Table 2 Sizes of *Cryptosporidium* oocysts (in μm) [a]

Host	Species of *Cryptosporidium*	Oocyst measurements			
		n	Length	Width	Shape index
Large Japanese field mouse	*Cryptosporidium* field isolate	50	7.66 ± 0.64	5.70 ± 0.59	1.36 ± 0.17
Norway rat	*C.muris* RN66	50	8.02 ± 0.72*	5.90 ± 0.61	1.37 ± 0.18
Cattle	*C.andersoni* Kawatabi isolate	50	7.91 ± 0.74	5.58 ± 0.55**	1.43 ± 0.19**
Human	*C.parvum* HNJ-1	50	5.46 ± 0.52**	4.88 ± 0.41**	1.12 ± 0.11**

[a] For each dimension the mean ± standard deviasion is provided
* Indicates statistically significant differences between the *Cryptosporidium* field isolate and *C. muris* RN66 ($P < 0.05$)
** Indicates statistically significant difference between the *Cryptosporidium* field isolate and *C. parvum* HNJ-1 ($P < 0.01$)

Table 3 Experimental infection of *Cryptosporidium* spp. to mice

Mice	*Cryptosporidium* species	Inoculation doses	Number of mice inoculated	Number of mice discharged oocysts
Large Japanese field mice	*Cryptosporidium* field isolate	5×10^5	3	1
	C. muris RN66	1×10^6	3	0
		5×10^6	3	2
		1×10^7	3	1
	C. andersoni Kawatabi isolate	2×10^5	5	0
		5×10^5	3	0
		1.3×10^7	3	0
Infants of large Japanese field mice	*Cryptosporidium* field isolate	1×10^5	3	1
	C. muris RN66	1×10^5	3	0
	C. andersoni Kawatabi isolate	1×10^5	3	0
ICR mice	*Cryptosporidium* field isolate	1×10^5	3	3
	C. muris RN66	1×10^5	3	0
	C. andersoni Kawatabi isolate	1×10^5	3	0
SCID mice	*Cryptosporidium* field isolate	1×10^4	3	3
	C. muris RN66	1×10^5	3	3
	C. andersoni Kawatabi isolate	1×10^5	3	3

Fig. 2 Hematoxylin and eosin staining of the stomach of a large Japanese mouse infected with the *Cryptosporidium* field isolate. The gastric glands are filled with cryptosporidial developing stages (*arrowhead*). *Scale bar* = 20 μm

sequences (Figs. 3, 4). The lengths of the sequences analyzed in this study ranged from 1,673 to 1,728 bp. The tree resulting from the neighbor joining analysis was identical in overall topology to the tree obtained from the maximum likelihood analysis. *Cryptosporidium* species examined in this study formed two clades. This result was in agreement with that reported in a previous study (Xiao et al. 1999). One clade contained the gastric *Cryptosporidium* parasites, while the other contained *C. baileyi* and intestinal parasites. In phylogenetic analysis of the 18S rRNA gene, the *Cryptosporidium* field isolate belonged to the gastric clade and clustered with parasites isolated from a bactrian camel, a rock hyrax, and a house rat.

Discussion

We isolated *Cryptosporidium* oocysts from the large Japanese field mouse, *A. speciosus*. The large Japanese field mice were captured on the farm where *C. andersoni* had been constantly detected from several beef cattle;

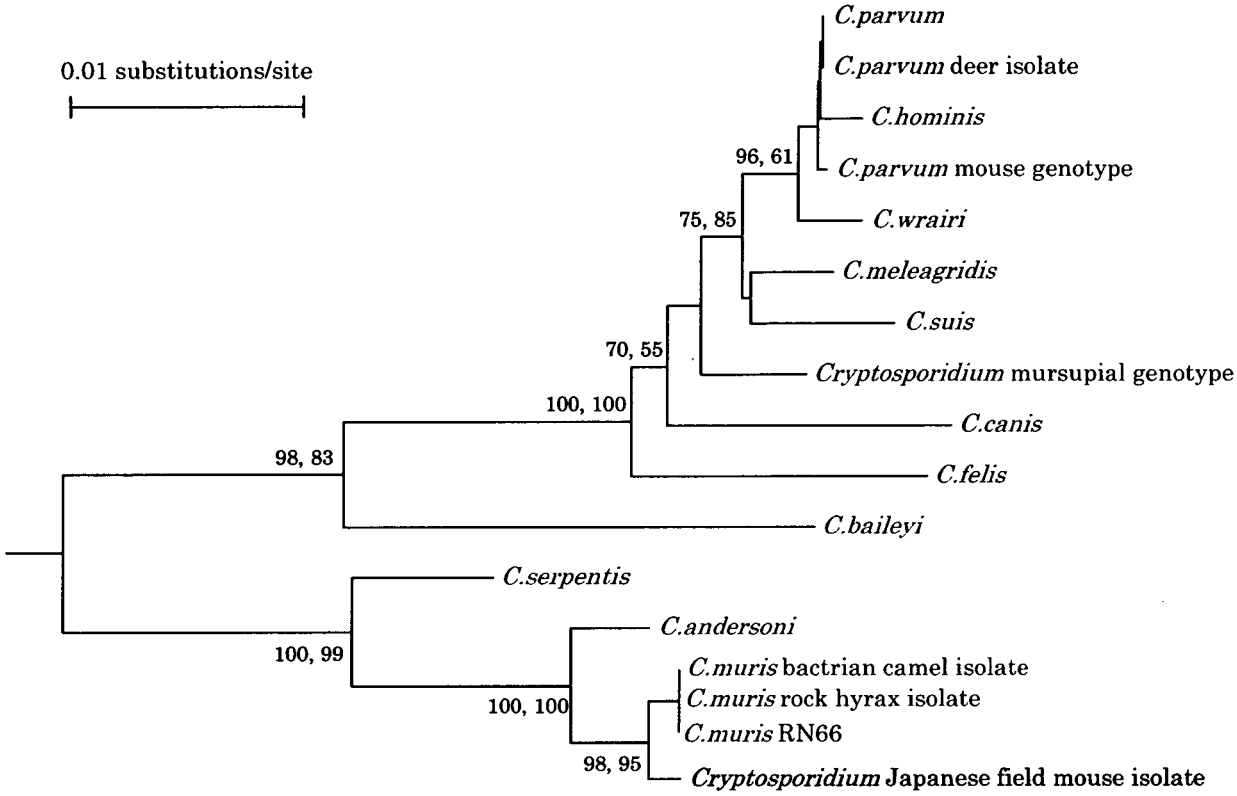
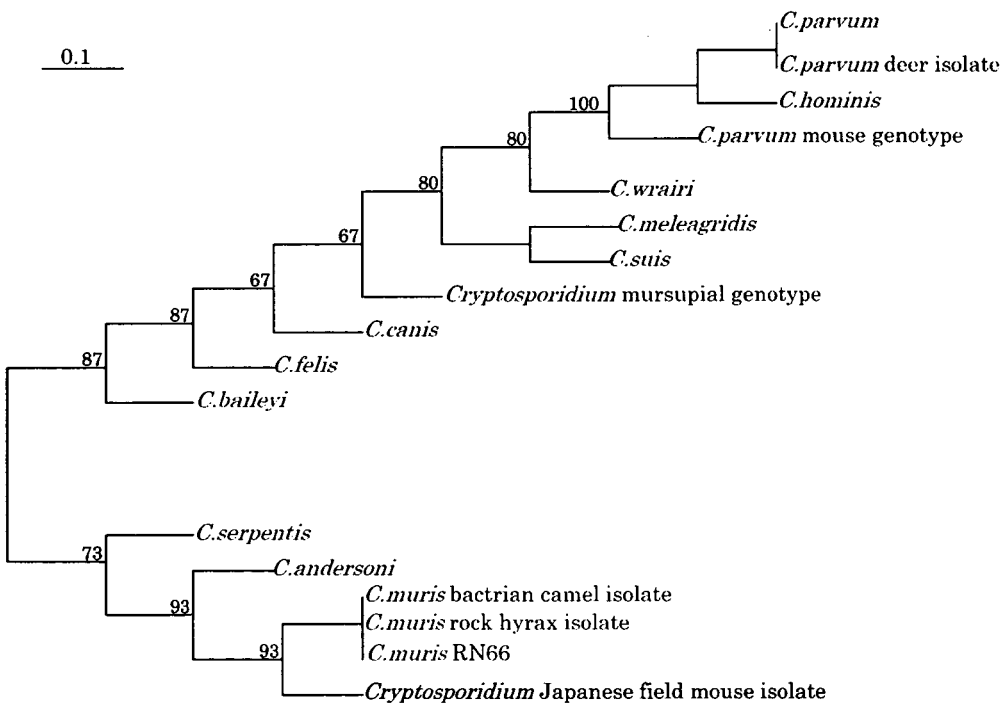


Fig. 3 Phylogenetic relationships (neighbor joining tree based on Kimura's 2-parameter analysis) between the *Cryptosporidium* field isolate and the *Cryptosporidium* species inferred from the 18S rRNA sequences. The tree was rooted with the 18S rRNA sequence from *Eimeria tenella* (AF26388), and the root was removed. Bootstrap values (in percentage) above 50 from 1,000 pseudo-replicates are shown for both the neighbor joining (the first value) and maximum parsimony analyses (the second value)

Fig. 4 Phylogenetic relationships (maximum likelihood tree) between the *Cryptosporidium* field isolate and the *Cryptosporidium* species inferred from the 18S rRNA sequences. The tree was rooted with the 18S rRNA sequence from *Eimeria tenella* (AF26388), and the root was removed. Bootstrap proportions (in percentage) at each node were estimated using the RELL method



however, the infectivity to some mice and the DNA sequence of the 18S rRNA gene of the field isolate markedly differed from those of the *C. andersoni* Kawatabi isolate. Further, the oocyst size and the infectivity of the field isolate to ICR mice slightly differed from those of *C. muris* RN66.

A large number of developing stages of field isolates was observed only in the gastric gland of the stomach of large Japanese field mice and SCID mice. Recently, *C. muris*, *C. andersoni*, and *C. serpentis* have been identified as gastric *Cryptosporidium* parasites. The infectivity of *C. muris* RN66 (Iseki 1986) and *C. andersoni* Kawatabi isolate (Satoh et al. 2003) to SCID mice has been confirmed. Although the infectivity of the field isolate to ICR mice and large Japanese field mice differed slightly from that of *C. muris* and *C. andersoni* in the experimental infection, the differences may not be significant. Iseki et al. (1989) have reported that *C. muris* RN66 are infective to 3-week-old ICR mice inoculated with 1×10^6 oocysts. We consider the difference in the results between the previous study and the present study to be due to the number of inoculation oocysts and the age of the mice. Oocysts isolated from cattle in the US, which were previously referred to as *C. muris*-like oocysts and which may be *C. andersoni*, did not infect mice or even cattle (Lindsay et al. 2000). Oocysts similar to those obtained from cattle in the US were not transmissible to neonatal or adult BALB/c mice and SCID mice (Koudela et al. 1998). However, several successful transmissions of bovine-derived large-type oocysts, referred to as *C. andersoni*, to SCID mice have been reported in Japan (Kaneta and Nakai 1998; Satoh et al. 2003; Matsubayashi et al. 2004a; Koyama et al. 2005). In the present study, we also confirmed the ability of the *C. andersoni* Kawatabi strain to infect SCID mice.

In phylogenetic analysis of the 18S rRNA gene, the *Cryptosporidium* field isolate belonged to the gastric clade and was located in the *C. muris* cluster. At the 18S rRNA locus, the similarity between this isolate and *C. muris* RN66 and between this isolate and the *C. andersoni* Kawatabi isolate was 99.5 and 99.1%, respectively. A previous study showed that the genetic similarity between *C. hominis* and *C. parvum*, which are considered to be distinct species, is 99.7% (Morgan et al. 2002). *Toxoplasma gondii* and *Neospora caninum* are classified into different genera, but there is 99.8% similarity at this locus. Furthermore, the similarity between *C. muris* and *C. andersoni* is 99.1% at the 18S rRNA locus (Lindsay et al. 2000). However, they are considered to be valid species based on the differences in their infectivity to laboratory mice and differences in their DNA sequences. The percentage similarity between this isolate and *C. muris* at this locus suggests that the field isolate and *C. muris* were different species. Further, high bootstrap values (98 and 95%) obtained by parsimony and neighbor joining analysis suggested that the sequences of *C. muris* and the field isolate clearly branched.

Based on the characteristics of the oocysts, the parasitized organ, the host specificity, and the DNA sequence in the 18S rRNA gene, the field isolate from large Japanese field mice was considered to be a distinct species from *C. muris*. However, biological differences between this isolate and *C. muris* were unclear, and this isolate clustered with *C. muris* in the phylogenetic analysis. Therefore, evidence for classifying this isolate as a novel species was insufficient. Thus, we proposed that this isolate is a novel genotype of *C. muris* and denoted it as *C. muris* Japanese field mouse genotype.

Recently, there have been many reports on new species and genotypes of *Cryptosporidium* on the base of molecular studies. *C. parvum* has several genotypes, for instance, mouse, rabbit, monkey, and bovine. *C. canis* has three genotypes, namely, coyote, dog, and fox (Xiao et al. 2002). In contrast to the small oocyst types of *Cryptosporidium*, there are few reports on the genotypes present in the large oocyst type of *Cryptosporidium* (Ryan et al. 2003). Our study is the first report of a novel genotype of *C. muris*.

Phylogenetic analysis has been used to study the diversity of *Cryptosporidium* spp. from humans, and wild and domestic animals (Hajdušek et al. 2004). *C. parvum* was found in a raccoon dog in Japan (Matsubayashi et al. 2004b). In this study, a novel *Cryptosporidium* genotype was isolated from large Japanese field mice, the most common wild mouse found in forests in Japan. Different *Cryptosporidium* species and genotypes may be distributed in various wild animals. Since human infection of *C. muris* has been reported (Katsumata et al. 2000; Gatei et al. 2002) and it is a potential zoonotic parasite, further epidemiological investigation and molecular and biological analysis of the *C. muris* group should be performed.

The taxonomic status of this isolate and *C. muris* should be clarified by finding closely related species of *C. muris* and accumulation of biological and genetic data of these strains.

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SHORT COMMUNICATION

Characterization of *Cryptosporidium canis* isolated in Japan

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Abstract Oocysts that morphologically resemble *Cryptosporidium canis* oocysts were isolated from a stray dog captured in the northeastern part of the main island of Japan. The DNA sequence of the 18S rRNA gene of the isolate showed high homology to the published sequence of *C. canis* that was isolated in USA by Fayer et al., *J Parasitol*, 87:1415–1422, (2001). The isolate phylogenetically belonged to the *C. canis* cluster; however, its DNA sequence showed two base substitutions. This suggests the genetic diversity of *C. canis*.

Introduction

Cryptosporidium oocysts were isolated from a dog with persistent diarrhea, and the 18S rRNA gene sequence was deposited as *Cryptosporidium parvum* dog genotype (Morgan et al. 1999; Xiao et al. 1999). Because the gene showed low homology, Fayer et al. (2001) proposed that it be classified as *Cryptosporidium canis*. However, it is still unclear whether the major species of *Cryptosporidium* isolated from dogs are *C. canis* or genetic diversity exists among these species.

Cryptosporidiosis accompanied by persistent diarrhea in dogs was first reported by Wilson et al. (1983), and it had been considered as a *C. parvum* dog genotype based on the nucleotide sequence of genes for the 18S rRNA, a 70-kD heat shock protein (HSP 70), and the oocyst wall protein (COWP) (Sulaiman et al. 2000; Xiao et al. 1999, 2000). Fayer et al. (2001) proposed that the new species should be classified as *C. canis* based on the following observations: (1) infectivity to cattle and no infectivity to mice, (2) the genetic distance of *C. canis* from *Cryptosporidium hominis* (previously considered to be the *C. parvum* human genotype or genotype 1) or *C. parvum* (previously known as the *C. parvum* bovine genotype or genotype 2) was greater than that of other genotypes, namely, *C. parvum*, *Cryptosporidium wrairi*, and *Cryptosporidium meleagridis*. *C. canis* is now considered as a valid species (Xiao et al. 2004).

In this study, *Cryptosporidium* oocysts that were isolated from a stray dog were characterized on the basis of morphological, biological, and genetical analyses.

Materials and methods

Fecal samples were collected from 294 dogs and 31 cats that were captured or carried to an animal center in the northern part of Miyagi prefecture. Miyagi prefecture is located in the northeastern part of the main island of Japan. Oocysts were detected by using the sugar centrifugal flotation method (Satoh et al. 2003). In brief, 1 g of fecal sample was mixed with a sodium acetate-acetic acid-formalin (SAF) solution, centrifuged, and then the supernatant was removed. Next, the supernatant was mixed with a 56% sugar solution and centrifuged. The surface of this

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mixture was observed under a microscope. The detected oocysts were collected and purified by the sugar centrifugal flotation method (Nakai et al. 2004). After five cycles of freezing and thawing the oocysts, DNA from the sample was extracted by using the MagExtractor Genome kit (Toyobo, Osaka, Japan). A primer set was used to amplify the complete sequence of the 18S rRNA gene (Xiao et al. 1999). As the reference strain, we used *C. parvum* HNJ-1 that was originally isolated from a Japanese woman (Masuda et al. 1991). This strain was previously classified as *C. parvum* bovine genotype or genotype 2, and it has recently been defined as a *C. parvum* (Satoh et al. 2005). In our laboratory, this strain was passaged in SCID mice.

The polymerase chain reaction (PCR)-amplified products were cloned by using the pT7Blue-2 Perfectly Blunt Cloning Kit (Novagen) according to the manufacturer's instructions. Three clones were sequenced by using an automated DNA sequencer (ABI 310; Applied Biosystems Japan, Tokyo, Japan). The sequence accuracy of the data was confirmed by bidirectional sequencing. To compare this sequence with the sequences of *C. parvum* genotypes registered in GenBank, we performed a distance-based analysis using Kimura's distance formula and constructed a phylogenetic tree using MEGA version 2.1 (Kumar et al. 2001). For phylogenetic analysis, a tree was constructed by using the neighbor-joining method on the basis of the arithmetic mean that was obtained from the aligned sequences of the 18S rRNA genes from various known genotypes of *C. parvum*.

To assess the infectivity of the isolate, two 4-week-old SCID mice were orally inoculated with 10^5 purified oocysts. The fecal sample of each mouse was collected and the amount of oocysts discharged was monitored microscopically for 28 days, as described previously (Satoh et al. 2003).

Results and discussion

In this study, although no oocysts were detected in any of the cats, we detected *Cryptosporidium* oocysts in 1 of the 294 dogs (0.3%). The dog that discharged the oocysts was captured from a mountainous area in a village, where agricultural farms were located and dairy and beef operations were carried out. The average size of the oocyst was $4.4 \pm 0.5 \times 3.2 \pm 0.4$ μm (length to width ratio: 1.34, $n = 50$). We referred to these oocysts as the Hanayama isolate. The average size of these oocysts was smaller than that of the *C. parvum* HNJ-1 strain (average size: $4.8 \pm 0.4 \times 4.2 \pm 0.5$ μm, $p < 0.05$) and *C. canis* (average size: 4.95×4.71 μm) (Fayer et al. 2001). The length to width ratio of the Hanayama isolate was 1.34 but that of *C. parvum* and *C. canis* was 1.04–1.06. This indicated that the isolate had an elliptical shape. This suggested the existence of morphological diversity with regard to the shape and size of oocysts in *C. canis*.

PCR was performed to amplify the 1741 bp sequence of the 18S rRNA gene of the Hanayama isolate; the determined sequence has been deposited in GenBank (accession no. AB210854). Two base pair substitutions at positions 1,684 and 1,685 were observed on comparison with the *C. parvum* dog genotype that is registered in GenBank (accession no. AF112576). Because Fayer et al. (2001) have proposed this registered genotype as a new species *C. canis*, we also considered the *C. parvum* dog genotype as *C. canis*. The constructed phylogenetic tree revealed that the Hanayama isolate belongs to the cluster of *C. canis* (Fig. 1). PCR was also performed using primer sets targeting HSP 70 and COWP of *C. parvum* that were used in a previous study (Satoh et al. 2005); however, no PCR fragments were obtained (data not shown). Morgan et al. (2000) have reported the heterogeneity of the HSP 70

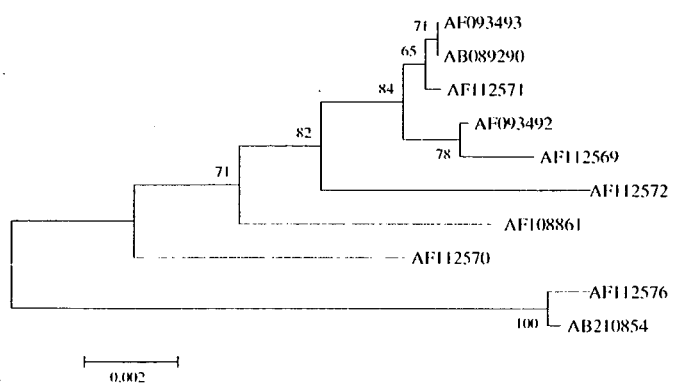

Fig. 1 The evolutionary relationship of our isolate in this study inferred by NJ analysis of Tamura-Nei distances calculated form pairwise comparisons of gene sequences for 18S rRNA using MEGA version 2.1. Percentage of bootstrap support from 1,000 replicate samples is indicated at each node. Each accession number indicates the 18S rRNA sequence registered in GenBank: AB210854, Hanayama isolate; AF093492, *C. hominis*; AF093493, *C. parvum*; AB089290, *C. parvum* HNJ-1 strain; AF112576, *C. canis*; and AF112571, AF112569, AF112572, AF108861, and AF112570 indicate mouse genotype, rhesus monkey genotype, ferret genotype, pig genotype, and kangaroo genotype of *C. parvum*, respectively

gene among the two dog genotypes isolated in USA and Australia, respectively. They observed partial 18S rRNA gene sequences of both isolates (nucleotide position: 1 to 713, corresponding to our data), and reported that these were identical. Abe et al. (2002a,b) isolated canine *Cryptosporidium* from a western region of Japan at a distance of approximately 800 km from Miyagi prefecture, where our investigations were carried out. The partial 18S rRNA gene sequence of this isolated canine *Cryptosporidium* was identical to that of *C. canis* (nucleotide position 445 to 734, corresponding to our data). Hanayama isolate and Fayer's isolate were identical to the species isolated in USA and Australia with regard to the positions from 1 to 713 and to Abe's isolate with regard to the positions from 445 to 734. To distinguish the isolates of *C. canis*, positions 1,684 and 1,685 were considered as the candidate sequence for DNA analysis. Xiao et al. (2004) observed that the genetic distance (0.38% in 18S rRNA) between the marsupial genotype from Australia and the opossum genotype from North America was small; this small genetic distance was attributed to the geographical distribution that had resulted from the continental drift. Widmer et al. (1998) demonstrated genetic diversity in the gene for β-tubulin among the same genotype of *C. parvum*, suggesting the population structure within the same genotype. Furthermore, we demonstrated that the *C. parvum* HNJ-1 strain has a heterogenous structure when compared to the other *C. parvum* strain (Satoh et al. 2005). Thus, the genetic difference (0.11%) in the 18S rRNA gene between the geographically separated Hanayama isolate and *C. canis* isolated in USA suggests the intraspecific diversities of *C. canis*. To clarify the extent of intraspecific diversity due to geographical distribution, further survey and molecular analyses of *C. canis* from different areas and countries must be conducted.

In this study, none of the SCID mice discharged oocysts for 28 days after the oral inoculation of the isolate. In this experiment, we used fresh oocysts isolated from a dog, and a considerably high dose of oocyst (10^5) was used for inoculation. Therefore, the Hanayama isolate may have low or no infectivity to SCID mice.

The characteristics of our isolate with regard to its infectivity in the laboratory mice was consistent with that of *C. canis*, as described previously; however, the two differed in terms of their morphological characteristics.

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ORIGINAL PAPER

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Gene analysis of *Cryptosporidium parvum* HNJ-1 strain isolated in Japan

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Abstract We analyzed genetically *Cryptosporidium parvum* HNJ-1 strain, which is the Japanese reference strain isolated from human in Japan. DNA sequences of genes for thrombospondin-related adhesive protein of *Cryptosporidium*-1 and *Cryptosporidium*-2 (TRAP-C1, TRAP-C2), heat shock protein 70 (HSP70), oocyst wall protein (COWP), beta-tubulin, alpha-tubulin, polythreonine-region (Poly-T), elongation factor 1 alpha (EF-1α), and 18S rRNA of this strain were determined. They showed high rate of homology to published sequences of genotype 2 strains, which were considered to be infective to both humans and animals. However, HNJ-1 had synonymous and non-synonymous substitutions in the nucleotide sequence of TRAP-C1 and beta-tubulin among HNJ-1 and published sequences of genotype 2 strains. These results implied that HNJ-1 strain was an unique subpopulation of genotype 2 strain of *C. parvum*.

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Introduction

Cryptosporidium parvum, a protozoan parasite, is an important etiologic agent of acute gastroenteritis in mammals. *C. parvum* infection is self-limiting in immunocompetent humans; however, it leads immunocompromised humans to chronic and sometimes lethal symptoms (Casemore et al. 1997). It is now recognized that two separated transmission cycles of *C. parvum* have been postulated, based on phenotypic and genotypic characterization of oocysts obtained from human and animal hosts. It is generally believed that *C. parvum* genotype 1 is restrictedly infective to humans, and genotype 2 is infective to both animals and humans.

Recent molecular and genetic methods allow to apply for not only tracking the source of pathogens epidemiologically, but also identifying pathogens at species and subspecies levels phylogenetically. Previous studies discriminated genotypes 1 and 2 using variation of nucleotide sequences of genes for 18S rDNA (Xiao et al. 1999), beta-tubulin (Widmer et al. 1998; Rochelle et al. 1999), polythreonine motifs (Carraway et al. 1997), oocyst wall protein (Spano et al. 1997; Xiao et al. 2000), thrombospondin-related anonymous protein of *Cryptosporidium*-1 (TRAP-C1, Pedraza-Diaz et al. 2000), thrombospondin-related anonymous protein of *Cryptosporidium*-2 (TRAP-C2, Peng et al. 1997), and heat shock protein 70 (HSP70, Sulaiman et al. 2000).

Cryptosporidium parvum HNJ-1 was isolated from an adult woman in Japan (Masuda et al. 1991) and this isolate has been maintained mainly in Osaka City University using SCID mice. This strain was kindly provided by Drs. Iseki and Kimata (Osaka City University), and propagated and maintained in SCID mice in our laboratory since 2000. Although this strain has been widely used as one of the reference strain in Japan, determination of type based on genotypic and phenotypic character such as infectivity to domestic animal has not been clear. Thus, in order to identify the type of this strain, we attempted to examine genetic characterization.

We report here genetic analysis of nucleotide sequence of some genes of HNJ-1 strain by comparing with those of known strains of *C. parvum*.

Materials and methods

Oocysts purification and DNA extraction

Oocysts were purified from fecal sample of experimentally infected SCID mice using sugar flotation method (Nakai et al. 2004). DNA from oocysts was extracted with MagExtractor -Genome- (Toyobo, Osaka, Japan) after five rounds of freezing and thawing of oocysts as described previously (Satoh et al. 2003).

PCR amplification and sequencing

We performed PCR to amplify full-length of the gene for 18S rDNA and fragments of genes for 70-kDa heat shock protein (HSP70), polythreonine motifs (Poly-T), thrombospondin-related anonymous protein of *Cryptosporidium*-1 (TRAP-C1), thrombospondin-related anonymous protein of *Cryptosporidium*-2 (TRAP-C2), alpha-tubulin, beta-tubulin, *Cryptosporidium* COWP and elongation factor 1 alpha (EF-1α). To amplify these fragments, we designed sets of primer based on sequences registered in the GenBank, respectively (Table 1). PCR amplification was performed in one time PCR buffer, 1 mM $MgSO_4$, 0.2 mM dNTPS (each), 0.3 μM each primer, and 1.0 unit KOD -plus- polymerase (Toyobo, Osaka, Japan) (final concentrations). This polymerase has high fidelity because it contains 3′ → 5′ exonuclease accompanying proofreading activity. Each samples was preheated at 94°C for 2 min, then subjected to 35 cycles of 94°C for 20 s, 56°C for 30 s, and 68°C for adequate times for length of target products (approximately 1 kb/min), followed by a final extension at 68°C for 10 min. Products of amplification by PCR were subjected to electrophoretic separation using 1.5% agarose gels, stained with ethidium bromide, and visualized on a UV transilluminator.

These products were purified using MagExtractor -PCR & Gel clean up- (Toyobo, Osaka, Japan) according to manufacture's instruction and sequenced using an ABI PRISM Big Dye Terminator Cycle Sequencing FS Ready Reaction kit (Applied Biosystems Japan Ltd, Tokyo, Japan) on an automated sequencer (ABI 310; Applied Biosystems Japan Ltd, Tokyo, Japan). The accuracy of data was confirmed by two-directional sequencing.

Sequence analysis

We aligned our data with sequence of genotypes 1 and 2 strains of *C. parvum* registered in the GenBank (Table 2) using MEGA (Version 2.1) (Kumar et al. 2001).

Results and discussion

Comparison of nucleotide sequences of HNJ-1 strains with both genotypes 1 and 2 was shown in Tables 3 and 4. Comparing with genotype 1 strains, non-synonymous substitutions were observed in the fragments of genes for TRAP-C1, TRAP-C2, and HSP70, and synonymous substitutions were in the fragments of genes for COWP. Substitution (Table 3) and deletions in the intron and synonymous substituions in the exon 2 regions were observed in the gene for beta-tubulin, respectively (Table

Table 1 Sets of primer used in this study

Primer name	Sequence (5′ to 3′)	Target gene	Length (bp)	Origination (Genbank accession number)
CPTRAPF	GGATGGGTATCAGGTAA	thrombospondin-related adhesive protein of *Cryptosproidium-1* (TRAP-C1)	469	AF248744
CPTRAPR	TTCCGCATCCACAAGTTAC			
PTRAP2F	ATATTCCCTGTCCCTTGAGTTGT	thrombospondin-related adhesive protein of *Cryptosproidium-2* (TRAP-C2)	355	AF082524
PTRAP2R	TGCAGACCTAAACAATCTGAA			
CPHSPF	AGTGATATGACTCACTGGCATT	*C. parvum heat shock protein*70	758	U71181
CPHSPR	ACAACATCATGTACAGATCTCTT			
COWPF	CCCAACATTCCTGGTGTAGCTTCC	*C. parvum* Oocyst wall protein gene	1030	Z22537
COWPR	GAACGCACCTGTTCCCACTCAATG			
BETUBF	AGAAATTGTTCATATTCAGGGAG	*C. parvum* Beta tublin gene	572	AF115398
BETUBR	AAAATACGATCTGGGAATTC			AF323577
Cry44	CTCTTAATCCAATCATTACAAC	*C. parvum* polythreonine-rich glycoprotein	518	CPU83169
Cry373	AGCAGCAAGATATGATACCG			
ALPTUBF1	GGGCCATTATACAGTAGGGAAG	*C. parvum* Alpha tublin gene	961	AF013984
ALPTUBR1	TAAGTCTTCTCTTGCCTCGCTAA			
ELOGF	ATATAATTGAACTAAGTGAGGGG	*C. parvum* elongation factor1 gene	1718	U69697
ELOGR	AAAATTAAATAGTAAAAGCCCGT			
Cry-18F	AGTCATATGCTTGTCTCAAA	*C. parvum* 18S-rDNA	1746	X64341
Cry-18R	GAATGATCCTTCCGCAGGTT			

Table 2 Referred sequence data in this study registered in the GenBank

Target gene	GenBank accession number	Name of strain or isolate name	Host	Isolated Country
TRAP-C1	Genotype1			
	AF248744	unknown	human	UK
	Genotype2			
	AF248745	unknown	human	UK
	AF017267	moredun	deer	UK
TRAP-C2	Genotype1			
	AF082522	HGMO7	human	Guatemala
	AF082523	HWA1	human	USA
	Genotype2			
	AF082521	HMOB3	human	USA
	AF017267	AGA75	calf	USA
HSP70	Genotype1			
	AF221535	497	human	Kenya
	Genotype2			
	AF221528	11	calf	USA
COWP	Genotype1			
	AF266265	181	human	USA
	Genotype2			
	AF266273	6	calf	USA
Beta-tubulin	Genotype1			
	AF115399	unknown	human	unknown
	AF323578	1119	human	UK
	AF323579	1664	human	UK
	AF323580	LD10	human	UK
	Genotype2			
	AF115398	unknown	calf	Unknown
	AF323576	A104	calf	UK
	AF323577	A22	calf	UK
	BX538353	IOWA	calf	USA
Polythreonine	U83169	GCH1	human	USA
Alpha-tubulin	AF013984	unknown	unknown	unknown
Elongation factor1	U69697	unknown	unknown	unknown
18S rRNA	Genotype1			
	AF108865	H7	human	Australia
	AF222998	NEMC1	human	USA
	L16997	unknown	unknown	unknown
	AF093489	HCNV4	human	USA
	AF093491	HFL2	human	USA
	AF093492	HFL5	human	USA
	Genotype2			
	AF093490	BOH6	calf	USA
	AF093493	GCH1	human	USA
	AF093494	DR1	deer	USA
	AF108864	C1	calf	Australia
	AF161856	MT	unknown	USA
	AF161857	UCP	calf	USA
	AF161858	TAMU	horse	USA
	AF164102	IOWA	calf	USA
	L16996	AUCP-1	calf	USA

4). Comparing with genotype 2 strains, sequences of HNJ-1 strain were coincident with genes for TRAP-C2, HSP70, and COWP (Table 3) except for variations of nucleotide sequence of genes for TRAP-C1 and beta-tubulin. Synonymous substitution in TRAP-C1 gene and non-synonymous substitution in beta-tubulin gene were observed in the exon 2 region, respectively (Table 4). Sequence of genes for polythreonine-region of HNJ-1 was coincident with that of GCH1 strain, which is genotype 2 strain (U83169), and sequences of alpha-tubulin and elongation factor 1 alpha of HNJ-1 were identical to registered strains of *C. parvum* (AF082877, AF013984, data not shown, Table 2).

Sequence of full-length of 18S rDNA of HNJ-1 strain showed to be identical to that of genotype 2 strains of *C. parvum* (AF093490, AF093493, AF108864, AF164102, AF164856, AF161857, AF161858, L16996, AF093494, Table 2). For phylogenetic analysis, we constructed a neighbor-joining tree using MEGA 2 with sequences of other genotypes and HNJ-1 strain of *C. parvum*. In the phylogenic tree, the position of HNJ-1 strain clustered with genotype 2 strains (Fig. 1). Our data suggested that HNJ-1 strain belonged to genotype 2 strain of *C. parvum* and had potential infectivity to human and other animals such as cattle. The sequence of beta-tubulin of HNJ-1 strain had been identical to that of *C. parvum*

Table 3 Nucleotide changes of genes for TRAP-C1, -C2, HSP 70, and COWP comparing *C. parvum* HNJ-1 with genotype 1 and genotype 2 strain. " Parenthese following codon indicates putative amino acid. Substitution of nucleotide and amino acid comparing those of *C. parvum* HNJ-1 indicate bold face

Trap-C1											Number of substitution with HNJ-1 strain	
	43–45	121–123	139–141	166–168	232–234	250–252	301–303	316–318	349–351		nucleotide	Amino acid
Genotype1												
AF248744	GAA(**E**)	AGC(**S**)	AAG(**K**)	ACA(**T**)	AAA(**K**)	GGT(**G**)	GTT(**V**)	GTT(**V**)	ACA(**I**)		9	7
Genotype2												
AF248745	GAT(D)	AGC(S)	AAA(K)	ATA(I)	GAA(E)	AGT(S)	ATT(I)	ATT(I)	AAA(K)		1	0
AF017267	GAT(D)	AGC(S)	AAA(K)	ATA(I)	GAA(E)	AGT(S)	ATT(I)	ATT(I)	AAA(K)		1	0
C. parvum HNJ-1	GAT(D)	AGT(S)	AAA(K)	ATA(I)	GAA(E)	AGT(S)	ATT(I)	ATT(I)	AAA(K)			
TRAP-C2												
	49–51	76–78	100–102	145–147	280–282							
Genotype1												
AF082522	TCG(S)	GGC(G)	**TTA (L)**	ACC(T)	CAT(H)						4	1
AF082523	TCG(S)	GGC(G)	**TTA (L)**	ACC(T)	**TAT (Y)**						5	2
Genotype2												
AF082521	TCA(S)	GGT(G)	GTA(V)	ACT(T)	CAT(H)						0	0
AF082524	TCA(S)	GGT(G)	GTA(V)	ACT(T)	CAT(H)						0	0
C.parvum HNJ-1	TCA(S)	GGT(G)	GTA(V)	ACT(T)	CAT(H)							
HSP 70												
	19–21	109–111	145–147	184–186	436–438	487–489	490–492	526–528	592–594	625–627		
Genotype1												
AF221535	GAT(D)	GAA(E)	AAT(N)	CAA(Q)	GTG(V)	ACT(T)	TCA(S)	TGT(C)	GAG(E)	GCC(A)	10	1
Genotype2												
AF221258	GAC(D)	GAG(E)	AAC(N)	CAG(Q)	GTA(V)	ACC(T)	ACA(T)	TGC(C)	GAA(E)	GCT(A)	0	0
C. parvum HNJ-1	GAC(D)	GAG(E)	AAC(N)	CAG(Q)	GTA(V)	ACC(T)	ACA(T)	TGC(C)	GAA(E)	GCT(A)		
COWP												
	138–140	423–425	486–488	498–500	594–596	735–755	828–830	834–836				
Genotype1												
AF266265	CCC(P)	GTC (V)	GGT (T)	GAA(E)	AAC(N)	GAT(D)	CAG(Q)	ATT(I)			8	0
Genotype2												
AF266273	CCT (P)	GTT (V)	GGC (T)	GAG(E)	AAT(N)	GAC(D)	CAA(Q)	ATA(I)			0	0
C.parvum HNJ-1	CCT (P)	GTT (V)	GGC(T)	GAG(E)	AAT(N)	GAC(D)	CAA(Q)	ATA(I)				

Table 4 Nucleotide changes of genes for beta-tubulin comparing *C. parvum* HNJ-1 with genotype 1 and genotype 2 strain. "Parenthese following codon indicates putative amino acid. '-' indicates deletion. Substitution of nucleotide and amino acid comparing those of *C. parvum* HNJ-1 indicate bold face

Beta-tublin													Number of substitution with HNJ-1 strain	
	138 (intron)	154–157 (intron)	160 (intron)	243–245 (exon2)	267–269 (exon2)	357–359 (exon2)	384–386 (exon2)	417–419 (exon2)	468–470 (exon2)	471–473 (exon2)	483–485 (exon2)	513–515 (exon2)	525–527 (exon2)	531–533 (exon2)	nucleotide	Aminoacid
Genotype1																
AF115399	C	T–T	C	TCT(S)	GCA(A)	GGC(G)	GCT(A)	TTG(L)	CTA(L)	CAA(Q)	ATC(I)	Unknown	Unknown	Unknown	11	0
AF323578	C	T–T	C	TCT(S)	GCA(A)	GGC(G)	GCT(A)	TTG(L)	CTA(L)	CAA(Q)	ATT(I)	TCG(S)	ACT(T)	CTG(L)	14	0
AF323579	C	T–T	C	TCT(S)	GCA(A)	GGC(G)	GCT(A)	TTG(L)	CTA(L)	CAA(Q)	ATT(I)	TCG(S)	ACT(T)	CTG(L)	14	0
AF323580	C	T–T	C	TCT(S)	GCA(A)	GGC(G)	GCT(A)	TTG(L)	CTA(L)	CAA(Q)	ATT(I)	TCG(S)	ACT(T)	CTG(L)	14	0
Genotype2																
AF115398	T	TTTT	T	TCG(S)	GCG(A)	GGT(G)	GCC(A)	TTA(L)	TTA(L)	CAG(Q)	ATC(I)	unknown	unknown	unknown	0	0
AF323576	T	TTTT	T	TCG(S)	GCG(A)	GGT(G)	GCC(A)	TTA(L)	TTA(L)	CAG(Q)	ATC(I)	TCA(S)	**CCT(P)**	TTG(L)	1	1
AF323577	T	TTTT	T	TCG(S)	GCG(A)	GGT(G)	GCC(A)	TTA(L)	TTA(L)	CAG(Q)	ATC(I)	TCA(S)	**CCT(P)**	TTG(L)	1	1
BX538353	T	TTTT	T	TCG(S)	GCG(A)	GGT(G)	GCC(A)	TTA(L)	TTA(L)	CAG(Q)	ATC(I)	TCA(S)	ACT(T)	TTG(L)	0	
C. parvum HNJ-1	T	TTTT	T	TCG(S)	GCG(A)	GGT(G)	GCC(A)	TTA(L)	TTA(L)	CAG(Q)	ATC(I)	TCA(S)	ACT(T)	TTG(L)		

isolated in the United States (IOWA strain: BX538353, Bankier et al. 2003); however, some of the sequence differed from published data of genotype 2 strains (AF323576 and AF323577) isolated in United Kingdom. Widmer et al. (1998) described extensive sequence polymorphisms in intron and exon 2 sequences of beta-tubulin among the same genotypes isolated from different areas, whereas Rochelle et al. (1999) demonstrated that the same genotypes derived from the United States and Australia could not be differentiated based on geographic origin in comparison to region of exon 1-intron-exon 2. In addition, it was reported that the infectivity to human was differed among genotype 2 strains, such as IOWA, UCP, and TAMU strains (Okhuysen et al. 1999). It is implicated that sequence polymorphism of beta-tubulin reflects the extent of diversity of subpopulation within the genotype, and sequence of beta-tubulin has the potential for utilization as the genetic marker for identification at subpopulation level.

Accordingly, it was concluded that HNJ-1 strain was a strain of genotype 2 of *C. parvum*, which has unique genetic polymorphisms in genes of TRAP-C1 and beta-tubulin.

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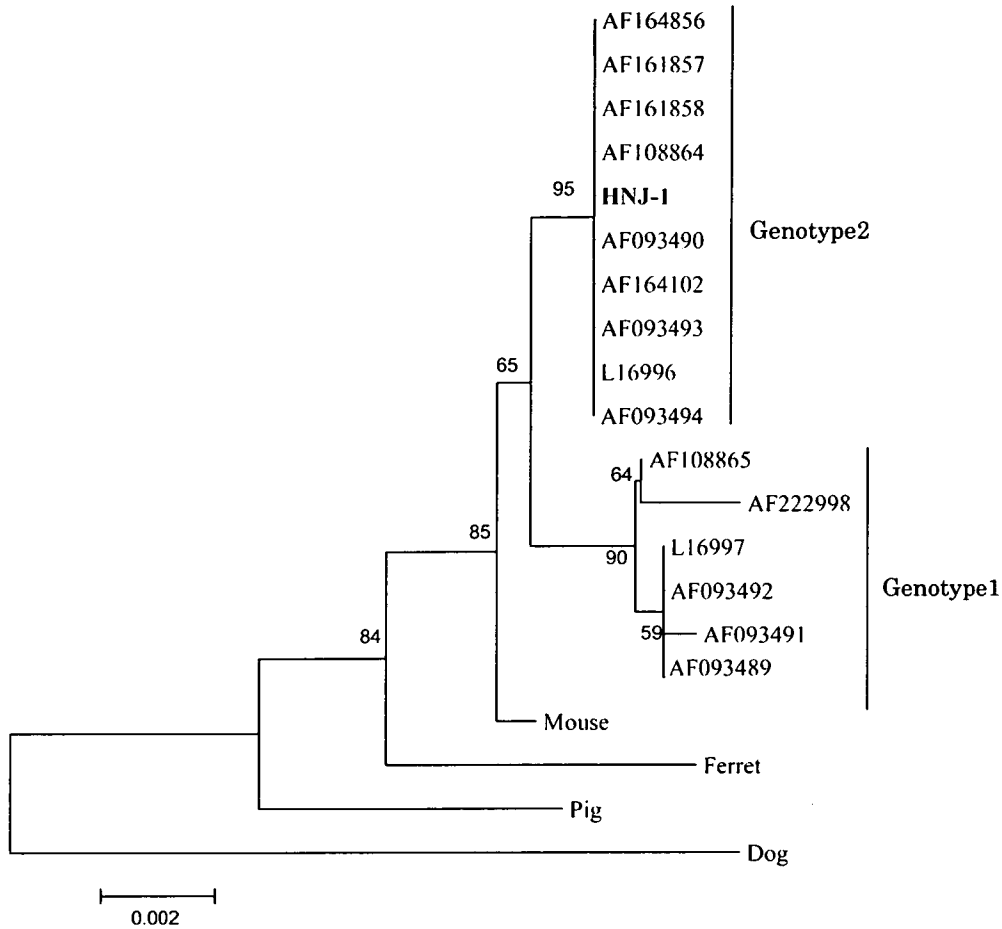

Fig. 1 Evolutionary relationship of *C. parvum* HNJ-1 and some genotypes inferred by NJ analysis of Tamura- Nei distances calculated form pair wise comparisons of sequences of the gene for18S rRNA using MEGA Version 2.1. Percentage of bootstrap support from 1,000 replicate samples is indicated in each node. Each genotype of mouse, ferret, pig, and dog, which are names of hosts of *C. parvum*, was referred from the GenBank (AF112571, AF112572, AF115377, and AF112576), respectively

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SHORT COMMUNICATION

Genetical identification of coccidia in red-crowned crane, *Grus japonensis*

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Abstract We genetically analyzed eimerian oocysts isolated from the red-crowned crane (*Grus japonensis*) in Hokkaido, a northern island of Japan. Two types of oocysts of which shapes were similar to *Eimeria gruis* and *E. reichenowi* were found. Nearly the total length of the 18S ribosomal RNA gene (about 1.7 kbp in length) was amplified from single oocyst of each type and was sequenced. The respective sequences showed high similarity to those of published partial sequences (349 bp) of *E. gruis* type oocyst and *E. reichenowi* type oocyst isolated from the hooded crane (*G. monacha*) and the white-naped crane (*G. vipio*) in Izumi, in the southern island of Japan. Phylogenetic analysis indicated that *E. gruis* type and *E. reichenowi* type are different species, and suggested that these crane coccidia have evolved independently from the intestinal parasitizing *Eimeria* species.

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Introduction

Flocks of red-crowned cranes (*Grus japonensis*) inhabit the eastern part of Hokkaido, a northern island of Japan. The flocks, numbering approximately 1,000 birds each, remain in Hokkaido all year round and are the only crane species that reproduce in Japan. About 12,000 hooded (*G. monacha*) and white-naped (*G. vipio*) cranes migrate to Izumi in the southern island of Japan in winter, but there is no contact between the flocks in Hokkaido and those in Izumi.

Eimeria species in the Eimeriidae family parasitize a wide variety of birds and mammals and have strict host, tissue, and cell specificity. They have been classified based on oocyst morphology, life cycle features, and host specificity (Levine 1982). Crane coccidia, *Eimeria gruis* and *E. reichenowi*, were reported in the USA (Courtney et al. 1975; Forrester et al. 1978; Carpenter et al. 1980, 1984). These parasites differ from other *Eimeria* species in that they do not parasitize only the intestinal tract but also other tissues and cause disseminated visceral coccidiosis (Parker et al. 1986; Novilla et al. 1989; Novilla and Carpenter 2004). The infection is often lethal to young birds. Crane coccidiosis is transmitted by oral ingestion of contaminated feces to every bird in the flock. Because it is not easy to clean up a given environment once contaminated, crane coccidiosis is a major parasitic disease not only in zoo flocks but also in flocks living in the wild.

In Japan, oocysts morphologically similar to *E. gruis* or *E. reichenowi* oocysts have been detected from *G. monacha* and *G. vipio* in Izumi in Kagoshima prefecture and from *G. japonensis* in Akan in Hokkaido (Shimizu et al. 1987; Watanabe et al. 2003). Partial 18S ribosomal RNA (rRNA) genes (349 bp in length) of two types of oocysts isolated in Izumi were sequenced (Matsubayashi et al.

2005). We investigated the morphological and genetic characteristics of coccidian oocysts in crane feces in Hokkaido.

Materials and methods

Fresh crane feces were collected from a male red-crowned crane (*G. japonensis*), which was captured with an injured leg in Hokkaido on February 24, 2005. The body weight was 5.12 kg, and the age was estimated to be 10 months. Fecal samples were stored at 4°C for about 6 months until use when they were found to contain 183 oocysts per gram feces. The oocysts used in this study were purified by sugar centrifugal flotation method (Nakai et al. 1993). They were morphologically observed, and the size of the oocysts was measured by microscopic analysis.

As the sample contained two types of oocysts, a single oocyst of respective pear or round shape was transferred to a polymerase chain reaction (PCR) tube using a micropipette under an inverted microscope (Nikon Eclipse TE300) for genetic analysis. After five rounds of freezing and thawing, 5 μl of reaction buffer (10 mM Tris–HCl [pH 8.3 at 20°C], 1.5 mM $MgCl_2$, 50 mM KCl, 0.01% Proteinase-K, 0.01% sodium dodecyl sulphate) was added to the PCR tube, which was then incubated at 37°C for 60 min and heated at 95°C for 10 min. To amplify about 1.7 kbp of 18S rRNA gene by PCR, we used two sets of primer pairs, ERIB 1–ERIB 10 (Barta et al. 1997) and E18SF–E18SR. ERIB 1–ERIB 10 (5′-ACCTGGTTGATCCTGCCAG-3′ and 5′-CTTCCGCAGGTTCACCTACGG-3′) was used for the first PCR and E18SF–E18SR (5′-GATTAAGCCATG CATGTCTAA-3′ and 5′-AGGGCTCTATTTACGCAACT-3′) was used for the second PCR. It was confirmed that the complete sequence of the 18S rRNA gene of *Eimeria* species was amplified using primer pair ERIB 1–ERIB 10 (Barta et al. 1997). The primers E18SF and E18SR were designed using OLIGO 5.0 (National BioScience, Plymouth, MN) to bind within the products of the first PCR, and their sequences were based on the sequence of *E. tenella* (GenBank accession number U67121). After DNA extraction, 45 μl of reaction mixture containing 1× PCR Gold Buffer, 2 mM $MgCl_2$, 0.2 mM dNTP, 0.25 μM of each primer solution of the first PCR (ERIB 1-ERIB 10), and 1.25 U AmpliTaq Gold (Applied Biosystems) was added to the template extract. The primary reaction program was as follows: 1 cycle at 94°C for 10 min, 45 cycles at 94°C for 30 s and 67°C for 2 min, and 1 cycle at 72°C for 10 min. The secondary reaction was performed using a 1-μl aliquot of the 1/100 diluted first PCR products in a second PCR (49 μl) containing new primers (E18SF–E18SR) for 30 cycles, during which 67°C was changed to 60°C. The PCR products were evaluated by electrophoresis in a 1.2% agarose gel followed by staining with ethidium bromide solution and visualization on ultraviolet transilluminator. The PCR products were purified using MagExtractor-PCR & Gel Clean up- (Toyobo, Osaka, Japan). The purified PCR products were sequenced using the ABI PRISM BigDye Terminator Cycle Sequencing Kit (Applied Biosystems) on an ABI 310 Genetic Analyzer (Applied Biosystems) according to the manufacturer's instructions. The primers E18SF, E18SR, and seven other primers were used for sequencing (Table 1). The primers for sequencing were designed as described above. The sequences determined from our isolates were aligned against homologous sequences of closely related species of coccidia registered in the GenBank by Clustal W multiple alignment program (Thompson et al. 1994), and then a phylogenetic tree was constructed using the MEGA version 2.1 program (Kumar et al. 2001), and the genetic distance was calculated using Kimura's two-parameter model.

Table 1 Primers for sequencing

Primer name	Sequence (5′–3′)	Location, *E. tenella* (U67121)
299-21F	ATCAGCTTTCGACGGTAGGGT	299–319
608-22F	AAAGCTCGTAGTTGGATTTCTG	608–629
1291-23F	CTGTCTGGTTAATTTCGATAACG	1291–1313
410-19R	TAATTTGCGCGCCTGCTGC	410–429
768-21R	TGCAGTATTCAGGGCGACAAG	768–788
1105-21R	AAGTTTCAGCCTTGCGACCAT	1105–1125
1456-21R	GTTGCATGCATCAGTGTAGCG	1456–1476

Results and discussion

Two types of oocysts were detected, pear and round, whose respective average dimensions were 17.7×12.4 and 17.2×15.8 μm. The morphological characteristics, the size and outer/inner structure, of the pear- and round-shaped oocysts (Fig. 1) were similar to the characteristics of *E. gruis* and *E. reichenowi*, respectively (Courtney et al. 1975). As we have not observed enough number of sporulated oocysts in the sample, we tentatively mentioned the pear-shaped oocysts as *E. gruis* type and the round-shaped oocysts as *E. reichenowi* type at the present time.

We chose two oocysts of *E. gruis* type or *E. reichenowi* type under the microscope and applied each single oocyst to PCR. Therefore, we obtained two PCR products for each oocyst type. As each PCR product showed a single band after electrophoresis, the PCR products were sequenced directly. The sequences of the 18S rRNA gene (about 1.7 kbp in length) from isolates were determined and submitted to GenBank (*E. gruis* type isolate GA and GC:

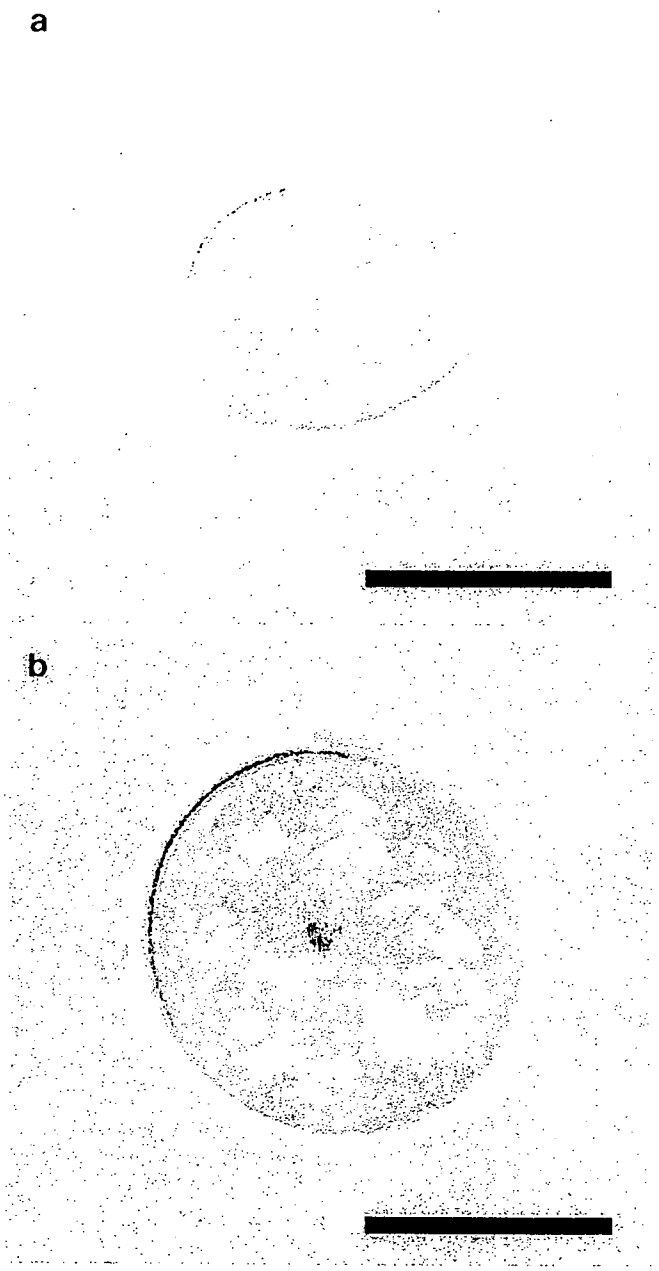

Fig. 1 Sporulated oocysts of *E. gruis* type (**a**) and *E. reichenowi* type (**b**) found in a crane fecal sample. Scale bar=10 μm

GenBank accession numbers AB243081 and AB243082; *E. reichenowi* type isolate RB and RD: GenBank accession numbers AB243083 and AB243084). Two of *E. reichenowi* type oocysts showed entirely the same sequence of 18S rRNA gene, but two of *E. gruis* type oocysts showed 5-bp difference from each other. Matsubayashi et al. (2005) published partial sequences (349 bp) of 18S rRNA genes of *E. reichenowi* type and *E. gruis* type oocysts isolated from *G. monacha* or *G. vipio* in Izumi, in the southern island of Japan. When we compared the number of different bases in homologous region (349 bp in length), the sequences of *E. reichenowi* type in this study differed by 10 bp from those of the respective oocysts in Izumi, and the sequences GA and GC of *E. gruis* type oocyst differed by 0 and 3 bp from those of the *E. gruis* type oocyst in Izumi, respectively (Matsubayashi et al. 2005). It was revealed that *E. gruis* type oocyst and *E. reichenowi* type oocyst isolated from *G. japonensis* in Hokkaido, respectively, were related to the respective type of oocyst isolated from *G. monacha* or *G. vipio* in Izumi. Although there may exist the same or related strain of *E. gruis* type in Hokkaido and Izumi, there may also exist different strains or genotypes of *E. reichenowi*

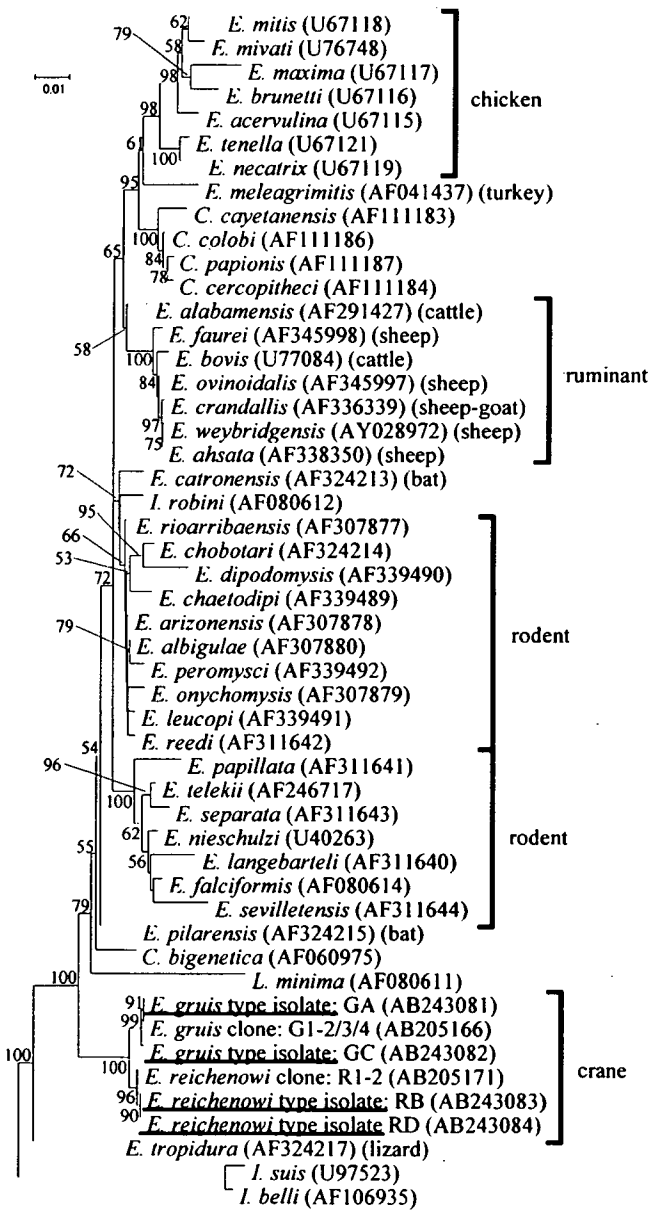

Fig. 2 Phylogenetic tree illustrating relationships among *Eimeria* species inferred using the neighbor-joining method on 18S rRNA gene sequences. The tree was rooted with the 18S rRNA gene sequences from *Isospora* species. *Underlined* species indicate isolates in this study. *Bootstrap* values are based on 1,000 replicates and are shown where larger than 50. The GenBank accession numbers of each isolate are shown in *parentheses*. Also indicated are the known hosts of each parasite taxon

type in the two areas or in the different crane species. In the future, it should be necessary to compare isolates in Hokkaido with isolates in Izumi by analyzing a much longer or complete sequence of 18S rRNA gene. *G. japonensis* remains in Hokkaido all year round and does not have contact with *G. monacha* or *G. vipio* coming to Izumi from Eurasia. It may therefore be assumed that crane coccidia adapted to cranes at the early stages of their evolution and have coevolved with them.

The DNA sequences were phylogenetically compared with other species in the genus *Eimeria* (Fig. 2). The phylogenetic tree did not include crane coccidia presented by Matsubayashi et al. (2005) because they determined only a short length of sequences (349 bp). Crane coccidia formed one clade with the other species of *Eimeria*. In the clade of *Eimeria*, crane coccidia formed a different cluster from those of chicken, rodent, or ruminant *Eimeria* species, which exclusively parasitize the intestine. Phylogenetic analysis also showed that isolates of *E. gruis* type or *E. reichenowi* type each formed their own clusters. Visceral coccidia such as crane coccidia were phylogenetically distinct from the intestinal coccidia.

A more detailed genetic analysis will give valuable information not only from a veterinary viewpoint, e.g., the control of crane coccidia and the treatment of infected birds, but also from ecological or phylogenetical viewpoints.

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