* (added as the host), Kushiro River, 7 May 1977. Although the remainder was not made available to us (perhaps already lost), we determine all of the specimens to be *P. skrjabini* from our reexamination of the specimens and the present study.

In diplozoids, it is said that two adult individuals fuse together in pairs in permanent copula to exchange sperm with each other on the host gills. According to Goto (1891) and Avenant-Oldewage *et al.* (2014), the sperm duct of one individual opens to the common vitelline duct of the other individual, and vice versa. We failed to confirm this opening in the present permanent preparations.

It was observed that the intestine of one individual communicated by anastomosis with that of the other individual in the body fusion part in living worms of *P. skrjabini* found on *Tribolodon hakonensis* from Ueda City (Ogawa, 2013, unpublished data). However, we could not confirm this anastomosis in the present permanent preparations.

Diplozoid worms feed on (or suck) the blood from the gills of host fish (Llewellyn, 1954). Anemia and emaciation of host fish may result from diplozoid infection in long captivity, due to loss of the blood. When several fish of *T. hakonensis* (60–90 mm in standard length) collected in the Hiroi River at Kotobuki, Iiyama City, Nagano Prefecture, were kept on sufficient food in an aquarium for about five months, their whole bodies turned pale, gradually losing weight. At autopsy, several worms of *P. skrjabini* each were found on the pale gills of the fish (Shimazu, 1998, unpublished data).

Acknowledgments

We are grateful to Dr. Satoshi Yamamoto (Nagano Prefectural Fisheries Experimental Station, Azumino), Dr. Koh Hasegawa (Hokkaido National Fisheries Research Institute, Sapporo), Dr. Hirofumi Hayano (Abashiri Fisheries Research Institute, Abashiri), Mr. Jun'ichi Naito (Fuchu Town, Hiroshima Prefecture), Mr. Masamichi Yamamoto (Faculty of Science, Shinshu University, Matsumoto), Mr. Ken'ichi Hashimoto (Saitama) and Dr. Kazunori Hasegawa (Department of Zoology, National Museum of Nature and Science, Tsukuba) for helping us obtain the specimens studied. Thanks are also due to Dr. Sergey. G. Sokolov (Center of Parasitology, Severtsov Institute of Ecology and Evolution, Russian Academy of Sciences, Moscow) and Dr. Pavel I. Gerasev (Laboratory of Parasitic Worms, Zoological Institute of the Russian Academy of Sciences, Sankt Petersburg) for reexamining the museum specimens of *Paradiplozoon skrjabini*, Dr. Tomáš Scholz (Biology Centre,

ASCR, Institute of Parasitology, Branišovská, České Budějovice, Czech Republic) for the loan of the specimens of *P. homoion*, Prof. Teruaki Nishikawa (Toho University, Funabashi) for helpful suggestions for the nomenclature and Prof. Annemarie Avenant-Oldewage (Department of Zoology, University of Johannesburg, Johannesburg, South Africa) for reviewing a draft of the manuscript.

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