〔Med. Entomol. Zool. Vol. 65 No. 1 p. 13–21 2014〕
DOI: 10.7601/mez.65.13

Construction of a DNA database for ticks collected in Japan: application of molecular identification based on the mitochondrial 16S rDNA gene

Ai Takano*, [1)], Hiromi Fujita[2)], Teruki Kadosaka[3)], Mamoru Takahashi[4)], Takeo Yamauchi[5)], Fubito Ishiguro[6)], Nobuhiro Takada[7)], Yasuhiro Yano[7)], Yosaburo Oikawa[8)], Toshirou Honda[9)], Mutsuyo Gokuden[10)], Takashi Tsunoda[11)], Miyako Tsurumi[12)], Shuji Ando[13)], Masako Andoh[13)], Kozue Sato[14)] and Hiroki Kawabata[14)]

* Corresponding author: *Department of Veterinary Medicine, Joint Faculty of Veterinary Medicine, Yamaguchi University, 1677–1 Yoshida, Yamaguchi 753–8515, Japan* (E-mail: a-takano@yamaguchi-u.ac.jp)
[1)] *Department of Veterinary Medicine, Joint Faculty of Veterinary Medicine, Yamaguchi University, 1677–1 Yoshida, Yamaguchi 753–8515, Japan*
[2)] *Mahara Institute of Medical Acarology, Anan, Tokushima 779–1510, Japan*
[3)] *Department of Infection and Immunity, Aichi Medical University, Nagakute, Aichi 480–1195, Japan*
[4)] *Department of Anesthesiology, Saitama Medical University, Iruma-gun, Saitama 350–0495, Japan*
[5)] *Toyama Institute of Health, Imizu, Toyama 939–0363, Japan*
[6)] *Fukui Prefectural Institute of Public Health and Environmental Science, Fukui, Fukui 910–8551, Japan*
[7)] *Faculty of Medical Sciences, University of Fukui, Yoshida-gun, Fukui 910–1193, Japan*
[8)] *Department of Medical Zoology, Kanazawa Medical University, Kahoku-gun, Ishikawa 920–0293, Japan*
[9)] *Kaseda Public Health Center, Minamisatsuma, Kagoshima 897–0001, Japan*
[10)] *Kagoshima Prefectural Institute for Environmental Research and Public Health, Kagoshima, Kagoshima 892–0836, Japan*
[11)] *Department of Vector Ecology and Environment, Institute of Tropical Medicine, Nagasaki University, Nagasaki, Nagasaki 852–8523, Japan*
[12)] *Yamashina Institute for Ornithology, Abiko, Chiba 270–1145, Japan*
[13)] *Department of Virology-I, National Institute of Infectious Diseases, Shinjuku-ku, Tokyo 162–8640, Japan*
[14)] *Department of Bacteriology-I, National Institute of Infectious Diseases, Shinjuku-ku, Tokyo 162–8640, Japan*

(Received: 15 August 2013; Accepted: 6 December 2013)

Abstract: Tick identification is important in control of tick-born diseases because tick-borne pathogens are often transmitted by specific tick species. In this study, we determined partial DNA sequences of the mitochondrial 16S rDNA gene (mt-*rrs*) for ticks including 7 genera and 39 species, and these ticks were allocated to 113 sequence types. Of the 39 species of ticks, 36 species (92.3%) were distinguishable by phylogenetic analysis of mt-*rrs*. This result suggests that species identification of ticks based on mt-*rrs* is a viable alternative to morphological identification. In order to establish a DNA database for identification of ixodid and argasid ticks in Japan, we deposited all sequence data in GenBank (from AB819156 to AB819268).

Key words: tick, mt-*rrs*, molecular identification

Introduction

Ticks are hematophagous ectoparasites of vertebrates. Since they have an ability to transmit several pathogens to humans and livestock, there are medical and veterinary needs for accurate tick identification (Bowman and Nuttall, 2008). A total of 47 species of ticks have been reported from Japan (Fujita and Takada, 2007). Of these, 21 species have been shown to parasitize humans (Okino et al., 2010). For identification of ticks, morphological identification is the worldwide gold standard. However, in fact, morphological discrimination of ticks is often difficult in medical fields for a number of reasons (e.g. lack of tick parts important for identification, and/or technical difficulties owing to insufficient training experience for morphological identification). We, therefore, developed an alternative method of morphological identification. We constructed a DNA database based on the mitochondrial 16S rDNA gene (mt-*rrs*) using ticks collected in Japan and South Korea.

Ticks have been phylogenetically analyzed using the nuclear 18S rDNA gene, mt-*rrs*, mitochondrial 12S rDNA gene, cytochrome *c* oxidase I (COI), internal

Table 1. List of tick species and sequence types.

No	Tick species [synonym]	Tick sequence type	Tick stage	Location	Collected source Host animals or Vegetation	Sampling date (year or yyyy.mm.dd)	Acc. No.
1	*Argas japonicus* Yamaguti, Clifford and Tipton, 1968	AJ-A	Female	Tahara, Tsuru-shi, Yamanashi	*Delichon urbica* (Bonaparte, 1850)*1	2006*2	AB819156
		AJ-B	Female	Tahara, Tsuru-shi, Yamanashi	*Delichon urbica* (Bonaparte, 1850)*1	2006*2	AB819157
2	*Argas vespertilionis* (Latreille, 1796) [*Carios vespertilionis*]	AV-A	Nymph	Azumino, Matsumoto-shi, Nagano	*Eptesicus japonensis* Imaizumi, 1953*3	2001.08.25	AB819158
3	*Amblyomma geoemydae* (Cantor, 1847)	AG-A	Larva	Haiminaka, Taketomi-cho, Yaeyama-gun, Okinawa	Vegetation	2004.12.05	AB819159
		AG-B	Larva	Fukai, Ishigaki-shi, Okinawa	Vegetation	2004.12.06	AB819160
		AG-C	Nymph	Ada, Kunigami village, Kunigami-gun, Okinawa	*Geoemyda japonica* Fan, 1931	2006.07.12	AB819161
		AG-D	Male	Ada, Kunigami village, Kunigami-gun, Okinawa	*Geoemyda japonica* Fan, 1931	2006.07.12	AB819162
		AG-E	Male	Ada, Kunigami village, Kunigami-gun, Okinawa	*Geoemyda japonica* Fan, 1931	2006.07.12	AB819163
		AG-F	Nymph	Uehara, Taketomi-cho, Yaeyama-gun, Okinawa	*Cuora flavomarginata evelynae* (Gray, 1863)	2010.06.14	AB819164
4	*Amblyomma nitidum* Hirst and Hirst, 1910	AN-A	Female	Kuroshima, Taketomi-cho, Yaeyama-gun, Okinawa	*Laticauda colubrina* (Schneider, 1799)	2011.04.22	AB819165
		AN-B	Nymph	Kuroshima, Taketomi-cho, Yaeyama-gun, Okinawa	*Laticauda colubrina* (Schneider, 1799)	2011.04.22	AB819166
5	*Amblyomma testudinarium* Koch, 1844	AT-A	Male	Kuchinoshima, Toshima village, Kagoshima-gun, Kagoshima	Vegetation	2008.01.13	AB819167
6	*Dermacentor taiwanensis* Sugimoto, 1935	DT-A	Larva	Ooya-cho, Yabu-shi, Hyogo	*Apodemus speciosus* (Temminck, 1844)	2007.11.04	AB819168
		DT-B	Larva	Gosesan, Fukui-cho, Anan-shi, Tokushima	Vegetation	2007.11.10	AB819169
7	*Haemaphysalis campanulata* Warburton, 1908	Hca-A	Male	Aratano-cho, Anan-shi, Tokushima	*Canis lupus familiaris* Linnaeus, 1758	2004.07.30	AB819170
8	*Haemaphysalis concinna* Koch, 1844	Hcon-A	Nymph	Kushiro-shi, Hokkaido	Vegetation	2010.07.29	AB819171
		Hcon-B	Nymph	Shibetsu-cho, Shibetsu-gun, Hokkaido	Vegetation	2010.07.30	AB819172
		Hcon-C	Nymph	Shibetsu-cho, Shibetsu-gun, Hokkaido	Vegetation	2010.07.30	AB819173
9	*Haemaphysalis cornigera* Neumann, 1897	Hcor-A	Nymph	Yagiumamawari, Minamiawaji-shi, Hyogo	Vegetation	2004.09.06	AB819174
10	*Haemaphysalis japonica* Warburton, 1908	HJ-A	Nymph	Karouji, Oowani-machi, Minamitsugaru-gun, Aomori	Vegetation	2004.07.29	AB819200
		HJ-B	Nymph	Matsukami, Fukaura-machi, Nishitsugaru-gun, Aomori	Vegetation	2007.07.30	AB819201
11	*Haemaphysalis japonica douglasi* Nuttall and Warburton, 1915 [*Haemaphysalis douglasi*]	HD-A	Female	Furano-shi, Hokkaido	Vegetation	2004.06.06	AB819175
		HD-B	Nymph	Asyoro-cho, Asyoro-gun, Hokkaido	Vegetation	2009.10.04	AB819176
12	*Haemaphysalis flava* Neumann, 1897	Hfl-A	Nymph	Nimyou, Kumakougen-cho, Kamiukena-gun, Ehime	*Emberiza spodocephala* Pallas, 1776	2006.11.08	AB819177
		Hfl-B	Nymph	Oimatsu, Echizen-cho, Nyuu-gun, Fukui	*Turdus pallidus* Gmelin, 1789	2007.10.31	AB819178
		Hfl-C	Larva	Tahara, Tsuru-shi, Yamanashi	*Syrmaticus soemmerringii* (Temminck, 1830)	2006.09.21	AB819179
		Hfl-D	Larva	Tahara, Tsuru-shi, Yamanashi	*Syrmaticus soemmerringii* (Temminck, 1830)	2006.09.21	AB819180
		Hfl-E	Nymph	Karouji, Oowani-machi, Minamitsugaru-gun, Aomori	Vegetation	2004.07.29	AB819181
		Hfl-F	Nymph	Yamada-machi, Kokurakita-ku, Kitakyusyu-shi, Fukuoka	*Turdus pallidus* Gmelin, 1789	2007.11.27	AB819182
		Hfl-G	Larva	Oimatsu, Echizen-cho, Nyuu-gun, Fukui	*Turdus pallidus* Gmelin, 1789	2007.10.22	AB819183
		Hfl-H	Larva	Oimatsu, Echizen-cho, Nyuu-gun, Fukui	*Turdus pallidus* Gmelin, 1789	2007.10.22	AB819184

No	Tick species [synonym]	Tick sequence type	Tick stage	Location	Collected source Host animals or Vegetation	Sampling date (year or yyyy.mm.dd)	Acc. No.
12	*Haemaphysalis flava* Neumann, 1897	Hfl-I	Nymph	Akusekijima, Toshima village, Kagoshima-gun, Kagoshima	Vegetation	2007.07.25	AB819185
		Hfl-J	Nymph	Akusekijima, Toshima village, Kagoshima-gun, Kagoshima	Vegetation	2007.07.25	AB819186
		Hfl-K	Nymph	Akusekijima, Toshima village, Kagoshima-gun, Kagoshima	Vegetation	2007.07.25	AB819187
		Hfl-L	Nymph	Akusekijima, Toshima village, Kagoshima-gun, Kagoshima	Vegetation	2007.07.25	AB819188
		Hfl-M	Nymph	Akusekijima, Toshima village, Kagoshima-gun, Kagoshima	Vegetation	2007.07.25	AB819189
		Hfl-N	Nymph	Akusekijima, Toshima village, Kagoshima-gun, Kagoshima	Vegetation	2007.07.25	AB819190
		Hfl-O	Nymph	Nakanoshima, Toshima village, Kagoshima-gun, Kagoshima	Vegetation	2007.07.26	AB819191
		Hfl-P	Nymph	Nakanoshima, Toshima village, Kagoshima-gun, Kagoshima	Vegetation	2007.07.26	AB819192
		Hfl-Q	Nymph	Kouchi, Yamamoto-cho, Mitoyo-shi, Kagawa	Vegetation	2007.10.05	AB819193
13	*Haemaphysalis formosensis* Neumann, 1913	Hfo-A	Male	Minami-cho, Kaifu-gun, Tokushima	Vegetation	2004.09.29	AB819194
		Hfo-B	Nymph	Mihonoseki-cho, Matsue-shi, Simane	*Ficedula narcissina* (Temminck, 1835)	2008.04.16	AB819195
14	*Haemaphysalis hystricis* Supino, 1897	HH-A	Larva	Mugiura, Mugi-cho, Kaifu-gun, Tokushima	Vegetation	2004.09.28	AB819196
		HH-B	Larva	Mugiura, Mugi-cho, Kaifu-gun, Tokushima	Vegetation	2004.09.28	AB819197
		HH-C	Larva	Mugiura, Mugi-cho, Kaifu-gun, Tokushima	Vegetation	2004.09.28	AB819198
		HH-D	Female	Otoutojima, Ogasawara village, Tokyo	*Capra aegagrus* Erxleben, 1777	2007.06.24	AB819199
15	*Haemaphysalis kitaokai* Hoogstraal, 1969	HK-A	Female	Nishiide, Ooizumi-cho, Hokuto-shi, Yamanashi	Vegetation	2004.06.05	AB819202
		HK-B	Female	Tennyosan, Nishiide, Ooizumi-cho, Hokuto-shi, Yamanashi	Vegetation	2004.06.05	AB819203
		HK-C	Female	Nishiide, Ooizumi-cho, Hokuto-shi, Yamanashi	Vegetation	2004.06.05	AB819204
16	*Haemaphysalis longicornis* Neumann, 1901	HL-A	Nymph	Shiriya, Higashidoori village, Shimokita-gun, Aomori	Vegetation	2008.07.23	AB819205
		HL-B	Nymph	Yagiumamawari, Minamiawaji-shi, Hyogo	Vegetation	2004.09.01	AB819206
		HL-C	Female	Jindaiurakabe, Minamiawaji-shi, Hyogo	Vegetation	2004.09.01	AB819207
		HL-D	Female	Jindaiurakabe, Minamiawaji-shi, Hyogo	Vegetation	2004.09.01	AB819208
		HL-E	Female*4	Chiba	Unknown	Unknown	AB819209
		HL-F	Nymph	Shiriya, Higashidoori village, Shimokita-gun, Aomori	Vegetation	2008.07.23	AB819210
17	*Haemaphysalis mageshimaensis* Saito and Hoogstraal, 1973	Hmag-A	Female	Mageshima, Nishinoomote-shi, Kagoshima	Unknown	1999.05.01	AB819211
		Hmag-B	Male	Mageshima, Nishinoomote-shi, Kagoshima	Unknown	1999.05.01	AB819212
		Hmag-C	Nymph	Akusekijima, Toshima village, Kagoshima-gun, Kagoshima	Vegetation	2007.07.25	AB819213
18	*Haemaphysalis megaspinosa* Saito, 1969	Hmegaspi-A	Nymph	Hirauchi, Yakushima-cho, Kumage-gun, Kagoshima	Vegetation	2004.06.26	AB819214
		Hmegaspi-B	Nymph	Hirauchi, Yakushima-cho, Kumage-gun, Kagoshima	Vegetation	2004.06.26	AB819215
		Hmegaspi-C	Female*4	Chiba	Unknown	Unknown	AB819216
		Hmegaspi-D	Nymph	Uchiura, Kamogawa-shi, Chiba	Vegetation	2004.12.07	AB819217

No	Tick species [synonym]	Tick sequence type	Tick stage	Location	Collected source Host animals or Vegetation	Sampling date (year or yyyy.mm.dd)	Acc. No.
19	*Haemaphysalis pentalagi* Pospelova-Shtrom, 1935	Hpen-A	Female*[4]	Amamiooshima, Kagoshima	*Pentalagus furnessi* (Stone, 1900)	2003.09.28	AB819218
		Hpen-B	Male *[5]	Amamiooshima, Kagoshima	*Pentalagus furnessi* (Stone, 1900)	2003.09.28	AB819219
20	*Haemaphysalis phasiana* Saito, Hoogstraal and Wassef, 1974	Hpha-A	Female*[4]	Shnan-ri, Haenam gun, South Korea	*Emberiza chrysophrys* Pallas, 1776	2007.05.04	AB819220
21	*Haemaphysalis wellingtoni* Nuttall and Warburton, 1908	HW-A	Male	Mihonoseki, Mihonoseki-cho, Matsue-shi, Simane	*Emberiza variabilis* Temminck, 1835	2007.04.29	AB819221
22	*Haemaphysalis yeni* Toumanoff, 1944	HY-A	Nymph	Anbou, Yakushima-cho, Kumage-gun, Kagoshima	Vegetation	2004.06.26	AB819222
		HY-B	Female	Koseda, Yakushima-cho, Kumage-gun, Kagoshima	*Canis lupus familiaris* Linnaeus, 1758	2008.08.08	AB819223
23	*Ixodes acutitarsus* (Karsch, 1880)	Iac-A	Nymph	Sai, Sai village, Shimokita-gun, Aomori	Vegetation	2008.07.23	AB819224
24	*Ixodes angustus* Neumann, 1899	Ian-A	Female	Minato, Wakkanai-shi, Hokkaido	*Apodemus speciosus* (Temminck, 1844)	2008.06.14	AB819225
25	*Ixodes asanumai* Kitaoka, 1973	Ias-A	Nymph	Takarajima, Toshima village, Kagoshima-gun, Kagoshima	*Ateuchosaurus pellopleurus* (Hallowell, 1861)	2007.07.26	AB819226
		Ias-B	Nymph	Takarajima, Toshima village, Kagoshima-gun, Kagoshima	*Ateuchosaurus pellopleurus* (Hallowell, 1861)	2007.07.26	AB819227
		Ias-C	Nymph	Takarajima, Toshima village, Kagoshima-gun, Kagoshima	*Ateuchosaurus pellopleurus* (Hallowell, 1861)	2007.07.26	AB819228
		Ias-D	Nymph	Nazekominato, Amami-shi, Kagoshima	*Turdus pallidus* Gmelin, 1789	2006.12.17	AB819229
		Ias-E	Female	Kuchinoshima, Toshima village, Kagoshima-gun, Kagoshima	Vegetation	2008.01.13	AB819230
		Ias-F	Female	Kuchinoshima, Toshima village, Kagoshima-gun, Kagoshima	Vegetation	2008.01.13	AB819231
		Ias-G	Male	Kuchinoshima, Toshima village, Kagoshima-gun, Kagoshima	Vegetation	2008.01.13	AB819232
26	*Ixodes columnae* Takada and Fujita, 1992	IC-A	Nymph	Oozasou, Fukushima-shi, Fukushima	Vegetation	2007.11.03	AB819233
		IC-B	Larva	Niimachi, Kawamata-machi, Date-gun, Fukushima	Vegetation	1985.11.21	AB819234
27	*Ixodes granulatus* Supino, 1897	IG-A	Female	Okinawa	*Rattus rattus* (Linnaeus, 1758)	Unknown	AB819235
		IG-B	Female	Okinawa	*Rattus rattus* (Linnaeus, 1758)	Unknown	AB819236
		IG-C	Female	Chichijima, Ogasawara village, Tokyo	*Rattus rattus* (Linnaeus, 1758)	2007.07.30	AB819237
28	*Ixodes monospinosus* Saito, 1968	IM-A	Female	Sai, Sai village, Shimokita-gun, Aomori	Vegetation	2007.09.23	AB819238
29	*Ixodes nipponensis* Kitaoka and Saito, 1967	IN-A	Nymph	Koyamaike, Tottori-shi, Tottori	*Locustella ochotensis* (Middendorff, 1853)	2007.06.04	AB819239
		IN-B	Nymph	Koyamaike, Tottori-shi, Tottori	*Locustella ochotensis* (Middendorff, 1853)	2007.06.04	AB819240
30	*Ixodes ovatus* Neumann, 1899	IO-A	Female	Nishiide, Ooizumi-cho, Hokuto-shi, Yamanashi	Vegetation	2004.06.05	AB819241
		IO-B	Male	Tennyosan, Nishiide, Ooizumi-cho, Hokuto-shi, Yamanashi	Vegetation	2004.06.05	AB819242
		IO-C	Male	Nishiide, Ooizumi-cho, Hokuto-shi, Yamanashi	Vegetation	2004.06.05	AB819243
		IO-D	Female	Karouji, Oowani-machi, Minamitsugaru-gun, Aomori	Vegetation	2004.07.29	AB819244
31	*Ixodes pavlovskyi* Pomerantzev, 1946	Ipav-A	Nymph	Takaoka, Tomakomai-shi, Hokkaido	*Turdus cardis* Temminck, 1831	2008.08.10	AB819245
		Ipav-B	Female	Takaoka, Tomakomai-shi, Hokkaido	*Turdus cardis* Temminck, 1831	2008.08.10	AB819246
		Ipav-C	Larva	Takaoka, Tomakomai-shi, Hokkaido	*Turdus cardis* Temminck, 1831	2008.08.10	AB819247

No	Tick species [synonym]	Tick sequence type	Tick stage	Location	Collected source Host animals or Vegetation	Sampling date (year or yyyy.mm.dd)	Acc. No.
32	*Ixodes persulcatus* Schulze, 1930	Iper-A	Female	Nishiide, Ooizumi-cho, Hokuto-shi, Yamanashi	Vegetation	2004.06.05	AB819248
		Iper-B	Female	Nishiide, Ooizumi-cho, Hokuto-shi, Yamanashi	Vegetation	2004.06.05	AB819249
		Iper-C	Female	Imaizumi, Nakadomari-machi, Kitatsugaru-gun, Aomori	Vegetation	2004.08.02	AB819250
		Iper-D	Nymph	Imaizumi, Nakadomari-machi, Kitatsugaru-gun, Aomori	Vegetation	2004.08.02	AB819251
		Iper-E	Male	Imaizumi, Nakadomari-machi, Kitatsugaru-gun, Aomori	Vegetation	2004.08.02	AB819252
		Iper-F	Nymph	Takaoka, Tomakomai-shi, Hokkaido	*Turdus cardis* Temminck, 1831	2008.08.14	AB819253
33	*Ixodes philipi* Keirans and Kohls, 1970	Iphi-A	Female	Minamigou, Mikurajima village, Tokyo	*Calonectris leucomelas* (Temminck, 1835)	2004.08.25	AB819254
34	*Ixodes signatus* Birula, 1895	Isig-A	Female	Hamamatsu, Nemuro-shi, Hokkaido	*Larus schistisagus* Stejneger, 1884	2007.06.12	AB819255
35	*Ixodes tanuki* Saito, 1964	Ita-A	Female	Ookubo, Higashinagura, Shitara-cho, Kitashitara-gun, Aichi	*Mustela itatsi* Temminck, 1844	2003.02.23	AB819256
		Ita-B	Male	Kuroda-cho, Toyota-shi, Aichi	*Nyctereutes procyonoides viverrinus* Temminck, 1838	2002.11.05	AB819257
36	*Ixodes turdus* Nakatsuji, 1942	Itur-A	Nymph	Kuchinoshima, Tomakomai-shi, Hokkaido	Vegetation	2008.01.13	AB819258
		Itur-B	Female	Sekiya, Tyuuou-ku, Niigata-shi, Niigata	*Parus major* Linnaeus, 1758	2008.04.09	AB819259
		Itur-C	Nymph	Nara, Kihoku-cho, Kitauwa-gun, Ehime	*Turdus pallidus* Gmelin, 1789	2007.09.29	AB819260
		Itur-D	Nymph	Takaoka, Tomakomai-shi, Hokkaido	*Turdus cardis* Temminck, 1831	2008.05.13	AB819261
		Itur-E	Female	Sekiya, Tyuuou-ku, Niigata-shi, Niigata	*Turdus cardis* Temminck, 1831	2008.04.30	AB819262
		Itur-F	Larva	Hidaka-cho, Hidaka-gun, Wakayama	*Turdus pallidus* Gmelin, 1789	2007.12.16	AB819263
		Itur-G	Female	Fusaheiwadai, Abiko-shi, Chiba	*Turdus naumanni* Temminck, 1820	2004.01.05	AB819264
		Itur-H	Female	Fusaheiwadai, Abiko-shi, Chiba	*Turdus naumanni* Temminck, 1820	2004.01.05	AB819265
37	*Ornithodoros capensis* Neumann, 1901 [*Carios capensis*]	OC-A	Male	Mukojima, Ogasawara village, Tokyo	*Puffinus pacificus* (Gmelin, 1789)	2008.08.22	AB819266
38	*Ornithodoros sawaii* Kitaoka and Suzuki, 1973 [*Carios sawaii*]	OS-A	Female	Kutsujima, Maizuru-shi, Kyoto	*Oceanodroma monorhis* (Swinhoe, 1867)	2007.08.25	AB819267
39	*Rhipicephalus microplus* (Canestrini, 1888) [*Boophilus microplus*]	RM-A	Male	Otoutojima, Ogasawara village, Tokyo	*Capra aegagrus* Erxleben, 1777	2007.04.24	AB819268

*[1] Ticks were collected from the nest of *Delichon urbica*. *[2] Ticks were collected from August to September of 2006. *[3] Ticks were collected from under the nest of *Eptesicus japonensis*. *[4] Engorged ticks. *[5] Molted ticks.

transcribed spacer 2 (ITS2), and the large subunit of RNA polymerase II (Pol II) (Black and Piesman, 1994; Black et al., 1997; Mangold et al., 1998; Fukunaga et al., 2000; Beati and Keirans, 2001; Murrell et al., 2001). In this study, we used mt-*rrs* as the target gene because the mt-*rrs* is variable enough to differentiate species, and the registered sequences of this gene in GenBank were more abundant than those of other genes.

Materials and Methods

Ticks

From 1985 to 2011, 39 species of ticks were collected from vegetation or vertebrates, including mammals, birds, and reptiles (Table 1). After morphological identification, total DNA was prepared from whole ticks using a DNA extraction kit (DNeasy tissue kit; Qiagen, Hilden, Germany) according to the manufacturer's instructions with minor modification (The column was incubated for 10 min at 70°C after adding elution buffers to it). The DNA was stored at −30°C until use. The tick species were described according to Guglielmone et al. (2010). More than one tick was used for each species where possible.

PCR and sequencing analysis

PCR was performed using a set of primers (mt-rrs1: 5′-CTG CTC AAT GAT TTT TTA AAT TGC TGT GG-3′, mt-rrs2: 5′-CCG GTC TGA ACT CAG ATC AAG TA-3′), previously described by Ushijima et al. (2003). The PCR assays were performed using 25 μL of reaction mixture for 30 cycles (10 s at 94°C, 30 s at 55°C, and 30 s at 72°C) with puRe *Taq* Ready-To-Go PCR Beads (GE Healthcare UK, Ltd., Buckinghamshire, UK) or Takara *Ex Taq* (Takara Bio Inc., Otsu, Japan). Subsequently, the PCR products were purified using a High Pure PCR Product Purification Kit (Roche Diagnostics, Basel, Switzerland), and all PCR products were directly sequenced using an ABI Prism 3130 or 3130xl Genetic Analyzer (Life Technologies Corporation, Gaithersburg, MD).

Phylogenetic analysis

Sequence data were analyzed using MEGA5.2 software (http://www.megasoftware.net) (Tamura et al., 2011). After alignment using the CLUSTAL-W (ver. 1.6) software package, the neighbor joining (NJ) phylogenetic tree construction and bootstrap tests were carried out according to the Kimura 2-parameter distance method (Kimura, M., 1980; Saitou and Nei, 1987). Pairwise alignments were performed with an open-gap penalty of 15, and a gap extension penalty of 6.66. Multiple alignments were also performed using the same values. All positions containing alignment gaps and missing data were eliminated in pairwise sequence comparisons (pairwise deletion).

Results

In total, 113 sequence types (designated as "AJ-A" to "RM-A" in Table 1) were detected from 39 species of ticks and all sequence data were deposited in GenBank as listed in Table 1. The length of amplicons ranged from 401 to 416 bp. In this study, sequence data were collected from more than one specimen of each species except in the case of *Haemaphysalis cornigera* Neumann, *Haemaphysalis phasiana* Saito, Hoogstraal and Wassef, and *Ixodes signatus* Birula. Of the 39 species, 36 (92.3%) were distinguishable by phylogenetic analysis based on mt-*rrs* sequences (Fig. 1). On the other hand, three *Haemaphysalis* spp. (*Haemaphysalis japonica* Warburton, *Haemaphysalis japonica douglasi* Nuttall and Warburton and *Haemaphysalis megaspinosa* Saito) could not be differentiated by this analysis. Sequence types of *H. japonica* (HJ-A and HJ-B) and *H. japonica douglasi* (HD-A and HD-B) showed 100% sequence identity, and sequence types of *H. japonica* and *H. megaspinosa* (from Hmegaspi-A to Hmegaspi-D) showed 99.3%～99.8% sequence similarity. In this study, each of 6 tick species, *Amblyomma geoemydae* (Cantor), *Haemaphysalis flava* Neumann, *Haemaphysalis longicornis* Neumann, *Ixodes asanumai* Kitaoka, *Ixodes persulcatus* Schulze, and *Ixodes turdus* Nakatsuji, had over 6 sequence types, and the intraspecific sequence similarity within these 6 species was 92.9%～99.8%, 97.3%～99.8%, 99%～99.8%, 99%～99.8%, 99.3%～99.8% and 98.8%～99.8%, respectively.

Discussion

In this study, we used mt-*rrs* as the target gene for tick identification which had high variation among species and a large number of registered sequences in GenBank. Of the ticks examined, 92.3% were distinguishable by the phylogenetic analysis based on mt-*rrs* sequences. Even this analysis could not differentiate 3 tick species (or subspecies), *H. japonica*, *H. japonica douglasi* and *H. megaspinosa*. Fukunaga (2007) suggested *H. japonica* and *H. megaspinosa* also could not be differentiated by NADH dehydrogenase subunit 2 (ND2) gene sequences, which was one of variable genes on tick mitochondrial genomes. On the other hand, the registered sequence data of other variable genes such as ITS2, mitochondrial 12S rDNA gene and COI of the *Haemaphysalis* ticks were quite few. Thus, for improvement in sensitivity for the *Haemaphysalis* ticks, further enrichment of the database is necessary.

The database we developed covered 83% (39/47), and identified 77% (36/47) of the tick species previously recorded in Japan. In addition, there were the sequence data for mt-*rrs* of *Rhipicephalus sanguineus* (Latreille), *Ixodes uriae* White and *Ixodes vespertilionis* Koch in GenBank (Accession Numbers were L34302, AB030017 and U95910, respectively). Taken together, 83% (39/47) of ticks found in Japan could be identified by this analysis when morphological identification proved difficult (Fig. 1).

Our analysis detected intraspecific variation among

Fig. 1. Phylogenetic analysis based on mt-*rrs* of ticks in Japan. The phylogenetic trees were constructed based on NJ methods and bootstrap tests carried out according to the Kimura 2-parameter distances method. The percentage of replicate trees in which the associated taxa are clustered together in the bootstrap test (1,000 replicates) was calculated. The phylogenetic branches were supported with more than 70% bootstrap values in this analysis. The length of the bar corresponds to the degree of sequence divergence. All positions containing alignment gaps and missing data were eliminated in pairwise sequence comparisons (pairwise deletion). Phylogenetic analyses were conducted in MEGA5.2.

A. geoemydae (Fig. 1). Several *A. geoemydae* individuals were collected from tortoises on the Okinawa islands and Yaeyama Archipelago in Okinawa Prefecture in the southern part of Japan. The ticks collected from the Okinawa islands and Yaeyama Archipelago were considered to belong to the same species morphologically. However, genetic analysis revealed that ticks originated from the two regions formed distinct monophyletic groups. Further analysis will be needed to resolve this discrepancy between DNA sequence variation and morphological similarity.

In contrast, although the Okinawa islands and Ogasawara Archipelago are separated by over 1,500 km over ocean, *Ixodes granulatus* Supino collected from each location formed a monophyletic group. Since *I. granulatus* often infested black rats [*Rattus rattus* (Linnaeus, 1758)], this result might be the consequence of the artificial or non-artificial diffuseness of the host animals.

Though DNA sequence-based analysis, with such as mt-*rrs* in this study, sometimes did not correlate with morphology-based analysis for phylogeny (Black et al., 1997), we believe that the former could be a useful alternative to the latter for identification of tick species (Jizhou et al., 2013).

In this study, we were not able to collect specimens of the remaining five tick species of Japan, *Dermacentor silvarum* Olenev, *Haemaphysalis fujisana* Kitaoka, *Haemaphysalis megalaimae* Rajagopalan, *Ixodes lividus* Koch, and *Ixodes simplex* Neumann. These ticks were considered to be rare species in Japan. In fact, ticks collected from human cases are possible to be identified by the method presented in this study, which uses the database as ours (Kawabata et al., 2011). This suggests that our method is helpful for surveillance on tick-borne diseases for humans and livestock. In reference to this study, there is currently worldwide development of DNA barcoding database, such as The Barcode of Life Data System (BOLD) (Ratnasingham and Hebert, 2007). Further examination for updating the DNA database would be beneficial for public health to control the tick-borne infectious diseases through the process of identification of ticks to be involved.

Acknowledgements

We would like to thank K. Miyamoto (Asahikawa Medical University), M. Takashi and H. Torikai (Amami Wild Bird Society of Japan), S. Yamamoto (Kagoshima University), N. Koizumi (National Institute of Infectious Diseases: NIID), Y. Nitta (Okinawa Prefectural Institute of Animal Health), T. Kasuya (University of Tsukuba), T. Kuramoto (Kagoshima Prefectural Institute for Environmental Research and Public Health), A. Nakamoto (Univ. of the Ryukyus), S. Kawada (National Museum of Nature and Science) and K. Ozaki (Yamashina Institute for Ornithology) for tick collection. We are also grateful to the members of Bird banders of Yamashina Institute for Ornithology. We also thank Y. Ito (Yamaguchi Univ.), A. Sakata and M. Muto (NIID) for their excellent technical assistance. We appreciate Dr. Kyle R. Taylor (University of Florida, Department of Infectious Diseases and Pathology) for his critical comments on this manuscript.

The authors would like to acknowledge the technical expertise of The DNA Core facility of the Center for Gene Research, Yamaguchi University, supported by a Grant-in-Aid from the Ministry of Education, Science, Sports and Culture of Japan. This work was supported in part by a grant for research on emerging and reemerging infectious diseases from the Japanese Ministry of Health, Labor, and Welfare, and Program to Disseminate Tenure Tracking System, MEXT, Japan.

References

Beati, L. and Keirans, J. E. 2001. Analysis of the systematic relationships among ticks of the genera *Rhipicephalus* and *Boophilus* (Acari: Ixodidae) based on mitochondrial 12S ribosomal DNA gene sequences and morphological characters. *J. Parasitol.*, 87: 32–48.

Black, W. C. 4th, Klompen, J. S. and Keirans, J. E. 1997. Phylogenetic relationships among tick subfamilies (Ixodida: Ixodidae: Argasidae) based on the 18S nuclear rDNA gene. *Mol. Phylogenet. Evol.*, 7: 129–144.

Black, W. C. 4th and Piesman, J. 1994. Phylogeny of hard- and soft-tick taxa (Acari: Ixodida) based on mitochondrial 16S rDNA sequences. *Proc. Natl. Acad. Sci. U.S.A.*, 91: 10034–10038.

Bowman, A. S. and Nuttall, P. A. 2008. Ticks: biology, disease and control. 506 pp., Cambridge University Press, Cambridge.

Fujita, H. and Takada, N. 2007. Identification of immature ticks in Japan. *In*: Acari and Emerging/Reemerging Infectious Diseases (ed. Yanagihara, Y.), pp. 53–68, Zenkoku Noson Kyoiku Kyokai Publishing, Tokyo (In Japanese).

Fukunaga, M. 2007. Molecular phylogeny of ticks. *In*: Acari and Emerging / Reemerging Infectious Diseases (ed. Yanagihara, Y.), pp. 69–75, Zenkoku Noson Kyoiku Kyokai Publishing, Tokyo (In Japanese).

Fukunaga, M., Yabuki, M., Hamase, A., Oliver, J. H. Jr. and Nakao, M. 2000. Molecular phylogenetic analysis of ixodid ticks based on the ribosomal DNA spacer, internal transcribed spacer 2, sequences. *J. Parasitol.*, 86: 38–43.

Guglielmone, A. A., Robbins, R. G., Apanaskevich, D. A., Petney, T. N., Estrada-Peña, A., Horak, I. G., Shao, R. and Barker, S. C. 2010. The Argasidae, Ixodidae and Nuttalliellidae (Acari: Ixodida) of the world: a list of valid species names. *Zootaxa*, 2528: 1–28.

Jizhou, L., Wu, S., Zhang, Y., Zhang, T., Feng, C., Jia, G. and Lih, Z. 2013. Development of a barcode system for the Ixodida (Acari: Ixodida). *Mitochondrial DNA*, [Internet] Early online in Journal home [accessed Sep 13, 2013]. Available from: http://informahealthcare.com/doi/abs/10.3109/19401736.2013.792052.

Kawabata, H., Takano, A., Ohnishi, M., Ando, S., Ogasawara, Y., Fujita, H., Kadosaka, T., Wada, Y., Baba, S. and Seishima, M. 2011. Investigation of tick bite cases that visited medical facilities in Japan, from 2005 to June 2011. *IASR*, 32: 226–228, In Japanese.

Kimura, M. 1980. A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. *J. Mol. Evol.*, 16: 111–120.

Mangold, A. J., Bargues, M. D. and Mas-Coma, S. 1998. 18S rRNA gene sequences and phylogenetic relationships of European hard-tick species (Acari: Ixodidae). *Parasitol. Res.*, 84: 31–37.

Murrell, A., Campbell, N. J. and Barker, S. C. 2001. A total-evidence phylogeny of ticks provides insights into the evolution of life cycles and biogeography. *Mol. Phylogenet. Evol.*, 21: 244–258.

Okino, T., Ushirogawa, H., Matoba, K. and Hatsushika, R. 2010. Bibliographical Studies on Human Cases of Hard Tick (Acarina:

Ixodidae) Bites in Japan (7) Cases of Unidentified Tick Infestation. *Kawasaki Medical Journal*, 36: 127–141, In Japanese with English abstract.

Ratnasingham, S. and Hebert, P. D. N. 2007. BOLD: The Barcode of Life Data System (www.barcodinglife.org). *Mol. Ecol. Notes*, 7: 355–364.

Saitou, N. and Nei, M. 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol. Biol. Evol.*, 4: 406–425.

Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M. and Kumar, S. 2011. MEGA5: Molecular Evolutionary Genetics Analysis using Maximum Likelihood, Evolutionary Distance, and Maximum Parsimony Methods. *Mol. Biol. Evol.*, 28: 2731–2739.

Ushijima, Y., Oliver, J. H. Jr., Keirans, J. E., Tsurumi, M., Kawabata, H., Watanabe, H. and Fukunaga, M. 2003. Mitochondrial sequence variation in *Carios capensis* (Neumann), a parasite of seabirds, collected on Torishima Island in Japan. *J. Parasitol.*, 89: 196–198.