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Author(s)	Konnai, Satoru; Saito, Yoichi; Nishikado, Hideto; Yamada, Shinji; Imamura, Saiki; Mori, Akina; Ito, Takuya; Onuma, Misao; Ohashi, Kazuhiko
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Establishment of a laboratory colony of taiga tick *Ixodes persulcatus* for tick-borne pathogen transmission studies

Satoru Konnai^{1)*}, Yoichi Saito¹⁾, Hideto Nishikado¹⁾, Shinji Yamada¹⁾, Saiki Imamura¹⁾, Akina Mori¹⁾, Takuya Ito²⁾, Misao Onuma¹⁾ and Kazuhiko Ohashi¹⁾

¹⁾Department of Disease Control, Graduate School of Veterinary Medicine, Hokkaido University, Sapporo 060-0818, Japan

²⁾Hokkaido Institute of Public Health, Sapporo, 060-0819, Japan

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Abstract

Ixodes persulcatus Schulze (*I. persulcatus*) is distributed in Russia and Far East Asia including Japan, and has been implicated as the vector of several human pathogens. In particular, *I. persulcatus* acts as the only tick vector for human lyme borreliosis in Japan. In order to elucidate the mechanism of transmission of *I. persulcatus*-borne pathogens, we developed a laboratory colony of *I. persulcatus*. Ticks were fed on Syrian hamster and engorged ticks that had dropped off the animals were collected and maintained to allow them to molt. Tick rearing was performed in incubator at 20°C with 95% relative humidity and 12-hour light/dark photo-period regimen. We found out that adult females fed for 8±2 days and had a pre-oviposition period lasting for 7±2 days. The minimum egg incubation period was 1 month with the hatched larvae feeding for 3±1 days and molting to nymphs 3-4 months thereafter. Meanwhile, the nymphs fed for 4±1 days and molted to adult 2-3 months thereafter. For future analysis of gene expression profiles in *I. persulcatus*, we cloned and sequenced the *actin* gene (a housekeeping gene), and found that it is 92.7% to 98.6% homologous to the published sequences of related ixodid ticks. This laboratory colony of *I. persulcatus* will facilitate investigations on the role of tick-derived molecules on the transmission of *I. persulcatus*-borne pathogens and will be important for identification of potential anti-tick vaccine and acaricide target molecules.

Key Words: ixodid tick, *I. persulcatus*, laboratory colony, life cycle, *actin* gene, vector

Introduction

Lyme disease and tick-borne encephalitis are two emerging tick-borne diseases transmitted by

the widely distributed ixodid ticks^{3,10)}. The ixodid tick found in Russia and in North and Far East Asia, belongs to the species *Ixodes persulcatus* (*I. persulcatus*), whereas in Europe and the

*Corresponding author: Satoru Konnai, Department of Disease Control, Graduate School of Veterinary Medicine, Hokkaido University, Sapporo 060-0818, Japan
Phone: +81-11-706-5215. Fax: +81-11-706-5217. E-mail: konnai@vetmed.hokudai.ac.jp

Americas, ixodid tick species include *Ixodes scapularis* (*I. scapularis*), *Ixodes pacificus* (*I. pacificus*) and *Ixodes ricinus* (*I. ricinus*). Lyme disease is a multi-systemic disorder caused by infection with at least three spirochete species including *Borrelia burgdorferi* (*B. burgdorferi*), *B. garinii* and *B. afzelii*. There are mainly four types of vector ticks for human lyme borreliosis, namely *I. scapularis*, *I. ricinus*, *I. pacificus* and *I. persulcatus*. In Japan, Lyme disease in humans is due to infection with *B. garinii* or *B. afzelii*, which are biologically and specifically transmitted by *I. persulcatus*¹³⁾.

Recently, there have been several reports on the identification and characterization of tick-derived molecules from the closely related ixodid tick species, *I. scapularis* and *I. ricinus*. Some of the functions associated with the identified molecules include acting as specific receptors for pathogens¹⁴⁾, anti-coagulants¹²⁾, pathogen-associated proteins^{15,16)} and immunosuppressants^{1,4,5,7,11)}. However, no sequence information and function analysis of such molecules has been reported for *I. persulcatus* mainly because of the lack of an established laboratory tick colony. In spite of this, reports on the prevalence and diversity of pathogens associated with *I. persulcatus* are increasing.

Here, we report the successful establishment of an *I. persulcatus* laboratory colony that will aid to elucidate the tick's biology at molecular level as well as to understand the events of *I. persulcatus*-borne pathogens transmission.

Materials and Methods

Tick collection

Host-seeking *I. persulcatus* adult ticks (Fig. 1 A) were collected from lower vegetation at peripheral and inner parts of deciduous and mixed forests in Nissho area located in Hidaka district, Hokkaido, Japan. These samples were gathered by flagging with cotton flannel. After collection, the ticks were placed in 50 ml centrifuge tubes containing damped sterile filter paper and packed in a cooler box containing ice packs to maintain viability

of the ticks in transit. Tick species were determined in the laboratory by examining the morphology of the ticks under a dissection light microscope (Fig. 1B). *I. persulcatus* ticks were then maintained in 50 ml centrifuge tubes packed with 5 ml of 10% plaster mixture (Wako, Tokyo) including 0.5 g activated charcoal (Wako) at 4°C to prevent bacterial and fungal spoilage until use.

Maintenance and development of various stages of *I. persulcatus* tick

The stored ticks were reactivated by incubation for 1 h at room temperature. Feeding was initiated by placing 10-20 adult ticks in a net and attaching them to a pathogen-free Syrian hamster (Japan SLC Inc., Shizuoka). Adult *I. persulcatus* males were placed with females at 1:1 ratio to ensure mating and feeding. The tick-infested animals were maintained in an isolated experimental animal facility which is under a strict acaricidal control regimen of the Hokkaido University, follow-

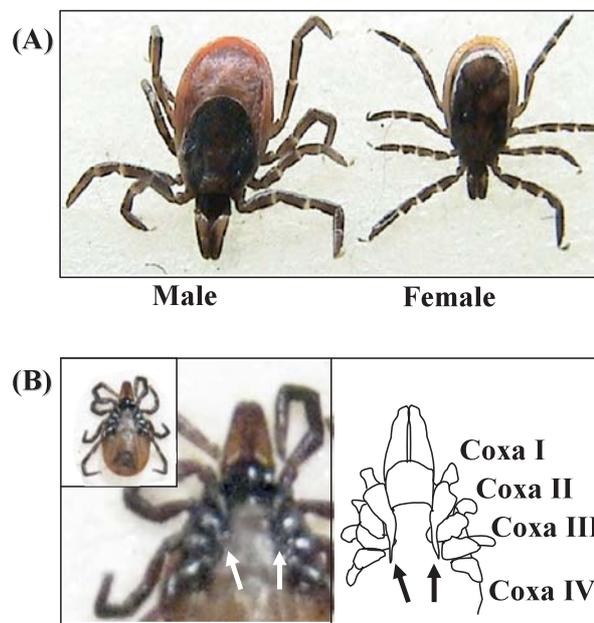


Fig. 1. *Ixodes persulcatus*. (A) Adult ticks (dorsal view). (B) Photography and diagram illustrating coxae I-IV (ventral view of female tick). Arrow indicates the long internal spur on coxa I, indicating one of morphological characteristics of *I. persulcatus*.

ing guidelines of the Institutional Animal Care and Use Committee of the Graduate School of Veterinary Medicine, Hokkaido University. Engorged adult ticks were collected daily starting from day 5 post-attachment, placed individually in clean containers with dampened sterile filter paper and incubated at 20°C to initiate oviposition. Hatched larval and nymphal ticks were fed to repletion on hamsters as described above to allow them to molt into succeeding stages. All tick rearing was performed in an incubator (Sanyo, Osaka) at 20°C and with a 12-hour light/dark photo period regimen.

Cloning of I. persulcatus actin gene

Total RNA was extracted from salivary glands of pathogen free-adult females (day 4 post-blood feeding) by using TRIZOL™ reagent according to the manufacturer's protocol (Invitrogen, Carlsbad, CA, USA). cDNA was synthesized with the reverse transcriptase-MMLV (Takara, Otsu) according to the manufacturer's instructions. One microliter of the cDNA template was added to a total volume of 20 µl containing PCR buffer, oligonucleotide primers (at 0.2 µM each of tick actin sense: 5'-ATGTGT GACGACGAGGTTGCCG-3' and tick actin anti-sense: 5'-TTAGAAGCACTTGCGGTGGACAATG-3')²⁾, and 2.5 units of rTaq polymerase (Takara). The PCR product was purified by the Gene-clean-III Kit (MP Biomedicals, Solon, OH, USA) and cloned into the pGEM T-Easy-vector (Promega Corp., Madison, WI, USA) for sequencing. The purified plasmids were sequenced using the CEQ 2000 Dye Terminator Cycle Sequencing method with the Quick Start kit (Beckman Coulter, Inc., Fullerton, CA, USA) according to the manufacturer's instructions. The sequences obtained were analyzed using the CEQ 2000 DNA analysis system (Beckman Coulter, Inc.).

Sequence analyses

Analyses of nucleotide and deduced amino acid sequences were carried out as previously described²⁾. Alignment of the sequences was performed by the Clustal W program, and phylogenetic analyses using the Neighbor Joining Method

were carried out by the Clustal X program. The EMBL/GenBank accession numbers are: AJ889837 (*Ixodes ricinus*), AY254898 (*Haemaphysalis longicornis*), AY255624 (*Rhipicephalus (Boophilus) microplus*), AY254899 (*Rhipicephalus appendiculatus*), AF426178 (*Ixodes scapularis*), X05185 (*Bombyx mori*), NM079076 (*Drosophila melanogaster*), AF358264 (*Drosophila virilis*), AF300705 (*Litopenaeus vannamei*), L13764 (*Manduca sexta*), AB055975 (*Marsupenaeus japonicus*) and AY547732 (*Ornithodoros moubata*).

Results and Discussion

The life cycle of I. persulcatus under laboratory conditions

To initiate reproduction, adult male and female ticks were kept together during feeding as well as during storage (Fig. 2). The life cycle (exact pre-feeding period not determined) had a duration of approximately 250 days (Fig. 3). We found out that adult female ticks fed for 6-10 days and had a pre-oviposition period lasting for 5-9 days. The minimum egg incubation period was 30-40 days with the hatched larvae feeding for 2-4 days and molting to nymph, 90-120 days thereafter. The nymphs fed for 3-5 days and molted to adult, 60-90 days thereafter (Table 1). Troughton and Levin have recently documented the biological data and life cycle for several Ixodid tick species, including *I. scapularis*, *I. pacificus*, *Amblyomma americanum*, *Dermacentor occidentalis*, *Dermacentor variabilis*, *Hemaphysalis leporispalustris* and *Rhipicephalus sanguineus* under the laboratory conditions¹⁸⁾. In the present study, the conditions established for the maintenance of *I. persulcatus* in the laboratory, though not compared directly to those reported for other Ixodid ticks, were somewhat similar to those reported for *I. scapularis* and *I. pacificus*, which are closely related to *I. persulcatus*. Interestingly, the pre-molting periods of larval and nymphal *I. persulcatus* ticks were longer than those of the other tick species (Fig. 3).

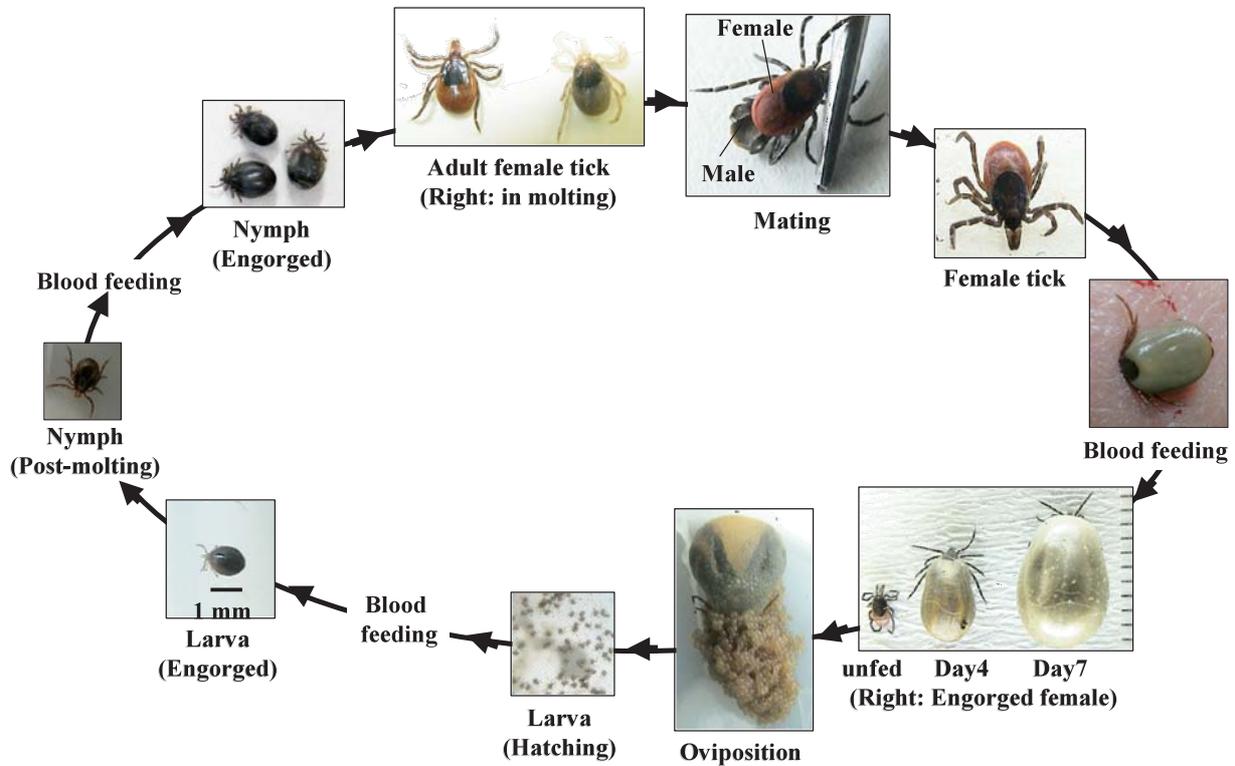


Fig. 2. The life cycle of *I. persulcatus* under laboratory conditions. All tick rearing was performed in incubator at 20°C with 95% relative humidity and 12-hour light/dark photo-period regimen.

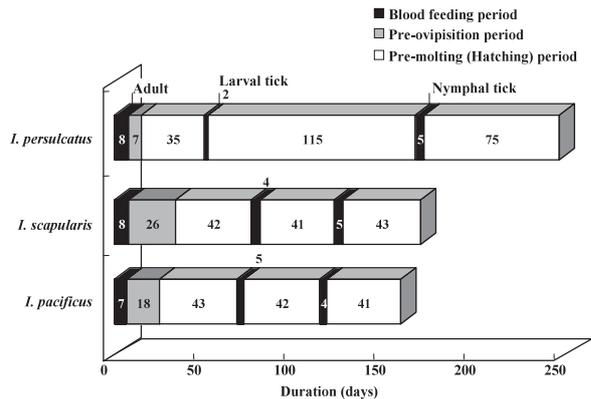


Fig. 3. Comparison of mean duration of feeding and development under laboratory conditions for *I. persulcatus* with those of *I. scapularis* and *I. pacificus*. Biological data of life cycle of *I. scapularis* and *I. pacificus* were taken from the report of Troughton and Levin¹⁸.

Sequencing of actin cDNA from I. persulcatus

Actin is commonly used for gene expression profiling studies due to its high conservation as an endogenous housekeeping gene. Thus, we cloned

Table 1. Feeding period of *I. persulcatus* and reproductive data

Trait	Biological data (n=20)
Feeding period (Adult female)	6-10 days
Engorged female weight	120-402 mg
Pre-oviposition period	5-9 days
Egg mass weight	55-116 mg
Egg incubation period	30-40 days
Feeding period (Larva)	2-4 days
Pre-molt to nymph	90-120 days
Feeding period (Nymph)	3-5 days
Pre-molt to adult	60-90 days

and characterized the *actin* gene from the established *I. persulcatus* laboratory colony. The cloned gene was 1131 nucleotide long with an open reading frame (ORF) encoding of 376 amino acids. The present sequence data have been submitted to GenBank under accession no. AB374098. Comparison of the nucleotide and amino acid sequence homology of the cloned *actin* gene with other pub-

	1	60
<i>I. persulcatus</i>	MCDDEVAALVVDNNGSGMCKAGFAGDDAPRAVFP	IVGRPRHQVMVGMGQKDSYVGDEAQ
<i>I. ricinus</i>	-----	-----
<i>H. longicornis</i>	-----	-----
<i>R. appendiculatus</i>	-----	-----
<i>R. microplus</i>	-----	-----
<i>I. scapularis</i>
	61	120
<i>I. persulcatus</i>	SKRGILTLKYPIDIEHGIVTNWDDMEKIWHHTFYNELRV	APPEHPVLLTEAPLNPKASREKM
<i>I. ricinus</i>	-----	-----N-----
<i>H. longicornis</i>	-----	-----N-----
<i>R. appendiculatus</i>	-----	-----N-----
<i>R. microplus</i>	-----	-----N-----
<i>I. scapularis</i>
	121	180
<i>I. persulcatus</i>	TQIMFETFNTPAMYVAIQAVLSLYASGR	TTGIVLDSGDGVSHTVPIYEGYALPHAILRLD
<i>I. ricinus</i>	-----	-----
<i>H. longicornis</i>	-----	-----
<i>R. appendiculatus</i>	-----	-----
<i>R. microplus</i>	-----	-----
<i>I. scapularis</i>	-----	-----
	181	240
<i>I. persulcatus</i>	<u>L</u> AGRDLTDYLMKILTERGYSFTTTAEREIVRD	<u>I</u> KEKLCYVALDFEQEMATAASSSSLEKS
<i>I. ricinus</i>	-----	-----
<i>H. longicornis</i>	-----	-----
<i>R. appendiculatus</i>	-----	-----
<i>R. microplus</i>	-----	-----
<i>I. scapularis</i>	-----	-----S-----
	241	300
<i>I. persulcatus</i>	YELPDGQVITIGNERFRCPEALFQPSFLGME	SCGIHETTYNSIMKCDVDIRKDLANTVL
<i>I. ricinus</i>	-----	-----
<i>H. longicornis</i>	-----N-----	-----
<i>R. appendiculatus</i>	-----	-----P-----
<i>R. microplus</i>	-----	-----
<i>I. scapularis</i>
	301	360
<i>I. persulcatus</i>	SGGTTMYPGIADRMQKEITALAPSTMKIKII	APPERKYSVRIGGSILASLSTFQQMWISK
<i>I. ricinus</i>	-----	-----W-----
<i>H. longicornis</i>	-----	-----W-----
<i>R. appendiculatus</i>	-----	-----W-----
<i>R. microplus</i>	-----	-----W-----
<i>I. scapularis</i>
	361	376
<i>I. persulcatus</i>	QEYDESGPSIVHRKCF	
<i>I. ricinus</i>	-----	
<i>H. longicornis</i>	-----	
<i>R. appendiculatus</i>	-----	
<i>R. microplus</i>	-----	
<i>I. scapularis</i>	

Fig. 4. Alignment of the actin gene amino acid sequences of *I. persulcatus* with other ixodid ticks. EMBL/GenBank accession numbers: BAF98180 (*I. persulcatus*), CAI63975 (*I. ricinus*), AAP81255 (*Haemaphysalis longicornis*)²⁾, AAP79880 (*Rhipicephalus (Boophilus) microplus*)²⁾, AAP81256 (*Rhipicephalus appendiculatus*)²⁾, AAL30373 (*Ixodes scapularis*)¹²⁾. Underlines indicate two nuclear export signals (NES-1, residues 170-181; NES-2, residues 211-222), which contain four hydrophobic residues (in bold) (leucine, isoleucine or valine) with the characteristic spacing present in established NES sequences²⁾. The dash indicates identity to the *I. persulcatus* actin sequence. The dot indicates unidentified amino acid in the sequence of *I. scapularis*.

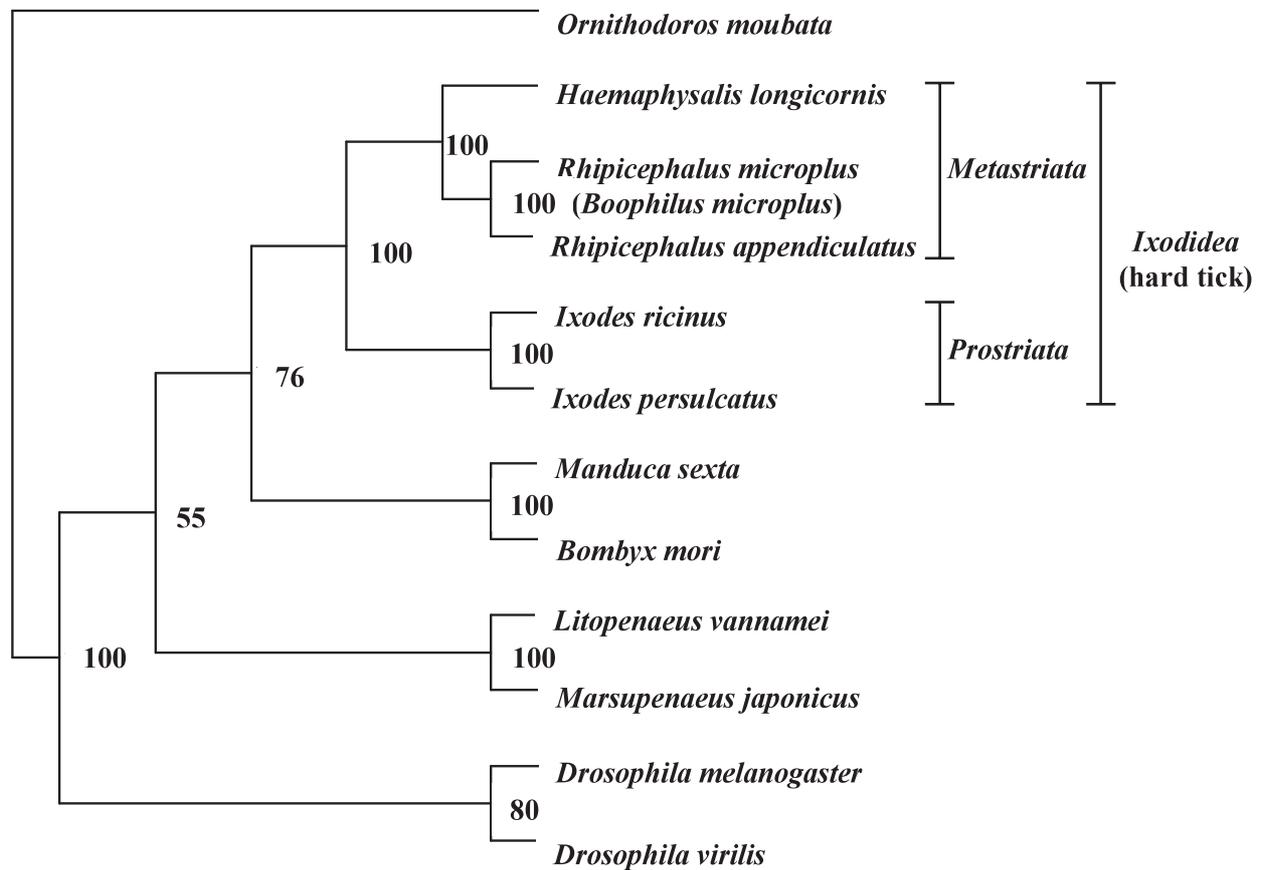


Fig. 5. Dendrogram based on actin coding sequences of *I. persulcatus*, *I. ricinus*, *Rhipicephalus* (*Boophilus*) *microplus*, *Haemaphysalis longicornis*, *Rhipicephalus appendiculatus* and other arthropod species. GenBank accession numbers for the *actin* gene sequences are shown in the Material and Methods.

Table 2. The nucleic and amino acid sequences similarities of *I. persulcatus* actin gene (AB374098)

	Accession No.	Identity	
		Nucleotide	Amino acid
<i>I. ricinus</i>	AJ889837	98.6%	99.5%
<i>H. longicornis</i>	AY254898	92.7%	99.2%
<i>R. appendiculatus</i>	AY254899	93.8%	99.2%
<i>R. (B). microplus</i> ^a	AY255624	93.6%	99.4%
<i>I. scapularis</i>	AF426178 ^b	98.5% ^c	99.3% ^c

^a *Rhipicephalus microplus* (*Boophilus microplus*)

^b Partial sequence

^c Partial comparison

lished sequences revealed 92.7-98.6% and 99.2-99.5%, respectively (Table 2). Two nuclear export signals (NES) (NES-1, residues 170-181; NES-2, residues 211-222), which are leucine-rich domains necessary for the shuttling between the nucleus

and cytoplasm, were well conserved as in other ixodid ticks²⁾ (Fig. 4). The phylogenetic tree demonstrated that the *I. persulcatus* actin coding sequence is more closely related to the *I. ricinus* sequence than to the other ixodid ticks sequence. In

addition, the tree illustrated that hard ticks including *I. persulcatus* can be classified into a separate cluster away from other arthropod species (100% bootstrap value) as our previous analysis²⁾ (Fig. 5).

Studies of transmission, maintenance, infectivity, virulence, and pathogenicity of tick-borne pathogens require the use of large numbers of laboratory-raised ticks. The *I. persulcatus* tick in Far East Asia and Russia is well known as an important vector of Lyme borreliosis, ehrlichiosis and tick-borne encephalitis^{3,10,13)}. The emergence of these diseases particularly Lyme disease invigorates the interest of researchers to tick-borne pathogens¹⁵⁾. Nevertheless, the understanding of events in the transmission of pathogens associated with *I. persulcatus* has not been well studied because of the lack of established tick laboratory colony. Despite that several tick-derived immunomodulatory factors¹⁷⁾ including Salp 15^{1,4,7,15)}, Salp 16¹⁶⁾ and TROSPA¹⁴⁾ of *I. scapularis* and *I. ricinus*^{5,11)} have been cloned and characterized, no such analyses have been done yet in *I. persulcatus*. It is only recently that cloning and sequencing of calreticulin²⁰⁾ and ribosomal RNA¹⁹⁾ genes were reported in this type of tick. In the present study, laboratory colony of *I. persulcatus* was established and developed for investigation of the vector-phase of tick-borne disease agents *in vivo*. In comparison to our previously established conditions for rearing laboratory colonies of *Hemaphysalis longicornis*⁶⁾, *Rhipicephalus* (*Boophilus*) *microplus*²⁾ and *Rhipicephalus appendiculatus*^{8,9)}, we observed that *I. persulcatus* demands more attention. Therefore, a more efficient and optimal condition to maintain the viability of tick is needed. The established tick colony will necessitate the elucidation of the biology of *I. persulcatus* at molecular level and will give impetus to the identification and characterization of potential anti-tick vaccine and acaricide target molecules. Furthermore, the colony will facilitate the unraveling of the intricate host-pathogen relationship between the tick and its mammalian hosts as well between the tick and the pathogens it transmits.

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