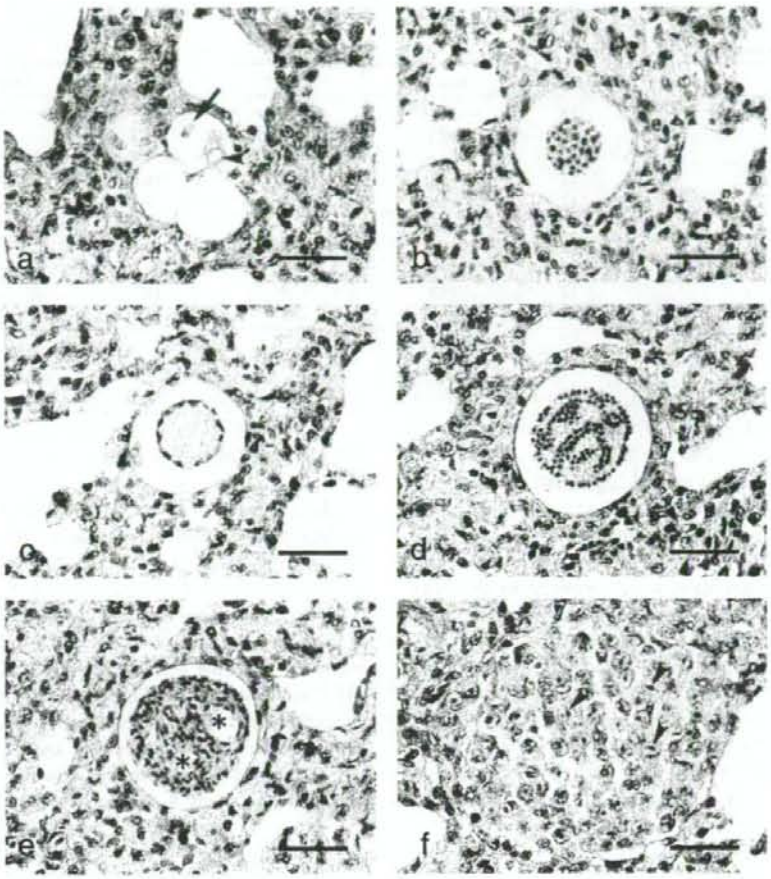

Fig. 2. Various developmental stages of *Hepatozoon ursi* n. sp. detected in the lung of bear N4. a) A trophozoite (arrow) within the cytoplasm of the host cell. Arrowhead indicates the nucleus of the host cell. b–d) Immature schizonts. e) A mature schizont consisting of numerous merozoites and two residual bodies (asterisks). f) A nodule consisting of the accumulation of macrophages. Each macrophage contains a merozoite or a gametocyte (arrowheads). H&E stain. All bars: 30 μm.

In the lungs, various degrees of thickening of alveolar wall and small foci of pyogranulomatous inflammation with infiltrations of neutrophils and eosinophils were observed.

All 14 examined blood samples of bears had *H. ursi* gametocytes (Fig. 3), but obvious hematological changes related to *H. ursi* infection were not observed. Some hematological values with the parasitism rate of gametocytes are shown in Table 3. In two bears (N5 and E2), the parasitism rate was not calculated because of the poor condition of the blood smear.

3.3. Morphological features

3.3.1. Trophozoites, schizonts and merozoites

Trophozoites were found in the parasitophorous vacuoles of the host cells, which had characteristic foamy cytoplasm and resembled swollen macrophages (Fig. 2a). Schizonts were located in the centers of the enlarged parasitophorous vacuoles of the host cells (Fig. 2b–e). Mature and premature schizonts were subspherical in shape and 45.7±4.6×42.7±4.5 μm (37.4–55.4×34.0–52.4 μm, $n=18$) in size (Fig. 2d–e). Each mature schizont contained approximately 80–130 merozoites and had 0–5 residual bodies (Fig. 2e). The merozoites were 7.0±0.7×1.8±0.3 μm (5.7–7.8×1.5–2.4 μm, $n=8$) in size.

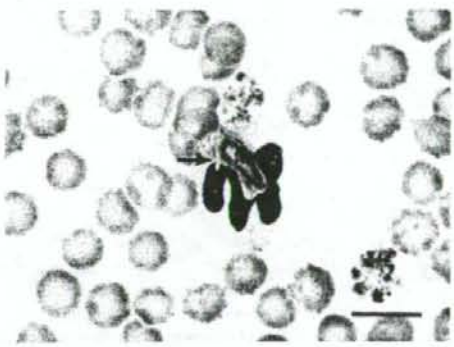

Fig. 3. A *Hepatozoon ursi* n. sp. gametocyte in the cytoplasm of the neutrophil detected in the peripheral blood smear of bear Sh2. Note the beak-like protrusion (arrow). Wright Giemsa stain. Bar: 10 μm.

Table 3
Hematological values of the examined bears

	N	Mean	SD[a]	SE[b]	Minimum	Maximum
Red blood cells ($\times 10^6$/μl)	13	10.385	3.258	0.904	6.18	18.87
Packed cell volume (%)	14	40.3	6.4	1.7	21	50
White blood cells (/μl)	14	9771.4	5753.6	1537.7	4800	28600
Neutrophil segmented (/μl)	12	6675.1	2482.9	716.8	3240	12126
Band (/μl)	12	46.8	30.3	8.7	22	130
Lymphocyte (/μl)	12	1214.8	369.2	106.6	504	1841
Monocyte (/μl)	12	292.0	140.8	40.6	132	657
Eosinophil (/μl)	12	342.5	370.4	106.9	2	1277
Basophil (/μl)	12	45.5	47.4	13.7	3	159
Hepatozoon ursi gametocytes (/10^3 WBCs)	12	13.18	11.87	3.43	1.3	42.6

[a] SD: Standard deviation.
[b] SE: Standard error.

3.3.2. Gametocytes

Gametocytes were slightly curved, cigar-like in shape and had a beak-like protrusion at one end (Fig. 3). The size of the gametocytes, excluding the protrusion, was 10.9±0.3×3.3±0.2 μm (10.5–11.5×2.9–3.6 μm, n=18). Occasionally, only unstained capsule-like structures were observed in the cytoplasm of the leukocytes. The morphological appearance of the host leukocytes resembled that of neutrophils.

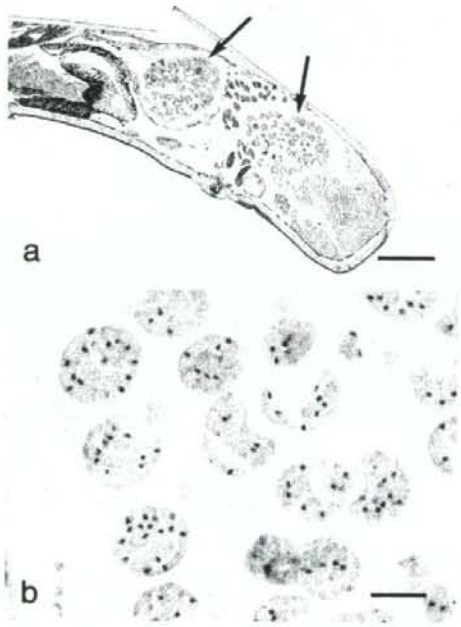

Fig. 4. H&E stained section of the male *Haemaphysalis flava* collected on bear Sh6. a) Two mature *Hepatozoon* oocysts (arrows) within the hemocoel. Bar: 200 μm. b) Higher magnification of the oocyst. The shapes of some sporocysts have been changed by the process of fixation and following histopathological methods. Bar: 20 μm.

3.4. Oocysts detected in ticks

Mature *Hepatozoon* oocysts were detected in a male *H. flava* (collected on bear Sh6) and a male *H. japonica* (collected on bear Sh5). In the *H. flava*, two oocysts were observed within the hemocoel (Fig. 4). It was not possible to observe details of an oocyst detected in the *H. japonica* because of the poor condition of the specimen. Only two oocysts found in the *H. flava* were measured, with the sizes being 263.2×234.0 μm and 331.8×231.7 μm, respectively (Fig. 4a). The oocysts contained approximately 40 and 50 sporocysts, respectively (Fig. 4a). The sporocysts were sub-spherical in shape and 31.2±2.5×27.0±2.9 μm (28.0–34.6×23.7–32.0 μm, n=5) in size (Fig. 4b). Each sporocyst contained at least 8–16 sporozoites (Fig. 4b). The sporozoites were 12.2±1.4×3.5±0.5 μm (10.0–14.0×2.9–4.2 μm, n=4) in size. The H&E stained section of *H. flava* containing *Hepatozoon* oocysts is deposited in the National Museum of Nature and Science under the accession numbers NSMT-Pr 240.

3.5. Immunohistochemistry

Trophozoites, schizonts, merozoites/gametocytes within macrophage nodules and oocysts showed strongly positive reaction with anti-*H. americanum* antiserum (Fig. 5).

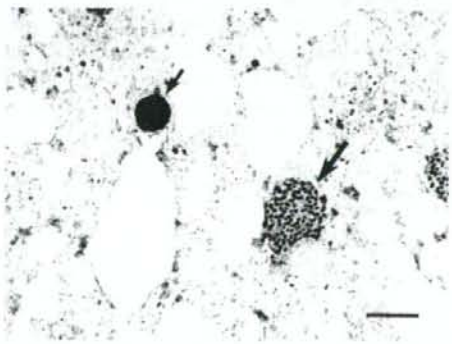

Fig. 5. The lung of bear E3. A schizont (small arrow) and merozoites/gametocytes within a macrophage nodule (large arrow) are positively stained with anti-*Hepatozoon americanum* antiserum. Immunohistochemistry, counterstained with Meyer's hematoxylin. Bar: 75 μm.

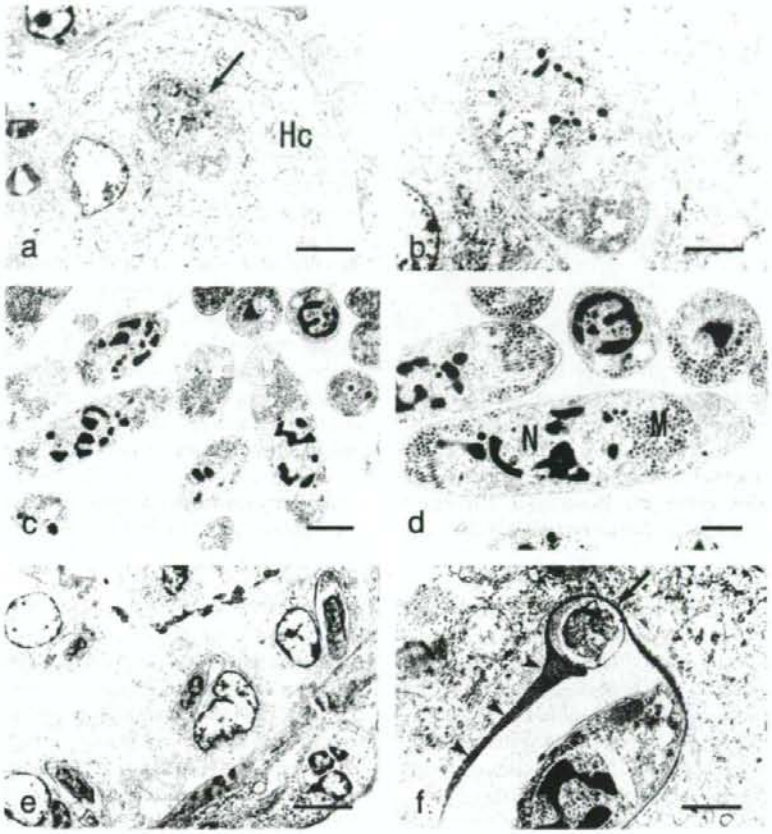

Fig. 6. Transmission electron micrograph of *Hepatozoon ursi* n. sp. detected in the lung of bear N4. a) Trophozoite (arrow). Note the swollen cytoplasm of the host cell (Hc). Bar: 4 μm. b) Higher magnification of the trophozoite Bar: 2 μm. c) Merozoites within mature schizont. Bar: 2 μm. d) Higher magnification of merozoites. Merozoite has an elongated nucleus (N) and numerous micronemes (M). Bar: 1 μm. e) Nodule of gametocyte-laden macrophages. Five gametocytes are shown. Bar: 5 μm. f) Higher magnification of gametocyte. Note the thick electron dense layer (arrowheads) and a beak-like protrusion (arrow). Bar: 1 μm.

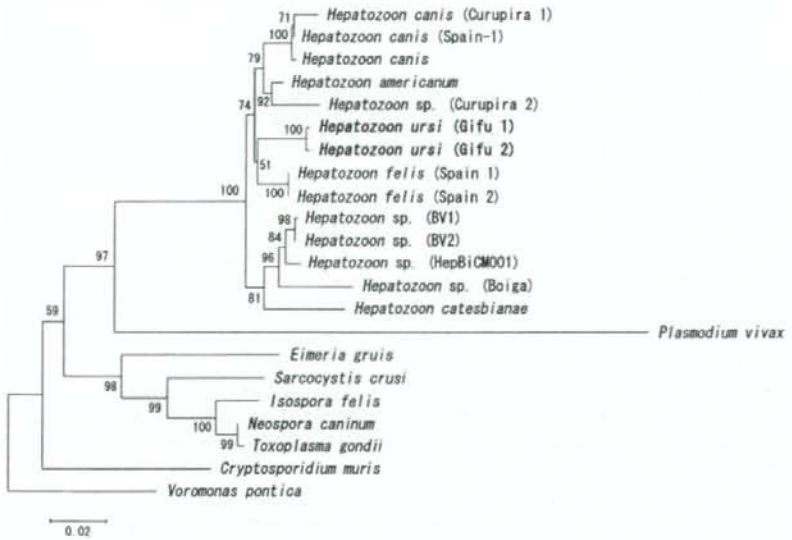

Fig. 7. The phylogenetic tree based on the 18S rRNA gene sequences of *Hepatozoon* species and some related protozoa, constructed using the neighbor-joining method. Numbers on branches are bootstrap values. Scale bar indicates an evolutionary distance of 0.02 nucleotide substitutions per nucleotide site.

3.6. Ultrastructural features

Trophozoites were oval in shape and located in the parasitophorous vacuoles of the host cells (Fig. 6a–b). Merozoites within mature schizonts had an elongated nucleus, a few rhoptries, numerous micronemes and a number of other organelles (Fig. 6c–d). Gametocytes within macrophage nodules were enclosed by an electron dense layer and had an elongated nucleus (Fig. 6e–f). The gametocytes, in contrast to the merozoites within schizonts, had few micronemes.

3.7. Genetic analyses

On PCR assay, both primer sets (HepF/R and BmF1/R1) produced positive results (the sizes of PCR products were approximately 625 bp and 1,110 bp, respectively) in all nine samples. After combining two sequences, which were amplified with these primer sets, the multiple sequence alignment and construction of phylogenetic tree were performed (of the consensus sequences, approximately 1240 bp, 1209 bp or 1207 bp fragments were used). These nine sequences were classified into two genotypes (Gifu 1 and Gifu 2), with the Gifu 2 genotype being obtained from only two bears (E3 and Ge2). These two sequences were 99% identical to each other. The sequences were deposited in GenBank under the accession numbers EU041717 and EU041718, respectively.

In comparison with the 18S rRNA gene sequences of other *Hepatozoon* species, *H. ursi* was most closely related to *H. felis* (96% homology). The phylogenetic tree indicated that the analyzed *Hepatozoon* species were classified into two clades (Fig. 7): the species infecting carnivores and the species infecting anuran, snake and rodents. *H. ursi* was included in the carnivore-related *Hepatozoon* group, and although it was supported only by a relatively low bootstrap value (51%), constituted a sub-group with *H. felis* (Fig. 7).

4. Discussion

The features of schizonts, the histopathological alterations in the lungs and the prevalence of infection of *H. ursi* closely resembled previous descriptions of *Hepatozoon* infection in Japanese black bears [2]. Considering the high prevalence (100%) and the fact that juvenile cubs were also infected, *H. ursi* may be one of the most common parasites in Japanese black bears in central Japan. In contrast, *Hepatozoon* species have not been reported in American black bears (*U. americanus*) in the United States [11] or in European brown bears (*U. arctos*) in Sweden [12], despite examinations of blood smears and/or histopathological examinations of lungs. Moreover, Gjerde et al. did not mention *Hepatozoon* sp. in their report of trypanosomes in polar bears (*U. maritimus*) in Svalbard, Norway [13].

Aside from *H. ursi*, a number of *Hepatozoon* species which prefer the lung for the site of schizogonic development have been reported in mammals: i.e. *H. griseisciuri* in grey squirrel (*Sciurus carolinensis*) [14,15] and unnamed *Hepatozoon* sp. in mink (*Mustela vison*) [16]. However, the size of the schizonts ($45.7\pm4.6\times42.7\pm4.5$ μm) and the number of merozoites in each schizont (approximately 80–130) of *H. ursi* are markedly larger than those of *H. griseisciuri*, being 8–24 μm in diameter with 6–19 merozoites [14], or those of *Hepatozoon* sp. in mink, 29–38 × 19–24 μm in size with 34–38 merozoites [16]. These morphometric features of *H. ursi* schizonts are relatively similar to those of *H. americanum* schizonts, being 48–68 × 40–63 μm in size and having 61–117 merozoites [17]. However, this *Hepatozoon* species principally parasitizes the skeletal muscles of American canids [3,17,18]. In addition, the size of *H. ursi* merozoites, at $7.0\pm0.7\times1.8\pm0.3$ μm, is slightly smaller than that of *H. americanum*, $7.5\pm0.85\times2.7\pm0.67$ μm [17].

The shape (slightly curved and cigar-like, with a beak-like protrusion) and the size ($10.9\pm0.3\times3.3\pm0.2$ μm) of *H. ursi* gametocytes are considerably different from those of other *Hepatozoon* species gametocytes. *H. americanum* gametocytes were described as being elongated and measuring $8.8\pm0.57\times3.9\pm0.54$ μm in size [17]. *H. canis* gametocytes detected in a Japanese dog were oval to elliptical in shape and 8.9 × 5.2 μm in mean size [19]. The beak-like protrusion of gametocyte was one of the most characteristic morphological features of *H. ursi*. Such a structure has been reported for *H. procyonis* gametocyte [20–22]. Not only the shape but also the size of *H. ursi* gametocyte is relatively similar to that of *H. procyonis*, 10.9 × 5.4 μm recorded by Richards [20] or $10.43\pm0.68\times4.03\pm0.18$ μm and $10.20\pm0.66\times3.70\pm0.48$ μm recorded by Rodrigues et al. [22]. However, *H. procyonis* schizonts were principally detected in the myocardium of raccoons (*Procyon lotor*) and measured 50 × 85 μm [20] or $31.2\pm7.8\times22.7\pm5.5$ μm [21] in size. Additionally, bears may not be infected with *H. procyonis*. In a study on endoparasites of American black bears in the southeastern United States, including Georgia, *Hepatozoon* species were not found in the peripheral blood despite the evidence that the bears were infected with some species of helminths which were common in raccoons [11]. On the other hand, *H. procyonis* was seen in 16.7–40% of raccoons in Georgia [20,23,24].

A number of mature *Hepatozoon* oocysts were detected in two tick species, *H. flava* and *H. japonica*. The morphological and morphometric features of these oocysts were relatively similar to those of *H. canis* oocysts detected in *Haemaphysalis longicornis* and *H. flava* which were collected from Japanese dogs [25]. Unfortunately, we did not perform the experimental transmission and the genetic analysis of oocyst in the present study. Therefore, these oocysts were strongly suspected, but not determined, as those of *H. ursi*.

The results of immunohistochemistry confirmed that *H. ursi* is a member of the genus *Hepatozoon*. Although Panciera et al. reported that the antiserum used in this study cross-reacted to some other apicomplexans, those reactions were weak [3].

Ultrastructurally, the gametocytes within macrophage nodules were quite distinct from merozoites within schizonts. Although we detected only gametocytes in macrophage nodules, Cummings et al. found both merozoites and developing gametocytes of *H. americanum* in similar granulomas [26]. *Hepatozoon* merozoites may develop into gametocytes in such nodules.

According to Smith [1], the classification of *Hepatozoon* with classical criteria, such as host species, is relatively difficult. Recently, the 18S rRNA gene sequence has been used as a useful criterion for identification of *Hepatozoon* species [4,27,28]. In the

present study, the analyses of the 18S rRNA gene sequences supported the conclusion that *H. ursi* was a novel species of carnivore-related *Hepatozoon*. The phylogenetic relationship within the genus *Hepatozoon* of the present study basically agreed with that reported by Criado-Fornelio et al. [27].

The pathogenicity of *H. ursi* against Japanese black bears has not yet been completely clarified. However, interstitial pneumonia, characterized by thickening of alveolar wall, and small foci of pyogranulomatous inflammation may cause respiratory disorders.

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