

## NOTE Parasitology

***Dirofilaria immitis* Infection of a Snow Leopard (*Uncia uncia*) in a Japanese Zoo with Mitochondrial DNA Analysis**Koichi MURATA<sup>1)</sup>, Tokuma YANAI<sup>2)</sup>, Takeshi AGATSUMA<sup>3)</sup> and Shigehiko UNI<sup>4)</sup>

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**ABSTRACT.** Three dog heartworms (*Dirofilaria immitis*) were detected in the lumen of the right cardiac ventriculus and of the pulmonary artery of a captive female snow leopard (*Uncia uncia*) that died of pancreatic carcinoma at a zoo in Japan. Neither clinical respiratory nor circulatory symptoms caused by the heartworm infection were observed. The filarial worms were identified as *D. immitis* from the morphologic characteristics of the esophagus, the presence of faint longitudinal ridges on the cuticular surface, the situation of vulva posterior to the esophagus, and the measurements of the body. The heartworms from the snow leopard were identical to that of *D. immitis* from dogs in the sequence of the cytochrome oxidase I region in the mitochondrial DNA. This host record is the first of *D. immitis* in *U. uncia*.

**KEY WORDS:** *Dirofilaria immitis*, Japan, snow leopard.

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The snow leopard (*Uncia uncia*) inhabits mountainous areas at elevations of 3,000–4,500 m in Central Asia and is listed as an endangered species in the International Union for Conservation of Nature and Natural Resources (IUCN) Red List of Threatened Animals; the wild population is estimated from 4,500 to 7,400 [8]. The animal is still decreasing in number because of scanty suitable habitats and poaching. A captive-propagation plan at zoos has been organized internationally and about 700 snow leopards are kept at zoos worldwide.

An adult female snow leopard kept in a zoo in Kobe (34°41'N, 135°11'W), Japan, died of prolonged depressed appetite for about one month. Necropsy revealed *Dirofilaria immitis* infection together with pancreatic carcinoma, the main cause of death. Morphologic characteristics and the sequence of the mitochondrial DNA were examined of the filarial parasites. The finding of *D. immitis* constitutes a new host record in *U. uncia*. Our report of heartworm infection in a snow leopard will contribute to the veterinary care of captive felids.

The animal, a 13-year-old female snow leopard named Yukie (International Studbook Number, 1163) was born on April 27, 1987 at Sapporo Municipal Maruyama Zoo (43°03'N, 141°21'W) and transferred to the Kobe Municipal Oji Zoo on February 10, 1988. Depressed appetite was first observed on November 28, 2000 (day 0 of sickness) and vomiting on days 11, 16, and 19. Anorexia and disturbance with gait were seen on day 25 and the leopard died on day 27, although her physical condition was slightly improved after the administration of enrofloxacin (5.5 mg/kg, Bayer Yakuhin, Ltd., Tokyo, Japan), ketoprofen (2 mg/kg, Chugai Pharmaceutical Co., Ltd., Tokyo), and cimetidine (4.4 mg/kg, Fujisawa Pharmaceutical Co., Ltd., Tokyo) on days 5 to 24. No clinical signs involving the respiratory or

circulatory system such as coughing or anemia were detected.

The animal was necropsied about 5 hr after death. The body weight was 45.5 kg. A female filarioid was found in the right ventricle of the heart (Fig. 1-a), immediately examined under a stereoscopic light microscope, and preserved at -30°C for DNA analysis. Two female filarioids were found in the lumen of pulmonary artery and fixed in 10% neutral buffered formalin. In the head of the pancreas was found a round nodule measuring 50 × 30 mm. Visceral organs and tissues were fixed in 10% neutral buffered formalin. The tissues were then dehydrated through a graded series of alcohol and toluene concentrations before embedded in paraffin, sectioned 5 μm thick and stained with hematoxylin and eosin. Well-differentiated adenocarcinoma originating from the endocrine glands infiltrated the head of pancreas. No pathological changes were found in the lungs nor in the heart, and no heartworms were detected in the other organs.

Differential interference microscopy was used to examine the morphologic characteristics of the three filarioids: the worms had no lips in the mouth (Fig. 1-b); small labial and cervical papillae were present on the head, and amphids were situated on the sides of the head. Esophagus was divided into muscular and glandular parts. Vulva (Fig. 1-c) was located posterior to the esophagointestinal junction. Eggs were found in the uterus (Fig. 1-d) but no microfilariae were found. Short, low longitudinal ridges were found on the cuticle of the midbody when three worms were examined without a cover slip (Fig. 1-e). Posterior extremity rounded. Measurements of two filarioids obtained from the pulmonary artery were as follows (the first of the paired values shows that of the shorter worm): body length, 19.0 and 28.0 cm; body width, 0.77 and 0.99 mm; the distance of nerve ring from anterior end, 0.54 and 0.55 mm; esophagus

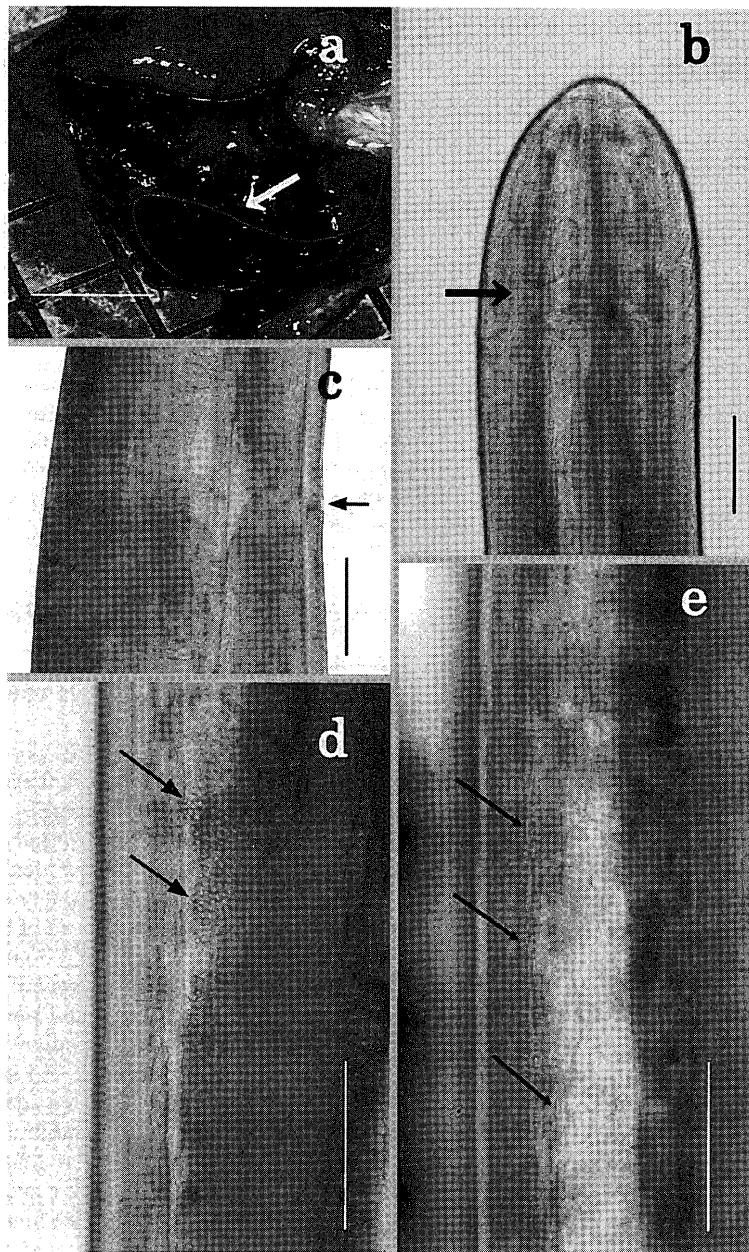


Fig. 1. a: Female *D. immitis* found in the right cardiac ventricle of a snow leopard. Bar, 3.0 cm. b: Differential-interference contrast-microscopic image of the head of the female worm. Nerve ring (arrow). Bar, 0.5 mm. c: Vulva (arrow). Bar, 0.4 mm. d: Eggs (arrows) in the uterus. Bar, 1.0 mm. e: Surface of the cuticle with low, short longitudinal ridges (arrows) on the cuticle of three worms examined without a cover slip. Bar, 1.0 mm.

in length, 1.78 and 1.75 mm (muscular part, 0.90 and 0.77 mm and glandular part, 0.88 and 0.98 mm), vulva from anterior end, 2.78 and 4.45 mm, and tail, 0.24 mm in length in both specimens. The female filarioids from the right cardiac ventricle measured 26.2 cm and 0.90 mm in body length and width, respectively. In venous blood sampled before death

and postmortem, no microfilariae were detected. The nematode specimens were deposited at the Meguro Parasitological Museum, Tokyo, Japan with the accession number of MPM-Collection No. 18806.

The worms were identified as *D. immitis* (Leidy, 1856) (Nematoda: Filarioidea) with such morphological features

as the esophagus consisting of muscular and glandular parts, the presence of postesophageal vulva, and no marked longitudinal ridges on the surface of cuticle of female worms [2, 12]. The measurements of the present specimens were closely akin to those of *D. immitis* taken from dogs [12]. The cuticular surface of female and male *D. immitis* specimens was compared with that of *D. ursi* by scanning electron microscopy, and the short, low longitudinal ridges were found on *D. immitis* of both sexes [14]. Our specimens had the same cuticular characteristics when examined without cover slips. On the other hand, *D. ursi* has conspicuous longitudinal ridges on the cuticle in both sexes. *Dirofilaria ursi* and *D. immitis* are known to occur in Japan, and the former species is frequently found in black bears [13].

DNA was examined to clarify the phylogenetic difference between both *D. immitis* specimens from the leopard and domestic dog. Genomic DNA was extracted from the whole worm as follows. The worm was incubated using Invitrogen extraction kit, Invitrogen Japan K. K., Tokyo in extraction buffer containing sodium dodecyl sulfate and proteinase K. The solubilized sample was treated with phenol and chloroform. The cytochrome oxidase I (COI) region in the mitochondrial DNA of the worm was examined because the sequence from several nematode species is known. A 656-nucleotide fragment of COI region was amplified by the polymerase chain reaction (PCR) with primers COI intF (5'-TGATTGGTGGTTTTGGTAA-3') and COI intR (5'-ATAAGTACGAGTATCAATATC-3'). Amplification was done in 30 cycles of 94 °C for 1 min, 50 °C for 2 min, and 72 °C for 3 min with a final volume of 50  $\mu$ l containing 3.2 pmol of each primer, deoxynucleoside triphosphates (0.2 mM each), and Taq polymerase (1.75 U). Products of PCR were precipitated with ethanol and suspended in distilled water, and a portion was sequenced with a Prism kit (ABI, Foster City, CA). Sequencing was done with the same primers as for PCR and two additional primers, T7-2 (5'-TAATGAGTAGTTTTGAATGGC-3') and SP6-2 (5'-ACTCGACTCCGGCAACTAGC-3'). The reaction mixture was examined with a DNA sequencer (ABI Prism 310). Sequence alignment was done with the programs CLUSTAL V [5] and GENETYX.MAC ver. 6.0 (Genetyx, Tokyo).

The sequences of *D. immitis* from the snow leopard and domestic dogs deposited in a database with the accession number AJ271613 [4] were compared and found 100% identical to each other.

*D. immitis* has been detected not only from canids but also from many other carnivorous mammals [1]. *D. immitis* infection has been found in several species of wild felids: the tiger (*Panthera tigris*), panther (*P. pardus*), and clouded leopard (*Neofelis nebulosa*) [6, 9, 15]. Serum antibodies against the parasite were detected in a wild mountain lion (*Felis concolor*) [10]. The snow leopard is a newly reported host of *D. immitis*.

In felids, the rates of *D. immitis* infection are lower than those in canids, and microfilariae are rarely detected in the peripheral blood even if the parasite is present [9]. In our case, no microfilariae were found in the uterus of worms, possibly because no male worms were present. However, *D. immitis* becomes more easily detected in domestic cats (*F. catus*) because of the improvement of diagnostic techniques such as ELISA and tests for serum antibodies and antigens [7, 11]. Respiratory and circulatory diseases, and hepatosis in domestic cats caused by *D. immitis* with or without microfilariae were clinically reported [3]. Although *D. immitis* infection did not seem the direct cause of death in our case, it may cause some diseases also in this species of animal as well as in domestic cats, as already known. Veterinary management may be necessary to protect captive snow leopards from heartworm infection in zoos where various carnivorous animals that are possible hosts of *D. immitis* are kept.

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