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北海道のライム病ボレリア伝播におけるエゾアカネズミのレゼルボア能力

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Reservoir competence of the wood mouse, *Apodemus speciosus ainu*, for the Lyme disease spirochete, *Borrelia burgdorferi*, in Hokkaido, Japan*

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Key words: *Borrelia burgdorferi*, reservoir, *Apodemus speciosus ainu*, vector, *Ixodes persulcatus*.

Abstract: To evaluate the reservoir competence of feral rodents for the Lyme disease spirochete *Borrelia burgdorferi* transmitted by the ixodid tick *Ixodes persulcatus*, 523 rodents of 4 species were captured from 2 endemic sites in Furano and Nemuro, Hokkaido, during 1991. A total of 581 larvae and 144 nymphs of *I. persulcatus* were collected from these rodent specimens. The principal host of *I. persulcatus* immatures was the wood mouse *Apodemus speciosus ainu*. The immature ticks collected were examined for spirochetal infections by culturing their internal tissues in BSK medium. The spirochetes were isolated from 35 (20.6%) of 170 *I. persulcatus* larvae derived from 53 *A. speciosus ainu* in Furano, and 82 (69.5%) of 118 *I. persulcatus* larvae derived from 26 *A. speciosus ainu* in Nemuro. We also demonstrated the transstadial infections in *I. persulcatus* nymphs that had fed as larvae on *A. speciosus ainu* in Nemuro. Xenodiagnosis indicated that the mice retained the long-term infectivity for the vector ticks. The laboratory experiment was conducted to determine whether the infected nymphs of *I. persulcatus* transmit spirochetes to the uninfected *A. speciosus ainu*. The mice readily became infected by nymphal feeding and showed no morbidity during the infection. These data clearly indicate that *A. speciosus ainu* is a suitable reservoir host for *B. burgdorferi* transmitted by *I. persulcatus* in Hokkaido.

Lyme disease is a recently recognized spirochetosis with various inflammatory manifestations involving the skin, joints, heart, and central nervous system (Steere, 1989). It has been generally assumed that ticks belonging to the *Ixodes ricinus* (L.) species complex are important arthropod vectors of the Lyme disease spirochete, *Borrelia burgdorferi* Johnson, Schmid, Hyde, Steigerwalt et Brenner, to humans (Piesman, 1989; Lane *et al.*, 1991). Rodents have been implicated as vertebrate reservoirs of this spirochete in the United States and in Europe. In highly endemic regions of the northeastern United States, the white-footed mouse, *Peromyscus leucopus* (Rafinesque), is the principal reservoir of *B. burgdorferi* (Levine *et al.*, 1985; Anderson *et al.*, 1987; Donahue *et al.*, 1987).

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The wood rat, *Neotoma fuscipes* Baird, appears to be the reservoir in north coastal California (Lane and Brown, 1991; Brown and Lane, 1992). Similarly, the rodents of genus *Apodemus* and *Clethrionomys* have been incriminated as reservoirs in Europe (Aeschlimann *et al.*, 1986; Hovmark *et al.*, 1988; Matuschka *et al.*, 1990).

In Japan, the host seeking adults of *Ixodes persulcatus* Schulze belonging to the *I. ricinus* complex appear to be highly infected with spirochetes in Hokkaido and Nagano districts where the majority of human cases of Lyme disease are concentrated (Uchikawa *et al.*, 1991; Miyamoto *et al.*, 1992; Nakao *et al.*, 1992c). Several spirochetal isolates similar to those from *I. persulcatus* were also obtained from the patients of Lyme disease with erythema chronicum migrans in Hokkaido (Nakao *et al.*, 1992a). Accordingly, female *I. persulcatus* that aggressively attack humans is thought to be most important for transmitting the infection to humans. However, the transmission dynamics of *B. burgdorferi* in nature remain to be determined. Since the transovarial infections are negligible in *I. persulcatus* (Nakao and Miyamoto, 1992), its immatures may acquire the spirochetes by feeding on reservoir hosts. The reservoir is presumably the Japanese wood mouse, *Apodemus speciosus ainu* Thomas, because *B. burgdorferi* has already been isolated from this mouse in Hokkaido (Miyamoto *et al.*, 1991).

This study was designed to clarify the transmission cycle of *B. burgdorferi* at two focuses in Hokkaido. The main objective was to evaluate the reservoir competence of *A. speciosus ainu* for spirochetes transmitted by *I. persulcatus*.

Materials and Methods

Study sites. Field studies were carried out at forests in Furano (43°14′N, 142°24′E) and in Nemuro (43°15′N, 145°24′E), Hokkaido. Vegetation of these sites was characterized by deciduous and coniferous trees and underbrush dominated by bamboo grass. Previous studies in Furano showed that the host seeking adults of *I. persulcatus* were abundant on low-lying vegetation in April–July season (Miyamoto and Nakao, 1991), and were highly infected with spirochetes (Miyamoto *et al.*, 1992). The same conditions have been observed in Nemuro (unpublished data). Sika deer, *Cervus nippon yesoensis* Heude, is numerous in these sites and is thought to be one of the hosts for adult *I. persulcatus*.

Collection of rodents. Rodents were captured in Sherman box traps (7×9×29 cm) baited with raw peanuts. The traps were set in woodland-grass in the afternoon and recovered in the following morning. The site in Furano was studied systematically from April through October, 1991. The traps were set monthly and their number ranged from 170 to 193. The site in Nemuro was studied 2 times in May and October, 1991. The numbers of traps set were 110 in May and 184 in October. The live-trapped rodents were transported to the laboratory as soon as possible. The species of rodents collected were *A. speciosus ainu, Apodemus argenteus* Temminck, *Clethrionomys rufocanus bedfordiae* Thomas, and *Clethrionomys rutilus mikado* Thomas.

Collection of ticks from rodents. After killing with chloroform inhalation, the rodents were identified by species and sex, and examined for ticks. All ticks attached on the body (mainly ears and around nose and eyes) were removed with tweezers under a magnifying glass with illumination. Twelve *A. speciosus ainu* from Nemuro were caged over pans of water, and ticks dropped in water were recovered. The collected ticks were identified by species and stages, and kept in small petri dishes until further processing. Species identification of ticks was based upon the keys of Fujita and Takada (1979), Kitaoka (1980), Takada (1990), and Nakao *et al.* (1992b).

Spirochetal culture. After tick collection, blood from heart and/or tissues from spleen of the rodents were removed aseptically and inoculated individually into 6 ml of Barbour-Stoenner-Kelly (BSK) medium (Barbour, 1984) containing rifampin (50 μg/ml) in a culture tube. Spirochetal cultures were also conducted in the ticks within 48 hr after removal from the rodents. According to the methods of Miyamoto *et al.* (1992), each tick was dissected and their internal tissues (mainly midgut) were inoculated individually into 6 ml of BSK medium. The remaining

tick bodies after dissection were preserved in 70% ethanol to reconfirm the species identification later. All cultures were kept at 31°C and examined weekly for spirochetes by dark-field microscopy for 4 weeks. The positive cultures without bacterial contaminants were passaged and used for immunochemical analysis.

SDS-PAGE and western blots. The spirochetal isolates established in culture were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and western blots according to the methods described previously (Nakao *et al.*, 1992c). The North American B31 strain (ATCC 35210) of *B. burgdorferi* (Johnson *et al.*, 1984) and the JEM2 and JEM3 isolates from the patients of Lyme disease in Hokkaido (Nakao *et al.*, 1992a) were used as controls. Monoclonal antibodies, H9724 (Barbour *et al.*, 1986) and H5332 (Barbour *et al.*, 1983) supplied by Alan G. Barbour (University of Texas Health Science Center, San Antonio, Texas, U.S.A.), were used as probes for western blots.

Molting of ticks. Of engorged larval and nymphal *I. persulcatus* that had parasitized rodents in Nemuro, the part were reared for development into the later stages to determine the spirochetal infections through the molting process. These immatures were placed in plastic boxes (60×30×15 mm) containing a hardened plaster of Paris base and kept at 25°C in a saturated humidity with a photoperiod of 12:12 (L:D) for 2–5 months until molting was completed. The molted nymphs and adults were examined for spirochetal infections as described above.

Transmission experiment 1 (xenodiagnosis). Eight *A. speciosus ainu* trapped in Nemuro (designated by numbers 1–8) were housed individually in small cages. Food (MF, Oriental Yeast Co., Ltd., Tokyo, Japan) and water were provided ad libitum. Larval *I. persulcatus* were allowed to feed on these mice 2, 8, and 16 weeks after trapping. More than 50 larvae were placed on each mouse which was confined in a tube (50 mm diameter, 100 mm long) made of a wire net of 3 mm meshes. The parasitized mice were caged over pans of water, and the dropped larvae were recovered. These engorged larvae were examined for spirochetal infections as described above. The larvae (F_1 generation) used in this experiment were derived from field-collected females fed in the laboratory on Japanese white rabbits, and determined to be free from prior infection of spirochetes by the methods of Nakao and Miyamoto (1992). The remaining engorged larvae from the mouse no. 8 (2 weeks after trapping) were reared as described above, and the molted nymphs were used for the following experiment.

Transmission experiment 2. Three *A. speciosus ainu* bred in our laboratory (designated by numbers 9–11) were used as hosts for ticks and spirochetes. According to the methods of Sinsky and Piesman (1989), these mice were determined to be free from prior infection of spirochetes by culturing the biopsy tissues of earlobes in BSK medium. The above mentioned nymphs were allowed to feed on each mouse (12 nymphs/mouse). Four weeks after nymphal feeding, these mice were examined for spirochetal infections by the xenodiagnostic method as described above. The earlobe tissues of each mouse were cultured in BSK medium 7 weeks after nymphal feeding.

Statistical analysis. In the field data, mean numbers of ticks/rodent were analyzed by Student's *t*-test. Chi-square test was used to analyze the proportional data on spirochetal infections in ticks and rodents. Both tests were conducted at $p<0.1$–0.01 levels of significance when sample sizes were adequate ($n>20$).

Results

Rodent data

The numbers of rodents captured in the study sites are summarized in Table 1. In Furano, a total of 454 rodents of 4 species were captured in 1,287 trap settings throughout the sampling period. The majorities of rodents trapped were the wood mice, *A. argenteus* (326; 71.8%) and *A. speciosus ainu* (105; 23.1%). Other rodents collected were the microtine voles, *C. rufocanus bedfordiae* (18; 4.0%) and *C. rutilus mikado* (5; 1.1%). Of these, all were examined for ticks, 429 were bled and all were taken spleen for spirochetal culture. In Nemuro,

Table 1 Number of rodents captured in the sites of Furano and Nemuro in 1991.

Site	Month	No. trap settings	Rodent species*				Total
			Apodemus		*Clethrionomys*		
			Asa	Aa	Crb	Crm	
Furano	APR	173	3	7	1	0	11
	MAY	170	2	21	1	0	24
	JUN	190	5	43	2	0	50
	JUL	189	17	69	2	0	88
	AUG	188	25	57	4	0	86
	SEP	184	24	59	4	0	87
	OCT	193	29	70	4	5	108
	Total	1,287	105	326	18	5	454
Nemuro	MAY	110	5	1	0	4	10
	OCT	184	42	0	0	17	59
	Total	294	47	1	0	21	69

* Asa, *A. speciosus ainu*; Aa, *A. argenteus*; Crb, *C. rufocanus bedfordiae*; Crm, *C. rutilus mikado*.

a total of 69 rodents of 3 species were captured in 294 trap settings. The majorities of rodents trapped were *A. speciosus ainu* (47; 68.1%) and *C. rutilus mikado* (21; 30.4%). Other rodent collected was *A. argenteus* (1; 1.5%). Of these, all were examined for ticks, 48 were bled and 57 were taken spleen for spirochetal culture. Twelve *A. speciosus ainu* trapped in Nemuro were reared in the laboratory; however, 4 mice died soon.

Apodemus spp. were dominant in our study sites. The woodland environment appeared suitable for these mice. In Furano both *A. argenteus* and *A. speciosus ainu* were more abundant in July–October than in April–June, which fact implied that the breeding seasons of these mice were during spring and early summer.

Tick data

All ticks on rodents trapped were identified and counted. Three species of ticks, *I. persulcatus, Ixodes ovatus* Neumann, and *Ixodes angustus* Neumann were found. The majority of ticks collected was *I. persulcatus*. In Furano, 371 larvae and 36 nymphs of *I. persulcatus* were collected from 159 rodents during the seasons (Table 2). Of these larvae, 216 (58.2%) were found on *A. speciosus ainu* and 128 (34.5%) were found on *A. argenteus*. Nymphs of *I. persulcatus* were also recovered frequently from *A. speciosus ainu*. In Nemuro, 210 larvae and 108 nymphs of *I. persulcatus* were collected from 48 rodents in May and October (Table 2). These immatures were recovered mostly from *A. speciosus ainu*. Of all ticks collected, 476 (343 *I. persulcatus* and 133 other species) in Furano and 203 (195 *I. persulcatus* and 8 other species) in Nemuro were examined immediately for spirochetal infections.

Mean numbers of immature *I. persulcatus*/rodent were compared among the species of rodents (Table 2). In Furano both means of larvae and nymphs were significantly higher in *A. speciosus ainu* than in *A. argenteus* (Student's t-test, $p<0.01$). The significant differences in both means of larvae and nymphs were also observed between *A. speciosus ainu* and *C. rutilus mikado* in Nemuro ($p<0.01$).

The seasonal distribution of immature *I. persulcatus* on *A. speciosus ainu* was analyzed (Fig. 1). In Furano the monthly mean numbers of larvae peaked in September. The nymphs were found in low numbers during April to October. In Nemuro both larvae and nymphs were numerous in May and October.

Table 2 Abundance of *Ixodes persulcatus* on rodents captured in the sites of Furano and Nemuro during the seasons in 1991.

Site	Rodent species*	No. rodents examined	*I. persulcatus*						Other ticks**
			No. rodents infested	Larvae collected		Nymphs collected		No. ticks per rodent	
				No.	% of total	No.	% of total	Larvae Mean±S.D. (range)	Nymphs Mean±S.D. (range)	
Furano	Asa	105	60	216	58.2	22	61.1	2.06±3.82 (0–23)	0.21±0.53 (0–3)	a, b
	Aa	326	87	128	34.5	11	30.6	0.39±0.85 (0–7)	0.03±0.18 (0–1)	c
	Crb	18	7	14	3.8	2	5.6	0.78±1.47 (0–6)	0.11±0.46 (0–2)	d, e
	Crm	5	5	13	3.5	1	2.8	2.60±1.85 (1–6)	0.20±0.40 (0–1)	f, g
	Total	454	159	371		36				
Nemuro	Asa	47	40	193	91.9	107	99.1	4.11±5.45 (0–27)	2.28±2.73 (0–10)	h, i
	Crm	21	8	17	8.1	1	0.9	0.81±1.14 (0–4)	0.05±0.21 (0–1)	j
	Aa	1	0	0	0	0	0			
	Total	69	48	210		108				

* Asa, *A. speciosus ainu*; Aa, *A. argenteus*; Crb, *C. rufocanus bedfordiae*; Crm, *C. rutilus mikado*.
** a) *I. ovatus*: 27 larvae, 17 nymphs; b) *I. angustus*: 1 larva, 1 nymph, 2 females; c) *I. ovatus*: 74 larvae, 1 nymph; d) *I. ovatus*: 1 larva, 13 nymphs; e) *I. angustus*: 1 female; f) *I. ovatus*: 2 nymphs; g) *I. angustus*: 1 nymph; h) *I. ovatus*: 2 larvae; i) *I. angustus*: 1 larva; j) *I. angustus*: 2 nymphs, 3 females.

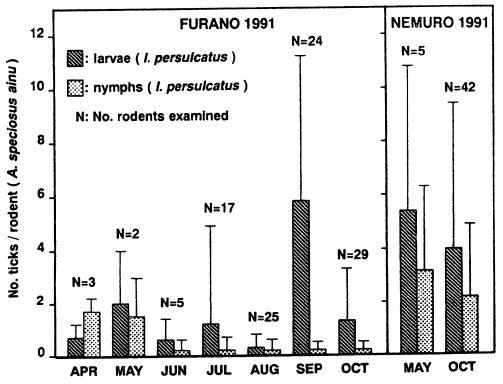

Fig. 1 Seasonal abundance (mean±S.D.) of immature *I. persulcatus* on *A. speciosus ainu* in Furano and Nemuro.

Spirochetal culture in rodents

Spirochetal infections were demonstrated in 4 species of rodents by culturing their blood and/or spleen tissues in BSK medium (Table 3). In *A. speciosus ainu* captured in Nemuro, the spirochetes were frequently detected in the spleen cultures; however, no positive results were obtained in the blood cultures. The detectable rates of spirochetes in the blood and spleen cultures were compared among the species of rodents by chi-square test. In the spleen cultures, the significant differences were observed between *A. speciosus ainu* and *A. argenteus* in Furano ($p<0.1$) and between *A. speciosus ainu* and *C. rutilus mikado* in Nemuro ($p<0.1$).

Spirochetal culture in ticks

Ticks collected from rodents were screened

Table 3 Detection of spirochetes from rodents captured in Furano and Nemuro by culturing their blood and spleen tissues in BSK medium.

Site	Rodent species*	Blood		Spleen	
		No. examined	No. (%) positives	No. examined	No. (%) positives
Furano	Asa	100	2 (2.0)	105	6 (5.7)
	Aa	306	1 (0.3)	326	6 (1.8)
	Crb	18	1 (5.6)	18	1 (5.6)
	Crm	5	1 (20.0)	5	1 (20.0)
	Total	429	5 (1.2)	454	14 (3.1)
Nemuro	Asa	32	0 (0)	35	17 (48.6)
	Crm	15	1 (6.7)	21	4 (19.0)
	Aa	1	0 (0)	1	0 (0)
	Total	48	1 (2.1)	57	21 (36.8)

* Asa, *A. speciosus ainu*; Aa, *A. argenteus*; Crb, *C. rufocanus bedfordiae*; Crm, *C. rutilus mikado*.

Table 4 Detection of spirochetes from immature *Ixodes persulcatus* fed on the rodents in Furano and Nemuro by culturing their internal tissues in BSK medium.

Site	Rodent species*	No. rodents with		Stage of ticks removed			
				Larva		Nymph	
		larvae	nymphs	No. examined	No. (%) positives	No. examined	No. (%) positives
Furano	Asa	53	14	170	35 (20.6)	19	6 (31.6)
	Aa	75	10	120	0 (0)	10	1 (10.0)
	Crb	4	1	12	0 (0)	2	0 (0)
	Crm	3	1	9	0 (0)	1	1 (100)
	Total	135	26	311	35 (11.3)	32	8 (25.0)
Nemuro	Asa	26	23	118	82 (69.5)	60	33 (55.0)
	Crm	8	1	16	4 (25.0)	1	0 (0)
	Total	34	24	134	86 (64.2)	61	33 (54.1)

* Asa, *A. speciosus ainu*; Aa, *A. argenteus*; Crb, *C. rufocanus bedfordiae*; Crm, *C. rutilus mikado*.

for spirochetal infections by culturing their internal tissues in BSK medium. The result of cultures in *I. persulcatus* is summarized in Table 4. In Furano, 35 (20.6%) of 170 larvae derived from 53 *A. speciosus ainu* were infected with spirochetes. In contrast, the cultures of 120 larvae from 75 *A. argenteus* revealed no spirochetes. Chi-square test indicated a significant effect of host species on the percentage of larvae infected with spirochetes ($p<0.01$). The numbers of infected nymphs on *A. speciosus ainu* also exceeded those on *A. argenteus*. In Nemuro, 82 (69.5%) of 118 larvae derived from 26 *A. speciosus ainu* were infected with spirochetes. The detectable rate of spirochetes was also prominent in the nymphs that had fed on *A. speciosus ainu*.

The records of spirochetal detection from other ticks are shown in Table 5. The few positive cultures were obtained from immature *I. ovatus*. No spirochetes were detected

Table 5 Detection of spirochetes from *Ixodes ovatus* and *Ixodes angustus* fed on the rodents in Furano and Nemuro by culturing their internal tissues in BSK medium.

Site	Rodent species*	*I. ovatus*				*I. angustus*					
		Larva		Nymph		Larva		Nymph		Female	
		No. examined	No. (%) positives	No. examined	No. (%) positives	No. examined	No. (%) positives	No. examined	No. (%) positives	No. examined	No. (%) positives
Furano	Asa	25	1 (4.0)	17	2 (11.8)	1	0 (0)	1	0 (0)	2	0 (0)
	Aa	69	1 (1.4)	1	0 (0)	0		0		0	
	Crb	1	0 (0)	13	6 (46.2)	0		0		0	
	Crm	0		2	0 (0)	0		1	0 (0)	0	
	Total	95	2 (2.1)	33	8 (24.2)	1	0 (0)	2	0 (0)	2	0 (0)
Nemuro	Asa	2	1 (50.0)	0		1	0 (0)	0		0	
	Crm	0		0		0		2	0 (0)	3	0 (0)
	Total	2	1 (50.0)	0		1	0 (0)	2	0 (0)	3	0 (0)

* Asa, *A. speciosus ainu*; Aa, *A. argenteus*; Crb, *C. rufocanus bedfordiae*; Crm, *C. rutilus mikado*.

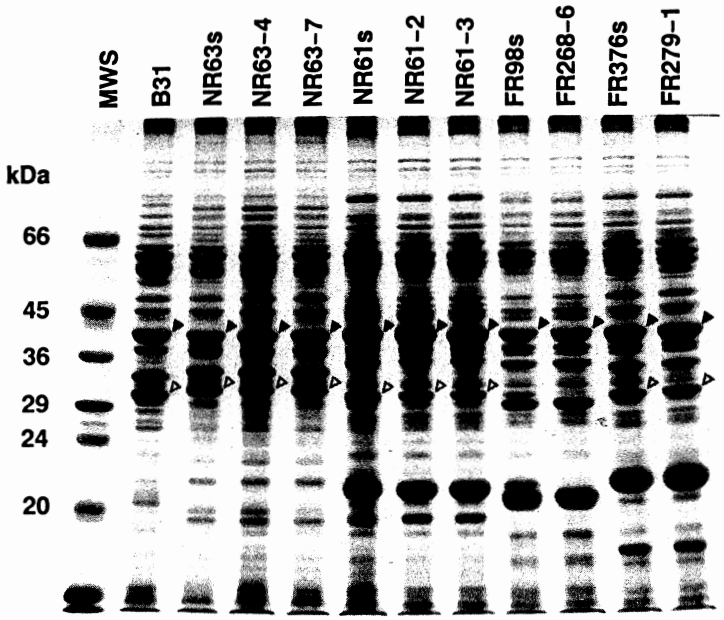

Fig. 2 Similarity of protein profiles among the spirochetes isolated from *A. speciosus ainu* and from *I. persulcatus* larvae fed on the mice.

The whole cell lysates were separated by SDS-PAGE (12.5%) and stained with Coomassie brilliant blue R-250. MWS, molecular weight standard; B31, the type strain of *B. burgdorferi*; NR63s, NR61s, FR98s, and FR376s, the isolates from spleen of mice; NR63-4, NR63-7, NR61-2, NR61-3, FR268-6, and FR279-1, the isolates from larval ticks. Closed and open arrowheads indicate the flagellin and OspA proteins, respectively.

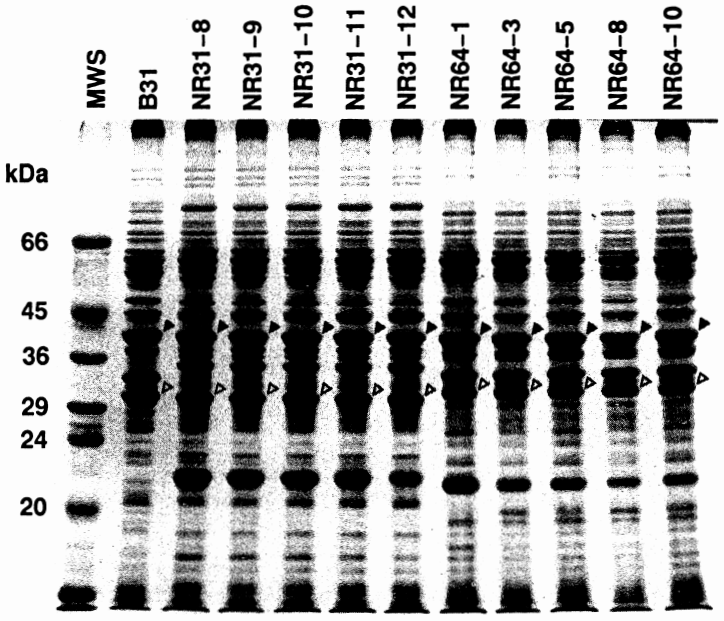

Fig. 3 Homogeneity of protein profiles among the spirochetes isolated from *I. persulcatus* larvae concurrently fed on the same hosts of *A. speciosus ainu*.

MWS, molecular weight standard; B31, the type strain of *B. burgdorferi*; NR31-8, NR31-9, NR31-10, NR31-11, and NR31-12, the isolates from larval ticks fed on the same mouse; NR64-1, NR64-3, NR64-5, NR64-8, and NR64-10, the isolates from larval ticks fed on the same mouse. Closed and open arrowheads indicate the flagellin and OspA proteins, respectively.

in all stages of *I. angustus*.

Characterization of spirochetes

All the spirochetal isolates from rodents and immature ticks established in culture were analyzed by SDS-PAGE and western blots. Numbers of the isolates examined were 37 from rodents and 173 from immature ticks. None of the 210 isolates examined in this study were identical to the North American B31 strain of *B. burgdorferi* used in the comparison of protein composition. The rodent isolates possessed the major proteins variable at the range from 35 to 21 kDa, and showed similar protein profiles to the isolates from larvae and nymphs of *I. persul-*

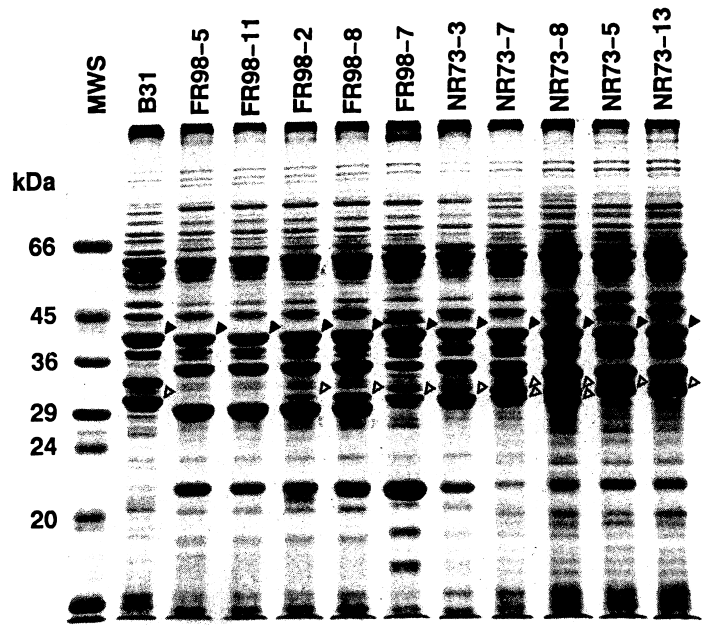

Fig. 4 Heterogeneity of protein profiles among the spirochetes isolated from *I. persulcatus* larvae concurrently fed on the same hosts of *A. speciosus ainu*.

MWS, molecular weight standard; B31, the type strain of *B. burgdorferi*; FR98-5, FR98-11, FR98-2, FR98-8, and FR98-7, the isolates from larval ticks fed on the same mouse; NR73-3, NR73-7, NR73-8, NR73-5, and NR73-13, the isolates from larval ticks fed on the same mouse. Closed and open arrowheads indicate the flagellin and OspA proteins, respectively.

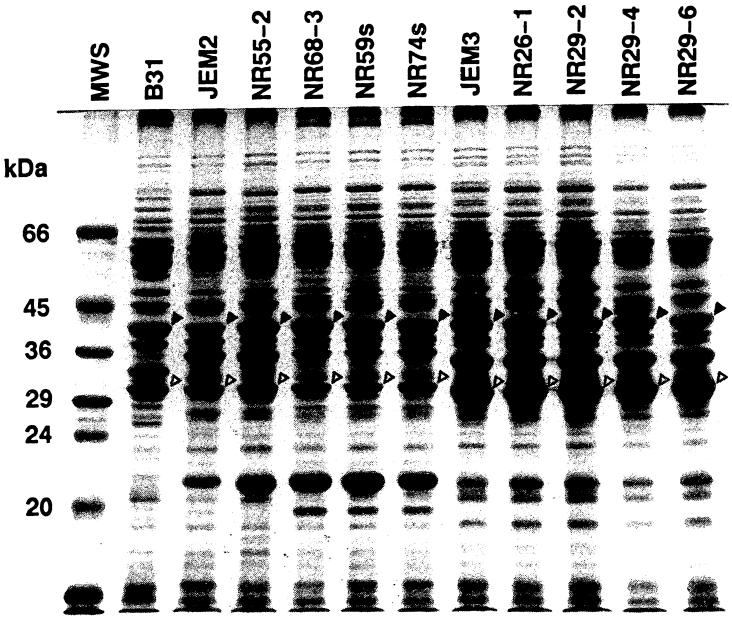

Fig. 5 Similarity of protein profiles among the spirochetes isolated from human patients, *I. persulcatus* larvae, and *A. speciosus ainu*.

MWS, molecular weight standard; B31, the type strain of *B. burgdorferi*; JEM2 and JEM3, the isolates from human patients; NR55-2, NR68-3, NR26-1, NR29-2, NR29-4, and NR29-6, the isolates from larval ticks fed on *A. speciosus ainu*; NR59s and NR74s; the isolates from spleen of mice. Closed and open arrowheads indicate the flagellin and OspA proteins, respectively.

catus (Fig. 2). The reactivities of monoclonal antibodies H9724 and H5332 against the rodent and tick isolates are summarized in Tables 6 and 7 and in Figs. 2–5. The epitope for H9724 was present in the 41 kDa flagellin protein of all isolates except 2 isolates from blood of *A. argenteus* in Furano and from a nymph of *I. persulcatus* fed on *A. speciosus ainu* in Furano. These 2 isolates possessed the 38 kDa flagellin protein that reacted with H9724. Twenty-three (62.2%) of 37 isolates from rodents and 150 (86.7%) of 173 isolates from immature ticks were reactive with H5332. These reactive isolates possessed the 30–32 kDa heterogeneous OspA proteins of *B. burgdorferi*. The H5332-non-

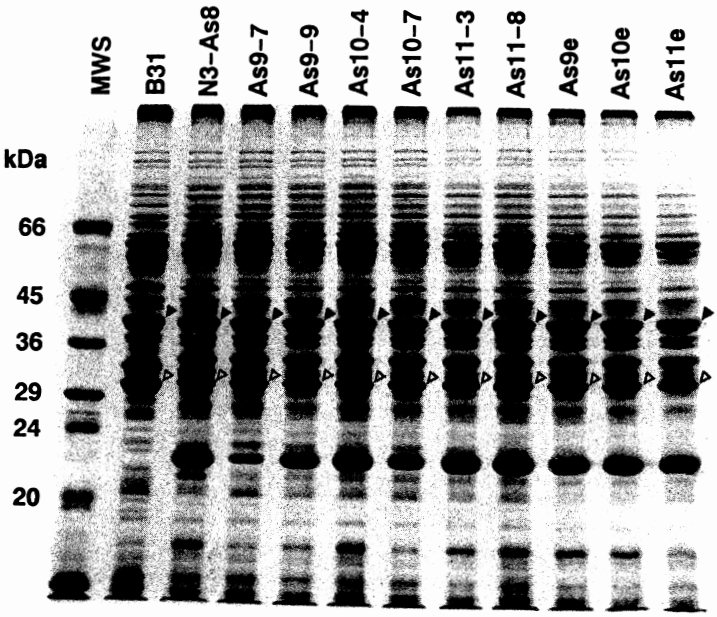

Fig. 6 Identity of protein profiles among the spirochetes obtained in transmission experiment 2.

MWS, molecular weight standard; B31, the type strain of *B. burgdorferi*; N3-As8, the isolate from a nymphal tick of the same lot used in this experiment; As9-7 and As9-9, the isolates from larval ticks fed on mouse no. 9; As10-4 and As10-7; the isolates from larval ticks fed on mouse no. 10; As11-3 and As11-8, the isolates from larval ticks fed on mouse no. 11; As9e, As10e, and As11e, the isolates from earlobe tissues of mice nos. 9, 10, and 11. Closed and open arrowheads indicate the flagellin and OspA proteins, respectively.

Table 6 Reactivity of monoclonal antibodies H9724 and H5332 against the isolates from rodents by western blots.

Site	Rodent species* (culture source)	No. examined	No. reactive isolates	
			H9724 (%)	H5332 (%)
Furano	Asa (spleen)	6	6 (100)	1 (16.7)
	Asa (blood)	1	1 (100)	0 (0)
	Aa (spleen)	5	5 (100)	2 (40.0)
	Aa (blood)	2	2 (100)**	1 (50.0)
	Crb (spleen)	1	1 (100)	1 (100)
	Crb (blood)	1	1 (100)	1 (100)
	Crm (blood)	1	1 (100)	1 (100)
	Total	17	17 (100)	7 (41.2)
Nemuro	Asa (spleen)	17	17 (100)	13 (76.5)
	Crm (spleen)	2	2 (100)	2 (100)
	Crm (blood)	1	1 (100)	1 (100)
	Total	20	20 (100)	16 (80.0)
	Grand total	37	37 (100)	23 (62.2)

* Asa, *A. speciosus ainu*; Aa, *A. argenteus*; Crb, *C. rufocanus bedfordiae*; Crm, *C. rutilus mikado*. ** One isolate possessed the 38 kDa flagellin protein that reacted with H9724.

reactive isolates from rodents and immature ticks are presumably *B. burgdorferi,* since the 41 kDa flagellin protein was present in both H5332-reactive and H5332-nonreactive isolates. The spirochetes that possessed the 38 kDa flagellin protein were unidentified.

The spirochetal isolates from several *I. persulcatus* larvae concurrently fed on the same *A. speciosus ainu* were homogeneous in their protein profiles (Fig. 3); however,

Table 7 Reactivity of monoclonal antibodies H9724 and H5332 against the isolates from immature ticks by western blots.

Site	Host rodents*	Tick species	Tick stage	No. examined	No. reactive isolates	
					H9724 (%)	H5332 (%)
Furano	Asa	*I. persulcatus*	larva	35	35 (100)	25 (71.4)
			nymph	6	6 (100)**	4 (66.7)
		I. ovatus	larva	1	1 (100)	0 (0)
			nymph	2	2 (100)	1 (50.0)
	Aa	*I. persulcatus*	nymyh	1	1 (100)	1 (100)
		I. ovatus	larva	1	1 (100)	1 (100)
	Crb	*I. ovatus*	nymph	6	6 (100)	3 (50.0)***
	Crm	*I. persulcatus*	nymph	1	1 (100)	1 (100)
			Total	53	53 (100)	36 (67.9)
Nemuro	Asa	*I. persulcatus*	larva	82	82 (100)	78 (95.1)
			nymph	33	33 (100)	31 (93.9)
		I. ovatus	larva	1	1 (100)	1 (100)
	Crm	*I. persulcatus*	larva	4	4 (100)	4 (100)
			Total	120	120 (100)	114 (95.0)
			Grand total	173	173 (100)	150 (86.7)

* Asa, *A. speciosus ainu*; Aa, *A. argenteus*; Crb, *C. rufocanus bedfordiae*; Crm, *C. rutilus mikado*.
** One isolate possessed the 38 kDa flagellin protein that reacted with H9724. *** Two isolates showed similar protein profiles to the isolates from unfed adults of *I. ovatus*.

Table 8 Prevalence of spirochetes in the laboratory-reared nymphs and adults of *Ixodes persulcatus*, which had been collected as immatures from rodents in Nemuro.

Host rodents*	Ticks collected		Ticks reared to later stage		
	No. hosts	Stage	Stage	No. examined	No. (%) positives
Asa	15	Larva	Nymph	48	32 (66.7)
Asa	12	Nymph	Adult	24	12 (50.0)

* Asa, *A. speciosus ainu*.

in some cases, they were heterogeneous (Fig. 4). Two isolates from *I. ovatus* nymphs fed on *C. rufocanus bedfordiae* showed similar protein profiles to the isolates from unfed adults of *I. ovatus* (data not shown). The remaining all isolates examined were equivalent to the spirochetes from unfed adults of *I. persulcatus* (data not shown). Part of the isolates from *A. speciosus ainu* and from *I. persulcatus* larvae fed on this mice were identical with the JEM2 and JEM3 isolates from the patients of Lyme disease (Fig. 5).

Retention of spirochetal infections through molting process

Fifty-five engorged larvae and 40 engorged nymphs of *I. persulcatus* collected from 18 *A. speciosus ainu* in Nemuro were reared in the laboratory to determine the transstadial transmission of spirochetes. Of these ticks, 48 larvae and 24 nymphs successfully molted to the later stages. Spirochetes were detected

from 32 (66.7%) of 48 nymphs and 12 (50.0%) of 24 adults by culturing their internal tissues in BSK medium (Table 8).

Table 9 Ingestion of spirochetes in larval *Ixodes persulcatus* by feeding on the field-trapped *Apodemus speciosus ainu* (Experiment 1).

Mouse no.	Weeks after trapping	Engorged larvae examined	
		No. examined	No. (%) positives
1	2	3	3 (100)
	8	5	1 (20.0)
	16	5	0 (0)
2	2	4	3 (75.0)
	8	5	2 (40.0)
	16	5	2 (40.0)
3	2	2	0 (0)
4	2	2	2 (100)
	8	5	2 (40.0)
	16	5	3 (60.0)
5	2	2	1 (50.0)
	8	NT*	
	16	5	2 (40.0)
6	2	5	4 (80.0)
	8	5	4 (80.0)
	16	5	5 (100)
7	2	4	0 (0)
8	2	5	3 (60.0)
	8	5	1 (20.0)
	16	5	0 (0)

* Not tested.

Among individual rodents from which molted nymph were examined, 10 (66.7%) of 15 *A. speciosus ainu* produced the spirochete-positive nymphs. These data certified that the spirochetes transmitted transstadially from engorged immatures to the later stages through the molting process. All spirochetal isolates from the molted nymphs and adults were analyzed by SDS-PAGE and western blots and identified as *B. burgdorferi.*

Transmission of spirochetes from rodents to larvae (Experiment 1)

Eight *A. speciosus ainu* trapped in Nemuro were reared in the laboratory, and *I. persulcatus* larvae were allowed to feed on these mice for specified intervals to examine the transmissible duration of spirochetes. The first xenodiagnostic screening to select the infected mice was performed 2 weeks after trapping. Six mice appeared to be infected with spirochetes in this screening because the cultivable spirochetes were detected from the engorged larvae (Table 9). These infected mice were further examined 8 and 16 weeks after trapping. Even 16 weeks passed, 4 mice still remained the potential to transmit spirochetes to larvae. All spirochetal isolates obtained in this experiment were identified as *B. burgdorferi.*

Transmission of spirochetes from nymphs to rodents (Experiment 2)

This laboratory experiment was conducted to determine whether *I. persulcatus* nymphs transmit spirochetes to *A. speciosus ainu* by their feeding. Prior to the experiment, part of the usable nymphs were examined for spirochetal infections by culturing their internal tissues in BSK medium. Four (80.0%)

Table 10 Spirochetal infection of the laboratory-bred *Apodemus speciosus ainu* by feeding of nymphal *Ixodes persulcatus* (Experiment 2).

Mouse no.	Spirochetal culture of earlobe tissue		Xenodiagnosis by larval *I. persulcatus* (4 weeks after nymphal feeding)	
	Before nymphal feeding	7 weeks after nymphal feeding	Engorged larvae examined	
			No. examined	No. positives (%)
9	Negative	Positive	10	2 (20.0)
10	Negative	Positive	10	6 (60.0)
11	Negative	Positive	10	2 (20.0)

of 5 nymphs were infected with spirochetes. The 12 nymphs were allowed to feed on each of three non-infected mice. After nymphal feeding, all mice were positive for spirochetes by the xenodiagnosis and by culturing the earlobe tissues (Table 10). No morbidity due to the spirochetal infection was observed in all mice during the experiment period. All spirochetal isolates obtained in this experiment were identified as *B. burgdorferi*. The protein profiles of spirochetes isolated from the unfed nymphs, the larvae fed on mice, and the ears of mice were similar to each other (Fig. 6). These similarity certified that the agent within nymphs was transmitted to mice by nymphal feeding on mice.

Discussion

Although several wild mammals and birds have been implicated as reservoirs for *B. burgdorferi*, the rodent species are of outstanding importance among them (Anderson, 1989; Jaenson, 1991). The suitability of reservoir rodents mainly depends on the intensity of their ecological relationship with the immature stages of vector ticks. In Japan, *I. persulcatus* is known as a vector of the agent to humans (Kawabata *et al.*, 1987; Miyamoto *et al.*, 1990); however, little information on the host preference of its immature ticks is available considering the transmission cycle of spirochetes in nature. In this study, we examined the following epidemiologic parameters on rodents at two focuses in Hokkaido: (1) the relative abundance of rodents in woodland; (2) the degree of tick-rodent contact; (3) the prevalence of spirochetes in rodents; (4) the prevalence of spirochetes in ticks feeding on rodents. In addition to the field study, we conducted the laboratory experiments to confirm the transmission of spirochetes from rodents to ticks, and from ticks to rodents. Based on these data, the reservoir competence of rodents for *B. burgdorferi* was evaluated.

The relative abundance of rodents weights their importance as hosts for ticks and as reservoirs for *B. burgdorferi*. In our study sites of woodlands densely inhabited by *I. persulcatus*, the wood mice of *Apodemus* spp. were more abundant than other rodents such as *Clethrionomys* spp., which fact suggested that *Apodemus* spp. were fitting as the hosts for *I. persulcatus*. Indeed, its immature ticks were found frequently on *A. speciosus ainu*. Although immature *I. persulcatus* exhibit a broad host range (Yamaguti *et al.*, 1971; Ai *et al.*, 1991), we believe that *A. speciosus ainu* is the most important host for immature *I. persulcatus* in Hokkaido.

Frequent attachment of immature ticks to the abundant rodents maximizes the transmission of spirochetes as a general rule. We demonstrated that both larvae and nymphs of *I. persulcatus* removed from *A. speciosus ainu* were highly infected with spirochetes. The clustering of infected larvae on *A. speciosus ainu* suggests that *I. persulcatus* may acquire spirochetes by feeding on this mouse. In contrast, *A. argenteus* was inadequate as the reservoir host because no spirochetes were detectable from the feeding larvae. Many engorged larvae and nymphs of *I. persulcatus* collected from *A. speciosus ainu* succeeded in molting to the later stages. These molting successes prove that *A. speciosus ainu* is adequate as the blood source for the tick development. The detection of spirochetes from these newly-molted nymphs and adults demonstrates that the transstadial infection occurs in *I. persulcatus* through the molting process.

Spirochetal cultures were successful in rodents by using their blood and spleen as culture sources. Although the spirochetes were detected principally from *A. speciosus ainu*, the detectable rates from blood were lower than those from spleen. This fact indicates that spirochetemia occurs rarely in the mice. Moreover, we noticed that the spirochete-positive *I. persulcatus* larvae were recovered from the spirochete-negative *A. speciosus ainu*. This discrepancy implied that the culture sources such as blood and spleen were unsuitable to determine the infection status in rodents. Sinsky and Piesman (1989) proved that the earlobe tissue of rodents was superior as a culture source to detect the spirochetal infections. We are now attempting this method to estimate the prevalence of spirochetes in field-collected rodents.

The spirochetal isolates obtained from rodents and immature ticks were characterized by SDS-PAGE and western blots. These isolates possessed the 30–32 kDa heterogene-

ous OspA proteins, and the isolates nonreactive with monoclonal antibody H5332 were also present. Similar heterogeneity had been observed in the isolates from unfed adults of *I. persulcatus* (Nakao *et al.,* 1992c). The spirochetes isolated from rodents and immature ticks were identified as *B. burgdorferi* according to their reactivity to the monoclonal antibodies and constituent protein profile. However, their heterogeneity strongly suggests that the species of *B. burgdorferi* includes several strain types or new species. Recently, Baranton *et al.* (1992) delineated three genomic species of *Borrelia* associated with Lyme disease, *B. burgdorferi* sensu stricto, *B. garinii,* and group VS461 by whole DNA hybridization and rRNA gene restriction patterns involving the European and North American isolates. Considering their taxonomic standpoint, no *B. burgdorferi* may be distributed in Japan because many spirochetes that have been isolated from ticks, rodents, and humans are apparently different from the type strain of *B. burgdorferi* (ATCC 35210) in their phenotypic protein profiles. Further studies on the genomic analysis of spirochetes are necessary to better understand the taxonomic status in Japan.

The spirochetal isolates from *A. speciosus ainu* frequently showed similar protein profiles to the isolates from immature *I. persulcatus* that had fed on this mouse. This similarity strongly suggests that the spirochetal transmission occurs between the mice and the ticks. Additionally, we observed the homogeneity or heterogeneity of spirochetes isolated from *I. persulcatus* larvae concurrently fed on the same mouse. These phenomena implied that the single or mixed infections of various types of *B. burgdorferi* occurred in *A. speciosus ainu* due to frequent contacts with nymphal *I. persulcatus.*

In the previous study, we found that the unfed adults of *I. ovatus* harbored a homogeneous type of *B. burgdorferi* which was distinguishable from the heterogeneous types of *B. burgdorferi* isolated from *I. persulcatus* (Nakao *et al.,* 1992c). This phenomenon strongly suggests that the transmission cycle of spirochetes in *I. ovatus* is different from that seen in *I. persulcatus.* In this study, most spirochetal isolates from immature *I. ovatus* that had fed on rodents showed similar protein profiles to the isolates from immature *I. persulcatus.* Ryder *et al.* (1992) demonstrated experimentally that a non-*I. ricinus* complex tick, *Ixodes cookei* Packard, ingested *B. burgdorferi* by larval feeding on infected animals; however, no spirochetes transmitted to the nymphs after molting. The detection of spirochetes from immature *I. ovatus* in this study indicates the possibility that the ticks ingest spirochetes accidentally by feeding on rodents. Further studies to determine the reservoir hosts for spirochetes transmitted by *I. ovatus* may provide useful information on the competition of ixodid ticks for the variants of *B. burgdorferi.*

Our evaluation of reservoir competence in the field study depends on the assumption that all recovered larvae were uninfected with spirochetes prior to feeding. The spirochetal transmission from *A. speciosus ainu* to *I. persulcatus* was verified xenodiagnostically by using the uninfected larvae. Ingestion of spirochetes was demonstrated in the larvae by feeding on the field-trapped mice. These mice retained the long-term infectivity for the vector ticks. The laboratory experiment was also conducted to determine whether the infected nymphs of *I. persulcatus* transmit the spirochetes to the uninfected *A. speciosus ainu.* These mice readily became infected by nymphal feeding, and showed no morbidity during the infections. The long-term continuance and the tolerance of infections are important factors of reservoir host for *B. burgdorferi.* Our experimental data showed that *A. speciosus ainu* satisfied these reservoir requirements.

In conclusion, the data presented in this report clearly indicate that *A. speciosus ainu* is a suitable reservoir host for *B. burgdorferi* transmitted by *I. persulcatus* in Hokkaido. In our study sites this reservoir mouse was numerous in autumn, and *I. persulcatus* larvae were frequently found on the mice in this season. We can speculate that the spirochetal transmission from the reservoir mice to *I. persulcatus* larvae mainly occurs in autumn, and the molted nymphs serve as a vector of the agent to the newly-rising generation of mice in the next year.

The pathogenicity of various types of *B. burgdorferi* isolated from rodents and ticks remains to be determined. However, the

similarity of protein profiles observed among the spirochetal isolates from *A. speciosus ainu*, immature *I. persulcatus*, and human patients, suggests that the strains of *B. burgdorferi* pathogenic to humans are maintained in "*I. persulcatus–A. speciosus ainu*" transmission cycle.

Apodemus speciosus ainu is an insular subspecies of *A. speciosus speciosus* (Temminck) distributed throughout the mainland of Honshu, Japan (Saitoh *et al.*, 1989). Further studies on the reservoir competence of *A. speciosus speciosus* are required in the central and northern regions of Honshu where the Lyme disease spirochetes were found from *I. persulcatus*.

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摘　要

北海道のライム病ボレリア伝播における エゾアカネズミのレゼルボア能力

北海道富良野市と根室市の森林で捕獲した野鼠のマダニ寄生状況を調査し，野鼠および野鼠寄生マダニからライム病ボレリアを検出した．森林環境に生息する野鼠は *Apodemus* 属が優占し，エゾアカネズミに多数のシュルツェマダニ幼若虫が寄生していた．ライム病ボレリアはエゾアカネズミの脾臓からの分離率が高く，エゾアカネズミに寄生していたシュルツェマダニ幼若虫からも高率にボレリアを分離できた．野外捕獲のエゾアカネズミに実験室内で飼育したボレリアフリーのシュルツェマダニ幼虫を暴露したところ，飽血幼虫がボレリアを保有するようになった．また，ボレリア保有シュルツェマダニ若虫を実験室内で繁殖させたボレリアフリーのエゾアカネズミに暴露したところ，ネズミへの感染が成立した．野外調査と感染実験の結果から，北海道におけるライム病ボレリアの主要なレゼルボアはエゾアカネズミであると結論した．