Nonaka et al., 1996; Yamashita et al., 1956). Coproantigen detection methods have been developed for the diagnosis of *Echinococcus* species using either monoclonal or polyclonal antibodies (Deplazes et al., 1999; Sakashita et al., 1995). Although a certain level of cross-reactivity with *Taenia* spp. infections has been recognized (Allan and Craig, 2006; Malgor et al., 1997), the validity of the assays was ascertained in *E. multilocularis* (Deplazes et al., 1999; Morishima et al., 1999), *Echinococcus granulosus* (Malgor et al., 1997), and *Echinococcus vogeli* infections (Matsuo et al., 2000). However, the assays were based on sandwich enzyme-linked immunosorbent assay (ELISA); thus, it takes one to several days to obtain results using a commercial kit or by outsourcing, respectively. Reliable DNA diagnostic methods are available; however, on-site diagnosis is difficult in most cases. We developed a latex agglutination test for detecting *E. multilocularis* coproantigen for on-site diagnosis.

2. Materials and methods

2.1. Parasitological examination and fecal sample collection

The samples used were obtained from 82 foxes shot in and around Sapporo (Ebetsu, Kita Hiroshima, Nanporo and Otaru) during 1997 to 1999 and were frozen at −80 °C for more than 10 days to sterilize infectious eggs of *E. multilocularis*. The intestinal tract of each fox was removed, and parasitological examination was performed on the intestinal contents and the scraping of the mucosa of the whole small intestine and colon under a stereomicroscope. The number of *E. multilocularis* found was counted. Fecal samples from the rectum were mixed with approximately equal volumes of 1% formalin, incubated at 70 °C for 12 h, and kept at room temperature. They were used for latex agglutination tests and sandwich ELISA.

2.2. Sensitization of latex particles

Polybead carboxylate microspheres were coupled with EmA9, a monoclonal antibody raised against adult *E. multilocularis* somatic antigen (Kohno et al., 1995), according to the manufacturer's instructions for the Carbodiimide Kit for Carboxylated Microparticles (Polysciences, Inc.). Briefly, 0.5 mL of a 2.5% carboxylated latex particle (diameter 1.0 μm) solution was washed twice with carbonate buffer by centrifugation at 13,000 × *g* for 6 min, and the supernatant was removed after each wash. The sediment was washed three times with phosphate buffer in the same manner. The sediment was resuspended with 0.6 mL of phosphate buffer and then stirred for 3.5 h on a wave shaker (MINI WAVE, Iuchi) with an equal volume of carbodiimide solution. The mixture was washed three times with borate buffer. After the supernatant was removed, it was resuspended with 1.2 mL of phosphate buffer that contained 60 μg of EmA9 and stirred overnight at room temperature. It was then centrifuged, and the supernatant was removed. One milliliter of 0.1 M ethanolamine was added to the sediment and stirred for 30 min. The mixture was washed, and the sediment was stirred with 1 mL of BSA solution for 30 min in the same manner. After the supernatant was removed, it was resuspended with 1 mL of storage buffer (final concentration: 1.75%) and stored at 4 °C. The latex particles sensitized with EmA9 were used within 2 weeks.

2.3. Latex agglutination tests

Latex agglutination test 1 (LA 1): samples were diluted to 0.13 g/mL with 0.1% Tween 20 in PBS and centrifuged. The supernatant (15 μL) and sensitized latex particles (5 μL) were mixed on a glass slide. Agglutination was checked after 5 min, and the results were classified into three categories by degree of agglutination: −, no agglutination; +, small agglutination masses formed; ++, large agglutination masses formed. Samples that were + or ++ were considered positive for agglutination (Fig. 1).

Latex agglutination test 2 (LA 2): to increase the sensitivity of LA 1, fecal samples were diluted with buffer containing 4 ng/mL of excretory/secretory antigen of adult *E. multilocularis* (EmES antigen) (Sakashita et al., 1995), and a latex agglutination test similar to LA 1 was performed. However, agglutination was checked after 7 min.

2.4. Sandwich ELISA

To compare the results of LA 1 and LA 2 with those of a standard technique, a sandwich ELISA was performed for coproantigen detection using the method of Morishima et al. (1999). A cut-off value to discriminate between positive and negative samples was calculated as 0.111, which was the mean optical density (OD) plus three standard deviations of fecal samples from silver foxes uninfected with *E. multilocularis* (Kaji mink, Fukagawa).

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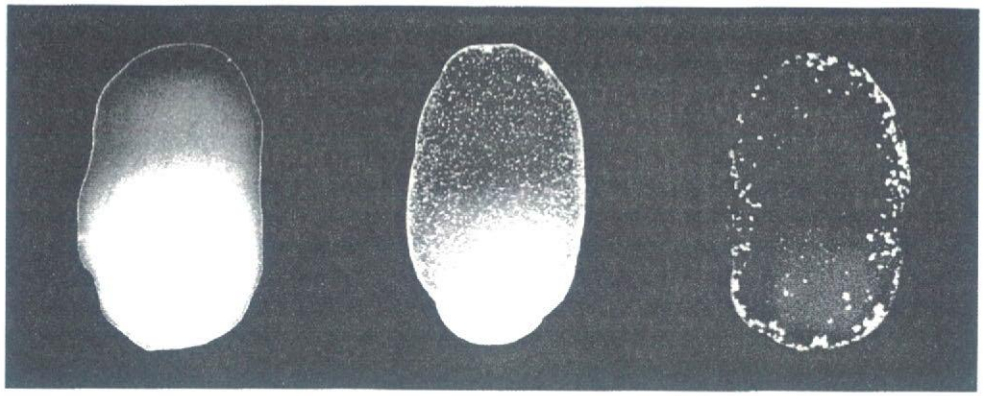

Fig. 1. Classification of the degree of agglutination. −: no agglutination observed; +: small agglutination masses formed; ++: large agglutination masses formed.

3. Results

3.1. Necropsy

E. multilocularis was found in 46 of 82 foxes necropsied. The intensity of worms ranged from 1 to 408,556, including 6 foxes with <10 worms, 11 foxes with 10–99 worms, and 29 foxes with ≥100 worms.

3.2. Latex agglutination tests

The results of LA 1 and LA 2 were compared with those of necropsy. The sensitivity of LA 1 was 61% (28/46) and the specificity was 86% (31/36), whereas the positive and negative predictive values (PPV and NPV) were 85% (28/33) and 63% (31/49), respectively (Table 1). When the sensitivity of LA 1 was evaluated by the level of worm intensity, the sensitivity became 29% (5/17) for samples with <100 worms found at necropsy and 79% (23/29) for samples with ≥100 worms (Table 2).

All of the 28 positives in LA 1 remained positive in LA 2 (Table 3). Fourteen of 18 samples that were positive in necropsy, but negative in LA 1, were positive in LA 2. However, 9 of 31 samples that were negative both in the necropsy and LA 1 were positive in LA 2 (Table 3). Accordingly, the sensitivity of LA 2 increased to 91% (42/46), but the specificity decreased to 61% (22/36) (Table 1). The PPV and NPV were 75% (42/56) and 85% (22/26), respectively. When the sensitivity of LA 2 was evaluated by the level of worm intensity, it was still low (50%) for samples with <10 worms, but it became higher for samples with ≥10 worms and was 100% for samples with ≥100 worms (Table 2).

3.3. Sandwich ELISA

The results of the sandwich ELISA were compared with those of necropsy. The sensitivity of the sandwich ELISA was 91% (42/46) and the specificity was 94% (34/36), whereas the PPV and NPV were 95% (42/44)

Table 1
Comparison of the results of necropsy with those of latex agglutination tests (LA 1 and LA 2) and sandwich ELISA

	LA 1			LA 2			Sandwich ELISA		
	Positive	Negative	Total	Positive	Negative	Total	Positive	Negative	Total
Necropsy									
Positive	28	18	46	42	4	46	42	4	46
Negative	5	31	36	14	22	36	2	34	36
Total	33	49	82	56	26	82	44	38	82
SE/SP[a]	61%/86%			91%/61%			91%/94%		
PPV/NPV[a]	85%/63%			75%/85%			95%/89%		

[a] SE: sensitivity; SP: specificity; PPV: positive predictive value; NPV: negative predictive value.

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Table 2
Sensitivity of latex agglutination tests (LA 1, LA 2) and sandwich ELISA at different levels of *Echinococcus multilocularis* infection intensity

Intensity (number of worms)	Number of tests positive/number of necropsies positive (%)		
	LA 1	LA 2	Sandwich ELISA
<10	2/6 (33)	3/6 (50)	3/6 (50)
10–99	3/11 (27)	10/11 (91)	10/11 (91)
≥100	23/29 (79)	29/29 (100)	29/29 (100)
Total	28/46 (61)	42/46 (91)	42/46 (91)

and 89% (34/38), respectively (Table 1). The sandwich ELISA showed 50% sensitivity for samples with <10 worms, but 91% and 100% sensitivity for samples with 10–99 and ≥100 worms, respectively (Table 2).

4. Discussion

The latex agglutination tests that we developed were simple, and the process could be completed within 10 min using minimal equipment. Effective latex agglutination tests for antigen detection have been developed for *Trichinella* (Choy et al., 1989) and *Trypanosoma* (Nantulya, 1994) infections with sensitivities of 89% and 88%, respectively. The sensitivity of LA 1 was lower than these results. For diseases like echinococcosis, low sensitivity is a serious disadvantage because the definitive hosts of *E. multilocularis* excrete eggs that are infectious to humans. To increase the sensitivity, a small amount of EmES antigen was added to the dilution buffer of the fecal samples (LA 2). As a result, the sensitivity of LA 2 increased to 91%, but the specificity decreased to 61%. A survey has shown that 92% of infected foxes in Hokkaido harbor >10 worms, and the median number of worms detected in infected foxes was 6400 (Yimam et al., 2002). Accordingly, infected foxes harboring <10 worms are of lesser importance in public health, not only because of their lower worm burden, but also because of their smaller proportion in the population. If only samples with ≥10 worms had been considered, the sensitivity of the LA 2 test would have been 97.5% (39/40) (100% with ≥100 worms).

The LA 1, LA 2, and sandwich ELISA produced a range of sensitivities and specificities. LA 1 had low sensitivity, but the specificity was high. Conversely, the sensitivity of LA 2 was high, but the specificity was low. Considering the predictive values, the PPV of LA 1 and NPV of LA 2 were sufficiently high (both were 85%) (Table 1). Therefore, it is effective to combine the two latex agglutination tests for practical diagnosis. In the diagnosis based on the combination of LA 1 and LA 2, samples that are positive in LA 1 are considered positive, samples that are negative in LA 2 are considered negative, and samples that are negative in LA 1 and

Table 3
Consistency in the results of necropsy, latex agglutination tests (LA 1 and LA 2), and sandwich ELISA

Necropsy	Latex agglutination tests			Sandwich ELISA		Number of worms found at necropsy
	LA 1	LA 2	No. of samples	Positive	Negative	
Positive	Positive	Positive	28	28	0	2 – 408,556
	Negative	Positive	14	12	2	4 – 408
		Negative	4	2	2	1 – 32
Negative	Positive	Positive	5	1	4	0
	Negative	Positive	9	0	9	0
		Negative	22	1	21	0

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positive in LA 2 are classified as suspicious. By this way, the chance for detecting infected case as either positive or suspicious in the combination test would become adequately high for preventing an accidental infection, although interpretation of the suspicious results should be carefully informed to dog owners because 39% (9/(14 + 9)) of samples that showed suspicious reaction in the combination test were those of uninfected cases (calculated from Table 3).

The high prevalence of *E. multilocularis* (approximately 40%) in foxes in Hokkaido and the fact that the habitat of foxes has been getting closer to or overlapping that of humans have resulted in the potential risk of infection to humans and companion animals (Eckert et al., 2000; Oku and Kamiya, 2003; Tsukada et al., 2000). Several surveys in Hokkaido showed that canine echinococcosis occurs in 0.3–1% of examined dogs (Kamiya et al., 2007; Nonaka et al., 2006); this, lead to the enforcement of a national reporting system for canine echinococcosis in Japan. Accordingly, a rapid and reliable screening system for canine echinococcosis is required.

Although the proposed assay was evaluated with fox samples, it would provide a rapid screening tool for infection in companion animals with an acceptable reliability. When the assay is performed on companion animals, the animals diagnosed as positive or suspicious must be further examined using more reliable tests such as coproantigen detection ELISA (Morishima et al., 1999) and DNA detection PCR (Dinkel et al., 1998; Trachsel et al., 2007) and should be dewormed to terminate a possible infection with *E. multilocularis*. Therefore, the assay developed here could contribute to the reporting system and to the risk management for echinococcosis. The assay could also be applied for field surveys where easy and quick diagnosis has a great advantage.

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Mitochondrial DNA Phylogeography of the Red Fox (*Vulpes vulpes*) in Northern Japan

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Mitochondrial DNA variation in the cytochrome *b* (cyt *b*) gene and the control region was examined in the red fox *Vulpes vulpes* from Japan, with special focus on the population divergence between Hokkaido and northern Honshu. Resultant haplotypes from Hokkaido were subdivided into two distinct groups (I and II), with an average genetic distance of 0.027 for cyt *b*. Divergence time is roughly estimated to be 1–2 million years ago, given that the conventional divergence rate of the mammalian cyt *b* gene is 2% per million years. Notably, Group II was only found in Hokkaido, whereas Group I comprised haplotypes from Honshu, Kyushu (Japan), eastern Russia, and Europe, as indicated by a comparison of our own data to the literature. On the other hand, judging from constructed trees, Group I haplotypes from Hokkaido appeared to differ from those from other parts of Japan, *i.e.*, Honshu and Kyushu. This implies that Blakiston's Line, which demarcates the boundary between Hokkaido and Honshu, has been an effective barrier and has allowed the structuring of genetic variation in maternal lineages. Thus, these results suggest that the Hokkaido population, which is sometimes referred to as the distinct subspecies *V. v. schrencki*, has its own genetic background with multiple migration events and differs from the parapatric subspecies *V. v. japonica* found in Honshu and Kyushu.

Key words: Japanese red fox, cytochrome *b*, control region, *Vulpes vulpes*, Blakiston's Line

INTRODUCTION

The red fox *Vulpes vulpes* has a wide geographical range and is distributed across the Northern Hemisphere from the Arctic Circle to North Africa, Central America, and the Asiatic steppes. In addition, it was introduced to Australia in 19th century. With this ubiquitous occurrence in a variety of habitats, 44 subspecies have been described (Lariviere and Pastischniak, 1996), although many of them are doubtful and their taxonomic status may require reconsideration (Macdonald and Reynolds, 2004). Until now, molecular phylogeographic analyses have been performed on a rather limited number of subspecies groups. Frati *et al.* (1998) examined cytochrome *b* gene variation in red foxes from ten European populations and found 18 distinct haplotypes, which appeared to be closely related and were perhaps generated by a rapid expansion during the last part of the Quaternary Period. However, the genetic structure of this species remains poorly understood over a large portion of its range, including the Japanese islands.

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In Japan, two subspecies have been described (Imaizumi, 1960). *Vulpes v. schrencki* (Kishida, 1924) is found in Hokkaido, as well as in the southern Kuriles and Sakhalin, Russia. *Vulpes v. japonica* (Gray, 1868) is found in Honshu, Kyushu, and Shikoku. In a study using a limited number of specimens, Imaizumi (1960) reported that *V. v. schrencki* has larger body size than *V. v. japonica* and that the two subspecies differ in coat color. In contrast, in a comparison of skull measurements, foxes from Hokkaido (Sasakawa, 1984) were smaller than those from Honshu (Takeuchi, 1994). On the other hand, the comparison of body measurements of red foxes from several regions in Japan showed no significant differences between Hokkaido and other islands (Tsukada, 1997). Therefore, the classification of the two subspecies is controversial and in need of phylogeographic investigation using reliable markers.

The border of the two subspecies is consistent with a biogeographic boundary known as Blakiston's Line. This boundary divides the much of the fauna (terrestrial vertebrates and arthropods) and flora of Japan into two distinct areas: Hokkaido, and the remaining major landmass of Japan consisting of the main islands of Honshu, Shikoku, and Kyushu and otherwise known as Hondo. In mammals, analyses of intraspecific variation in mitochondrial DNA sequences have shown that the barrier is effective in main-

taining genetic structuring in various species such as the sika deer (*Cervus nippon*; Nagata *et al.*, 1999) and the Japanese wood mice *Apodemus speciosus* and *A. argenteus* (Suzuki *et al.*, 2004). Genetic differentiation between the geographic regions is most apparent in *A. speciosus*, which exhibits a distinct composition of haplotypes that diverged up to half a million years ago. On the other hand, the differentiation is not as marked in *C. nippon* (Nagata *et al.*, 1999) or *A. argenteus* (Suzuki *et al.*, 2004). A better understanding of the subdivisions within red fox populations in Hokkaido and Honshu would provide new insight into Blakiston's Line.

In addition, population subdivisions within Hokkaido can be predicted even in large mammalian species. The brown bear *Ursus arctos*, for example, is geographically subdivided in Hokkaido on the basis of mitochondrial haplotype diversity (Matsuhashi *et al.*, 1999). Similar subdivisions are evident in small mammals. For example, in the house mouse *Mus musculus*, mitochondrial haplotype distributions vary greatly between the northern and southern populations of Hokkaido (Terashima *et al.*, 2006).

In Hokkaido, red foxes play an important role as a host of the small fox tapeworm *Echinococcus multilocularis*, which is the causative agent of a serious zoonosis, alveolar echinococcosis (Oku and Kamiya, 2003). During the early 1980s, the endemic area of *E. multilocularis* spread rapidly from eastern Hokkaido to the entire island, and the prevalence of the parasite in red foxes increased during the 1990s. In the last decade, the prevalence has been around 40% (Takahashi *et al.*, 2005). To control the situation, deworming trials have been conducted in Hokkaido by distributing bait containing an anthelminthic (Tsukada *et al.*, 2002; Takahashi *et al.*, 2002). To plan an effective deworming program, it is important to understand the population structure of red foxes in Hokkaido, such as the distribution of subpopulations and the dispersion pattern of individuals. Furthermore, although *E. multilocularis* has not yet been found in wild animals on other Japanese islands (Morishima *et al.*, 2005), slaughtered pigs infected with *E. multilocularis* were found in Aomori prefecture, the northernmost area of Honshu (Kamiya and Kanazawa, 1999). Thus, the expansion of the endemic area from Hokkaido to Honshu is a public concern (Doi *et al.*, 2000; Morishima *et al.*, 2006). It is not clear how the pigs became infected; since they were apparently not transferred from Hokkaido, they must have been infected by ingesting parasite eggs present in their locality in Aomori. However, no infected foxes or rodents were found in a subsequent intensive survey of this locality. Accordingly, it was hypothesized that the infectious source for the pigs could have been an infected fox or dog that moved from Hokkaido. It is thus necessary to evaluate whether the populations of foxes in Hokkaido are genetically different from those in Honshu. If the two populations are different, it could be possible to detect the movement of foxes from Hokkaido to Honshu (*e.g.*, by migration through the tunnel connecting the two islands, or by human introduction).

In this study, we investigated variation in mitochondrial DNA sequences in the Japanese red fox to shed light on its phylogeography, with a special focus on genetic relationships between the Hokkaido and Honshu populations.

MATERIALS AND METHODS

Sampling and DNA extraction

A total of 88 red foxes sampled between 1989 and 2006 were examined. Sampling localities and years are shown in Table 1 and Fig. 1. In Hokkaido, 56 individuals were collected at 20 sites. In northern Honshu, 24 individuals were collected at 16 sites in Aomori and Akita Prefectures. In central Honshu, four individuals were

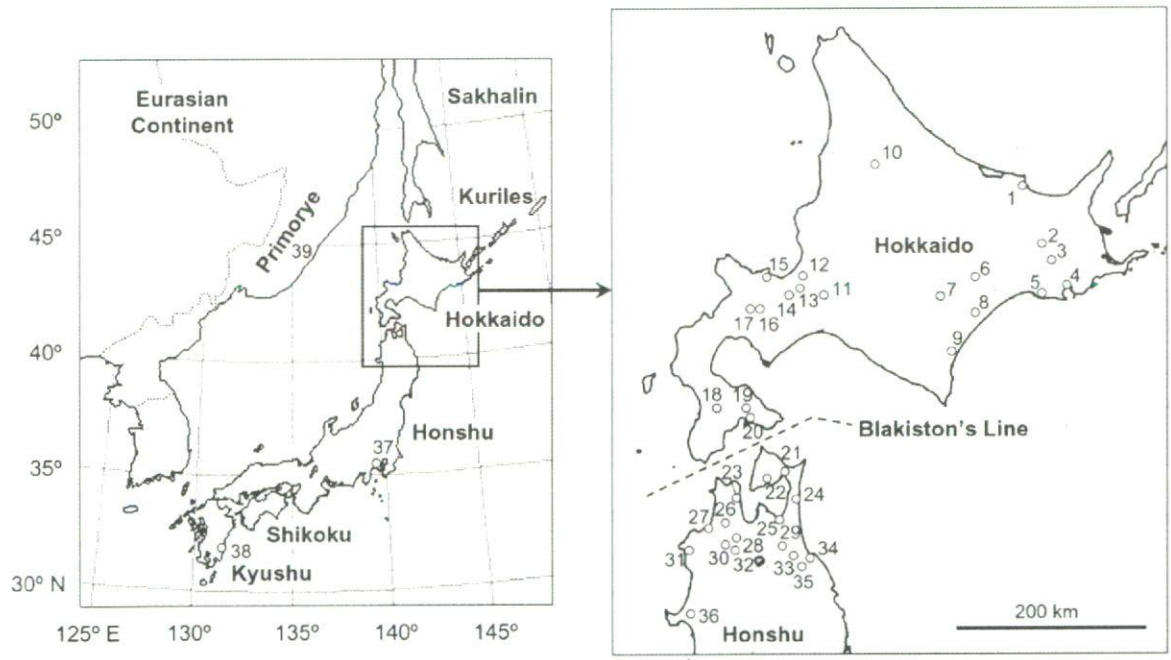

Fig. 1. Collection localities of red foxes, *Vulpes vulpes*, in Japan and Primorye, Russia. Numbers assigned to localities are listed in Table 1.

Table 1. Samples used and haplotypes of the mitochondrial DNA cytochrome *b* gene (375 bp) and control region (397bp)

Locality	Year of collection	No. examined	mt DNA haplotypes* (No. detected)
Hokkaido, Japan			
1. Abashiri	2004	4	C5-D1 (3)#, C14-D18 (1)#
2. Teshikaga	2005	4	C1-D3 (1)#, C5-D1 (2), C7-D8 (1)
3. Shibecha	2003	1	C6-D9 (1)#
4. Akkeshi	2003	2	C1-D3 (1) , C13-D18 (1)#
5. Kushiro	2003	2	C5-D1(1), C4-D2 (1)#
6. Ashoro	2004	1	C5-D1 (1)
7. Otofuke	2003, 2004	2	C1-D3 (1), C5-D4 (1)#
8. Toyokoro	2003	1	C13-D20 (1)#
9. Taiki	2003	1	C7-D8 (1)#
10. Shibetsu	2006	2	C6-D5 (1), C13-D19 (1)#
11. Kuriyama	2003	1	C6-D6 (1)#
12. Toubetsu	2003, 2004	2	C6-D5 (1), C6-D6 (1)
13. Ebetsu	2003	1	C6-D7 (1)#
14. Sapporo	2003, 2004	4	C1-D1 (1), C6-D5 (2)#, C6-D6 (1)
15. Otaru	2002	21	C1-D1 (14), C6-D6 (5) , C12-D18(2)#
16. Kyogoku	2005	2	C1-D1 (2)#
17. Kutchan	2005	1	C1-D1 (1)
18. Assabu	2003	1	C3-D1 (1)#
19. Nanae	2004	2	C2-D1 (2)
20. Hakodate	2004	1	C2-D1 (1)#
Honshu, Japan			
21. Mutsu, Aomori Pref.	2000, 2004	2	C9-D12 (2)
22. Kawauchi, Aomori Pref.	2000	1	C9-D12 (1)
23. Kanita, Aomori Pref.	2005	1	C9-D12 (1)#
24. Rokkasho, Aomori Pref.	2000, 2001, 2005	4	C9-D12 (3), C9-D14 (1)#
25. Noheji, Aomori Pref.	1999, 2000	2	C9-D12 (2)
26. Goshogawara, Aomori Pref.	2000	1	C9-D13 (1)
27. Ajigasawa, Aomori Pref.	1999	1	C9-D12 (1)
28. Kuroishi, Aomori Pref.	1999	1	C9-D12 (1)
29. Towada, Aomori Pref.	2006	1	C9-D13 (1)#
30. Hirosaki, Aomori Pref.	2001	1	C9-D12 (1)
31. Iwasaki, Aomori Pref.	1999, 2004	3	C9-D12 (3)
32. Hiraka, Aomori Pref.	2000	2	C9-D12 (2)
33. Gonohe, Aomori Pref.	2001	1	C9-D12 (1)
34. Hachinohe, Aomori Pref.	2006	1	C9-D13 (1)
35. Nango, Aomori Pref.	2004	1	C9-D12 (1)
36. Gojome, Akita Pref.	1999	1	C9-D12 (1)
37. Kanagawa Pref.†	unknown	4	C10-D15 (1)#, C10-D16 (2), C11-D17 (1)#
Kyushu, Japan			
38. Shintomi, Miyazaki Pref.	1989	1	C10-D16 (1)#
Primorye, Russia† 39.	unknown	3	C6-D10 (2)#, C8-D11 (1)#

* The combinations of the cytochrome *b* gene haplotype (C1-14) and the control region haplotype (D1-19) are shown.

\# One individual sample of each haplotype combination was used for the analysis of 1,672 bp containing the cytochrome *b* gene, the tRNA-threonine gene, the tRNA-proline gene and the 5' end of the control region.

† The exact localities where samples were collected were unknown.

collected in Kanagawa Prefecture. In Kyushu, one individual was collected in Miyazaki Prefecture. In Primorye, Far East Russia, three individuals were collected. Total DNA was isolated from blood or tissue of these animals using either the QIAamp DNA Mini Kit (QIAGEN) or by the conventional phenol-chloroform method (Sambrook *et al.*, 1989).

DNA sequencing

The nucleotide sequences of two mitochondrial DNA fragments were amplified and analyzed from each of the 88 samples. These fragments were a 375-bp fragment at the 5' end of the cytochrome *b* (cyt *b*) gene and a 397-bp fragment at the 5' end of the control region. To further define the genetic relationships among the haplotypes detected, a 1,672-bp sequence consisting of the entire cyt *b* gene, the tRNA-threonine gene, the tRNA-proline gene, and the partial control region were analyzed in one individual of each haplotype. Each DNA fragment was amplified by polymerase chain reaction (PCR), and both strands were directly sequenced. Primer pairs were as follows. To amplify the 5' end of the cyt *b* gene, primer pair 1 (5'-CAGAATGATATTTGTCCTCA-3', 5'-GATATGAAAAACCA-TCGTTG-3') was used; these primers were modified from primers H15149 and L14724, respectively (Irwin *et al.*, 1991; see Frati *et al.*, 1998). To amplify the fragment containing the 3' end of the tRNA-threonine, the tRNA-proline gene, and the 5' end of the control

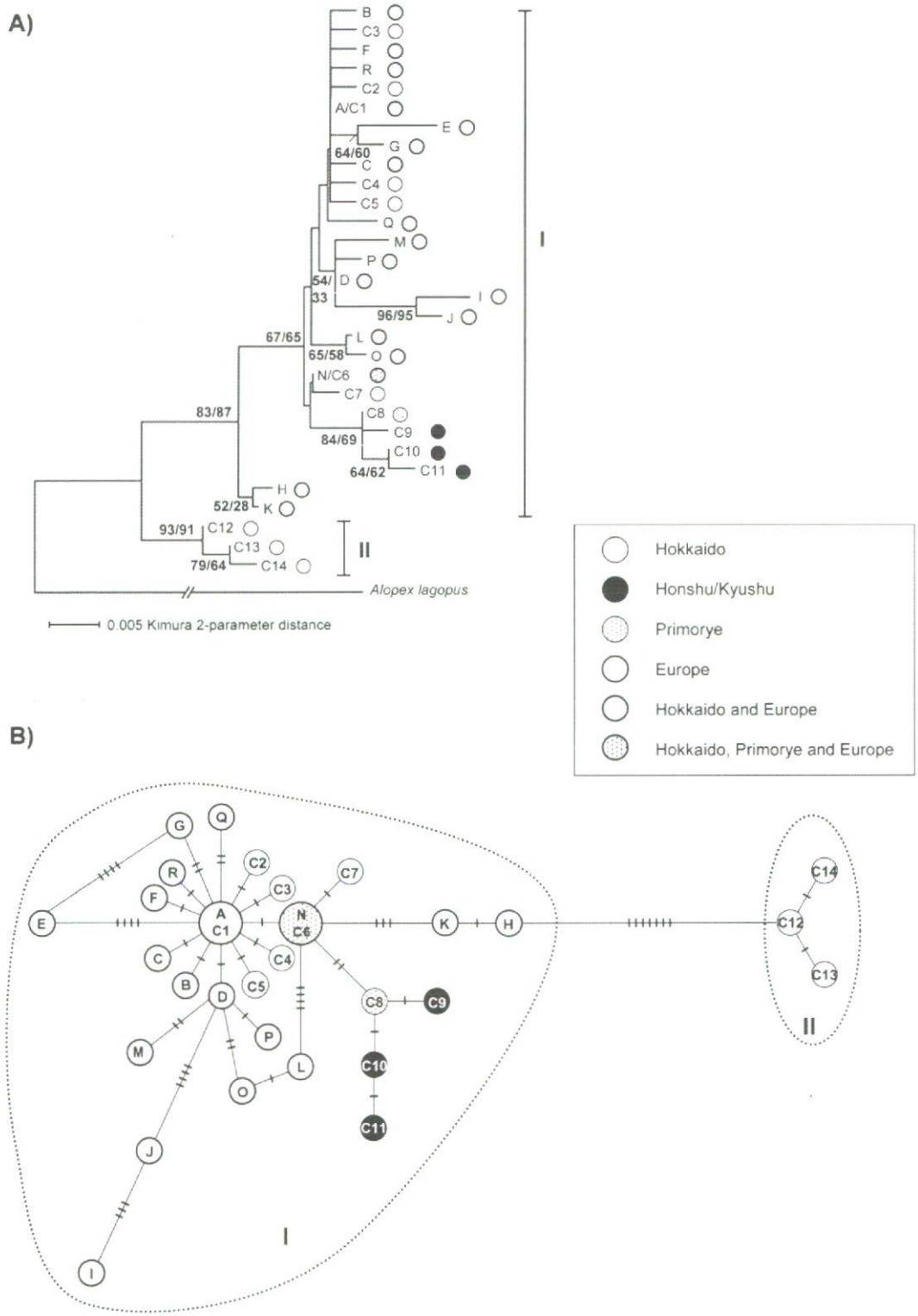

Fig. 2. Phylogenetic relationships among 24 sequences of the partial cytochrome *b* gene (375 bp) from *Vulpes vulpes*. Each taxon name corresponds to the name of the haplotype. Haplotypes C1–C14 were found in Hokkaido and Honshu, Japan, and in Primorye, Russia. Haplotypes A–Q were found in 10 regions of Europe (Spain, Italy, Austria, Bulgaria, and Israel) by Frati *et al.* (1998). Haplotypes C1 and C6 were identical to A and N, respectively. Shading of circles indicates the location where each haplotype was found. **(A)** Neighbor-joining (NJ) tree. The numbers near nodes are bootstrap values (%) for the NJ and maximum-parsimony analyses based on 1,000 pseudoreplicates. Values<50% are not shown. **(B)** Minimum spanning network. Each haplotype is represented by a circle. The number of base substitutions is indicated along each branch.

region, primer pair 2 (5'-CCAAATGCATGACACCACACAG-3', 5'-TACACTGGTCTTGTAAACC-3') was used (Stanley *et al.*, 1996; Mizuno *et al.*, 2003). To amplify the middle part of the cyt *b* gene, primer pair 3 (5'-GCTAGGACTCCTCCTAGTTTG-3', 5'-TGGAATTATCTTATTGTTCGC-3') was used. To amplify the 3' end of cyt *b* and tRNA-threonine, primer pair 4 (5'-GAATTTCAGCTTTGGGTGCT-3', 5'-ATCTTTTAGGAGACCCAGACAAC-3') was used. PCR reactions were conducted in 20-μl volumes containing 2 μl of 10X QIAGEN PCR buffer (final Mg^{2+} concentration was 1.5 mM; Qiagen), 200 μM each dNTP, 2.5U of *Taq* DNA polymerase (Qiagen), 1 μM each primer, and 2 μl of DNA extract. PCR conditions were an initial incubation at 94°C for 5 min; 30 cycles of 94°C for 30 s, 45°C (for primer pair 1) or 50°C (for primer pairs 2, 3, and 4) for 30 s, and 72°C for 30 s; and a final extension at 72°C for 7 min. PCR products were confirmed by electrophoresis in 3% agarose gels stained with ethidium bromide. After dNTPs and primers were cleaned up with EXOSAP-IT (USB), PCR products were sequenced with an automated sequencer, either CEQ 8000 (Beckman Coulter) with the CEQ DTCS–Quick Start Kit (Beckman Coulter) or an ABI PRISM™ 377 DNA Sequencer (Applied Biosystems) with the BigDye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems). The nucleotide sequences have been deposited in the DDBJ, EMBL, and GenBank nucleotide sequence databases under accession numbers AB292741–AB292765.

Phylogenetic analysis

Phylogenetic analyses were conducted using two data sets as follows: a 375-bp data set from the cyt *b* gene, including an additional 18 sequences of distinct haplotypes from 41 European red foxes (accession nos. Z80957–Z80997) from ten European populations (two populations in Spain, four in Italy, one in Austria, two in Bulgaria, and one in Israel) reported by Frati *et al.* (1998); and a 1,672-bp data set from the cyt *b* gene, tRNA-threonine gene, tRNA-proline gene, and part of the control region. From the 375-bp data set, a minimum spanning network was constructed according to the number of substitutions by using ARLEQUIN 3.01 software (Excoffier *et al.*, 2005). From the both data sets, trees were constructed with the neighbor-joining (NJ) method (Saitou and Nei, 1987), based on Kimura's two-parameter distances (Kimura, 1980), by using MEGA 3.1 (Kumar *et al.*, 2004), and the maximum parsimony (MP) method by using PAUP* 4.0b (Swofford, 2003). Bootstrap analyses for the NJ and the MP analyses were carried out with 1,000 pseudoreplicates. As outgroups, sequences available for related species were used: *Alopex lagopus* (accession no. AY598511; Delisle and Strobeck, 2005) in the analyses of 375-bp data set and *Canis familiaris* (accession no. U96639; Kim *et al.*, 1998) in the analyses of the 1,672-bp data set. Nucleotide gaps (insertions/deletions; indels) in the control region were excluded from analyses of the 1,672-bp data set.

RESULTS

The partial cyt *b* gene sequences (375 bp) and the partial control region sequences (397 bp) from 88 individuals revealed 14 (C1–14) and 20 (D1–20) haplotypes, respectively. When both of the sequences were combined, 25 combinations were found. The partial cyt *b* gene sequences differed by one to 13 substitutions at 21 variable sites; the

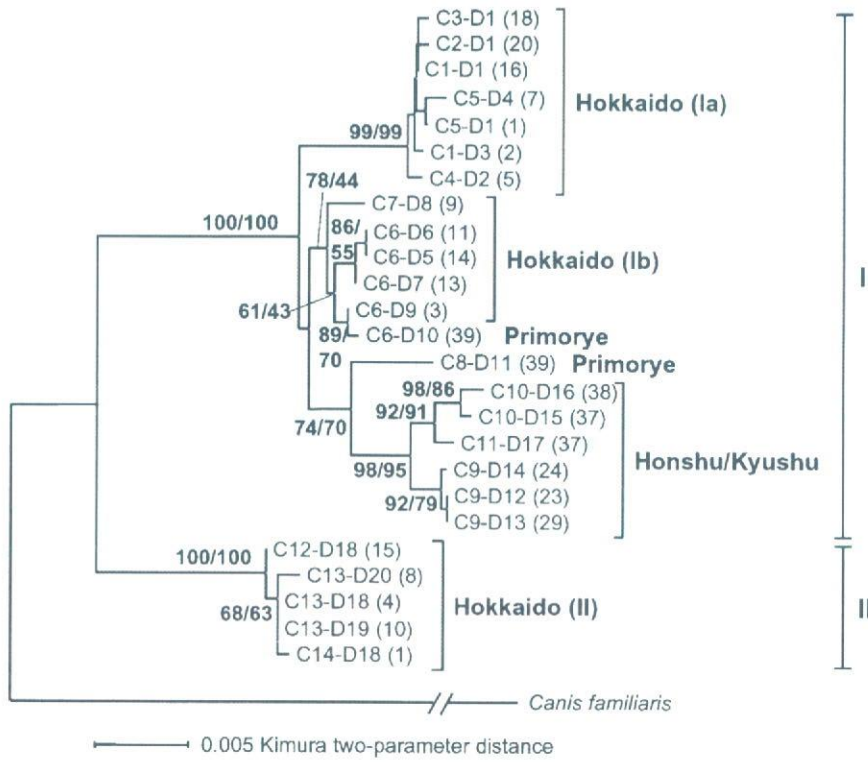

Fig. 3. Phylogenetic relationships among concatenated sequences of the cytochrome *b*, tRNA-threonine, and tRNA-proline genes, and the 5' end of the control region (1672 bp) from 25 *Vulpes vulpes* individuals collected in Hokkaido and Honshu, Japan, and in Primorye, Russia. The trees were constructed using Kimura two-parameter distances with the neighbor-joining (NJ) method. Numbers near nodes are bootstrap values (%) for the NJ and maximum parsimony analyses derived from 1,000 pseudoreplicates. Values<50% are not shown. Each taxon name corresponds to the name of the haplotype and the locality number (Table 1 and Fig. 1).

sequence divergence among haplotypes ranged from 0.003–0.035. The partial control region sequences differed by one to 16 substitutions at 26 variable sites and had indels at three sites; the sequence divergence without indels ranged from 0.003–0.040. Frequencies of haplotypes at each collection site are listed in Table 1. From one individual of each mtDNA haplotype combination, a 1,672-bp sequence consisting of the entire cyt *b*, tRNA-threonine, and tRNA-proline genes and the 5' end of the control region was determined. In the entire cyt *b* gene sequence (1,140 bp) from 25 individuals, 54 variable sites were detected; the sequence divergence ranged from 0–0.032. Some samples that had identical 375-bp sequences differed in other parts of the gene. One variable site in the tRNA-threonine gene (70 bp) and two variable sites in the tRNA-proline gene (66 bp) were detected.

The relationships among the partial cyt *b* gene sequences (375 bp) of the 14 haplotypes (C1–14) detected in this study and the 18 haplotypes (A–R) detected in 41 individuals are shown in a NJ tree (Fig. 2A) and a minimum spanning network (Fig. 2B). The two haplotypes from Hokkaido, C1 and C6, both of which were found at six collection sites, were identical to the two haplotypes from western Eurasia, A and N, respectively, and were also detected in more than two populations in Europe (Frati *et al.*, 1998). All the sequences included were divided into two major groups (Group I and II). This grouping was supported by bootstrap values of 83–93% in the NJ and MP analyses (Fig. 2A). Group I contained the 11 haplotypes (C1–11) from Hokkaido, Honshu/Kyushu, and Primorye and all 18 haplotypes from Europe, whereas Group II contained only three haplotypes (C12–14) from Hokkaido. Group I was built around a core of C1=A and C6=N; the sequence differences between the core haplotypes (C1 or C6) and other Group-I haplotypes without I and J were within four substitutions (Fig. 2B).

Phylogenetic trees were constructed using 25 sequences of the 1,672-bp data set between the cyt *b* gene and the control region (Fig. 3). As with the analysis of the partial cyt *b* gene, the sequences fell into two major groups (Groups I and II). Bootstrap analysis highly supported this grouping (100% in NJ and MP). The sequence divergence between the two groups ranged from 0.023–0.032 (mean 0.027) for the entire cyt *b* gene and from 0.025–0.040 (mean 0.031) for the partial control region without indel sites. Group I comprised haplotypes from Hokkaido, Honshu/Kyushu, and Primorye. Group II contained haplotypes unique to Hokkaido. In Group I, haplotypes from Honshu/Kyushu were clustered apart from others, with high bootstrap values (98% in NJ and 95% in MP). The sequence divergence between the haplotypes from Honshu/Kyushu and other members of Group I ranged from 0.007–0.013 (mean 0.010) for the cyt *b* gene and from 0.015–0.038 (mean 0.026) for the partial control region without indel sites. The haplotypes of Group I from Hokkaido were divided into two subgroups (Ia and Ib). The sequence divergence between the two subgroups ranged from 0.023–0.031 (mean 0.028) in the partial control region without indel sites. One subgroup was clustered with a haplotype from Primorye; the entire cyt *b* gene sequences of two individuals from Hokkaido and Primorye were identical. When the collection sites of haplotype-groups/subgroups were mapped in Hokkaido, no group/subgroup was separately distributed in Hokkaido (Fig. 4). Three haplotypes belonging to each of the three groups/subgroups were found at one site, Otaru, where 21 individuals were examined.

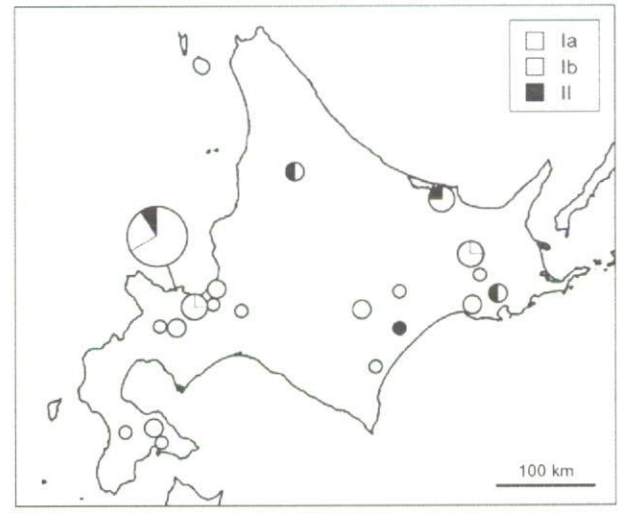

Fig. 4. Geographic distribution and frequency of mtDNA groups or subgroups of *V. vulpes* in Hokkaido. The group or subgroup is classified in Fig. 3. White, gray, and black sections of the pie charts show the frequencies of subgroups Ia and Ib, and Group II, respectively, in each location. The sizes of circles reflect sample sizes (1, 2, 4, or 21).

DISCUSSION

Genetic structure of red foxes in Eurasia

The data presented here show that the Hokkaido red foxes have uniquely composed mitochondrial lineages with two distinct haplotype groups, I and II (Figs. 2, 3). Group I was commonly detected in Hokkaido, Honshu, and Kyushu, Japan, and also in Primorye, Russia, and Europe, while Group II was detected only in Hokkaido, Japan. Genetic distance between the two groups is 2.7% on average in the cyt *b* sequences. Taking into account the reported divergence rate of 2% per million years for large mammals (Brown *et al.*, 1980; Avise *et al.*, 1998), the divergence time between Groups I and II is roughly estimated to be 1.4 million years ago. This implies that the geographic patterns of this species have been structured over a long evolutionary time frame, likely the entire period of the Quaternary. This further suggests that Hokkaido is an important phylogeographic area in the emergence and maintenance of genetic diversity, having an evolutionary history of at least 1–2 million years. This is in agreement with the fact that Hokkaido harbors an endemic species of red-backed vole, *Clethrionomys rex*, with an estimated divergence time of a few million years from the most closely related species (Wakana *et al.*, 1996; Kaneko *et al.*, 1998). The molecular data from foxes and voles thus imply that the mammalian fauna of Hokkaido has a long evolutionary history, even though Early and Middle Pleistocene fossils of extant mammals have not found in Hokkaido (Kamei *et al.*, 1988).

Group I haplotypes collected from Hokkaido and the

Eurasian continent (Primorye and Europe) were closely related (Figs. 2, 3). This suggests that a subsequent lineage introduction from the continent to Hokkaido occurred in relatively recent times. Similarly, closely related mitochondrial DNA haplotypes between populations in Hokkaido and those on the Eurasian continent have been found in other carnivores, such as the sable (*Martes zibellina*; Hosoda *et al.*, 1999) and brown bear (Matsuhashi *et al.*, 2001). In the sable, the minimum sequence difference between Hokkaido and the continent was only one substitution for partial cyt *b* gene sequences (402 bp; Hosoda *et al.*, 1999). These observations are congruent with the idea that Hokkaido and the continent were intermittently connected during the ice ages via land bridges (Ohshima *et al.*, 1990). Since the number of localities examined in this study was limited, further investigations will be necessary to verify that Group II haplotypes are confined to Hokkaido and to test the hypothesis just mentioned.

Frati *et al.* (1998) reported that a rapid expansion has occurred in *V. vulpes*, based on their analysis of samples collected in Europe. The present data show that red foxes from Europe and East Asia (Primorye and Japan) possess remarkably similar mitochondrial sequences. Similarly, mitochondrial lineages distributed throughout the entirety of Eurasia have been found in other wide-ranging carnivores such as the brown bear (Matsuhashi *et al.*, 2001) and gray wolf (Vila *et al.*, 1999; Sharma *et al.*, 2003). Furthermore, populations of the harvest mouse, *Micromys minutus*, share close mitochondrial DNA sequences that diverged an estimated several tens of thousands of years ago, even though the populations in question are spread throughout Europe and East Asia, including the Japanese islands (Yasuda *et al.*, 2005). As with these species, red foxes appear to have undergone a rapid population growth extending throughout Eurasia.

Geographic structuring across Blakiston's Line

In the tree based on 1,672-bp sequences between the cyt *b* gene and the control region (Fig.3), haplotypes from Honshu and Kyushu clustered with one another within Group I and were distinct from all haplotypes originating from Hokkaido and the continent. These results imply that the red fox population in Hondo has a different evolutionary background from that in Hokkaido. The cluster of Honshu/Kyushu haplotypes was closer to a haplotype from Primorye than to the Hokkaido haplotypes; the minimum sequence difference between Honshu/Kyushu and Primorye was only one substitution in the partial cyt *b* gene (375 bp) (Fig. 2). The mean sequence divergence between Honshu/Kyushu haplotypes and the other haplotypes of Group I was around 1% for the entire cyt *b* gene (1140 bp). Taking into account the standard divergence rate, the divergence time can be estimated to be 0.5 million years ago. This shows good agreement with fossil records indicating that red foxes could have colonized Hondo in the Middle Pleistocene (Dobson and Kawamura, 1998). Our data, therefore, suggest that the mitochondrial lineage structuring in Hondo may have started to form around half a million years ago, independent of the extant lineages of Hokkaido. This implies that Blakiston's Line has been functioning as a geographic boundary. Corresponding to Blakiston's Line, Tsugaru Strait is about 140 m deep, so that Hondo is considered to have been separated from Hokkaido, without the temporary formation of land or ice bridges, since the Middle Pleistocene. According to the estimation by Rohling *et al.* (1998), the sea level has not been 140 m lower than it is today for the past 0.45 million years. Therefore, the extant mitochondrial lineages that are distributed on both Hokkaido and the Eurasian continent would have not been established in Hondo.

The phylogenetic relationships among mitochondrial DNA sequences suggest that red fox populations in Hokkaido and Honshu/Kyushu have different evolutionary backgrounds. These two populations have been recognized as different subspecies: *V. v. schrencki* (Kita-kitsune in Japanese) in Hokkaido and *V. v. japonica* (Hondo-kitsune) in Hondo (Imaizumi, 1960). Because the collection sites and number of samples were limited in this study, further genetic and morphological studies using more specimens will be required to clarify the classification of the two subspecies and the population structure of Japanese red foxes.

Our current data set of the mitochondrial DNA sequences brings various insights to the issue of controlling *E. multilocularis*. The present survey of *V. vulpes* from Hokkaido and the northern tip of Honshu showed that genetic exchange across the Tsugaru Strait is not evident in the modern age. Our study clearly shows that mitochondrial DNA sequences would be useful as a diagnostic marker to distinguish the two populations of *V. vulpes,* making it possible to monitor the movements of individuals across the strait. However, it is not possible to evaluate the movements of males over generations, because this is not reflected in mitochondrial DNA variation. This suggests the need to survey with nuclear phylogeographic markers. The geographical distribution of mtDNA haplotype groups/subgroups of foxes in Hokkaido revealed no visible genetic structuring within Hokkaido during the course of evolution (Fig. 4). Therefore, it is necessary to conduct a deworming program against *E. multilocularis* throughout Hokkaido. Nevertheless, highly polymorphic markers such as microsatellite DNA (Lade *et al.*, 1996; Wandeler *et al.*, 2003; Swanson *et al.*, 2005), and the statistical approach of landscape genetics (Guillot *et al.*, 2005), would be useful to assess fine genetic structuring in populations of red foxes and would be useful in detailing individual movements in present-day Hokkaido. This additional information would help to establish a strategic deworming program.

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Characterization of *em*Y162 encoding an immunogenic protein cloned from an adult worm-specific cDNA library of *Echinococcus multilocularis*

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Abstract

A cDNA library based on mRNA from adult worms of *Echinococcus multilocularis* was constructed. One cDNA clone, *em*Y162, was isolated from this cDNA library. The putative protein from *em*Y162 cDNA consists of 153 amino acids and has a predicted molecular weight of 17.0 kDa. The amino acid sequences of EMY162 are predicted to be a hydrophobic N-terminus conserving a secretory signal, and a hydrophobic C-terminus encoding a transmembrane domain or glycosyl-phosphatylinositol membrane anchor, and to have single fibronectin type III-like domain. In addition, it was shown that the *emY162* gene (1738 bp) in the *E. multilocularis* genome DNA consists of three exons and two introns, and that *em*Y162 is expressed in all four stages (protoscoleces, cultured metacestodes, immature adult worms and mature adult worms). Moreover, immunity to recombinant EMY162, which comprises the fibronectin type III-like domain on the EMY162 protein, was examined. Immune responses to the recombinant EMY162 were studied by using serum from dogs infected with *E. multilocularis*. Strong IgG immune responses were detected in Western blots.

Keywords: *Echinococcus multilocularis*; Adult worm; cDNA library; *em*Y162 cDNA; Immunogenic protein; Recombinant EMY162 antigen

1. Introduction

Echinococcus multilocularis is a cestode parasite [1]. The larva, metacestode, develops in several mammalian intermediate host species, while the adult tapeworms develop mainly in dogs and foxes (definitive hosts). The life cycle of *E. multilocularis* generally occurs in foxes and rodents as intermediate hosts. Humans can be infected by accidental ingestion of the parasite eggs from an infected fox, or occasionally from infected dogs or cats. Invasion by *E. multilocularis* leads to destruction of the liver, and to damage of other organs via metastases [2]. Infection in humans causes alveolar hydatid disease [1]. Although human infection is uncommon in many countries, the disease has a high prevalence in the European latitudes north of the Alps [3] and south Gansu in China [4]. Similarly, the disease is endemic in the island of Hokkaido, Japan. In these areas the disease is a significant public health problem.

The basis of the strategy to reduce the risk of human infection is to break the cycle of transmission and avoid the production of infectious eggs. Protection against infection based on this strategy has been already achieved in the genus *Taenia*, which causes cysticercosis. Protective vaccination with either recombinant proteins or peptide epitopes of 45W, TO16, TO18 and TSA18 proteins had a high efficacy against *T. ovis* infection in sheep and *T. saginata* infection in cattle [5–8]. In addition, a recombinant vaccine has been developed for use in the control of cystic hydatid disease caused by *E. granulosus*. Lightowlers et al. [9] have demonstrated that the recombinant EG95 is a highly effective vaccine to prevent infection with *E. granulosus* in sheep in Argentina, Australia and New Zealand. These experimental results indicate that the prevention of the disease by vaccination, with a high degree of protective immunity against parasites, is possible. Vaccination of foxes and/or dogs (the definitive hosts) is postulated to be one of the most effective

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measures to control the spread of *E. multilocularis*. Gauci et al. [10] reported that EM95 recombinant protein induced significant levels of protection in mice (intermediate host) infected with *E. multilocularis* eggs. However, no information is available about the vaccination of dogs to prevent infection with *E. multilocularis* due to its biohazardous nature, and few studies have been conducted on immunity to *Echinococcus* infections in definitive hosts.

Meanwhile, many vaccine candidate proteins have been discovered that are secretory and anchored on the surface of parasites. They usually possess N-terminal hydrophobic signal peptides and C-terminal hydrophobic trans-membrane domains, and are involved in host–parasite interactions. These secreted and trans-membrane proteins participate in parasite functions including penetration and establishment in host tissues and modulation of host immune responses. These secretory proteins are candidates for development of an *E. multilocularis* vaccine, or for diagnostic use in canines. cDNAs encoding secreted and trans-membrane proteins from a parasite cDNA library have been isolated [11,12].

We attempted to clone cDNA of secretory proteins involved in immune defense systems in order to use them in the control of alveolar hydatid disease. One of the cDNA clone, *em*Y162, proved to be the first new secretory protein that acted as an antigen recognized by the canine immune system. In this paper, we report the analysis of *em*Y162 cloned from an adult worm-specific cDNA library, and the immune responses to recombinant EMY162 with the serum from *E. multilocularis*-infected dogs.

2. Materials and methods

2.1. Materials

E. multilocularis (Nemuro strain) was obtained from a dog–cotton rat life cycle maintained at the Hokkaido Institute of Public Health. Protoscoleces were taken from a cotton rat infected with the Nemuro strain and washed with PBS. Immature adult worms were collected on day 20 post-infection from a dog experimentally infected with *E. multilocularis* protoscoleces. Mature adult worms were also collected on day 60 post-infection. The worms were first released from the intestinal contents by soaking in PBS to remove canine intestinal mucus, then rinsed several times in PBS. The cultured metacestodes were obtained by an *in vitro* culture system basically following Hemphill and Gottstein [13] and Spiliotis et al. [14]. After washing with PBS, all parasite materials were immediately soaked in RNA Later (Ambion, Inc.) and stored in liquid nitrogen. In addition, 1×10^5 of *E. multilocularis* protoscoleces in 2 ml of PBS were used for oral infection. Five Beagle dogs (male, 16 months old) were bled at 40 days after infection, and sera were stored individually at −30 °C until examined for IgG response. The sera of 5 uninfected-Beagle dogs as the control were treated similarly. All experiments were performed in a specially designed safety facility, the Hokkaido Institute of Public Health (biosafety level 3).

2.2. cDNA library construction

Total RNAs from immature and mature adult worms were isolated using Isogen (Nippon Gene) according to the manufacturer's instructions. About 3 ml of parasite material was used for extracting total RNA. mRNAs in the total RNA were prepared using an Oligotex-dT30 (Super) mRNA Purification Kit (Takara Bio), and then reverse transcribed to cDNA using the SMART cDNA Library Construction Kit (BD Biosciences) for first-strand cDNA synthesis in a total reaction volume of 20 μl. The cDNA was inserted into the *Sfi*I site of the vector. The vector was used to prepare a cDNA library in bacteriophage λTriplEx2 (BD Biosciences) according to the manufacturer's recommendations. The library was screened by random cloning. Five hundred forty clones were identified from about 400,000 recombinant phages. The clone, designated *em*Y162 was excised from pTriplEx2 in host bacteria XL-Blue according to the manufacturer's recommendations (BD Biosciences).

2.3. Isolation of emY162 clone from cDNA library

The *em*Y162 clone was isolated as follows. Recombinant bacteriophages of the cDNA library were infected with XL1-Blue bacteria. After treatment for 15 min at 37 °C, the infected bacteria were spread on to a LB/$MgSO_4$ plate and then incubated at 37 °C overnight. Single plaques were transferred to SM solution and kept at 4 °C overnight. The SM solution containing the recombinant phage was treated with BM25.8 bacteria. After incubation for 1 h at 31 °C with shaking, the transformed bacteria were spread on a LB–carbenicillin (50 μg/ml) plate and incubated at 31 °C overnight. The cloned bacteria were grown in LB–carbenicillin (50μg/ml) medium at 31 °C overnight. The bacteria were harvested by centrifugation at 2600 rpm. Plasmid DNA was isolated by using QIAGEN Plasmid Tip 20 (Qiagen). The nucleotide sequences of the plasmid were analyzed on a Gene Analyzer, and the *em*Y162 clone was determined.

2.4. Amplification of emY162 gene in genome DNA

Genome DNA was extracted from protoscoleces of *E. multilocularis* using a DNeasy genomic Kit (Qiagen). One μl (1 ng μl) of the solution was used as the template DNA for PCR. Primers were designed according to the DNA sequence of *em*Y162 compiled by Gene Works sequence analysis software: upstream primer: 5′-ggaagatggtacttcgattctgt-3′, downstream primer: 5′-tgagggcctgtaagttccaact-3′. Two additional primers (5′-gagctaatagcaaagttg-3′ and 5′-cacgtgaatccatcggaagt-3′) were also designed to sequence a fragment of *em*Y162 DNA. PCR amplification was carried out by a Gene Amp PCR System 9700 (Applied Biosystems) in 50 μl of reaction mixture with Taq DNA polymerase (Roche Diagnostics). PCR conditions were as follows: 94 °C for 2 min, then 30 cycles of 93 °C for 30 s, 55 °C for 30 s, and 68 °C for 4 min, and finally 68 °C for 7 min. The amplified DNA fragment was purified with TaKaRa Easy Trap v. 2 (Takara Bio) after separation by agarose gel electrophoresis. This was used as the template DNA for the sequencing reactions.

2.5. Detection of emY162 cDNA in four stages

The *em*Y162 cDNA was amplified by reverse transcriptase (RT)-PCR from each of the four stages (protoscoleces, cultured metasestodes, immature adult worms, and mature adult worms) of *E. multilocularis*. Total RNA from the four stages was isolated using an RNeasy Mini Prep Kit (Qiagen) according to the manufacturer's instructions. About 0.1 μg of total RNA was used for RT-PCR amplification of the *em*Y162 cDNA by the SMART cDNA Library Construction Kit (BD Biosciences). The same primers (5′-ggaagatggtacttcgattctgt-3′and 5′-cacgtgaatccatcggaagt-3′) used for amplification of the *emY162* gene in genome DNA were used for RT-PCR. The predicted size of the RT-PCR product was 144 bp. In addition, two primers (5′-gttgtgctatgtggcactcgact-3′ and 5′-caatccagacagagtatttgcgttc-3′) were also designed to amplify a fragment of β-actin cDNA of *E. multilocularis* to monitor the integrity of the RNA from each stage. PCR amplifications were carried out using Gene Amp PCR System 9700 in 50 μl of the reaction mixture with Taq polymerase (Roche Diagnostics). The conditions for PCR were as follows: denaturation at 94 °C for 2 min, then 35 cycles of 93 °C for 30 s, 55 °C for 30 s, and 68 °C for 30 s, and finally 68 °C for 7 min. Fragments were visualized by ethidium bromide staining after agarose gel electrophoresis.

2.6. Preparation of recombinant EMY162

The *em*Y162 DNA fragment was amplified from *em*Y162-pTriplEx2. An up-stream primer (5′-agatctgtagacccagagctaatag-3′) with a *Bgl*II site and a downstream primer (5′-ctgcaggaatccgccagctctgtca-3′) with a *Pst*I site were designed for amplification of a 360-bp fragment of *em*Y162 DNA. PCR amplification was carried out using the Gene Amp PCR System 9700 in 50 μl of reaction mixture with Taq DNA polymerase (TakaraBio). Amplified DNA was subcloned into a *Bgl*II/*Pst*I-digested ThioHis vector (Invitrogen) and then

transformed into *Escherichia coli* Top10 strain (Invitrogen). The bacterial culture was incubated overnight at 37 °C. After cultivation, recombinant EMY162 was induced with 0.5 mM isopropyl-D-thiogalacto-pyranoside for 4–5 h at 32 °C, and following centrifugation, suspended in B-PER plus a protease inhibitor. The recombinant EMY162 expressed as a fusion protein with ThioHis was treated with ProBond™Affinity Resin (Invitrogen). The fusion protein was eluted with 20 mM sodium phosphate buffer (pH 6.0) containing 500 mM imidazole and 500 mM sodium chloride, and purified by AKTA Explorer (Amersham Biosciences) with a Hiload Superdex 75 pg column equilibrated with 20 mM Tris–HCl buffer (pH 7.8) containing 500 mM sodium chloride. Purified protein was used in Western blotting.

2.7. Detection of IgG response by Western blotting

Western blotting was carried out as follows. Approximately 1 μg of recombinant EMY162 was loaded onto a 12% SDS-polyacrylamide gel. After electrophoresis, proteins were transferred to a PVDF membrane (Amersham Biosciences), and then treated with PBS containing 10% skim milk and 0.1% Tween 20 for 1 h at room temperature. The recombinant EMY162 protein was detected with diluted canine serum (1:400) at 40 days after infection with *E. multilocularis* and diluted AP-labeled rabbit anti-dog IgG (1:2500), using the BCIP/NBT Immuno-detection Kit (PerkinElmer) according to the manufacturer's instructions.

2.8. Analysis of DNA and protein sequences

Plasmid DNA containing the parasite cDNA was prepared using a DNA Purification Kit (Qiagen) and used as a template in DNA sequencing reactions. Sequencing was performed using the 3130*xl* Gene Analyzer with a Dye-terminator Cycle Sequencing Kit (Applied Biosystems). DNA sequencing of the *emY162* gene was also performed by the same means. DNA and protein sequences were compiled using Gene Works (v. 2.5.1, Teijin) sequence analysis software. The sequence was aligned to other gene sequences available in a Basic Local Alignment Search Tool (BLAST) search of GenBank databases.

3. Results and discussion

cDNA library was constructed from immature adult *E. multilocularis* worms. In the cloning experiment, 540 cDNA clones were isolated. Their nucleotide and predicted amino acid sequences were aligned to other sequences available in GenBank databases. The alignment hit one protein sequence relating to secretory proteins containing EG95 and EM95 proteins that act as antigen.

The *em*Y162 cDNA (GenBank accession number: AB303298) cloned from the immature adult worm cDNA library of *E. multilocularis* comprised 776 nucleotides with an open reading frame of 462 bases. Comparison of nucleotide alignments showing the presence of ATG at the 5′ end and TGA (stop codon) at the 3′ end indicated that this cDNA represents a complete copy of the mRNA of the *emY162* gene. This cDNA sequence does not appear to be a copy of any other gene sequence available in a BLAST search.

The deduced amino acid sequence of *em*Y162 cDNA showed similarity to the previously described antigenic secretory proteins EM95 and EG95-1 (30% and 36%, respectively) [10,15]. When a BLAST search was also conducted for homology to antigenic proteins of *T. ovis* and *T. saginata* parasites, the EMY162 protein showed less than 30% identity to all antigens [5,7,16]. In addition, the amino acid sequence of EMY162 showed some similarity to a part of tenascin, collagen-like protein, protein tyrosine phosphatase (receptor type), and fibronectin 1, according to a BLAST search of GenBank databases. Comparison with amino acid sequences of the cDNAs that

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AAGATGGTAC TTCGATTCTG TCTTATTTTA CTGGCAACTT CAGTTATCGC TGAGGAAGTC GGGGTAGACC CAGGTAAGAT ACCTTTAAAA TTCAACTTGC   100
   M  V  L   R  F  C   L  I  L   L  A  T  S   V  I  A   E  E  V   G  V  D  P
ATCAGCATAC AATTTAAATT ATATAACAAG CTTGTATTCA TCTGCGTATG CATAGTATGT TGTGGTGGAT GCTTTTCAAC TCGTTAGTAG TTAACAGATG   200

ACCAGTTGGC TACTACCACT CATTTGATAA TATGTCACTG TCAAATCTAA AATGACGATT TTTAGCAGAA TCATTGACTC CATATTCCTA CTGCTGTCCT   300

CCTTCTGTAT CAGTTCGGGT AGATATTTGA GTCATCTGCG AAGTGTCATC CACGGGTAGG GAAATGCTTC AAAAATACAC CCTCACTTAG AAATGTAGTC   400

CCAAACACTG ATAGTAGACT GATTCACTGC ACCTCACGAC TACTTATTAT TGCACACAAT TTGTGTACCC TGCACTTGAT AGAACATTGC CTTGCTGATA   500

TTTCGCCTCA TTGTGTTCAC TCGCATAGTA CACTCCCACT TAAACAGCTC GTAAGGTAAA AGGCCTTCGG TATCTGGTCG CACCTACACA TCACCTCGTC   600

ACTGCGTGCA ACTTCAGCTG TAGCAAACCC ACCACTGACA CTATAACTCT CCGAAATCAG GATGTAGTGA CATCCATTTA CCTGCATGAT GGGTCCAGGA   700

TGACAGGAAG AAGTGCCTTG ACACACTTCA TATTGTTGGA ACACAATCCA CAAACTTAAC TTAAGCCTCA TGTTTAACCT TCTACCCAAG CATTCCGCTT   800

AATTTCGCTA AAAGCTTAGC CAAGAAGTGA CTGGAAAATT GGCTGAAATT GTTAATTTTG CAATGGGTTT GCTCCGGAAA AAAGGTTGCG CACGCGTGTG   900

TACACATGAC TGGCTATTGC GCATCAACGG ATGACACTCA GCAATTCTTA CCTCTGGTGG AACATACGGG TTAATTACTC CATCGTTTAG GGGAACACAG  1000

CAAAGTCCTG TTATCCACTG CCATCATTGT CATTATGTTT GGATAGAGGG TGCAACATTT CTGCCATTAA ATGGAATGGA ATCAACAAAA CATTATTGTT  1100

GAACAACGAA TTTTTCCGAT TCGTTTTTGC AGAGCTAATA GCAAAGTTGA CAAAGAAACT ACACAGACCA CTGCCAGAAC ACTTCCGATG GATTCACGTG  1200
                                E  L  I   A  K  L  T   K  K  L   H  R  P   L  P  E  H   F  R  W   I  H  V
GGTTCCCGCT CCCTTGAATT GGGTTGGAAT GCCACTGGTT TAGCCAATCT CCACGCGGAC CACATTAAAC TGACTGCAAA CCTTTATACA ACTTACGTTT  1300
G  S  R  S   L  E  L   G  W  N   A  T  G  L   A  N  L   H  A  D   H  I  K  L   T  A  N   L  Y  T   T  Y  V  S
CATTCAGGTA CAGAAATGTT CCTATCGAAC GTCAGAAACT CACTCTTGAG GGACTAAAGC CCAGTACATT CTACGAAGTG GTTGTGCAAG CACTCAAAGG  1400
  F  R  Y   R  N  V   P  I  E  R   Q  K  L   T  L  E   G  L  K  P   S  T  F   Y  E  V   V  V  Q  A   L  K  G
GGATTCCGAA GTTTATAAAT ACACTGGATT TATTAGAACA CTGGCTCCAG GTAAGCTAGC ATGCGATAGT AATGTGAACT TCACACCGAG GATGGATGCC  1500
D  S  E   V  Y  K  Y   T  G  F   I  R  T   L  A  P
CACCAAACTT CACCTTTGGC GGCAGTTCCT GTGTTATTAC ACTAGACCAC AAGTTAGACG AACCACAAGG CGTCAGTAGC TAATGCTCAA ATTTTGCAAA  1600

GTTTGCCGTT GAGTGGCACT GGTGTGCTGC GTAACGACAC TGACAGAGTG TCATTTTCGC ATTGCAGGGG AAGATGGCGC TGACAGAGCT GGCGGATGCG  1700
                                                                G  E   D  G  A   D  R  A   G  G  C  A
CCCTAATTTT TGCAATGGCT GGGCTCCTAT TACTTACTTG AGCCTTCCCG TAAGCCAATG AAGGTGGTCA ACTGTTGCAT TAGTTGGAAC TTACAGGCCC  1800
  L  I  F   A  M  A   G  L  L  L   L  T
TCA                                                                                                           1803
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Fig. 1. Nucleotide sequences (1803 bp) of *emY162* gene in *Echinococcus multilocularis*. Predicted amino acid sequence of the encoded protein is shown below the DNA sequence.

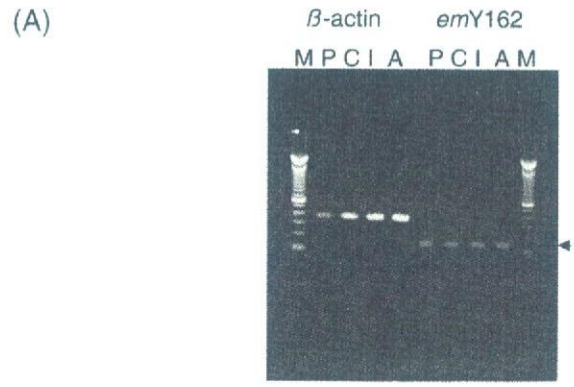
produce the known secretory protein in parasites of the genus *Taenia* did not identify significant homology.

The EMY162 protein consists of 153 amino acids and has a predicted molecular weight of 17.0 kDa. This protein has a relatively high proportion of leucine residues (16%) and one putative N-linked glycosylation site at amino acid position 83. The predicted structure from the amino acid sequence of EMY162 consists of a hydrophobic N-terminus (amino acids 1 to 16) predicted to be a secretory signal, a hydrophobic C-terminus encoding a trans-membrane domain or glycosyl-phosphatylinositol membrane anchor (amino acids 130 to 153), and one fibronectin type III domain (amino acids 84 to 129) [17–19]. Bork and Doolittle [20], Bork et al. [21] and Campbell and Spitzfaden [22] have indicated that proteins with fibronectin type III domains include the immunoglobulin superfamily, cell adhesion, surface receptors and carbohydrate-binding proteins. Analysis of the predicted amino acid sequence has revealed the presence of a conserved motif. The motif defines a fibronectin type III domain, having 40% homology compared to fibronectin sequences available in a BLAST search of GenBank databases.

The *emY162* gene (GenBank accession number: AB303297) in genome DNA was amplified by PCR from the *E. multilocularis* genome DNA. A 1738-bp DNA fragment encodes amino acid sequences of the EMY162 protein. The *em*Y162 nucleotide sequences (1803 bp) are shown in Fig. 1. The *emY162* gene consists of three exons and two introns, with similarities in structure to secretory proteins such as the *em95* gene [10]. The intron splice sites of the *emY162* gene are conserved in comparison with the cDNA. Exons 1, 2 and 3 of the gene and cDNA are identical in the DNA sequence. The lengths of exons 1, 2 and 3 are 70, 318 and 74 bp, respectively. The intron length of the *emY162* gene does not appear to be as conserved as that of the *em95* gene [10]. The lengths of intron 1 and 2 are 1059 and 217 bp, respectively.

The length of exon 1 in the *emY162* gene is similar to that of the *em95* gene, while exons 2 and 3 differ in these genes [10]. The lengths of exon 2 and 3 of *em95* were 306 and 92 bp, respectively [10]. The exon lengths of the *emY162* gene do not appear to be as conserved. In addition, the length of intron 1 of *em*Y162 also differs from the length of intron 1 in *em*95. These analyses clearly indicate that the *em*Y162 isolated from the cDNA library based on mRNA from adult *E. multilocularis* tapeworms codes for a novel secreted transmembrane protein that is different from the gene family of secretory proteins such as em95.

Meanwhile, the same putative size (144 bp) was detected in all four stages examined. The RT-PCR products are shown in Fig. 2(A). The amplified sequence covers exon 1 and a part of the N-terminus in exon 2 of EMY162 protein that includes the



(B)

```
P-emY162F1/Ra TGGAAGATGG TACTTCGATT CTGTCTTATT TTACTGGCAA CTTCAGTTAT 50
C-emY162F1/Ra TGGAAGATGG TACTTCGATT CTGTCTTATT TTACTGGCAA CTTCAGTTAT 50
I-emY162F1/Ra TGGAAGATGG TACTTCGATT CTGTCTTATT TTACTGGCAA CTTCAGTTAT 50
A-emY162F1/Ra TGGAAGATGG TACTTCGATT CTGTCTTATT TTACTGGCAA CTTCAGTTAT 50

P-emY162F1/Ra CGCTGAGGAA GTCGGGGTAG ACCCAGAGCT AATAGCAAAG TTGACAAAGA 100
C-emY162F1/Ra CGCTGAGGAA GTCGGGGTAG ACCCAGAGCT AATAGCAAAG TTGACAAAGA 100
I-emY162F1/Ra CGCTGAGGAA GTCGGGGTAG ACCCAGAGCT AATAGCAAAG TTGACAAAGA 100
A-emY162F1/Ra CGCTGAGGAA GTCGGGGTAG ACCCAGAGCT AATAGCAAAG TTGACAAAGA 100

P-emY162F1/Ra AACTACAGAC CACACTGCCA GAACACTTCC GATGGATTCA CGTG       144
C-emY162F1/Ra AACTACAGAC CACACTGCCA GAACACTTCC GATGGATTCA CGTG       144
I-emY162F1/Ra AACTACAGAC CACACTGCCA GAACACTTCC GATGGATTCA CGTG       144
A-emY162F1/Ra AACTACAGAC CACACTGCCA GAACACTTCC GATGGATTCA CGTG       144
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Fig. 2. Agarose gel electropherogram (A) and nucleotide sequences (B) of products amplified by reverse-transcriptase polymerase chain reaction (RT-PCR). (A) Total RNA from protoscoleces (P), cultured metacestodes (C), and immature (I) and mature adult (A) worms of *Echinococcus multilocularis* were used to amplify *em*Y162 cDNA with β-actin cDNA (379 bp) as the control. The position of the amplified *em*Y162 cDNA band of the expected size (144 bp) is indicated by an arrow. Molecular (M) size markers are shown in the left and right lanes. (B) Nucleotide sequences of 144-bp products of protoscoleces (P), cultured metacestodes (C), and immature (I) and mature adult worms (A) amplified by using RT-PCR with the primer pair of F1 and Ra are shown.

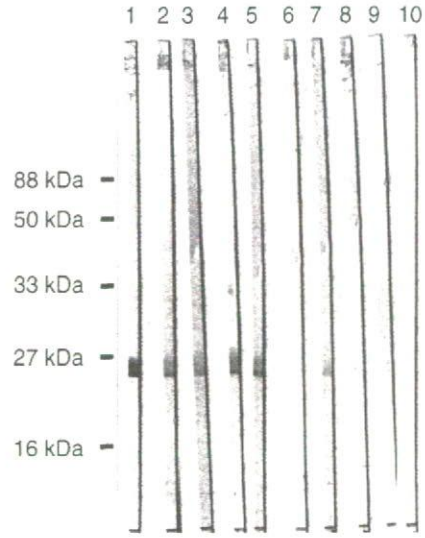

Fig. 3. Immunoblot analysis of recombinant EMY162 antigen using sera of dogs infected with *Echinococcus multilocularis*. Lanes 1–5: sera of dogs infected with the Nemuro strain (40 days after infection); lanes 6–10: sera of uninfected dogs.

peptide sequences (MVLRFCLILLATSVIAEEVGVDPE and LIAKLTKKLQTTLPEHF). The primers used in the RT-PCR analysis, according to the *em*Y162 mRNA sequence, span an intron of 1059 bp in the *emY162* gene. In a comparison of sequence alignment, PCR products from each of the four stages corresponded to positions 1 to 140 in *em*Y162 mRNA. The 144-bp RT-PCR products indicated that they contained cDNA sequences only, without introns. The results of RT-PCR indicated that *em*Y162 is expressed in all four stages. In addition, the nucleotide sequences of the 144-bp RT-PCR products are shown in Fig. 2(B). The sequences were all the same. No difference was detected in the peptide sequences of *em*Y162 mRNA expressed in all four stages.

As shown in Fig. 3, IgG responses to the recombinant EMY162 in dog serum at 40 days after infection were detected by using Western blotting. A blotting band was observed at a predicted molecular weight of 27 kDa, but it was not detected in the serum of the uninfected dogs used as the control. In this experiment, sera of five dogs that were infected with 1×10^5 *E. multilocularis* protoscoleces showed strong IgG response to recombinant EMY162. It is therefore possible that EMY162 could be used as a diagnostic antigen for serological evaluation of canine *E. multilocularis* infection.

Alignment of the amino acid sequences of *em*Y162, *em*95, *eg*95-5 and TSO45w-4B is shown in Fig. 4. The alignment data showed that amino acid differences are evident in the fibronectin type III-like domain and C-terminus, while these genes were 70% similar to amino acid sequences in the N-terminus. The significant differences are an insertion of four amino acids in the fibronectin type III-like domain and a deletion of seven amino acids in the C-terminus. In addition, the amino acid sequences of *em*Y162 were over 70% different in the linear immunogenic regions of *eg*95-5 and *em*95. The linear immunogenic regions of the EM95 and EM95 proteins include the peptide sequences TETPLRKHFNLTPV (peptide 6), SLKAVNPSDPLVYKRQTAKF (peptides 12/13), DIETPRAGKKESTVMTSGSA (peptides 21/22) and SALTSAIAGFVFSC (peptide 24) [23–25]. As shown Fig. 4, the amino acid sequences of the EM95 proteins are very similar to linear immunogenic regions of the EG95-5 protein. However, significant differences existed in the amino acid sequences of EMY162 protein as compared with two EM95 and EG95-5 proteins. In considering the use of EMY162 as a serodiagnostic

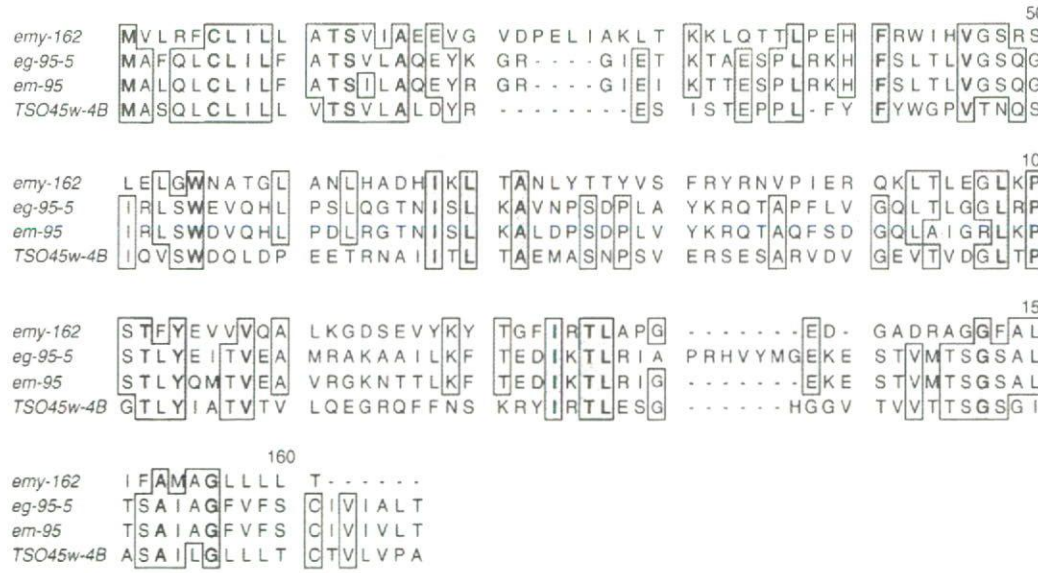

Fig. 4. Alignment of amino acid sequences of *em*Y162 and *em*95 from *Echinococcus multilocularis*, *eg*95-5 from *Echinococcus granulosus* and *TSO*45W-4B from *Taenia solium* (EM95, EG95-5 and TSO45W-4B sequences: GenBank accession numbers AJ420235, AF134378 and AF267119, respectively). The genes of each species have conserved amino acid sequences designated by gray and clear boxes.

tool, it will be essential to characterize host antibody responses, especially the kinetics of the specific antibody response after infection and subsequent chemical deworming, and class and subclass specificity against EMY162. In other parasitic infections in dogs, Deplazes et al. [26] and Nieto et al. [27] demonstrated that the analysis of IgG subsets in parasitized dogs provides evidence of a dichotomous response to infection: IgG2 is associated with asymptomatic protozoan infections and IgG1 is associated with helminth infections and diseases caused by protozoan infection.

Overall, *em*Y162 has features similar to those encoded by oncosphere antigens like *em*95, but does not share significant homology within its sequence. We demonstrated that EMY162 could target both mucosal and systemic immunity in dogs because it is predicted to be a protein with a fibronectin type III-like domain, while the serum of infected dogs showed strong IgG antibody responses to the recombinant EMY162. EMY162 could provide a potential route for the development of a practical vaccine to reduce the level of echinococcosis in canines. Future research will therefore focus on investigating the protective potential of the EMY162 protein or its peptide epitopes against infection with *E. multilocularis* eggs in intermediate and definitive hosts.

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