MOLECULAR IDENTIFICATION OF INFECTIVE LARVAE OF THREE SPECIES OF *ONCHOCERCA* FOUND IN WILD-CAUGHT FEMALES OF *SIMULIUM BIDENTATUM* IN JAPAN

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Summary:

Wild female black flies attracted to a man or an idling automobile were collected at Oita, Japan where five cases of zoonotic onchocerciasis had occurred. Among the five *Simulium* species captured, 2 % of *Simulium bidentatum*, the predominant species, were infected with filarial larvae. There were at least two types of infective larvae, types A and B, based on morphometric observation. Moreover, molecular analysis of the mitochondrial cytochrome *c* oxidase subunit 1 (CO1) gene revealed that types A and B were represented by a single unknown species of *Onchocerca* and two species, *i.e.*, *Onchocerca dewittei japonica* from wild boar, the causative agent of zoonotic onchocerciasis in Japan, and an undescribed *Onchocerca* sp. from wild boar, respectively. Phylogenetic analysis based on the sequences of the mitochondrial 12S ribosomal RNA (12S rRNA) gene also showed that type A is likely to be an unknown species of *Onchocerca*. Natural infection of black flies with infective larvae of *O. dewittei japonica* and *O.* sp. was demonstrated for the first time. The present study strongly suggests that *S. bidentatum* plays a role as a vector in the transmission of zoonotic onchocerciasis due to *O. dewittei japonica* in Japan.

KEY WORDS : *Onchocerca*, infective larvae, *Simulium*, vector, zoonosis, molecular identification.

Résumé : IDENTIFICATION MOLÉCULAIRE DES LARVES INFECTANTES DE TROIS ESPÈCES D'ONCHOCERQUES RÉCOLTÉES CHEZ DES FEMELLES SAUVAGES DE *SIMULIUM BIDENTATUM* AU JAPON

Des simulies sauvages, attirées par l'homme, une voiture ou les deux, ont été récoltées à Oita, Japon, où cinq cas d'onchocercose zoonotique ont été diagnostiqués. Parmi les cinq espèces de Simulium *capturées, 2 % des* S. bidentatum, *l'espèce dominante, avaient des larves de filaires. Deux types de larves, A et B, définis par leurs caractéristiques morphométriques, étaient présents. L'analyse moléculaire de la sous-unité du gène mitochondrial de la cytochrome oxidase c (CO1) montre que le type A correspond à une seule espèce, tandis que le type B correspond à deux espèces parasites du sanglier,* Onchocerca dewittei japonica, *agent de l'onchocercose zoonotique, et* Onchocerca *sp. L'analyse phylogénique basée sur les séquences du gène ribosomal mitochondrial 12S RNAr montre en outre que le type A est une espèce d'onchocerque inconnue. L'infection naturelle des simulies par les larves infectantes d'*O. d. japonica *et d'autres onchocerques est démontrée pour la première fois en analysant les séquences de CO1. L'étude suggère fortement que* S. bidentatum *joue un rôle dans la transmission de l'onchocercose zoonotique due à* O. d. japonica *au Japon.*

MOTS CLÉS : Onchocerca, *larves infectantes*, Simulium, *vecteur, zoonose, identification moléculaire.*

INTRODUCTION

Zoonotic onchocerciasis is a rare infection in humans. The *Onchocerca* species involved and their life cycles are poorly known. Out of 15 cases described so far, five have been reported from Oita, Japan (Beaver *et al.*, 1989; Hashimoto *et al.*, 1990; Takaoka *et al.*, 1996, 2001, 2004, 2005). During investigation of its transmission in Japan, we have proved that the causative agent of Japanese cases is *Onchocerca dewittei japonica* Uni, Bain & Takaoka, 2001 from wild boar *Sus scrofa* Linnaeus, 1758 (Takaoka *et al.*, 2001; Uni *et al.*, 2001), and that experimentally infected black flies (Diptera: Simuliidae) are able to support the larval development of the agent (Fukuda *et al.*, 2008). In addition, we have shown that mitochondrial cytochrome *c* oxidase subunit 1 (CO1) gene is useful to distinguish three *Onchocerca* species in Japan, *O. dewittei japonica*, an as yet unnamed another *Onchocerca* sp. from wild boar (abbreviated *O.* sp. hereafter) and *O. gutturosa* Neumann, 1910 from cattle (*Bos taurus* Linnaeus, 1758), all of which are morphologically indistinguishable from one another at the infective larval stage (Fukuda *et al.*, 2010).

To search for the natural vectors of *O. dewittei japonica*, we investigated infections of black flies with filarial larvae in Oita, Japan and attempted to identify infective larvae found on the basis of the analysis of the mitochondrial CO1 and 12S ribosomal RNA (12S rRNA) gene sequences.

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MATERIALS AND METHODS

Collection and dissection of adult black flies for filarial infective larvae

Adult black flies were collected at a site (about 33° 10' N and 131° 33' E: ca. 120 m in altitude) in Megusuno, Oita City, Oita Prefecture, Japan from May to December, 2006, and in September, 2008, for 1-3 hours in the morning. The site is 7.5 km from the area where the zoonotic *Onchocerca* infection (the fourth case in Japan) was presumed to have occurred (Takaoka *et al.*, 2004). Female flies attracted to a man or an idling automobile were captured by an insect net. A total of about 100 females were collected within six days almost every month. The flies collected in 2006 were housed individually, fed on 30 % sucrose solution at 25 °C for 10 days and stored at – 20 °C, while those collected in 2008 were stored at – 20 °C without being reared. All the flies were identified morphologically using the keys of Takaoka (1977), and dissected in 0.9 % saline solution. Morphological generic identification of infective larvae (third-stage larvae) recovered followed Bain & Chabaud (1986). In addition, infective larvae of *Onchocerca* species were grouped into two types (A with the body width over 23 µm and esophagus shorter than half of the body length; B with the body width narrower than 23 µm and esophagus longer than half of the body length) (Fukuda *et al.* (2008)).

DNA extraction of infective larvae

Total DNA was extracted from individual infective larvae with a DNeasy Blood & Tissue Kit (QIAGEN, Hilden, Germany). The final volume of each extract was 50-150 µl.

PCR and sequencing of the partial mitochondrial CO1 and 12S rRNA gene regions

The mitochondrial CO1 gene was amplified using the primer set CO1intF-CO1intR (Casiraghi *et al.*, 2001) as described elsewhere (Fukuda *et al.*, 2010). Briefly, PCR was performed in a final volume of 50 µl containing 1× Ex *Taq* buffer including 2 mM Mg^{2+}, 200 µM each of dNTPs, 0.1 µM each of primers, 1.25 units of Ex *Taq* Polymerase (TAKARA BIO INC., Otsu, Japan), and 3 µl of template DNA under the following thermal conditions: an initial denaturation at 94 °C for 3 min, followed by five cycles of 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 1 min and 37 cycles of 94 °C for 30 s, 48 °C for 30 s, and 72 °C for 1 min.

The mitochondrial 12S rRNA gene of three of nine samples was also amplified with the primer set 12SF-12SR described by Casiraghi *et al.* (2004) in the same reaction mixture as above. The thermal profile was as follows: 40 cycles of 94 °C for 45 s, 50 °C for 45 s, and 72 °C for 90 s (Casiraghi *et al.*, 2004).

PCR products were purified with a QIA quick PCR Purification Kit (QIAGEN) and directly sequenced using the PCR primers, a BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA), and an Applied Biosystems 3130 Genetic Analyzer (Applied Biosystems). Sequence data were deposited in DDBJ/EMBL/GenBank under the following accession numbers AB518872-AB518879.

Data analysis

All the sequences obtained from both genes were aligned with published sequences of *O. dewittei japonica*, other *Onchocerca* species, *Loxodontofilaria* species and other filaria species using clustal W (Thompson *et al.*, 1994) in BioEdit program ver. 7.0.5.3 with default setting (Hall, 1999). Alignment gaps were removed for the following analyses. Using these alignments, sequences were compared, neighbor-joining trees (Saitou & Nei, 1987) were constructed, and bootstrap probabilities were estimated, all by MEGA ver. 4.0.2 based on 585 bp of the CO1 gene and 375 bp of the 12S rRNA gene (Tamura *et al.*, 2007), respectively. The Kimura 2-parameter method was used to estimate evolutionary distances in the trees. Accession numbers of sequences taken from databases are as follows: *O. armillata* Railliet & Henry, 1909 (12S rRNA, DQ523736), *O. dewittei japonica* (CO1, AB518689, AB518691, AM749266; 12S rRNA, AM779815), *O. dukei* Bain, Bussiéras & Amégéé, 1974 (12S rRNA, DQ523740), *O. eberhardi* Uni & Bain, 2007 (CO1, AM749268; 12S rRNA, AM779810), *O. fasciata* Railliet & Henry, 1910 (12S rRNA, DQ523744), *O. flexuosa* (Wedl, 1856) (12S rRNA, DQ523746), *O. gibsoni* (Cleland & Johnston, 1910) (CO1, AJ271616; 12S rRNA, AJ544837), *O. gutturosa* (CO1, AJ271617; 12S rRNA, DQ523743), *O. jakutensis* (Gubanow, 1964) (12S rRNA, DQ523745), *O. lienalis* (Stiles, 1982) (12S rRNA, AY462927), *O. lupi* Rodonaja, 1967 (CO1, EF521409), *O. ochengi* Bwangamoi, 1969 (CO1, AJ271618; 12S rRNA, AJ544839), *O. ramachandrini* Bain, Wahl & Renz, 1993 (12S rRNA, DQ523737), *O. skrjabini* Ruklyadev, 1964 (CO1, AM749269; 12S rRNA, AM779804), *O.* sp. 'bushbuck' *sensu* Krueger *et al.*, 2007 (12S rRNA, DQ523739), *O.* sp. from wild boar *sensu* Fukuda *et al.* (CO1, AB518693, AB518694), *O.* sp. 'Siisa' *sensu* Krueger *et al.*, 2007 (12S rRNA, DQ523738), *O. suzukii* Yagi, Bain & Shoho, 1994 (CO1, AM749275; 12S rRNA, AM779811), *O. volvulus* (Leuckart, 1893) (CO1, AF015193; 12S rRNA, AM779855), *Loxodontofilaria caprini* Uni & Bain, 2006 (CO1, AM749237; 12S rRNA, AM779822), *Setaria digitata* (Linstow, 1906) (12S rRNA, AM779801), *Thelazia callipaeda* Railliet & Henry, 1910 (CO1, AM042549; 12S rRNA, AJ544858) and *Wuchereria bancrofti* (Cobbold, 1877) (CO1, AJ271612).

RESULTS

BLACK-FLY SPECIES COLLECTED

A total of 1,094 females of five black-fly species were collected (Table I). *Simulium bidentatum* (Shiraki, 1935) was the most abundant while four other species, *S. quinquestriatum* (Shiraki, 1935), *S. arakawae* Matsumura, 1915, *S. japonicum* Matsumura, 1931 and *S. oitanum* (Shiraki, 1935), were present in smaller numbers, especially from June to October.

NATURAL INFECTIONS WITH FILARIAL LARVAE

Table II shows the results of dissections of black flies collected. Natural filarial infections were found in 2 % (20/1,007) of *S. bidentatum*. No infection was found in the other species. Sixteen of 20 infected flies had one to eight infective larvae. The infective larvae were classified into two types (designated as types A and B) of *Onchocerca* species based on their morphology (*i.e.*, caudal morphology, body length, body width, and length of esophagus relative to body length, though the esophago-intestinal junction was unclear in many larvae). Of 25 infective larvae from 14 flies measured, six were type A with a thicker body (774.4-1,382.7 µm long by 24.6-26.7 µm wide) and shorter esophagus, while 19 were type B with a thinner body (718.3-1,117.9 µm long by 17.8-21.5 µm wide) and longer esophagus (Table III). The infective larvae of both types were recovered from the head, thorax and abdomen of the flies.

Two flies harbored one second-stage larva and one first-stage larva, respectively. One fly harbored both of them. All of these larvae were found in the thorax. Four flies harbored one to three microfilariae, all of which were found in the thorax, except one microfilaria which was found in the head.

	Date of collection									
	2006								**2008**	
***Simulium* spp.**	**May 30-Jun. 1**	**Jun. 29**	**Jul. 30-Aug. 2**	**Aug. 28-31**	**Sept. 30**	**Oct. 30**	**Dec. 2**	**Dec. 19**	**Sept. 17 & 22**	**Total (%)**
S. bidentatum	126	123	122	55	131	146	100	111	93	1,007 (92.0)
S. quinquestriatum	5	0	1	1	2	6	5	4	7	31 (2.8)
S. arakawae	7	0	0	0	0	0	9	4	0	20 (1.8)
S. japonoicum	11	0	0	0	2	0	4	1	0	18 (1.6)
S. oitanum	1	0	0	0	0	0	11	6	0	18 (1.6)
Total	150	123	123	56	135	152	129	126	100	1,094 (99.8)

Table I. – Numbers of female adult black flies collected in Oita, Japan in 2006 and 2008.

	Date of collection								
	2006								**2008**
	May 30-Jun. 1	**Jun. 29**	**Jul. 30-Aug. 2**	**Aug. 28-31**	**Sept. 30**	**Oct. 30**	**Dec. 2**	**Dec. 19**	**Sept. 17 & 22**
No. flies dissected	126	123	122	55	131	146	100	111	93
No. flies infected	5	1	1	2	6	1	0	0	4
No. & stage of larvae	9 L_3, 2 L_2, 1 L_1, 2 Mf	3 Mf	1 L_3	1 L_3, 1 L_1	20 L_3	2 L_3	–	–	2 L_3, 4 Mf
No. L_3/infected fly	1-3	0	1	1	1-8	2	–	–	1

Mf, microfilariae; L_1, first-stage larva; L_2, second-stage larvae; L_3, third-stage larva(e); –, relative data not obtainable.

Table II. – Natural filarial infections of *Simulium bidentatum*.

	Date of collection								
	2006								**2008**
	May 30-Jun. 1	**Jun. 29**	**Jul. 30-Aug. 2**	**Aug. 28-31**	**Sept. 30**	**Oct. 30**	**Dec. 2**	**Dec. 19**	**Sept. 17 & 22**
No. flies infected	4	0	1	1	5	1	0	0	2
No. & type of larvae	2 A, 5 B	–	1 B	1 B	2 A, 11 B	1 A	–	–	1 A, 1 B

–, relative data not obtainable.

Table III. – Morphological typing of infective larvae found in *Simulium bidentatum*.

Molecular identification of infective larvae from wild-caught black flies

Nine (L-1–L-9) of the 25 infective larvae morphologically classified as two types were used for determination of the mitochondrial CO1 gene region (Table IV). All the sequences were 649 bp long. Compared with other *Onchocerca* species available in databases, among six larvae of type B (L-1–L-6), five (L-1–L-5) were identical or similar to *O. dewittei japonica* (nucleotide difference: 0-0.7 %) and one larva (L-6) was identical or similar to *O.* sp. (nucleotide difference: 0-0.2 %), where differences between type B and other *Onchocerca* species were 7.5-11.8 %. The nucleotide and amino acid sequences of three larvae of type A (L-7–L-9) had difference of more than 9.1 % and 0.9 % from any *Oncho-*

L_3 No.	Date of collection	Types by morphological analysis	Body length × body width (µm)	Definitive identification of *Onchocerca* spp.	GenBank accession no.
L-1	Aug. 28, 2006	B	979.5 × 20.5	*O. dewittei japonica*	AB518872 (CO1)
L-2	Aug. 1, 2006	B	1,117.9 × 21.5	*O. d. j.*	AB518873 (CO1)
L-3	Sept. 30, 2006	B	967.0 × 20.3	*O. d. j.*	AB518874 (CO1)
L-4	Sept. 30, 2006	B	850.3 × 18.8	*O. d. j.*	–[a]
L-5	Sept. 22, 2008	B	1,020.5 × 20.5	*O. d. j.*	AB518875 (CO1)
L-6	Sept. 30, 2006	B	771.6 × 17.8	*O.* sp.	–[b]
L-7	Sept. 30, 2006	A	1,295.9 × 25.5	*O.* sp. (type A)*	AB518876 (CO1), AB518877 (12S rRNA)
L-8	Sept. 30, 2006	A	1,223.1 × 25.6	*O.* sp. (type A)*	AB518878 (12S rRNA)[c]
L-9	Sept. 22, 2008	A	1,220.5 × 25.6	*O.* sp. (type A)*	AB518879 (12S rRNA)[c]

* different from *O. suzukii* and *Loxodontofilaria caprini*. [a] The CO1 sequence was identical to that of AB518691 (Fukuda *et al.*, 2010). [b] The CO1 sequence was identical to that of AB518694 (Fukuda *et al.*, 2010). [c] The CO1 sequence was identical to that of *O.* sp. (type A) (L-7).

Table IV. – Molecular identification of infective larvae of *Onchocerca* spp. found in *Simulium bidentatum* in Oita, Japan.

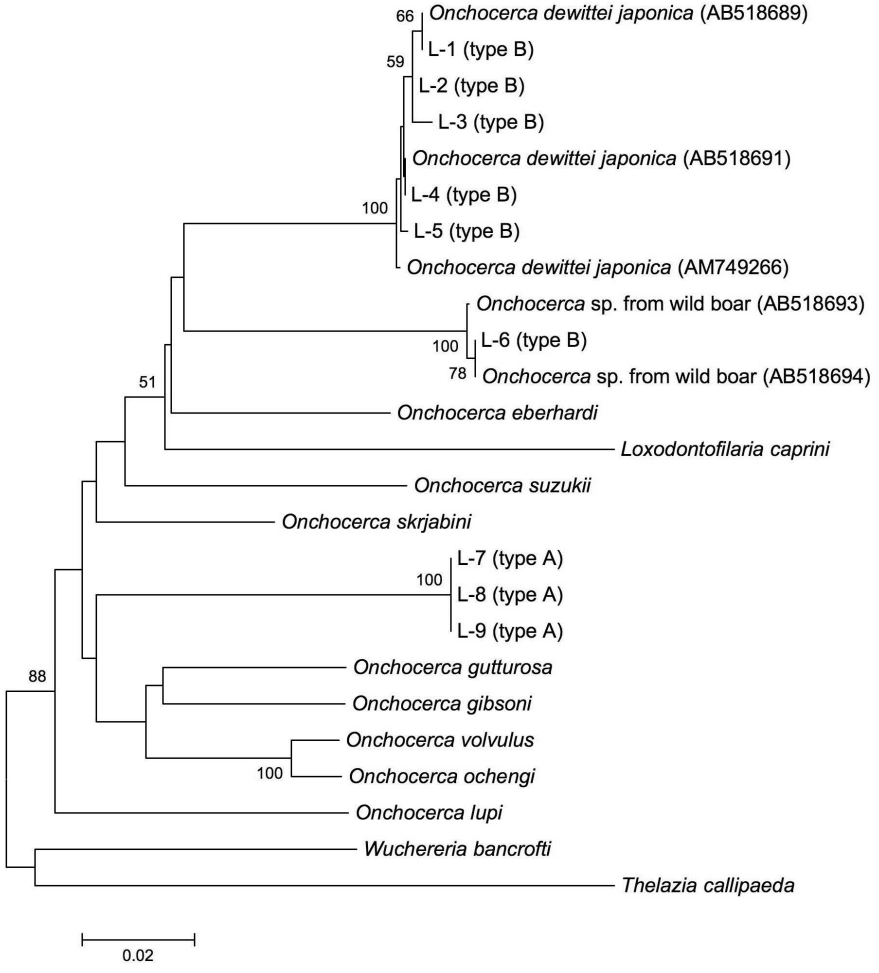

Fig. 1. – Neighbor-joining phylogenetic tree based on the partial mitochondrial CO1 gene sequences of *Onchocerca* spp. Numbers at the nodes are the bootstrap confidence values after 500 replicates. Values more than 50 % are shown. The scale bar indicates the distance in substitutions per nucleotide.

cerca sequence in databases, respectively. The phylogenetic relationship between type A, type B, 10 *Onchocerca* species, one *Loxodontofilaria* species, and two other filaria species (as outgroups), is shown in the neighbor-joining tree (Fig. 1).

For the three infective larvae of type A, the sequences of the mitochondrial 12S rRNA gene were also determined (Table IV). The lengths of the sequences were 471 bp for L-7 and L-8, and 470 bp for L-9. There was one nucleotide difference between L-7 and L-8. The sequences of L-7 and L-9 were identical to each other except for one nucleotide deletion in L-9. The sequences were compared with those of all the *Onchocerca* species and one *Loxodontofilaria* species available in GenBank. Table V shows the numbers of nucleotide differences over 375 sites. As was the case with the sequences of the CO1 gene, the sequences of type A were not identical or similar to any sequences in databases, where differences between type A and 18 filaria species (17 *Onchocerca* species and one *Loxodontofilaria* species) were 5.9-9.1 %. The phylogenetic relationship of type A with 17 other *Onchocerca* species, one *Loxodontofilaria* species, and two other filaria species (as outgroups), is shown in a neighbor-joining tree (Fig. 2).

DISCUSSION

In this study, two types (types A and B) of infective larvae classified by their morphology were recovered from *S. bidentatum*, the most abundant and anthropophilic black-fly species, near an area of zoonotic onchocerciasis in Japan. According to our previous study (Takaoka & Bain, 1990; Fukuda *et al.*, 2008), type B larvae are presumed to be equal to type II from black flies collected in a cattle shed, which includes *O. gutturosa* from cattle, *O. dewittei japonica* and *O.* sp. from wild boar. Using CO1 gene sequences, we were able to identify the morphologically similar infective larvae of type B as *O. dewittei japonica* (L-1–L-5) and *O.* sp. (L-6) (Table IV). It is noteworthy that L-5 which was recovered from a female fly not reared after the collection was identified as *O. dewittei japonica*. This means that *S. bidentatum* has an ability to support larval development of the parasite to the infective stage in nature as well as in the laboratory.

Type A is presumed to be identical to type I from black flies collected in a cattle shed (Takaoka & Bain, 1990). It had been suggested that type I was *L. caprini*, adult worms of which were found in a serow *Naemorhedus crispus* (= *Capricornis crispus*) (Temminck, 1845) in Oita (Uni *et al.*, 2006). However, in our previous study (Fukuda *et al.*, 2008) that possibility was excluded because of the morphometric difference of the microfilariae. As shown in figures 1 and 2, it was confirmed with DNA sequences of the CO1 and 12S rRNA genes that type A was not *L. caprini*. It was not *O. suzukii*, another parasite of the serow (Uni *et al.*, 1998), or any other described species. It is likely to be an unknown distinct *Onchocerca* species on the basis of the morphological characteristics and mitochondrial DNA sequences of the CO1 and 12S rRNA genes. The diver-

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23
1 L-7 (Type A)																							
2 L-8 (Type A)	1																						
3 L-9 (Type A)	0	1																					
4 *O. jakutensis*	28	29	28																				
5 *O. lienalis*	29	30	29	14																			
6 *O. fasciata*	23	24	23	14	15																		
7 *O. gutturosa*	31	32	31	20	21	18																	
8 *O. gibsoni*	28	29	28	14	17	19	19																
9 *O.* sp. 'bushbuck'	32	33	32	20	24	26	28	22															
10 *O. dukei*	32	33	32	20	22	22	25	20	8														
11 *O. volvulus*	30	31	30	22	23	21	23	18	9	7													
12 *O.* sp. 'Siisa'	30	31	30	21	24	22	20	17	12	6	5												
13 *O. ochengi*	33	34	33	22	25	19	23	18	13	7	6	3											
14 *O. skrjabini*	29	30	29	23	27	26	25	23	29	27	29	24	25										
15 *O. eberhardi*	31	32	31	29	37	30	37	27	32	32	31	28	27	23									
16 *O. flexuosa*	24	25	24	23	28	23	29	22	29	28	28	27	26	23	22								
17 *O. dewittei japonica*	24	25	24	32	34	29	32	27	36	38	35	34	33	29	33	27							
18 *O. ramachandrini*	27	28	27	25	31	21	31	29	29	29	30	29	26	32	30	23	26						
19 *O. suzukii*	22	22	22	33	37	31	36	36	36	38	37	36	37	29	35	28	33	24					
20 *O. armillata*	31	31	31	29	36	29	34	31	24	28	29	26	25	26	24	25	31	25	26				
21 *Loxodontofilaria caprini*	26	27	26	24	28	27	30	19	27	26	26	25	26	21	23	26	27	31	32	27			
22 *Setaria digitata*	57	58	57	53	55	51	60	57	58	58	57	58	55	52	56	54	57	52	59	52	54		
23 *Thelazia callipaeda*	70	71	70	71	75	69	75	65	73	74	74	75	72	70	64	64	66	67	75	63	65	74	

Table V. – Pairwise comparisons of the numbers of nucleotide differences over 375 sites of the 12S rRNA gene sequences among *Onchocerca* species and other filaria species.

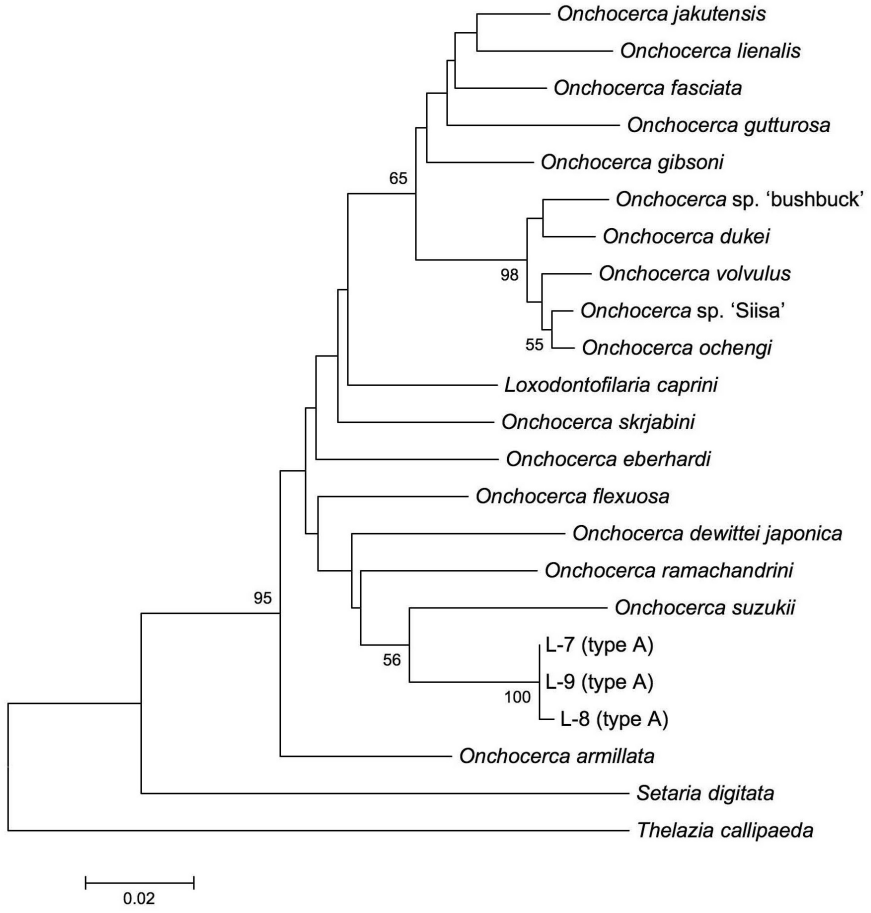

Fig. 2. – Neighbor-joining phylogenetic tree based on the partial mitochondrial 12S rRNA gene sequences of *Onchocerca* spp. Numbers at the nodes are the bootstrap confidence values after 500 replicates. Values more than 50 % are shown. The scale bar indicates the distance in substitutions per nucleotide. L-1-L-9, Infective larvae of *Onchocerca* spp. from wild-caught black flies. Alphabet in parenthesis indicates the type by morphometric classification.

sity of this genus in Japanese wild ruminants is still not completely understood.

In conclusion, we found for the first time that anthropophilic *S. bidentatum* was naturally infected with infective larvae of *O. dewittei japonica*, the causative agent of zoonotic onchocerciasis in Japan, and a related *O.* sp. Analysis of mitochondrial sequences enabled us to identify the infective larvae of three *Onchocerca* species, two of which are morphologically indistinguishable. This study is the first report of molecular identification of infective larvae of *Onchocerca* species found in wild-caught black flies. It strongly suggests that *S. bidentatum* plays a role as a vector in the transmission of zoonotic onchocerciasis due to *O. dewittei japonica* in Japan.

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