GENETIC EVIDENCE FOR THE PRESENCE OF TWO SPECIES OF *ONCHOCERCA* FROM THE WILD BOAR IN JAPAN

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Summary:

In order to clarify the genetic differences between *Onchocerca dewittei japonica*, the causative agent of zoonotic onchocerciasis in Japan and a related undescribed *Onchocerca* sp., both parasitizing wild boar (*Sus scrofa*) of which the infective larval stages are indistinguishable from each other, we compared the sequences of the mitochondrial cytochrome *c* oxidase subunit 1 (CO1) gene region from four infective larvae (recovered from experimentally infected black flies), one microfilaria, and one adult of *O. dewittei japonica*, and from one infective larva (recovered from an experimentally infected black fly), one microfilaria, and a pool of several microfilariae of *O.* sp. The length of the CO1 gene region was 649 bp for all samples but there was a difference of 8.8 to 9.4 % in the sequences between the two species although there were intraspecific variations of 0 to 0.5 %. The CO1 sequences of *O.* sp. did not correspond to any of those deposited in the databases. Our study provides evidence that *O. dewittei japonica* and *O.* sp. are genetically different from each other.

KEY WORDS : *Onchocerca*, wild boar, infective larvae, *Simulium*, vector, zoonosis, genetic differences.

Résumé : CONFIRMATION DE L'EXISTENCE DE DEUX ESPÈCES D'ONCHOCERQUES CHEZ LE SANGLIER AU JAPON PAR LES DIFFÉRENCES GÉNÉTIQUES

Pour confirmer l'existence de deux espèces d'onchocerques chez le sanglier Sus scrofa *au Japon,* Onchocerca dewittei japonica, *agent de l'onchocercose zoonotique au Japon, et* Onchocerca *sp., dont l'adulte est inconnu et qui a des larves infectantes semblables, nous avons déterminé et comparé les séquences de la région du gène mitochondrial de la sous-unité 1 de la cytochrome oxydase c (CO1) de quatre larves infectantes (récoltées chez des simulies infestées expérimentalement), une microfilaire et un adulte d'*O. dewittei japonica, *et d'une larve infectante (récoltée chez une simulie infestée expérimentalement), une microfilaire et un groupe de plusieurs microfilaires d'*Onchocerca *sp. La longueur de la région du CO1 est de 649 bp pour tous les lots mais il y a une différence de 8,8 à 9,4 % entre les séquences des deux espèces alors que les variations intraspécifiques sont de 0 à 0,5 %. Les séquences de CO1 d'O. sp. ne correspondent à aucune de celles déposées dans les banques de données. Notre étude apporte la preuve que* O. dewittei japonica *and O. sp. sont génétiquement différentes.*

MOTS CLÉS : Onchocerca, *sanglier, larve infectante,* Simulium, *vecteur, zoonose, différences génétiques.*

INTRODUCTION

Zoonotic onchocersiasis is rare in humans. Only 15 cases including five cases in Japan have been reported (Beaver *et al.*, 1989; Hashimoto *et al.*, 1990; Takaoka *et al.*, 1996, 2001, 2004, 2005). In all the Japanese cases, the infections were caused by *Onchocerca dewittei japonica* Uni, Bain & Takaoka, 2001, a common filarial parasite of wild boar *Sus scrofa* Linnaeus, 1758, in Japan (Takaoka *et al.*, 2001; Uni *et al.*, 2001), but the life cycle of which was unknown. Recently we have proved experimentally that six local black-fly species are suitable for the development of *O. dewittei japonica* microfilariae to the infective (third stage) larva, suggesting that *Simulium* species are vectors of the parasite (Fukuda *et al.*, 2008). During this investigation, we also found microfilariae of a species of *Onchocerca* (longer than those of *O. dewittei japonica*) from wild boar and demonstrated the complete larval development of this unnamed *Onchocerca* sp. in female black flies. Although the adult was unknown, this filaria was tentatively assumed to be a distinct species. Interestingly, the infective larvae of these two *Onchocerca* species are morphologically indistinguishable (Fukuda *et al.*, 2008).

To determine the natural vectors of *O. dewittei japonica*, accurate identification of the infective larvae of zoonotic *Onchocerca* species from wild-caught black flies is needed. In this study, we aimed to identify the two morphologically similar *Onchocerca* spp. of wild boar in Japan by differences in the sequences of the mitochondrial cytochrome *c* oxidase subunit 1 (CO1) gene.

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MATERIALS AND METHODS

Specimens examined

Table I shows the specimens examined in this study and their measurements. An adult worm of *O. dewittei japonica* was recovered from a wild boar, captured in Oita Prefecture, Kyushu, Japan by a licensed hunter. Species identification followed Uni *et al.* (2001). Five infective larvae (four *O. dewittei japonica* and one *O.* sp.) were recovered from the black flies experimentally infected in our previous study (Fukuda *et al.*, 2008). Microfilariae of *O. dewittei japonica* and *O.* sp. were isolated from skin snips of wild boar in Oita. Discrimination of microfilariae between *O. dewittei japonica* and *O.* sp. followed Uni *et al.* (2001) and Fukuda *et al.* (2008).

PCR and sequencing of the partial mitochondrial CO1 gene region

Total DNA was extracted from each sample with a DNeasy Blood & Tissue Kit (QIAGEN, Hilden, Germany). The final volume of each extract was 50-100 µl. The partial mitochondrial CO1 gene region was amplified with the primer set (CO1intF-CO1intR) described by Casiraghi *et al.* (2001). The reaction mixture (50 µl) contained 1 x 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 1-3 µl of template DNA. PCR was performed under the following 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. PCR products were purified with a QIAquick PCR Purification Kit (QIAGEN) and directly sequenced using a BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) and an Applied Biosystems 3130 Genetic Analyzer (Applied Biosystems). Sequences were deposited in DDBJ/EMBL/GenBank databases under accession numbers AB518689-AB518694 (Table I).

Data analysis

The sequences obtained were aligned with published sequences of nine *Onchocerca* species and two other filarial species. Using this alignment, sequences were compared, a neighbor-joining tree (Saitou & Nei, 1987) was constructed, and bootstrap probabilities were estimated, all by MEGA 4.0.2 based on 580 bp available for comparison (Tamura *et al.*, 2007). The Kimura 2-parameter method was used for estimation of evolutionary distances in the tree. Used GenBank database accession numbers were as follows: *O. dewittei japonica* (AM749266, AM749267), *O. eberhardi* Uni & Bain, 2007 (AM749268), *O. gibsoni* (Cleland & Johnston, 1910) (AJ271616), *O. gutturosa* Neumann, 1910 (AJ271617), *O. lupi* Rodonaja, 1967 (EF521409), *O. ochengi* Bwangamoi, 1969 (AJ271618), *O. skrjabini* Ruklyadev, 1964 (AM749269), *O. suzukii* Yagi, Bain & Shoho, 1994 (AM749275), *O. volvulus* (Leuckart, 1893) (AF015193), *Thelazia callipaeda* Railliet & Henry, 1910 (AM042549) and *Wuchereria bancrofti* (Cobbold, 1877) (AJ271612).

RESULTS

Sequences of the mitochondrial CO1 gene region of nine samples of *O. dewittei japonica* and *O.* sp. were determined (Table I). The length of this region in the two species was 649 bp. Interspecific nucleotide differences between *O. dewittei japonica* and *O.* sp. ranged from 8.8 to 9.4 %, while intraspecific variation within *O. dewittei japonica* and *O.* sp. was 0-0.5 % and 0-0.3 %, respectively. Interspecific amino acid difference between both species was 0.9 %, while no intraspecific variation was found in each species. The sequences were compared with those of all the *Onchocerca* species available in GenBank. Table II shows the numbers of nucleotide differences over 580

***Onchocerca* spp.**	**Stage of worm**	**No. of worm**	**Body length (µm)**	**Body width (µm)**	**GenBank accession no.**
O. dewittei japonica	Adult (A)	1	–	–	AB518689
	Third stage larva (L3-1)	1	943.6	17.9	–[a]
	Third stage larva (L3-2)	1	328.2*	20.5	–[a]
	Third stage larva (L3-3)	1	1,059.0	19.5	AB518690
	Third stage larva (L3-4)	1	806.8**	20.2	AB518691
	Microfilaria (Mf)	1	205.1	5.1	AB518692
O. sp. from wild boar	Third stage larva (L3)	1	964.1	22.6	AB518693
	Microfilaria (Mf)	1	364.1	6.7	–[b]
	Microfilariae (Mfs)	10	292.3-333.3	6.2-8.2	AB518694

* The length of one fragment. ** Total length of three fragments. [a] The sequence was identical to that of *O. d. j.* (A). [b] The sequence was identical to that of *O.* sp. (L3).

Table I. – Measurements and stages of specimens examined in this study.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
1 *O. dewittei japonica* (A)																					
2 *O. dewittei japonica* (L3-1)	0																				
3 *O. dewittei japonica* (L3-2)	0	0																			
4 *O. dewittei japonica* (L3-3)	1	1	1																		
5 *O. dewittei japonica* (L3-4)	2	2	2	3																	
6 *O. dewittei japonica* (Mf)	1	1	1	2	1																
7 *O. dewittei japonica* (AM749266)	2	2	2	3	0	1															
8 *O. dewittei japonica* (AM749267)	0	0	0	1	2	1	2														
9 *O.* sp. from wild boar (L3)	50	50	50	51	48	49	48	50													
10 *O.* sp. from wild boar (Mf)	50	50	50	51	48	49	48	50	0												
11 *O.* sp. from wild boar (Mfs)	51	51	51	52	49	50	49	51	1	1											
12 *O. eberhardi*	44	44	44	43	44	43	44	44	52	52	53										
13 *O. suzukii*	54	54	54	55	54	55	54	54	59	59	59	53									
14 *O. skrjabini*	48	48	48	49	48	47	48	48	53	53	54	42	49								
15 *O. gutturosa*	60	60	60	61	60	61	60	60	60	60	60	51	47	43							
16 *O. gibsoni*	62	62	62	63	60	61	60	62	47	47	47	53	59	39	36						
17 *O. volvulus*	58	58	58	57	58	59	58	58	56	56	56	40	49	49	36	41					
18 *O. ochengi*	57	57	57	56	57	58	57	57	53	53	53	38	47	50	38	40	10				
19 *O. lupi*	61	61	61	62	61	62	61	61	67	67	68	62	61	48	47	52	56	61			
20 *Wuchereria bancrofti*	71	71	71	72	71	72	71	71	67	67	67	70	69	66	62	59	67	68	66		
21 *Thelazia callipaeda*	95	95	95	96	94	94	94	95	97	97	97	89	94	78	82	83	91	96	88	84	

Table II. – Pairwise comparisons of the numbers of nucleotide differences over 580 sites among *Onchocerca* species and other filaria species.

sites of *Onchocerca* spp. Compared with differences among the other *Onchocerca* spp., equivalent differences were observed between the sequences of two *Onchocerca* species from wild boar. The sequences of *O.* sp. did not correspond with any of those registered in databases. The phylogenetic relationship between the two *Onchocerca* species from wild boar and eight other *Onchocerca* species is shown in Figure 1.

DISCUSSION

In this study, clear differences were shown in the CO1 gene sequence between *O. dewittei japonica* and *O.* sp. from wild boar. Ferri *et al.* (2009) suggested that the mitochondrial CO1 gene region was an appropriate genetic marker for DNA-based species identification of filarioid nematodes and detection of putative new species. According to them, if the genetic distance of the CO1 gene between two filarioid nematode specimens is greater than threshold value of 4.8 %, it is possible to conclude that they are different species. This standard applied to our case with a genetic distance of 8.8-9.4 %. It seems therefore reasonable to consider these two *Onchocerca* species from wild suids to be distinct species.

In our previous investigation to search for the potential vector of zoonotic onchocerciasis in Japan, we found three types of infective filarial larvae (designated as types I, II, and III based on their size and morphology) from wild black flies collected in a cattle shed (Takaoka & Bain, 1990). In addition, we presumed that type II infective larva might be *O. gutturosa* of cattle, *O. dewittei japonica*, or the unnamed *Onchocerca* sp. of wild boar on the basis of the morphometric observation of experimentally produced infective larvae (Fukuda *et al.*, 2008). The present study shows that morphologically similar infective larvae of these three *Onchocerca* species are clearly distinguishable by comparison of their CO1 gene sequences.

According to the original description of *O. dewittei japonica* (Uni *et al.*, 2001), the lengths of the microfilariae found from the uterus of female worms and skin of wild boar ranged between 183-210 µm. The longer *Onchocerca*-like microfilariae (about 1.5 times the length of *O. dewittei japonica*) found in wild boar were provisionally referred to *Onchocerca* sp. in our recent study (Fukuda *et al.*, 2008). The CO1 gene analysis of adult *O. dewittei japonica* confirmed that the short microfilariae from wild boar corresponded with this species. Co-infection of wild boar, *Sus scrofa*, and wart hog, *Phacochoerus aethiopicus* (Pallas, 1766), with two species of *Onchocerca* has been reported in Malaysia (Bain *et al.*, 1977) and Cameroon in Africa (Bain *et al.*, 1993). In these cases, as in Japan, microfilariae differred (long *versus* short) and the corresponding adults were sought but not found. In Malaysia, no transmission study was performed. In Cameroon, the two types of microfilariae had close but different *Simulium* vectors and the infective larvae were distinguishable (Wahl & Bain, 1995). The present molecular analysis of Japanese material, by confirming the morphological diagnosis based on microfilariae, also supports the interpretation of dual *Onchocerca* infections in suids.

In conclusion, we could reliably separate *O. dewittei japonica* from *O.* sp. at the infective stage by analysis

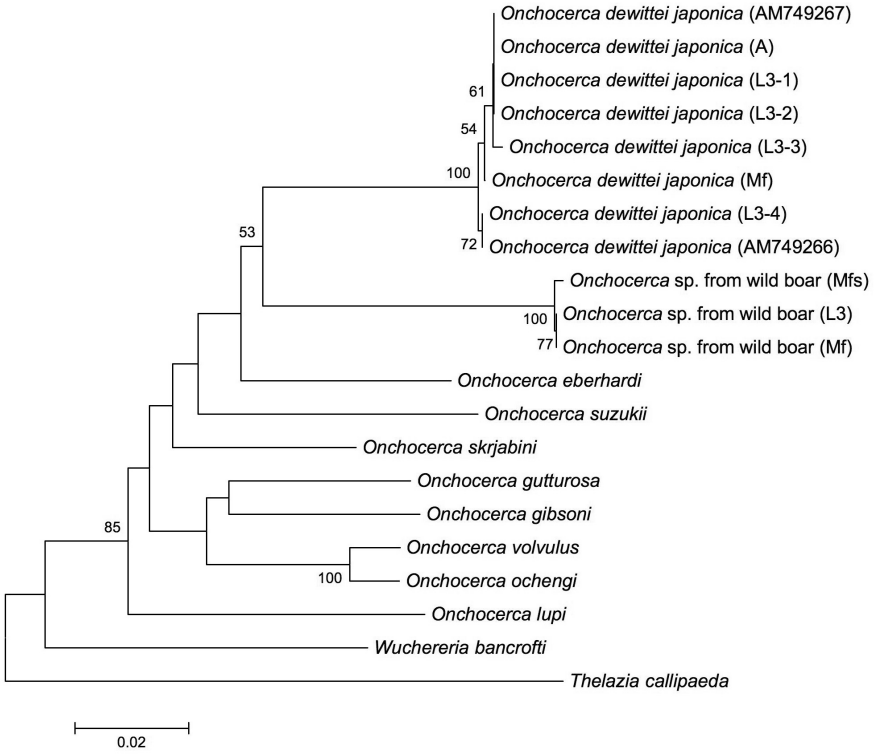

Fig. 1. – Neighbor-joining phylogenetic tree based on the partial mitochondrial CO1 gene sequences of *Onchocerca* spp. Numbers at the nodes are the bootstrap confidence values after 500 replicates. Values more than 50 % are shown. The scale bar indicates the distance in substitutions per nucleotide.

of the CO1 gene sequences. Molecular identification of three *Onchocerca* species including two species of wild boar, *O. dewittei japonica* and *O.* sp., from wild-caught black flies in Japan will be reported in a subsequent paper.

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REFERENCES

Bain O., Wahl G. & Renz A. *Onchocerca ramachandrini* n. sp. from the warthog in Cameroon. *Annales de Parasitologie Humaine et Comparée*, 1993, *68*, 139-143.

Bain O., Ramachandran C.P., Petter F. & Mak J.W. Description d'*Onchocerca dewittei* n. sp. (Filarioidea) chez *Sus scrofa* en Malaisie. *Annales de Parasitologie Humaine et Comparée*, 1977, *52*, 471-479.

Beaver P.C., Yoshimura H., Takayasu S., Hashimoto H. & Little M.D. Zoonotic *Onchocerca* in a Japanese child. *The American Journal of Tropical Medicine and Hygiene*, 1989, *40*, 298-300.

Casiraghi M., Anderson T.J.C., Bandi C., Bazzocchi C. & Genchi C. A phylogenetic analysis of filarial nematodes: comparison with the phylogeny of *Wolbachia* endosymbionts. *Parasite*, 2001, *122*, 93-103.

Ferri E., Barbuto M., Bain O., Galimberti A., Uni S., Guerrero R., Ferté H., Bandi C., Martin C. & Casiraghi M. Integrated taxonomy: traditional approach and DNA barcoding for the identification of filarioid worms and related parasites (Nematoda). *Frontiers in Zoology*, 2009, *6*, 1.

Fukuda M., Takaoka H., Uni S. & Bain O. Infective larvae of five *Onchocerca* species from experimentally infected *Simulium* species in an area of zoonotic onchocerciasis in Japan. *Parasite*, 2008, *15*, 111-119.

Hashimoto H., Murakami I., Fujiwara S., Takayasu S., Takaoka H., Uga S., Akao N., Kondo K. & Yoshimura H. A human case of zoonotic onchocerciasis in Japan. *The Journal of Dermatology*, 1990, *17*, 52-55.

Saitou N. & Nei M. The neighbor-joining method: A new method for reconstructing phylogenetic trees. *Molecular Biology and Evolution*, 1987, *4*, 406-425.

Takaoka H. & Bain O. Infections of blackflies (Diptera: Simuliidae) with three types of zoonotic *Onchocerca* larvae in Oita, Japan. *Japanese Journal of Tropical Medicine and Hygiene*, 1990, *18*, 1-10.

Takaoka H., Bain O., Tajimi S., Nakayama I., Kashima K., Aoki C., Korenaga M. & Otsuka Y. Second case of zoonotic *Onchocerca* infection in a resident of Oita, Japan. *Parasite*, 1996, *3*, 179-182.

Takaoka H., Bain O., Uni S., Korenaga M., Kozek W.J., Shirasaka C., Aoki C., Otsuka Y., Fukuda M., Eshita Y. & Daa T. Zoonotic onchocerciasis caused by a parasite from wild boar in Oita, Japan: A comprehensive analysis of morphological characteristics of the worms for its diagnosis. *Parasite*, 2004, *11*, 285-292.

Takaoka H., Bain O., Uni S., Korenaga M., Tada K., Ichikawa H., Otsuka Y. & Eshita Y. Human infection with

Onchocerca dewittei japonica, a parasite from wild boar in Oita, Japan. *Parasite*, 2001, *8*, 261-263.

TAKAOKA H., YANAGI T., DAA T., ANZAI S., AOKI C., FUKUDA M., UNI S. & BAIN O. An *Onchocerca* species of wild boar found in the subcutaneous nodule of a resident of Oita, Japan. *Parasitology International*, 2005, *54*, 91-93.

TAMURA K., DUDLEY J., NEI M. & KUMAR S. MEGA4: Molecular evolutionary genetics analysis (MEGA) software version 4.0. *Molecular Biology and Evolution*, 2007, *24*, 1596-1599.

UNI S., BAIN O., TAKAOKA H., MIYASHITA M. & SUZUKI Y. *Onchocerca dewittei japonica* n. subsp., a common parasite from wild boar in Kyushu Island, Japan. *Parasite*, 2001, *8*, 215-222.

WAHL G. & BAIN O. Development by injection in *Simulium damnosum* s. l. of two *Onchocerca* species from the wart hog to infective larvae resembling type D larvae (Duke, 1967). *Parasite*, 1995, *2*, 55-62.

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