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DNA barcoding of *Anoplocephala perfoliata* derived from a draft horse (Ban'ei horse) in Hokkaido, Japan

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A two-year-old male Japanese draft horse (known as a "Ban'ei horse") excreted eight cestodes. Based on their morphological features, they were identified as Anoplocephala perfoliata. The partial mitochondrial cytochrome c oxidase subunit 1 (COI) sequences of the worms were nearly identical to A. perfoliata isolated from horses in Europe. The results of phylogenetic analyses of COI revealed that our samples and the European isolates formed the same clade, which was separate from Chinese and Australian isolates. Ban'ei horses were developed by crossbreeding draft horses imported from European countries in the 1900s. Our results suggest that A. perfoliata was transported to Hokkaido with horses from Europe. To our knowledge, this is the first report of A. perfoliata infection in a Japanese draft horse.

Key words: *Anoplocephala perfoliata, DNA barcoding, draft horse, Equus caballus, Hokkaido*

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Adult tapeworms of the three cosmopolitan species, *Anoplocephala perfoliata*, *Anoplocephala magna*, and *Equinia mamillana* (syn. *Anoplocephaloides mamillana*, *Paranoplocephala mamillana*), are found in the intestines of equine definitive hosts [4]. The soil-dwelling mites belonging to Oribatida serve as intermediate hosts of them [5]. These three species can be easily differentiated based on the morphological characteristics of their adult worms. *Anoplocephala perfoliata* is distinguished from *A. magna* by the presence of four lappets protruding from the base of the scolex [13, 24]. According to the keys in the reviews by Haukisalmi [11], *E. mamillana* can be diagnosed based on features such as laterally directed suckers and the arrangement of genital organs. Among these three cestodes, *A. perfoliata* is the dominant species in horses worldwide [8, 24]. Histopathological lesions, such as mucosal damage and inflammation of the lamina propria and submucosa, have been observed at the site of attachment of adult worms of *A. perfoliata* [18]. These lesions were associated with ileocecal colic in horses [19]. Massive infection could cause intestinal ulceration, necrosis, rupture, and perforation [2, 12, 20].

A few reports have shown a high prevalence of *A. perfoliata* in thoroughbred and Anglo-Arabian breeds in Honshu and Hokkaido, Japan [7, 21, 28, 31]. In most previous studies in Japan, only one key morphological characteristic, the presence of lappets, has been used for diagnosis, and there are no molecular data on *A. perfoliata* derived from Japanese horses. In this study, we aimed to determine DNA barcodes of *A. perfoliata* in Japan and compare them with those of other countries to infer the origin of Japanese *A. perfoliata*. We have recently come across a case of a draft horse that excreted cestodes in Hokkaido, the northernmost island of Japan. The adult worms recovered from the horse were morphologically diagnosed as *A. perfoliata*, and DNA barcodes were determined based on the nuclear 28S ribosomal RNA gene (28S rDNA) and mitochondrial cytochrome *c* oxidase subunit 1 (COI). This is the first report of DNA barcoding on Japanese *A. perfoliata* and the first case of *A. perfoliata* infection in a Japanese draft horse.

In June 2023, a two-year-old male Japanese draft horse (known as a "Ban'ei horse") presented with a fever and was brought to Athena Integrative Veterinary Care in Obihiro, Hokkaido, Japan. The horse was born in Nakafurano, Hokkaido. It was reared individually, and no horses had

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recently been imported from foreign countries to the stable. During hospitalization, the horse excreted eight cestodes without anthelmintic administration. The adult worms were flattened between slide glasses and fixed in 70% ethanol after cutting off a small piece of the proglottids for DNA template preparation. The flattened worms were stained with Schneider's acetocarmine and mounted using Canada balsam. Morphological observations and measurements of objects were carried out using a microscope with a digital camera (DP74, Olympus, Japan) and the imaging software cellSens 1.16 (Olympus).

Morphological descriptions were based on three adult specimens. All measurements are presented as means, with the minimum-maximum range in parentheses. Whole body, 46 (31–54) mm in length and 8.8 (8.3–9.1) mm in maximum width. Scolex rounded, 1.3 (1.0–1.6) mm long by 2.1 (2.0–2.2) wide, with four suckers (Fig. 1A). Four lappets posterior to suckers, two on the dorsal and two on the ventral surface. Lappet 0.56 (0.50–0.61) mm long by 0.45 (0.33–0.52) mm wide. Segment craspedote. Mature proglottid 0.30 (0.23–0.34) mm long by 5.8 (4.9–6.9) mm wide (Fig. 1B). Genital apparatus single. Genital pore unilateral. Cirrus armed with small spines. Testes 126 (109–156) in number, scattered in medullary parenchyma. External and internal seminal vesicle, 1.2 (1.1–1.3) mm long by 6.0 (5.5–6.4) mm wide and 0.8 (0.7–1.0) mm long by 4.7 (4.1–5.1) mm wide, respectively. Ovary poral, 0.81 (0.72–0.89) mm long by 0.90 (0.71–1.1) mm wide. Vitellaria well lobed, 0.10 (0.092–0.11) mm long by 0.3 (0.26–0.34) mm wide. Uterus transverse, tubular, restricted to medulla. The morphological features of the cestodes in the present study were consistent with those of previous reports [3, 13, 24].

DNA barcodes based on the 28S rDNA and COI were generated for the eight recovered worms. DNA template preparation, PCR, and sequencing were performed as previously described [22]. Two primer sets were used for amplification of the genes: the primer set of XZ-1 (5′-ACCCGCTGAAYTTAAGCATAT-3′) [29] and 1500R (5′-GCTATCCTGAGGGAAACTTCG-3′) [23] for nuclear 28S rDNA and the primer set of COX-F (5′-GATGTTTTCTTTACATTTATCTGGTG-3′) and COX-R (5′-GCCACCACAAATCAAGTATC-3′) [10] for COI. Each PCR primer was used as a sequencing primer.

The DNA sequences obtained in the present study were compared using a BLAST homology search with those available in the NCBI database (https://blast.ncbi.nlm.nih.gov). The nucleotide dataset for the phylogenetic tree was prepared using the multiple aligner MAFFT [14]. Comparative sequences of related taxa were retrieved from the databases. The COI dataset used for the analyses was composed of 564 nucleotide sites. The genetic software MEGA 11 [25] was employed to select the best-fit substitution model and construct phylogeny. A phylogenetic tree of the COI genes was constructed using the maximum likelihood method under the TN + G model. The robustness of the trees was tested by bootstrapping with 500 replicates. The mean values of pairwise genetic divergence were computed using MEGA 11 under the p-distance model. The DNA sequences determined in this study have been deposited in the DDBJ/ENA/GenBank databases under accession numbers LC795781 (28S rDNA) and LC796742–796748 (COI).

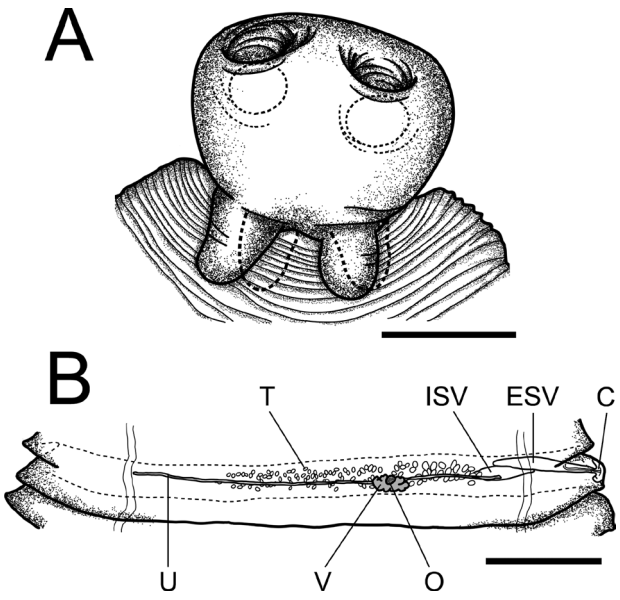

Fig. 1. An adult tapeworm recovered from a draft horse.
A, scolex; B, mature proglottid. T, testes; ISV, internal seminal vesicle; ESV, external seminal vesicle; C, cirrus; U, uterus; V, vitellaria; O, ovary. Bar=1 mm.

Only one sequence of *A. perfoliata* 28S rDNA has been previously deposited in the database, and it was derived from a horse in Australia [30]. The 28S rDNA sequence of the cestode in the present case showed 98.5% similarity to that of *A. perfoliata* from Australia (1234 nucleotide sites). The phylogenetic analyses of COI showed that our isolates and European species (*A. perfoliata* from the Czech Republic [6] and Poland [1]) formed the same clade, which was separate from Chinese [9] and Australian [30] species (Fig. 2). The pairwise distances of COI sequences between the species in the present case and European species were at the intraspecific level (0.007–0.000). We treated them as *A. perfoliata* sensu stricto (s. s.). In contrast, the pairwise divergence of COI between the species in this case and Chinese species and between the species in this case and Australian species reached 0.104 and 0.114, respectively. In a previous study, the difference between *A. perfoliata* s. s. and species from China or Australia has been considered to represent intraspecific diversity [1]. However, it is suggested that they

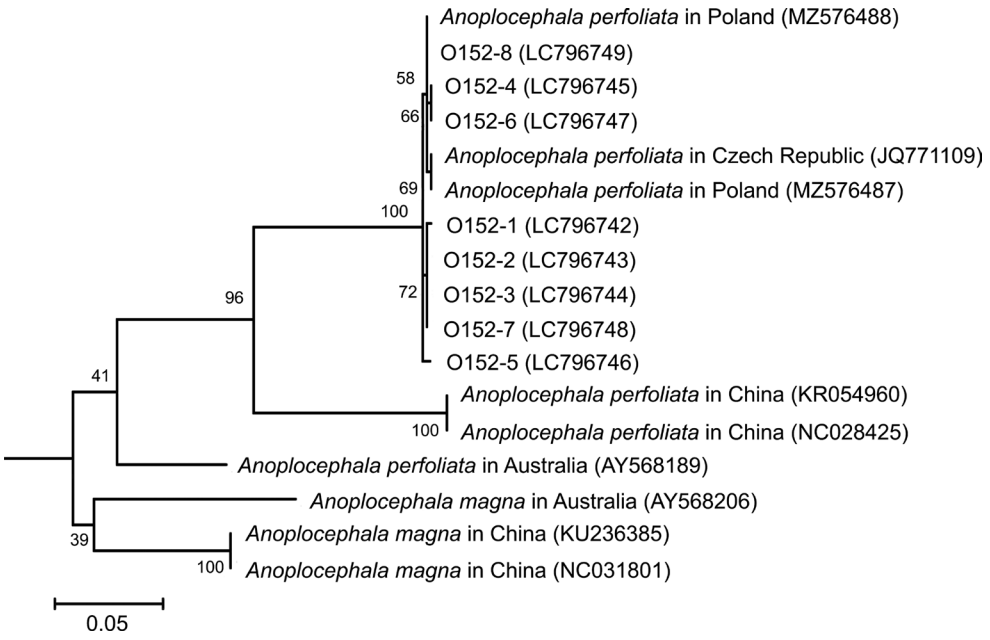

Fig. 2. A maximum-likelihood phylogenetic tree of the genus *Anoplocephala*.
The tree was made with sequences of COI (564 nucleotide sites). The taxa named O152-1–8 are isolates from this study. The DNA accession number of each taxon is shown in parenthesis. *Anoplocephaloides dentata* (accession no. AY568190) was used as an outgroup.

are distinct species because the p-distances between them are too high to regard them as conspecific. It is necessary to collect samples worldwide and perform phylogenetic analyses to determine the presence of cryptic species.

Horses in Hokkaido are not native species. Although it is uncertain when they were originally brought to Hokkaido, the introduction of horses to Hokkaido is assumed to have started in the 17th century with the immigration of people from Honshu, the main island of Japan [27]. These horses were adapted to cold climates and used as packhorses [15]. The import of draft horses from abroad to Hokkaido gained momentum in the 1900s, and they were used for agriculture and transportation [26]. The number of racehorses, such as thoroughbred and Anglo-Arab has increased since the 1960s [17]. Ban'ei Keiba is a Japanese local horse race that is now held only in Obihiro City, Hokkaido. The horses mainly used in the races are called Ban'ei horses, and they were developed by crossbreeding of draft horses, such as the Percheron, Breton, and Belgian, imported from France, Belgium, and other countries [16, 26]. Horse breeds from European countries might have brought *A. perfoliata* s. s. to Hokkaido. In addition, there has surely been many chances for the invasion of *A. perfoliata* directly from Europe or by route via Honshu and other countries. A further survey using DNA barcoding is needed to reveal the diversity of *A. perfoliata* in Japan, because a Chinese or Australian type of *Anoplocephala* might be found.

Tapeworm infections can spread easily by introduction of infected animals. It has been reported that the diagnosis of *A. perfoliata* infection by egg detection in stool samples showed low sensitivity and that the egg positive rate is too low to show the actual rate of infection [21]. Periodic deworming is required to reduce the risk of ileocecal colic and other symptoms caused by *A. perfoliata*.

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