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Seroepidemiological study of canine ehrlichial infections in Yamaguchi prefecture and surrounding areas of Japan

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Abstract

Randomly selected serum samples from 150 dogs from Yamaguchi and neighbouring prefectures were subjected to the indirect immunofluorescent assay to detect antibodies against *Ehrlichia canis, Ehrlichia chaffeensis, Ehrlichia muris* and *Ehrlichia* from *Ixodes ovatus.* A total of 30 out of the 150 serum samples reacted with at least one of the antigens at a titer of 1:20 or more. Considerable cross-reactivity was seen and most samples reacted with at least two different antigens. Fifteen (10.0%) dogs had higher titers to *E. canis* than any of the other antigens. Four (2.7%) dogs had higher titers to *Ehrlichia* from *Ixodes ovatus* and one (0.6%) dog had higher titers to *E. muris* compared to the other antigens. The findings suggest that these five dogs may be infected with the domestic *Ehrlichia* of Japan. The remaining ten dogs had similar high titers to two or more of the antigens. This is the first serological evidence obtained of canine infection with the domestic *Ehrlichia* of Japan.
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Keywords: Ehrlichia canis; Ehrlichia chaffeensis; Ehlrichia muris; Ehrlichia from Ixodes ovatus; Dog; IFA

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1. Introduction

Ehrlichia are small-gram negative, pleomorphic, obligatory intracellular bacteria that primarily infect leukocytes (Ristic and Huxsoll, 1984; Rikihisa, 1991) and cause disease of varying severity in humans, some domestic and wild animals. The genus *Ehrlichia* based on the 16SrRNA gene sequence includes *Ehrlichia canis, Ehrlichia chaffeensis, Ehrlichia ewingii, Ehrlichia ruminantium* and *Ehrlichia muris* (Dumler et al., 2001). *Ehrlichia* from *Ixodes ovatus* has not yet been included in this genus though analysis of the 16SrRNA sequence revealed greatest homology to *E. chaffeensis* and then to *E. muris* (Shibata et al., 2000). *Ehrlichia canis* is a well recognized pathogen of dogs, but in Japan there has only been one confirmed case of *E. canis*.(Suto et al., 2001). Various studies have been conducted to try and isolate *E. canis* from canine blood in Japan using the PCR method, but only one positive case has been reported thus far, following analysis of the 16SrRNA gene sequence (Inokuma et al., 2001; Unver et al., 2003). Antibodies to *E. canis* in dogs, however, have been previously reported in Yamaguchi (4.7%) (Inokuma et al., 1999), Kagoshima and Fukuoka (1.9%) (Yamamoto et al., 1994) and Okinawa prefectures (14.7%) (Inokuma et al., 1998). *Ehrlichia muris,* a relatively new *Ehrlichia,* is known to induce mild non-specific clinical signs and splenomegaly in experimentally infected mice (Kawahara et al., 1993). The *Ehrlichia* detected from *I. ovatus* is more pathogenic and causes mortality in experimentally infected mice, 8–10 days post infection (Shibata et al., 2000). Both *E. muris* and the *Ehrlichia* from *I. ovatus,* are as of yet not known to cause disease in dogs, however since their suspected tick vectors, *Haemaphysalis flava* and *I. ovatus*, respectively, are widespread throughout mainland Japan (Yamaguchi et al., 1971), the possibility of infection is very high. So far, there has been only one dog PCR positive with the *Ehrlichia* from *I. ovatus* (Inokuma et al., 2001). Dogs seropositive for *E. muris* in Gifu and Ibaraki prefecture, have been previously reported (Kawahara et al., 1999), however there have not been any such reports in Western Japan. It is often difficult to determine which of the *Ehrlichia* is causing seroreactivity, as there is significant cross-reactivity among species in the genus *Ehrlichia* (Wen et al., 1995; Kawahara et al., 1993). The aim of this study was to evaluate the seroprevalence of *E. canis, E. chaffeensis, E. muris* and *Ehrlichia* from *I. ovatus* in dogs from Yamaguchi prefecture and neighboring areas and to clarify cross-reactivities, that may have clouded the interpretation of previous studies, using the indirect immunofluorescent assay (IFA).

2. Materials and methods

2.1. Sera and serological analysis

150 random serum samples from dogs examined at the Veterinary Teaching Hospital, Yamaguchi University between February and June 2001, and kept at −20 °C, were used for serological analysis. The signalment, history, clinical signs and blood parameters of the dogs were noted for further analysis. IFA antigen slides were routinely prepared (Brouqui et al., 1994) using DH82 cells infected with *E. canis* (Israel Strain, supplied by Dr. Harrus, The Hebrew University of Jerusalem) and *E. chaffeensis* (Arkansas strain, supplied by J.

Dawson) and mice spleen infected with *E. muris* (Hyogo strain) and *Ehrlichia* from *I. ovatus* (HF639, supplied by Dr. Fujita, Ohara Hospital). Sera was screened at a 1:20 dilution in phosphate-buffered saline (pH 7.4) Tween 0.5% (PBST) and an optimized dilution (1:200) of fluorescein isothiocyanate-labelled IgG conjugate in PBST was used as the second antibody. Reactive antibodies were then detected using a fluorescence light microscope. Those samples that reacted with any one of the antigens at the screening dilution were then titrated using serial twofold dilutions to determine end titers. Positive sera and PBST were used as the positive and negative controls, respectively. The positive controls used were *E. canis* positive sera from a naturally infected dog, *E. muris* positive and *Ehrlichia* from *I. ovatus* positive sera from experimentally infected mice. *E. chaffeensis* positive sera was unavailable. Uninfected spleen and DH82 cell controls were also used to detect non-specific reactions.

2.2. *Statistical analysis*

The Chi-square test was used to evaluate any associations between seroreactivity and the sex, breed and age of the dogs (Statsview Version 5).

3. Results and discussion

A total of 30 canine serum samples reacted with at least one of the antigens at a dilution of 1:20 or more (Table 1). Cross-reactivity was seen and most samples reacted with at least two different antigens (Table 1). Twenty-seven out of 150 (18.0%) dogs seroreacted with *E. canis*, 28 out of 150 (18.7%) with *E. chaffeensis*, 17 out of 150 (11.3%) with *E. muris* and 20 out of 150 (13.3%) with *Ehrlichia* from *I. ovatus* (Table 2). Eleven of the 30 (37%) samples reactive at the screening dilution, reacted with all four IFA antigens. Fifteen of the total 150 samples had a higher titer to *E. canis* (10.0%), 4 to *Ehrlichia* from *I. ovatus* (2.7%) and 1 to *E. muris* (0.7%) as compared to the other antigens. Nine out of the 27 (33.3%) that seroreacted with *E. canis* did so at a dilution of 1:160, 3 out of the 28 (10.7%) that seroreacted with *E. chaffeensis* did so at a dilution of 1:160, 1 out of the 17 (5.9%) that seroreacted with *E. muris* did so at a dilution of 1:160 and 4 out of 20 (20.0%) that seroreacted with *Ehrlichia* from *I. ovatus* did so at a dilution of 1:160 or greater (Table 2). Most of the seroreactive dogs were presented to the hospital for a routine check-up and vaccination and only a few for specific disease conditions (Table 1).

The most common clinical signs, among the 30 seroreactive dogs, were pyrexia (20.0%) and lymphadenomegaly (16.7%). Thrombocytopenia was the most common hematological abnormality occurring in eleven of the thirty seroreactive dogs (36.7%) (5243, 2930, 5465, 80, 234, 409, 5000, 3914, 4575, 3975, 4729) with values ranging from 2×10^4 to $19.2 \times 10^4\ \mu L^{-1}$, followed by hypoproteinaemia in five dogs (16.7%) (5243, 1025, 1229, 1836, 5000) with values ranging from 5.2 to 5.8 g/dL (reference values; Aeillo et al., 1998).

The number of seroreactive males (25.0%) and females (14.5%) was not significantly different ($P = 0.1023$) (Table 3). There was no significant difference in the number of seroreactive pure-bred dogs (20.8%) and cross-bred dogs (16.7%) ($P = 0.6098$) (Table 3). The mean age of dogs that had antibodies to any one of the antigens was 4 years and 9

Table 1
IFA antibody titers and clinical signs of seroreactive dogs

Dog no.	Antibody titers				Clinical findings
	E. from *I. ovatus*	*E. muris*	*E. canis*	*E. chaffeensis*	
1014	320	160	<20	<20	Pyrexia, otitis externa, dermatitis
5339	320	20	80	40	Addison's disease
1229	160	80	80	80	None
3975	160	80	80	80	None
322	80	<20	80	80	LNE, coughing, vomiting, otitis externa
5365	80	20	80	40	Vomiting (ingestion of plastic bag)
2923	40	80	80	40	Pyrexia
5277	<20	<20	80	20	LNE
409	80	80	<20	40	None
5000	20	40	40	40	Vomiting after eating
1836	80	20	160	80	Otitis externa, dermatitis
1281	80	20	160	160	Otitis externa
1992	80	<20	160	80	None
234	20	<20	160	160	Pyrexia
3784	20	20	160	160	Mild otitis externa
4629	<20	20	160	80	Pyrexia, LNE
80	<20	20	160	80	Pyrexia
4575	<20	<20	160	80	Tenesmus, hematochezia
3914	20	20	160	80	None
5418	20	<20	80	20	Pyrexia, demodicosis
5535	<20	<20	80	20	Vomiting (ingestion of rubber glove)
4729	40	<20	80	40	None
3762	<20	<20	80	80	None
347	<20	<20	80	80	Pyrexia
1025	20	<20	80	40	None
2930	20	20	80	40	Otitis externa
2349	<20	<20	40	20	LNE
5243	<20	<20	40	20	None
5227	<20	20	<20	<20	Hypothyroidism
5465	20	20	80	40	LNE

LNE: lymph node enlargement.

months; however, there was no significant difference in the number of seroreactive dogs of different ages ($P = 0.4063$). However, the number of seroreactive dogs 2–5 years of age (29.2%) was higher than the other age groups (Table 3). Previous studies conducted on dogs with antibodies reactive with *E. canis,* showed there was no association between seroprevalence and sex, age or breed, (Matthewman et al., 1993; Inokuma et al., 1999), and similar findings were obtained in this study (Table 3).

Based on the high antibody titers against *Ehrlichia* from *I. ovatus* and *E. muris*, it can be concluded that the domestic *Ehrlichia* of Japan can infect dogs. This is the first serological evidence of canine infection with the domestic *Ehrlichia* in Yamaguchi prefecture and its surrounding areas. Three of the four dogs with highest antibody titers to *Ehrlichia* from *I. ovatus* were from Hiroshima prefecture. One of the four dogs (dog no. 1014) with highest antibody titers to *Ehrlichia* from *I. ovatus* was pyrexic and had a slightly elevated MCH value (24.7 pg), two showed no clinical signs one of which had mild thrombocytopenia

Table 2
Seroprevalence of various *Ehrlihia* in dogs from Yamaguchi prefecture and surrounding areas

Ehrlichial antigen	Total number dogs tested	Number reactive (%)[a]	Number with highest titer to respective antigen (%)	Number with titer ≥1:160 among reactive (%)
Ehrlichia canis	150	27 (18.0)	15 (10.0)	9 (33.3)
Ehrlichia chaffeensis	150	28 (18.7)	0 (0.0)	3 (10.7)
Ehrlichia muris	150	17 (11.3)	1 (0.7)	1 (5.9)
Ehrlichia detected from *I. ovatus*	150	20 (13.3)	4 (2.7)	4 (20.0)

[a] Seroreactivity based on a titer of 1:20 or greater.

(dog no. 3975, platelets = 17.7×10^4 μL^{-1}) and the other had slight hypoproteinaemia (dog no. 1229, plasma protein = 5.6 g/dL) and the fourth had Addison's disease (dog no. 5339). The dog (dog no. 5227) that had highest antibody titers to *E. muris* had hypothyroidism. Since thrombocytopenia is known to be the most common hematological finding in all stages of *E. canis* infection (Harrus et al., 1997), it is possible that for similar reasons it was seen in the dog seroreactive to *Ehrlichia* from *I. ovatus*. Possible causes include enhanced platelet sequestration, antiplatelet antibody production, or bone marrow hypoplasia (Shaw et al., 2001). Apart from thrombocytopenia, hyperglobulinaemia and hypoalbuminaemia are also commonly seen in dogs infected with *E. canis* due to various factors such as blood loss and protein loss following vasculitis (Woody and Hoskins, 1991) and decreased protein production due to concurrent liver disease (Reardon and Pierce, 1981). Nevertheless, since the pathogenesis of the new *Ehrlichia* in dogs is unknown, it cannot be assumed that the abnormalities were due exclusively to the ehrlichial infection.

The domestic *Ehrlichia* may be of low pathogenic importance to dogs as previous studies showed that experimental infection of dogs with *E. muris* did not produce any disease (Wen et al., 1995). However, as the suspected tick vectors of the new *Ehrlichia* are widespread throughout Japan and the possibility of infection has been established, there is a need to clarify the pathogenesis in dogs. Furthermore, since both of the domestic *Ehrlichia* are closely related to *E. chaffeensis* based on 16SrRNA gene sequences (Wen

Table 3
Differences in seroprevalence of *Ehrlichia* according to sex, breed and age

Criteria		Total no.	No. reactive (%)
		150	30
Sex	Male	79	20 (66.7)
	Female	69	10 (33.3)
	Unknown	2	0 (0.0)
Breed	Pure-bred	120	25 (83.3)
	Cross-bred	30	5 (16.7)
Age (years)	0–1	22	4 (13.3)
	2–5	48	14 (46.7)
	6–9	48	8 (26.7)
	10–13	19	3 (10.0)
	14–17	4	0 (0.0)
	Unknown	9	0 (0.0)

et al., 1995; Shibata et al., 2000) and that *E. chaffeensis* is a human pathogen, it is necessary to determine the zoonotic factor.

Antibodies to *E. canis* were also prevalent in the dog serum samples, the highest titer being 1:160. It is difficult to determine whether they are indeed antibodies to *E. canis* or some other *Ehrlichia* of similar antigenicity that is causing reactivity, especially since the presence of *E. canis* in Japan is still doubtful, except for two isolated cases, one from a dog brought from Indonesia (Suto et al., 2001) and the other from canine blood in Kagoshima prefecture (Unver et al., 2003). *Ehrlichia chaffeensis* meanwhile is the aetiological agent of human monocytic ehrlichiosis and is known to be transmitted and maintained in the tick vector, *Amblyomma americanum* (Dumler et al., 2001). Natural infection of dogs has been reported in the USA (Breitschwerdt et al., 1998) and experimental infections of dogs produce mild clinical and hematological abnormalities (Dawson and Ewing, 1992). However, since the tick vector of *E. chaffeensis* is not known to exist in Japan, it is unlikely that the organism has established itself in Japan. This was further supported by the findings in this study where none of the dogs showed highest titers to *E. chaffeensis* and the titers were either the same as or lower than those against the other antigens.

Cross-reactivity was seen and the majority of the serum samples reacted with two or more antigens, as is often the case with members of the genus *Ehrlichia* (Wen et al., 1995; Shibata et al., 2000). Therefore, to obtain an accurate picture of the seroprevalences of the different ehrlichial species using the IFA test, various ehrlichial antigens should be included. Then, based on which antigen stimulated the highest titer of antibodies, the *Ehrlichia* infecting the dog could be determined. The ability to differentiate between the *Ehrlichia* becomes important, especially with regards to *Ehrlichia* not yet known in Japan, to identify whether seroreactivity is due to its actual presence or due to cross-reactivity.

In conclusion, the presence of antibodies against *E. muris* and the *Ehrlichia* from *I. ovatus* in the serum of dogs in Yamaguchi prefecture and its surrounding areas, was demonstrated for the first time and is indicative of canine infection with these *Ehrlichia*.

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The mitochondrial genomes of soft ticks have an arrangement of genes that has remained unchanged for over 400 million years

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Abstract

There are two major groups of ticks: soft ticks and hard ticks. The hard ticks comprise the prostriate ticks and the metastriate ticks. The mitochondrial (mt) genomes of one species of prostriate tick and two species of metastriate ticks had been sequenced prior to our study. The prostriate tick has the ancestral arrangement of mt genes of arthropods, whereas the two metastriate ticks have rearrangements of eight genes and duplicate control regions. However, the arrangement of genes in the mt genomes of soft ticks had not been studied. We sequenced the mt genomes of two species of soft ticks, *Carios capensis* and *Ornithodoros moubata*, and a metastriate tick, *Haemaphysalis flava*. We found that the soft ticks have the ancestral arrangement of mt genes of arthropods, whereas the metastriate tick, *H. flava*, shares the rearrangements of mt genes and duplicate control regions with the other two metastriate ticks that have previously been studied. Our study indicates that gene rearrangements and duplicate control regions in mt genomes occurred once in the most recent common ancestor of metastriate ticks, whereas the ancestral arrangement of arthropods has remained unchanged for over 400 million years in the lineages leading to the soft ticks and the prostriate ticks.

Keywords: tick, Ixodida, Acari, arthropod.

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Introduction

Comparative studies of mitochondrial (mt) genomes help to elucidate the evolution of these genomes and the phylogenetic relationships of animals (Smith *et al.*, 1993; Boore *et al.*, 1995; Hwang *et al.*, 2001; Murrell *et al.*, 2003; Nardi *et al.*, 2003). The suborder Ixodida (ticks) of the subphylum Chelicerata has two major groups: soft ticks (family Argasidae) and hard ticks (Ixodidae). The hard ticks comprise the prostriate ticks (249 spp. of *Ixodes* ticks) and the metastriate ticks (12 genera and 464 spp.; Barker and Murrell, 2004). The mt genomes of a prostriate tick (*Ixodes hexagonus*, Black & Roehrdanz, 1998) and two metastriate ticks (*Boophilus microplus*, Campbell & Barker, 1999; *Rhipicephalus sanguineus*, Black & Roehrdanz, 1998) have been sequenced. The prostriate tick has the hypothetical arrangement of the common ancestor of arthropods, whereas the metastriate ticks have a translocation of seven genes (encoding ND1, tRNA-Leu [TTR], 16S rRNA, tRNA-Val, 12S rRNA, tRNA-Ile and tRNA-Gln), and a translocation and inversion of the tRNA-Cys gene (Black & Roehrdanz, 1998; Campbell & Barker, 1998, 1999). However, the mt genomes of the other major family of ticks, the soft ticks, are unknown.

We sequenced and characterized the mt genomes of two soft ticks, *Carios capensis* and *Ornithodorous moubata*, and a metastriate tick, *Haemaphysalis flava*. *H. flava* is from a lineage of metastriate ticks, the subfamily Haemaphysalinae, which has not been studied in this way before. The arrangement of mt genes in the soft ticks is identical to that of the prostriate tick, *Ixodes hexagonus* (Black & Roehrdanz, 1998) and the horseshoe crab, *Limulus polyphemus*, which has been inferred to be ancestral for arthropods (Staton *et al.*, 1997). The arrangement of mt genes in *H. flava* is identical to that of the other two metastriate ticks that have been sequenced, *B. microplus* (Campbell & Barker, 1999) and *R. sanguineus* (Black & Roehrdanz, 1998). Thus, the simplest explanation for the evolution of the mt genomes of ticks is that the translocation and/or inversion of the eight genes and the duplicate control regions occurred only once in the most recent common ancestor of the metastriate ticks, whereas the ancestral arrangement of arthropods has remained unchanged for

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over 400 million years in lineages leading to the soft ticks and the prostriate ticks (*Ixodes* spp.).

Results

General features of the mt genomes of C. capensis, O. moubata *and* H. flava

The mt genomes of the three ticks are circular and about 14.5 kbp long: 14 418 bp (*C. capensis*), 14 398 bp (*O. moubata*) and 14 686 bp (*H. flava*; Table 1). These mt genomes have the same gene content: 13 protein-coding genes, two rRNA genes and 22 tRNA genes. However, the mt genomes of the two soft ticks have one control region (the largest non-coding region) whereas the mt genome of the hard tick, *H. flava*, has two control regions which are nearly identical to each other in size and nucleotide sequence. For the two soft ticks, the majority strand encodes nine protein-coding genes (encoding ND2, ND3, ND6, CO1, CO2, CO3, ATP6, ATP8 and Cyt b) and 13 tRNA genes, whereas the minority strand encodes four protein-coding genes (encoding ND1, ND4, ND4L and ND5), nine tRNA genes and two rRNA genes. The distribution of genes between the two strands in *H. flava* is the same as that of the soft ticks, except that the tRNA-Cys gene is on the majority strand in *H. flava*.

Protein-coding genes

Three codons, ATA, ATG and ATT, appear to be used as start codons in the mt genomes of the three ticks. Four genes (encoding CO2, ND2, ND4 and ND5) of *C. capensis*, seven genes (encoding CO2, CO3, Cyt b, ND2-5) of *O. moubata*, and five genes (encoding ND2-4, CO3 and Cyt b) of *H. flava* have incomplete stop codons, T or TA. The ND4L gene of *O. moubata* and the CO2 gene of *H. flava* appear to have TAG as the stop codons whereas the other protein-coding genes of these ticks have TAA as the stop codon. Codons of the 13 protein-coding genes of these ticks showed biases toward A and T. This bias towards A and T is higher in the hard tick, *H. flava*, than in the two soft ticks, *C. capensis* and *O. moubata*. About 40% of the codons in these ticks have A or T at the first and second codon positions (41%, 40% and 46%, respectively, for *C. capensis, O. moubata* and *H. flava*; Table 2) and about 80% of the codons have A or T at the third codon position (81%, 80% and 85%, respectively, for *C. capensis, O. moubata* and *H. flava*; Table 2).

rRNA genes and tRNA genes

The putative 16S rRNA gene and 12S rRNA gene are 1225 bp and 695 bp for *C. capensis*, 1212 and 686 bp for *O. moubata*, and 1196 and 699 bp for *H. flava*. The lengths of the rRNA genes of the three ticks are similar to those of the other known ticks (Black & Roehrdanz, 1998).

The 22 tRNA genes of the three ticks range in size from 52 to 66 bp (Table 1). All of the putative tRNAs of these ticks can form conventional four-arm cloverleaf structures except the tRNAs for serine (AGN) and the tRNA for cystine of *H. flava*. The three tRNAs for serine (AGN) lack D-arms but have loops with four or five nucleotides. Most of the mt genomes of metazoans have this type of tRNA-Ser (AGN) (Wolstenholme, 1992). The putative tRNA for cystine also lacks a D-arm in *H. flava*; this also occurs in the other metastriate ticks, *B. microplus* and *R. sanguineus*.

Arrangements of mt genes

The two soft ticks have the same arrangements of mt genes (Fig. 1). This arrangement is identical to that of the prostriate tick, *Ixodes hexagonus* (Black & Roehrdanz, 1998) and the horseshoe crab, *Limulus polyphemus* (Staton *et al.*, 1997). The arrangement of mt genes present in *I. hexagonus* and *L. polyphemus* has been inferred to be ancestral for arthropods (Staton *et al.*, 1997). In contrast, *H. flava* has the arrangement seen in the other two metastriate ticks, *B. microplus* and *R. sanguineus*. Compared with the arrangement of the hypothetical ancestral arthropods, a block of seven genes (encoding ND1, tRNA-Leu [TTR], 16S rRNA, tRNA-Val, 12S rRNA, tRNA-Ile and tRNA-Gln) have translocated, and the tRNA-Cys gene has translocated and inverted in *H. flava* (Fig. 1).

Discussion

The suborder Ixodida of the subphylum Chelicerata comprises three families of ticks. The two major families are the Ixodidae (hard ticks) and the Argasidae (soft ticks). The third family, Nuttalliellidae, has only one species in one genus, *Nuttalliella namaqua*. The largest family, Ixodidae, has five subfamilies, the Ixodinae, Amblyomminae, Haemaphysalinae, Hyalomminae and Rhipicephalinae (Oliver, 1989). The latter four subfamilies are collectively called metastriate ticks. The mt gene arrangement in the Ixodinae tick, *I. hexagonus*, is the inferred ancestral arrangement for arthropods. However, the gene arrangement in the Rhipicephalinae ticks, *B. microplus* (Campbell & Barker, 1999) and *R. sanguineus* (Black & Roehrdanz, 1998) differs markedly with that of *I. hexagonus* since a block of seven genes (encoding ND1, tRNA-Leu [TTR], 16S rRNA, tRNA-Val, 12S rRNA, tRNA-Ile and tRNA-Gln) have translocated, and tRNA-Cys gene has translocated and inverted compared with the arrangement of *I. hexagonus* and the ancestral arrangement of arthropods. PCR tests suggested that these gene rearrangements may also occur in ticks of the other three subfamilies of Ixodidae: Amblyomminae, Haemaphysalinae and Hyalomminae (Black & Roehrdanz, 1998; Campbell & Barker, 1999). In the present study we showed that the Haemaphysalinae tick, *H. flava*, also has these rearrangements (Fig. 1).

Prior to the present study, nothing was known about the arrangement of mt genes in the family Argasidae (soft

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Table 1. The genes in the mitochondrial genomes of the soft ticks, *Carios capensis* and *Ornithodoros moubata* and the hard tick, *Haemaphysalis flava*

	C. capensis (14 418 bp)					*O. moubata* (14 398 bp)					*H. flava* (14 686 bp)				
Gene	Position From–to	Size nt	aa	Codon Start	Stop	Position From–to	Size nt	aa	Codon Start	Stop	Position From–to	Size nt	Codon aa	Start	Stop
tRNA-Met	1–60	60				1–60	60				1–63	63			
ND2	62–1017	956	318	ATA	TA	62–1023	962	320	ATA	TA	65–1023	959	319	ATT	TA
tRNA-Trp	1018–1078	61				1024–1083	60				1024–1082	59			
tRNA-Cys*	1072–1135	64				1077–1138	62				14631–14682	52			
tRNA-Tyr[C]	1139–1197	59				1139–1198	60				1092–1154	63			
CO1	1191–2729	1539	512	ATT	TAA	1192–2730	1539	512	ATT	TAA	1148–2686	1539	512	ATT	TAA
CO2	2733–3408	676	225	ATG	T	2739–3414	676	225	ATG	T	3691–3365	675	224	ATG	TAG
tRNA-Lys	3409–3474	66				3415–3482	58				3367–3432	66			
tRNA-Asp	3474–3531	58				3482–3539	58				3433–3492	60			
ATP8	3533–3688	156	51	ATT	TAA	3541–3696	156	51	ATA	TAA	3494–3655	162	53	ATT	TAA
ATP6	3682–4350	669	222	ATG	TAA	3690–4358	669	222	ATG	TAA	3649–4314	666	221	ATG	TAA
CO3	4350–5129	780	259	ATG	TAA	4358–5135	778	259	ATG	T	4318–5095	778	259	ATG	T
tRNA-Gly	5130–5190	61				5136–5196	61				5096–5156	61			
ND3	5191–5526	336	111	ATA	TAA	5197–5530	334	111	ATT	T	5157–5493	337	112	ATT	T
tRNA-Ala	5527–5586	60				5531–5592	60				5494–5557	64			
tRNA-Arg	5587–5643	57				5593–5652	60				5559–5617	59			
tRNA-Asn	5645–5705	61				5654–5715	62				5617–5677	61			
tRNA-Ser (AGN)	5708–5760	53				5713–5765	53				5681–5735	55			
tRNA-Glu	5761–5820	60				5766–5825	60				5738–5796	59			
tRNA-Phe[C]	5819–5877	59				5824–5882	59				9201–9262	62			
ND5[C]	5878–7537	1660	553	ATA	T	5883–7543	1661	553	ATT	TA	9263–10918	1656	551	ATT	TAA
tRNA-His[C]	7538–7598	61				7544–7604	61				10919–10982	64			
ND4[C]	7599–8914	1316	438	ATG	TA	7605–8925	1321	440	ATG	T	10983–12297	1315	438	ATG	T
ND4L[C]	8908–9186	279	92	ATG	TAA	8919–9197	279	92	ATG	TAG	12291–12566	276	91	ATG	TAA
tRNA-Thr	9194–9253	60				9203–9262	60				12569–12629	61			
tRNA-Pro[C]	9254–9314	61				9263–9322	60				12630–12689	60			
ND6	9317–9745	429	142	ATA	TAA	9325–9759	435	144	ATA	TAA	12693–13133	441	146	ATT	TAA
Cyt b	9751–10851	1101	366	ATT	TAA	9759–10858	1100	366	ATG	TA	13135–14201	1067	355	ATG	TA
tRNA-Ser (TCN)	10852–10914	63				10859–10919	61				14202–14262	61			
ND1[C]	10858–11835	978	325	ATT	TAA	10873–11847	975	324	ATG	TAA	5783–6730	948	315	ATG	TAA
tRNA-Leu (TTR)[C]	11839–11899	61				11851–11909	59				6731–6791	61			
tRNA-Leu (CTN)[C]	11902–11964	63				11914–11973	60				14262–14320	59			
16S-rRNA[C]	11965–13189	1225				11974–13185	1212				6792–7987	1196			
tRNA-Val[C]	13190–13254	65				13186–13243	58				7988–8048	61			
12S-rRNA[C]	13255–13949	695				13244–13929	686				8049–8747	699			
control region	13950–14291	342				13930–14271	342				8748–9057	310			
control region[D]											14321–14630	310			
tRNA-Ile	14292–14354	63				14272–14335	64				9058–9122	65			
tRNA-Gln[C]	14352–14417	66				14333–1	67				9123–9188	66			

*tRNA-Cys gene located on the minority strand (complement) in *C. capensis* and *O. moubata* but located on the majority strand in *H. flava*.
[C]Complement.
[D]Duplicated.

Table 2. Codons of protein-coding genes in the mitochondrial genomes of *Carios capensis* (*Cc*), *Ornithodoros moubata* (*Om*) and *Haemaphysalis flava* (*Hf*)

Codon	aa	*Cc*	*Om*	*Hf*	Codon	aa	*Cc*	*Om*	*Hf*	Codon	aa	*Cc*	*Om*	*Hf*	Codon	aa	*Cc*	*Om*	*Hf*
AAA	K	96	94	120	ACA	T	76	85	66	AGA	S	63	71	74	ATA	M	234	232	275
AAG	K	25	23	18	ACG	T	1	3	4	AGG	S	2	10	7	ATG	M	60	57	56
AAC	N	43	54	38	ACC	T	26	40	10	AGC	S	12	8	5	ATC	I	74	82	47
AAT	N	116	109	155	ACT	T	47	60	61	AGT	S	30	29	27	ATT	I	340	304	442
CAA	Q	54	54	43	CCA	P	48	58	58	CGA	R	30	32	29	CTA	L	73	70	50
CAG	Q	7	12	5	CCG	P	1	4	4	CGG	R	5	5	1	CTG	L	2	5	7
CAC	H	14	29	9	CCC	P	27	23	13	CGC	R	4	5	3	CTC	L	30	16	9
CAT	H	56	42	60	CCT	P	63	46	38	CGT	R	11	9	9	CTT	L	64	95	52
GAA	E	55	58	66	GCA	A	47	43	43	GGA	G	105	92	79	GTA	V	59	77	67
GAG	E	28	29	15	GCG	A	4	9	2	GGG	G	41	54	27	GTG	V	15	27	5
GAC	D	13	9	14	GCC	A	18	23	10	GGC	G	13	6	24	GTC	V	14	10	10
GAT	D	51	48	39	GCT	A	50	48	54	GGT	G	48	55	33	GTT	V	84	96	65
TAA	*	9	5	6	TCA	S	100	82	119	TGA	W	78	83	66	TTA	L	314	242	315
TAG	*	0	1	1	TCG	S	4	8	8	TGG	W	18	17	17	TTG	L	56	84	45
TAC	Y	32	32	24	TCC	S	46	23	26	TGC	C	6	5	9	TTC	F	62	62	63
TAT	Y	102	100	112	TCT	S	86	89	98	TGT	C	26	29	27	TTT	F	305	310	326
Total codons		3623	3622	3610															
A+T-rich		1489	1459	1676															
G+C-rich		515	512	427															
G+C-rich/A+T-rich		0.35	0.35	0.25															
A/T at the 3rd codon position		2920	2847	3047															

Abbreviations are 'aa' for amino acids; '*Cc*' for *Carios capensis*; '*Om*' for *Ornithodoros moubata*; '*Hf*' for *Haemaphysalis flava*.
*Stop codons.

ticks). The arrangements of mt genes in *C. capensis* and *O. moubata* show that the ancestral arrangement of the mt genes of arthropods has persisted in these soft ticks. Thus, the simplest explanation for the evolution of the mt genomes of ticks is that the translocation and/or inversion of the eight mt genes occurred only once in the most recent common ancestor of the metastriate ticks, whereas the ancestral arrangement of arthropods has remained unchanged for over 400 million years in the lineage leading to the soft ticks and one of the two lineages of hard ticks, the Prostriata (*Ixodes* spp.).

It is increasingly clear that in contrast to vertebrates, the arrangement of mt genes varies considerably among lineages of invertebrates. In the case of arthropods, rearrangements of mt genes have been found in the lineages leading to insects, crustaceans, myriapods and metastriate ticks (Chelicerata; Boore *et al.*, 1995; Shao *et al.*, 2001a,b; Lavrov *et al.*, 2002). Only the horseshoe crab, *L. polyphemus*, the prostriate tick, *I. hexagonus*, and the two soft ticks studied thus far have the arrangement of the hypothetical ancestor of the arthropods. Some lineages have extremely rearranged mt genomes (e.g. the lineages of the wallaby louse (Shao *et al.*, 2001a), the plague thrips (Shao & Barker, 2003) and the crustacean, *Tigriopus japonicus* (Machida *et al.*, 2002)); some lineages have mt genomes with only one or a few rearrangements (e.g. the lineages of the fruit fly, *Drosophila yakuba* (Clary & Wolstenholme, 1985), the water flea, *Daphnia pulex* (Crease, 1999)) whereas other lineages have mt genomes with no rearrangements (e.g. the soft ticks). Parasitism was thought to be a factor which might lead to rearranged mt genomes in some lineages (Dowton & Austin, 1999; Dowton & Campbell, 2001; Shao *et al.*, 2001b). However, this is not the case in the Diptera (Castro *et al.*, 2002) and does not appear to be the case for ticks either since soft ticks, prostriata ticks and metastriata ticks have similar parasitic lifestyles but have substantially different arrangements of genes in their mt genomes. Shao *et al.* (2003) proposed that an increased rate of mt nucleotide substitution might lead to an increased rate of mt gene rearrangement in insects. Whether this is the case in ticks and other chelicerates awaits further study.

Experimental procedures

Collection of ticks and extraction of DNA

Unfed adult *H. flava* were collected in Saitama, Japan by Dr K. Fujimoto, Saitama Medical School, and were stored in alcohol. These ticks were washed in 500 µl of saline-EDTA (0.15 M NaCl, 0.1 M EDTA, pH 8.0), air-dried and then used for DNA extraction. Laboratory-reared *O. moubata* were a gift from Dr Y. Chinzei, College of Medicine, Mie University. *C. capensis* were a gift from Dr M. Tsurumi, Yamashina Institute of Ornithology (Ushijima *et al.*, 2003). Genomic DNA was extracted by crushing individual ticks and then putting them in 200 µl of saline-EDTA containing 100 µg/ml proteinase K, and incubating for 30 min at 37 °C and for 10 min at 60 °C with 1% SDS. The aqueous phase was separated by centrifugation and was extracted twice using an equal volume of phenol (equilibrated with TE-buffer [10 mM Tris-HCl, 1 mM EDTA, pH 8.0], 2% 8-hydroxyquinoline). After centrifugation, the aqueous

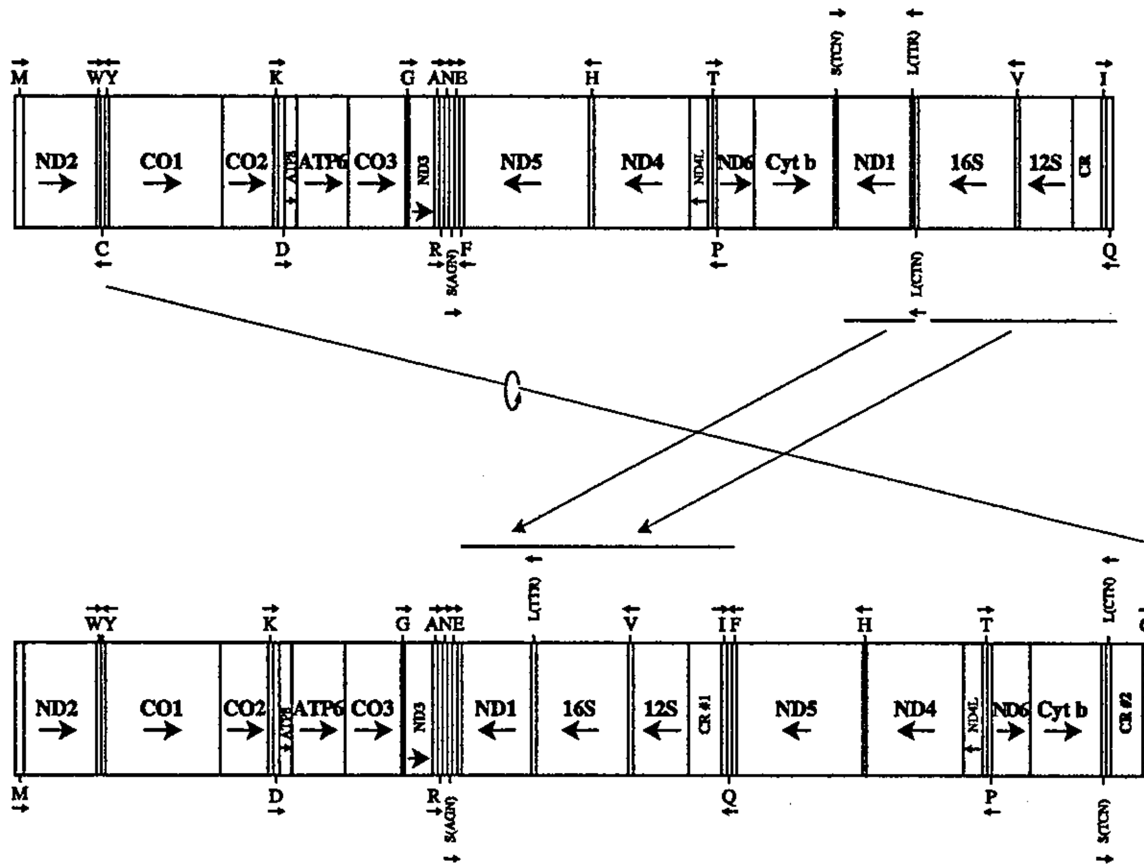

Figure 1. Arrangement of genes in the mitochondrial genomes of the soft ticks, *Carios capensis* and *Ornithodoros moubata*, and the hard tick, *Haemaphysalis flava*. Arrows under gene names show the direction of transcription. Long arrows link genes that have translocated in *H. flava*, relative to the arrangement of genes in the two soft ticks, which is identical to the inferred arrangement in the hypothetical ancestor of arthropods. The circular arrow indicates the inversion of the gene for tRNA-Cys in *H. flava*. Protein-coding genes and rRNA genes are represented by the abbreviations of the proteins or rRNAs they encode: CO1-3 for cytochrome oxidase subunits 1–3; Cyt b for cytochrome *b*; ND1 to ND6 for NADH dehydrogenase subunits 1–6; ND4L for NADH dehydrogenase subunit 4L; ATP6 and ATP8 for ATP synthase subunits 6 and 8; 16S and 12S for large and small ribosomal subunit RNAs. tRNA genes are labelled with single-letter amino acid abbreviations except for those encoding tRNAs leucine and serine which are labelled as L(CTN), L(TTR), S(AGN) and S(TCN). CR is the abbreviation for control region (the largest non-coding region).

phase was mixed with 2.5 volumes of 95% cold ethanol and the DNA was precipitated by centrifugation, and then dried and dissolved in 50 µl of TE-buffer containing 100 µg/ml ribonuclease. Five µl of this solution (approximately 10 ng of DNA) was used in a 50-µl PCR.

PCR amplification

The entire mt genomes of each of the three ticks were amplified in two overlapping fragments by PCR with the primers: (1) 16S-J, 5′-CCG GTC TGA ACT CAG ATC AAG TA-3′ with 12S-N, 5′-AAA CTA GGA TTA GAT ACC CTA TTA-3′ (1.6 kbp) and 12S-J, 5′-GGC GAT ATG TGC ATA TTC TAG AGC-3′ with 16S-N, 5′-GAC AAG AAG ACC CTA TGA AT-3′ (13.5 kbp) for *C. capensis* and *O. moubata*, (2) CO1-J, 5′-CCT GAT ATA GCT TTC CCT CG-3′ with 16S-N, 5′-CTG CTC AAT GAT TTT TAA ATT GCT GTG-3′ (5.9 kbp) and 16S-J, 5′-TTA CGC TGT TAT CCC TAG AGT ATT-3′ with CO1-N, 5′-GCT ATA TCA GGT GCT CCT-3′ (9.2 kbp) for *H. flava*. The names of the primers indicate the genes that the primers target and the strands that the primers locate. 'J' is for the majority strand whereas 'N' is for the minority strand. Each 50-µl PCR had 5 µl of template DNA, 200 µM of each deoxyribonucleotide triphosphate, 1 µM of each prime and 1.5 units of *Taq* polymerase (Takara Shuzo, Kyoto, Japan). Perkin-Elmer GeneAmp PCR System 9700 (PE Biosystems, Osaka, Japan) was used and the reaction conditions were: 30 cycles of 94 °C for 60 s, 48 °C for 30 s and 72 °C for 2–15 min (about 1 min/kbp).

Sequencing and sequence analysis

The short PCR fragment (1.6 kbp) was sequenced directly in both directions with custom synthesized primers. The long PCR fragments (5.9, 9.2 and 13.5 kbp, respectively; ≈2.5 µg DNA/250 µl of TE) were fragmented randomly by sonication (20 W, 30 s). DNA

fragments were then separated in an agarose gel and the fragments of 1.5–2.5 kbp size were excised from the gel. DNA purification, vector-ligation and transformations were as described in Tabuchi *et al.* (2002). Over 300 clones were sequenced from each long PCR fragment. Sequences were aligned with Auto Assembler Software (PE Biosystems). The sequences of long PCR fragments were then confirmed by direct sequencing with primers which were 500 bp apart. A Thermo Sequenase dye terminator cycle sequencing kit (Amersham Japan, Osaka, Japan) was used in the sequencing reactions. Nucleotide sequences were analysed with the DNASTAR program (DNASTAR Inc. Madison, Wisconsin). Protein-coding genes were identified by comparison with the nucleotide sequences and amino acid sequences of protein-coding genes of the mt genomes of the three known ticks, *Ixodes hexagonus*, *Boophilus microplus* and *Rhipicephalus sanguineus*. tRNA genes were identified from their putative secondary structures. rRNA genes were identified by sequence homology with these genes in the known ticks (see Black & Roehrdanz, 1998). The mtDNA sequences of the three ticks have been deposited in the DDBJ/GENBANK/EMBL data libraries; the DDBJ accession numbers are AB075955 (*C. capensis*), AB073679 (*O. moubata*) and AB075954 (*H. flava*).

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New World Relapsing Fever *Borrelia* Found in *Ornithodoros porcinus* Ticks in Central Tanzania

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Abstract: **Ticks were collected from 8 houses in Mvumi Mission village, near Dodoma, Tanzania. All ticks were examined for *Borrelia* infestation by flagellin gene-based nested polymerase chain reaction. All houses were highly infested with ticks, and all ticks collected were of the *Ornithodoros porcinus* species. Fifty-one out of 120 ticks were infected with spirochetes, and a flagellin gene sequence comparison showed that most of the spirochetes belonged to *Borrelia duttonii*, which is the causative agent of tick-borne relapsing fever in East Africa. The rest of the spirochetes were quite different from *B. duttonii* and instead resembled the New World tick-borne relapsing fever borreliae. Phylogenetic analysis using 16S ribosomal RNA gene sequences also supported the interpretation that the spirochete was a *Borrelia* species distinct from previously described members of the genus.**

Key words: **Phylogenetic analysis, *Ornithodoros porcinus*, Relapsing fever borreliae**

Tick-borne relapsing fever (TBRF) is found in Africa, Europe, western Asia, and North America (2). The disease is caused by several species of relapsing fever borreliae and is transmitted by various species of *Ornithodoros* and *Argas* ticks (15). Specific relationships often exist between *Borrelia* species and vector tick species, and certain *Borrelia* species are reported to be transmitted by a single tick species (18). *Ornithodoros moubata* complex ticks, such as *O. moubata* and *Ornithodoros porcinus*, are distributed in East Africa and are known to carry *Borrelia duttonii*, which is responsible for Old World TBRF (22). Ticks live in close proximity with humans, and the sole reservoir host for borreliae is thought to be humans. The disease is endemic in central Tanzania, where clinical and epidemiological studies have been conducted (20). TBRF causes a significant amount of morbidity and mortality in the Dodoma region, mainly in children under five and pregnant women (12). People in Dodoma Rural District live in traditional dwellings constructed of mud with earth-covered roofs, and house infestation with *O. moubata* complex ticks is very high (20). Residents sleep on the floor on animal skins, and it is probable that nocturnal tick bites are frequent and pass unnoticed because tick feeding takes only about 30 min.

Cutler et al. (3) have successfully cultivated *B. duttonii* in artificial BSKII medium from pediatric patients in Mvumi Hospital (which is 40 km southeast of the Tanzanian capital Dodoma), and five clinical isolates were characterized genetically. The sequences of 16S ribosomal RNA gene (*rrs*) and flagellin B gene (*flaB*) of *B. duttonii* were quite similar to those of *Borrelia recurrentis*, the causative agent of louse-borne relapsing fever, which is transmitted by the human louse, *Pediculus humanus* (4, 13). We have reported that *O. moubata* complex ticks collected from the houses in Dodoma Rural are highly infected with spirochetes, based on flagellin gene-based nested PCR (9). While most of the borrelia in ticks were *B. duttonii*, we have also found a different spirochete in *O. porcinus* ticks. In this study, we extended our work (8, 14) to elucidate the phylogenetic position of unidentified *Borrelia* found in *O. porcinus* ticks (9) and to screen spirochete infection rates in ticks collected from tick-infested houses in Mvumi village.

Materials and Methods

Ticks, collection sites, and tick species identification. Adult and nymphal ticks were collected from 8 houses

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Abbreviations: *flaB*, flagellin B gene; PCR, polymerase chain reaction; *rrs*, 16S ribosomal RNA gene; TBRF, tick-borne relapsing fever.

in Mvumi village (6° 34′60″ S, 37° 10′0″ E) in October and November 2002. Tick species were all *O. porcinus* with morphological characterization, and we also sequenced the mitochondrial *rrs* gene to confirm their species. Mitochondrial gene sequencing is as described previously (11). Ticks were all numbered based on the householder's initials (Table 1).

Culture. Ticks were washed in 3% hydrogen peroxide and dipped in 70% ethanol for 5 min. Ticks were then washed twice in distilled water and dissected individually in BSKII medium. Half the internal tissues were inoculated into 13 ml of BSKII medium within 15 ml culture tubes at 32 C and the rest were used for PCR examination. Cultures were examined for spirochetes with a dark-field microscope and passaged into fresh medium every week.

DNA preparation and PCR amplification. Individual ticks were frozen with liquid nitrogen, crushed manually, suspended in 50–200 µl of 0.02 N NaOH, and boiled 2 min as described previously (9). The aqueous phase was separated by centrifugation, and 1 µl of solution was used for PCR amplification. The primer sets were based on identical sequences in flagellin and 16S rRNA genes of *Borrelia* species (7). The positions listed refer to *B. duttonii flaB* and *rrs* genes: *Bfpad*, 5′-GAT CAA GCA CAA TAT AAC CAT ATG CA-3′ (376–401); *Bfpbu*, 5′-GCT GAA GAG CTT GGA ATG CAA CC-3′ (442–464); *Bfpcr*, 5′-TGA TCA GTT ATC ATT CTA ATA GCA-3′ (765–788); *Bfpdu*, 5′-AGA TTC AAG TCT GTT TTG GAA AGC-3′ (805–828); Bor16F, 5′-GCT GGC AGT GCG TCT TAA GCA TGC-3′ (26–49); Bor16SR, 5′-GTG ACG GGC GGT GTG TAC AAG-3′ (1373–1393). DNA was amplified using *Taq* polymerase (*Ex Taq*, Takara Bio, Japan) according to the manufacturer's protocol. The PCR was performed with the primer sets described above, each containing 50 µl of the reaction mixture, using 30 cycles (40 s at 94 C, 60 s at 50 C, 60 s at 72 C) with GeneAmp PCR system 9700 (Applied Biosystems Japan). If no PCR product was obtained at the first PCR, nested PCR was performed by taking 1 µl of the PCR product under the same conditions. A different set of conditions (40 s at 94 C, 30 s at 67 C, 90 s at 72 C, 35 cycles) was used for borrelial *rrs* amplification. Sequences of both *flaB* and *rrs* PCR products were determined directly in both directions by using custom-synthesized sequencing primers and a BigDye terminator cycle sequencing FS ready reaction kit (Applied Biosystems) as described previously (10, 19).

Sequence analysis. Nucleotide sequences were aligned using the CLUSTAL W software package (21), and neighbor-joining phylogenetic tree construction (17) and bootstrap analysis were done by the Kimura-2-parameter distance method (13) supplied on the web site (www.ddbj.nig.ac.jp). Pairwise alignments were performed with an open-gap penalty of 10, a gap extension penalty of 0.5, and a gap distance of 8. Multiple alignments were also done with the same values, and phylogenetic branches were supported by bootstrap analysis with 100 replications.

Accession numbers. The accession number of the mitochondrial genome sequence is AB105451 (*O. porcinus*), and the sequence determination procedures were the same as were described previously (1). The database accession numbers for the *flaB* reported in this paper are AB057546 (GC9), AB057548 (MK7), AB105128 (MK8), AB057547 (VS4), AB113313 (EM14), and AB113314 (MS3), and the *rrs* sequence accession number is AB113315 (VS4).

Results and Discussion

Borrelia *Infection Rates in Ticks*

Ticks used in this study were all nymphal and adult *O. porcinus* species. As shown in Table 1, each of the 8 houses was highly infested with *O. porcinus* ticks, and those ticks harbored spirochetes at levels from 19 to 86%, with an average of 43% (51 out of 120 ticks) as determined by PCR amplification of the targeted *flaB* gene of *Borrelia*. The sequences of the nested PCR products (341 nucleotides) were determined and aligned with the other flagellin gene sequences (6, 7, 16). Fifty-one *flaB* sequences obtained in this examination were classified into two major groups (Fig. 1). Forty-two of the sequences were *B. duttonii* and the remaining 9 were the new type *Borrelia*. Between the two groups there were 21 nucleotide substitutions, resulting in 7 amino acid substitutions, and 3 nucleotide deletions (data not shown).

Classification of Borrelia *Partial Gene Sequences*

The three sequences (VS4, EM14, and MS3) differed from each other by 1 nucleotide at each locus in the *flaB* gene. The deduced flagellin protein sequences were identical over 113 residues (Fig. 1). The sequences were branched deeply from the sequences of *B. duttonii*, *B. recurrentis*, *B. crocidurae*, and *B. hispanica*, all *Borrelia* species that cause Old World relapsing fever. The sequences of MK7, GC9, and MK8 were all included into the same cluster of Old World relapsing fever borreliae. The new type of borrelia found in *O. porcinus* ticks rather resembled *B. hermsii*, *B coriaceae*, *B. turicatae*, and *B. parkeri*, all of those borrelias distributed in North America. *B. miyamotoi* and *B. lonestari* are the species found in hard-bodied ixodid ticks exclusively, but they are closely related to relapsing

Table 1. Source of *Ornithodoros moubata* species complex ticks and detection of spirochetes in ticks by PCR and culturing of their internal tissues

Householder's initials	PCR		Culture	
	Borrelia positive	New type *Borrelia*	*Borrelia* positive	New type *Borrelia*
MK	12/18	1/18	1/27	0/27
DN	2/7	1/7	NT[a]	
EM	6/17	1/17 (EM14)	NT	
JA	4/21	0/21	NT	
MS	11/22	4/22 (MS3)	2/40	0/40
RC	3/5	1/5	NT	
GC	7/23	0/23	NT	
VS	6/7	1/7 (VS4)	NT	
Total	51/120 (42.5%)	9/120 (7.5%)	3/67 (4.5%)	0/67 (0%)

[a] NT, Not tested.

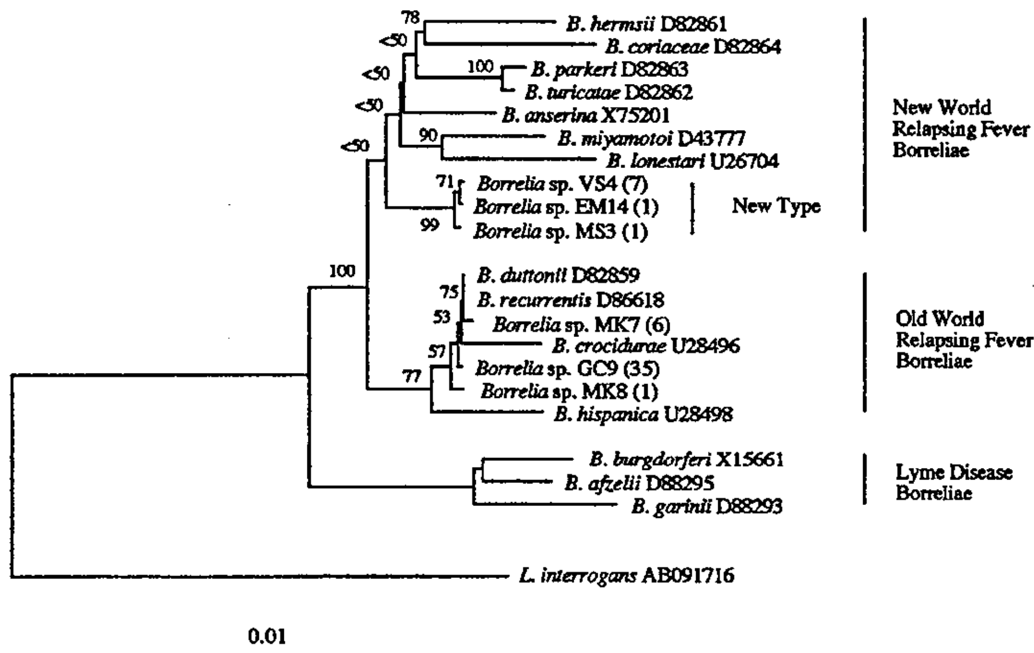

Fig. 1. Phylogenetic tree derived from the nucleotide sequences of the *flaB* gene of the new type of borrelia. The neighbor-joining tree was constructed using the sequences of partial flagellin genes. The bar represents the calculated distance value. Bootstrap values (100 duplications) are indicated to the left of each relevant branch. The numbers in parentheses indicate the number of tick-infected borrelia sequences determined. Accession numbers for the *flaB* shown in this tree are AB057546 (GC9), AB057548 (MK7), AB105128 (MK8), AB057547 (VS4), AB113313 (EM14), and AB113314 (MS3), and the other accession numbers of reference strains are all indicated in the figure. *Leptospira interrogans flaB* sequence (X17547) was used as the out group sequence.

fever borreliae (4, 6, 8). Most of the borrelias found in *O. porcinus* ticks are clustered into the *B. duttonii-B. recurrentis* clade.

The partial *rrs* sequence in VS4 was 1,368 nucleotides and there were 17 transitions between that of *B. duttonii* strain Ly. The *rrs* sequences in EM14 and MS3 were identical to that of the VS4. Further phylogenetic analysis was done by comparison of *rrs* sequences (Fig. 2). The *rrs* sequence found in tick (VS4) showed the following similarities to the other borrelial *rrs* sequences: *B. burgdorferi*, 95.7%; *B. duttonii*, *B. recurrentis*, *B. crocidurae*, *B. hispanica*, 98.5%; *B. anserina*, 98.2%; *B. hermsii*, 98.5%; *B. parkeri*, 98.7%; *B. turicatae*, 98.8%. The neighbor-

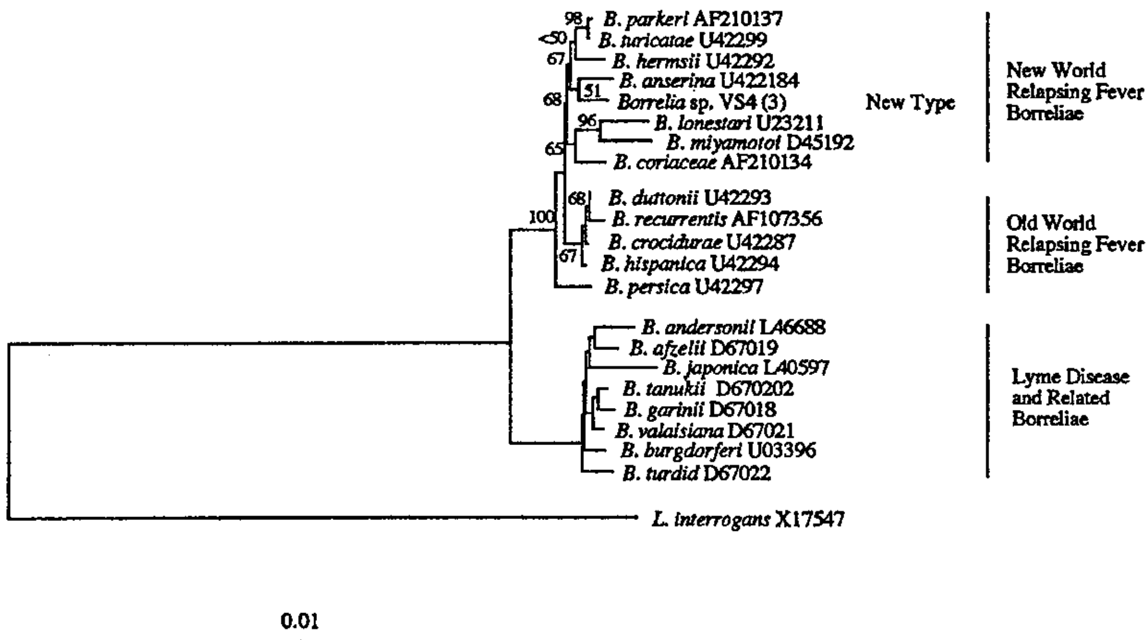

Fig. 2. Phylogenetic tree derived from the nucleotide sequences of the *rrs* gene of the new type of borrelia. The neighbor-joining tree was constructed using the sequences of partial 16S ribosomal RNA genes. The bar represents the calculated distance value. Bootstrap values (100 duplications) are indicated to the left of each relevant branch. The accession number for the tick *Borrelia rrs* sequence is AB113315 (VS4), and the other accession numbers are all shown in this figure. *Leptospira interrogans rrs* sequence (AB091716) was used as the out group sequence.

joining phylogenetic tree showed that the new type of borrelia found in *O. porcinus* ticks clustered with a group containing the New World relapsing fever borrelia species and a bird borreliosis agent. Bootstrap values in both trees supported the sequence being clustered with a non-Old World relapsing fever borreliae group and being most closely related to New World relapsing fever borreliae.

Borrelia *Culture*

Cutler et al. (3) successfully cultivated 5 TBRF borrelia strains from 12 blood samples of pediatric patients but these were all *B. duttonii*. To date, *B. duttonii* has only been cultivated from human blood specimens and not from ticks. Our recent investigation using the nested PCR for *flaB* gene for *Borrelia* detected the new type spirochetes in blood samples from both healthy children (5/11) and children with fever (1/6) in a village 12 km from Mvumi Mission (5, 14). This raises the possibility that the new *Borrelia* may be a causative agent for TBRF but successful culture will be necessary to confirm this.

To attempt *Borrelia* culture from *O. porcinus* ticks, two houses were chosen, and 67 ticks (27 ticks from the house MK and 40 ticks from the house MS) were used to cultivate the new type of *Borrelia* (Table 1). These ticks were estimated to be infected with new type of borrelia at 6% (1/18) and 18% (4/22), respectively, by PCR data (Table 1), and PCR examination of their internal tissues also showed 14/27 and 18/40 were *Borrelia* positive. However no new type spirochete grew in the BSKII media, and the three successful cultures from the ticks were all *B. duttonii*.

It seems likely that many of the spirochetes found in the ticks in this area are the new *Borrelia* species, not *B. duttonii*, and that *O. porcinus* acts as a competent vector for TBRF locally. Whether the new type *Borrelia* is an etiological agent for TBRF in central Tanzania remains to be determined. At least we must realize that *O. porcinus* ticks can transmit both *Borrelia* species in the Dodoma region and that one of these species resembles North American TBRF *Borrelia*. The evolution and adaptive radiation of borrelia is of interest.

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Evolution of Duplicate Control Regions in the Mitochondrial Genomes of Metazoa: A Case Study with Australasian *Ixodes* Ticks

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To investigate the evolution pattern and phylogenetic utility of duplicate control regions (CRs) in mitochondrial (mt) genomes, we sequenced the entire mt genomes of three *Ixodes* species and part of the mt genomes of another 11 species. All the species from the Australasian lineage have duplicate CRs, whereas the other species have one CR. Sequence analyses indicate that the two CRs of the Australasian *Ixodes* ticks have evolved in concert in each species. In addition to the Australasian *Ixodes* ticks, species from seven other lineages of metazoa also have mt genomes with duplicate CRs. Accumulated mtDNA sequence data from these metazoans and two recent experiments on replication of mt genomes in human cell lines with duplicate CRs allowed us to re-examine four intriguing questions about the presence of duplicate CRs in the mt genomes of metazoa: (1) Why do some mt genomes, but not others, have duplicate CRs? (2) How did mt genomes with duplicate CRs evolve? (3) How could the nucleotide sequences of duplicate CRs remain identical or very similar over evolutionary time? (4) Are duplicate CRs phylogenetic markers? It appears that mt genomes with duplicate CRs have a selective advantage in replication over mt genomes with one CR. Tandem duplication followed by deletion of genes is the most plausible mechanism for the generation of mt genomes with duplicate CRs. Once duplicate CRs occur in an mt genome, they tend to evolve in concert, probably by gene conversion. However, there are lineages where gene conversion may not always occur, and, thus, the two CRs may evolve independently in these lineages. Duplicate CRs have much potential as phylogenetic markers at low taxonomic levels, such as within genera, within families, or among families, but not at high taxonomic levels, such as among orders.

Introduction

The mitochondrial (mt) genomes of metazoa are typically circular, are about 15 to 20 kb long, and contain 37 genes and a large noncoding region (LNR [Boore 1999]). mt Genomes of metazoa are usually compact: there are no introns and few noncoding nucleotides, except in the LNR (Attardi 1985). For several metazoans, the LNR has been shown to contain elements that control transcription of mt genes and/or replication of mt genomes; this region is, therefore, commonly called the control region (CR). For other metazoans, the largest noncoding region in an mt genome is generally assumed to be the CR (Boore 1999).

CRs may contain the initiation sites of mt gene transcription. Two of the three transcription-initiation sites in the mt genome of *Homo sapiens* are in the CR (Taanman 1999). All of the transcription-initiation sites identified so far in other vertebrates are in the CR (Tracy and Stern 1995). Two of the five initiation sites in the mt genome of *Drosophila melanogaster* are in the CR (Berthier et al. 1986). The sea urchin, *Paracentrotus lividus*, is the only exception known so far: neither of its two known transcription-initiation sites is in the CR (Cantatore et al. 1990).

CRs may also contain the initiation sites for the replication of mt genomes. Two models have been proposed for the replication of mt genomes in mammals: the strand-displacement model (Clayton 1982) and the strand-coupled model (Holt, Lorimer, and Jacobs 2000). According to the strand-displacement model, replication of one strand (the leading strand) initiates at the CR, whereas replication of the other strand (the lagging strand) initiates at a site distant from the CR. According to the strand-coupled model, replications of both strands initiate at the CR. These two models agree that the replication of the leading strand initiates at the CR, although they disagree on the initiation sites of the replication of the lagging strand, and there is also debate over which model predominates in mammalian cells (Bogenhagen and Clayton 2003; Holt and Jacobs 2003). The replication mechanism of mammalian mt genomes is thought to be conserved in vertebrates (Shadel and Clayton 1997) but not conserved in invertebrates (Rubenstein, Brutlag, and Clayton 1977). However, it is known that replications of leading strands in the mt genomes of fruitflies also initiate at the CR (Goddard and Wolstenholme 1980).

The mt genomes of most metazoa studied to date have only one CR. However, the mt genomes of some snakes (Kumazawa et al. 1996), sea cucumbers (Arndt and Smith 1998), metastriate ticks (Black and Roehrdanz 1998; Campbell and Barker 1999), *Amazona* parrots (Eberhard, Wright, and Bermingham 2001), a fish (Lee et al. 2001), a thrips (Shao and Barker 2003), and a sea firefly (Ogoh and Ohmiya 2004) have duplicate CRs; that is, two separate CRs with identical or highly similar nucleotide (nt) sequences. The lineage of snakes has had duplicate CRs for over 70 Myr, whereas the lineage of metastriate ticks has had duplicate CRs for over 210 Myr (Kumazawa et al. 1996; Campbell and Barker 1999). Some humans with mt disorders also have mt genomes with duplicate CRs; these patients usually have a mixture of wild-type mt genomes (one CR), partially deleted mt genomes (one CR), and partially duplicated mt genomes (two CRs) in their clinically affected tissues (Schon, Bonilla, and DiMauro 1997).

The presence of duplicate CRs in the mt genomes of metazoa is an intriguing mutational phenomenon in light

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