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An Epidemiological Survey of Hemoplasma Infection in Iriomote Cats (*Prionailurus bengalensis iriomotensis*)

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ABSTRACT. An epidemiological survey of Iriomote cats (*Prionailurus bengalensis iriomotensis*) was conducted to understand the prevalence and molecular characteristics of hemotropic mycoplasma (hemoplasma). A series of ecological surveys of Iriomote cats were performed between November 2003 and September 2010. During this period, 31 Iriomote cats were captured or found, and 39 blood samples were collected. Polymerase chain reaction screening for hemoplasmas and BLAST searches revealed that 4 of the 31 cats were positive for hemoplasma infection (n=3, *Mycoplasma haemofelis* [Mhf]; n=1, '*Candidatus* M. turicensis' [CMt]). The 4 infected cats were captured or found in the northern area of the island of Iriomote. Phylogenetic analyses revealed close relationships between Mhf and CMt isolated from Iriomote cats compared with those from domestic cats and other wild felids. In our study, we identified two species of hemoplasma in Iriomote cats. The number and location of the hemoplasma-positive cats appeared to be limited; however, continuous surveillance of hemoplasma infection in Iriomote cats is necessary.

KEY WORDS: conservation, endangered species, hemotropic mycoplasma, Iriomote cat, wildcat.

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The Iriomote cat (*Prionailurus bengalensis iriomotensis*) is a Japanese wild felid that was discovered in 1965 [8]. This wildcat is an endemic subspecies that only inhabits Iriomote-jima in the Ryukyu Archipelago of Japan, and occupies the top of the ecological food chain on this island. The conservation of this felid is important not only in terms of national property, but also for the conservation of biodiversity. However, the Iriomote cat population is believed to be approximately 100, and is on the government's official endangered species list [9].

Several factors are threatening the continued existence of this cat on the island. One of the most important factors is the overlap between living spheres of Iriomote cats and humans. This situation gives rise to cats being killed by traffic. Furthermore, their exposure to infectious agents may also be increased because of the introduction of animals, especially companion dogs and cats, to the island with the growth of the human population. A case of interspecies transmission of feline immunodeficiency virus (FIV) was reported in another Japanese wildcat, the Tsushima leopard cat (*Prionailurus bengalensis euptilurus*) [16]. This virus is a common infectious agent in domestic cats. Phylogenic analysis revealed that the FIV isolate from the Tsushima leopard cat was closely related to those from domestic cats living in the vicinity. Therefore, it is essential for the conservation of Iriomote cats to monitor infectious diseases, even though keeping domestic cats in Iriomote-jima is currently controlled by law.

Hemoplasma (hemotropic mycoplasma) is a Gram-negative bacterium without a cell wall that infects the cell surface of erythrocytes [22]. The infected host occasionally develops hemolytic anemia, thrombocytopenia, pyrexia, and jaundice [22]. Two strains (large and small, or Ohio and California) of the rickettsia *Haemobartonella felis* are considered the etiological agents of feline hemobartonellosis [5, 6, 15, 16]. However, a recent study concerning taxonomic classification showed that *H. felis* is more closely related, genetically, to mycoplasma. These results are based on the sequence of the 16S rRNA gene [6]. Additionally, the two strains mentioned above have been reclassified as *Mycoplasma haemofelis* (Mhf) and '*Candidatus* M. haemominutum' (CMhm), respectively [5, 6, 15]. In 2004, a third feline hemoplasma was isolated in Switzerland and designated '*Candidatus* M. turicensis' (CMt) [29]. Currently, these three hemoplasma species are recognized as important pathogens in the field of feline practice. It has been suggested that Mhf is the most virulent species in domestic cats [6, 27].

Infection with hemoplasmas, including the three species mentioned above, has been reported in many wild felids,

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including Iberian lynxes (*Lynx pardinus*), Eurasian lynxes (*Lynx lynx*), European wildcats (*Felis silvestris silvestris*), African lions (*Panthera leo*), and Brazilian wild felids [30]. Therefore, the Iriomote cat is also likely to be susceptible to such hemoplasmas, with those pathogens likely to be virulent in this endangered cat. To understand the prevalence and molecular characteristics of hemoplasmas in Iriomote cats, we conducted an epidemiological survey as a part of on-going conservation activities.

MATERIALS AND METHODS

Examined Iriomote cats and collection of blood samples: A series of ecological surveys were performed between November 2003 and September 2010. During this period, 20 Iriomote cats were captured. Cat ID E-60 had been captured three times before suffering from severe emaciation and death. E-92J was found in the field in a severely emaciated condition and was rescued by the Iriomote Wildlife Conservation Center (IWCC), but died. The cause(s) of death remain unknown for E-60 and E-92J. E-84 and D-043J were found deceased, with cause of death also unknown. Further seven cats were found in moribund conditions or deceased after being run over by cars. The profiles of these cats, including gender, dates and location of capture, and rescue or salvation are shown in Table 1. From these 31 cats, 39 blood samples were collected and stored at −80°C until required.

Extraction of total DNA and detection of hemoplasma derived DNA by polymerase chain reaction (PCR) screening: Total DNA was extracted from 200 μl blood samples using a QIAamp DNA Blood Mini Kit (QIAGEN, Hilden, Germany) with a final elution volume of 200 μl. Extracted DNA was then used as a template to amplify the DNA from hemotropic mycoplasmas and as an internal control for PCR analyses.

Because hemoplasma infection in Iriomote cats has never been examined, we did not know whether it was sufficient to only analyze the three species of hemoplasmas (Mhf, CMhm and CMt) that infect domestic cats. For this reason, we used the universal primer set reported by Willi *et al.* for analysis, which enabled us to amplify the 16S rRNA gene from a broad range of hemoplasma species [31]. One forward primer (MY-F, 5'-AGC AAT RCC ATG TGA ACG ATG AA-3', nt 325–347, GenBank/EBML/DDBJ accession number DQ157160) and two reverse primers (MY-R1, 5'-TGG CAC ATA GTT TGC TGT CAC TT-3', nt 428–406, DQ157160; MY-R2, 5'-GCT GGC ACA TAG TTA GCT GTC ACT-3', nt 459–436, DQ157149) were used [31]. Reaction mixtures (50 μl) contained three primers (0.5 μM each), 2 μl of template DNA, *Taq* DNA polymerase (0.25 units), and reagents recommended by the manufacturer (Takara, Kyoto, Japan). The thermal cycling profile for the PCR involved 40 cycles of denaturation (94°C, 15 sec), annealing (60°C, 1 min), and polymerization (72°C, 1 min). The sensitivity of this PCR was determined using the method reported by Tanahara *et al.* [23]. In this analysis, three plasmid templates containing three feline hemoplasmas, Mhf, CMhm, and CMt, were used as representative hemoplasma species.

The PCR amplicons were electrophoresed on 2.5% (w/v) agarose gels and stained with ethidium bromide. If DNA bands of the expected size were obtained, those samples were then subjected to DNA sequencing analysis.

As an internal control, the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene was also amplified in each sample using the primers cGS (5'-CTC ATG ACC ACA GTC CAT GC-3', nt 514–533 in AB038240) and cGR (5'-TGA GCT TGA CAA AGT GG TCA-3', nt 925–906) [25]. The thermal cycling profile for the PCR involved 25 cycles of denaturation (94°C, 1 min), annealing (60°C, 1 min), and polymerization (72°C, 3 min). Only the samples in which the GAPDH gene was successfully detected were included in this study.

DNA sequencing and BLAST search: If the expected DNA fragments were obtained in the PCR screening, those PCR products were ligated into a pCR2.1 plasmid vector (Invitrogen, Carlsbad, CA, U.S.A.), and the nucleotide sequence of the inserted DNA fragments determined by the dideoxy chain termination method (ABI Prism BigDye Primer Cycle Sequencing Ready Reaction Kit, Applied Biosystems, Foster City, CA, U.S.A.) using primers constructed from the M13 region (Forward, 5'-GTA AAA CGA CGG CCA G-3', nt 404–389; Reverse, 5'-CAG GAA ACA GCT ATG AC-3', nt 205–221). Three clones from each sample were subjected to DNA sequencing. Obtained nucleotide sequence data were first subject to BLAST analysis in the DNA data bank of Japan (DDBJ, http://www.ddbj.nig.ac.jp/Welcome-j.html) to find similar sequences and to check whether the sequence was correctly derived from hemoplasma.

Phylogenic analysis: Hemoplasma-positive samples obtained from the PCR screening were then analyzed using a nested PCR that amplified the longer length of the 16S rRNA gene derived from hemoplasmas. This was carried out based on the methods reported by Dieckmann *et al.* and Willi *et al.* [3, 28]. In the first-round PCR, forward primer 5'-TCG AAC GGA YYT TGG TTT CG-3' and reverse primer 5'-CAA ATG AAT GTA TTT TTA AAT GCC CAC-3' were used [28]. Reaction mixtures (25 μl) contained primers (0.5 μM each), 2 μl of template DNA, *Taq* DNA polymerase (0.125 units), and the reagents recommended by the manufacturer (Takara). The thermal cycling profile involved 30 cycles of denaturation (98°C, 10 sec), annealing (54°C, 1 min), and extension (72°C, 1 min) [28]. In the second-round PCR, forward primer 5'-GGC CCA TAT TCC TRC GGG AAG-3' and reverse primer 5'-ACR GGA TTA CTA GTG ATT CCA-3' were used [3]. The reaction mixture contents were the same as for the first-round PCR except for the addition of 2 μl of first-round PCR product as the template. The second PCR reaction involved 30 cycles of denaturation (94°C, 30 sec), annealing (52°C, 30 sec), and extension (72°C, 1 min) [3]. If DNA bands of the expected size were detected, those samples were then subjected to the DNA sequencing analysis, as mentioned above. Based on the obtained DNA sequencing data of fragments approximately 930-bp from the 16S rRNA gene, phylogenetic analysis was then performed to determine the genetic relationship among hemoplasmas using the neighbor-joining method in the DNADIST pro-

Table 1. Profile of 31 Iriomote cats evaluated in this study

Cat ID	Gender	Date	Location[1)]	Alive/Dead	Sample ID
E-30	Male	11.23.03	E13	Alive	39
E-33	Male	11.26.04	E4	Alive	1
E-60	Male	11.27.04	E11	Alive	2
		11.04.05	E7	Alive	3
		02.04.07	E8	Alive	15
		02.08.10	E4	Dying>Dead	30
E-67	Male	11.04.05	E2	Alive	4
		12.17.06	E3	Alive	36
		01.27.08	E6	Alive	19
E-70	Male	02.06.06	E14	Alive	7
E-72	Male	02.07.06	E9	Alive	8
E-83	Female	11.17.08	E14	Alive	21
E-84	Male	12.03.08	E14	Dead	22
E-91	Male	02.14.10	E1	Runover>Dying	31
		02.17.10	E4	Dead	32
W-48	Male	02.07.06	E4	Alive	9
		10.20.06	E4	Alive	35
E-71	Male	01.28.06	E5	Alive	6
W-87	Female	02.07.07	E4	Alive	37
W-99	Male	01.27.08	N9	Runover death	20
W-101	Male	02.04.07	N2	Alive	16
W-106	Male	12.11.05	N4	Alive	5
		02.04.07	N5	Alive	17
W-108	Male	02.04.07	W2	Alive	18
W-113	Male	11.18.06	N1	Alive	12
W-118	Male	10.22.06	N7	Alive	10
W-119J	Female	11.14.06	N5	Runover death	11
W-120	Male	11.18.06	W2	Alive	13
W-121	Male	11.18.06	W2	Alive	14
W-126	Male	01.09.10	N7	Alive	26
W-127	Female	01.10.10	N8	Alive	29
W-129	Male	01.09.10	N8	Alive	27
W-131J	Male	07.22.09	N3	Runover death	23
W-134	Female	01.09.10	N9	Alive	28
W-135	Female	04.13.10	N6	Runover death	33
W-137J	Female	09.09.10	N8	Runover death	38
D-043J	Male	12.26.09	W1	Dead	24
E-89J	Male	01.03.10	E10	Runover death	25
E-92J	Male	05.07.10	E12	Dying>Dead	34

1) Details of locations are shown in Fig. 2.

gram from the PHYLIP software package [4].

RESULTS

Detection of hemoplasma-derived DNA fragments and sequencing analysis: As shown in Fig. 1A, the GAPDH gene was detected in 39 blood samples derived from 31 Iriomote cats. All samples were used for the molecular epidemiological survey. In addition, the detection limits of the PCR screening method used in this study for Mhf, CMhm, or CMt were one copy in one reaction mixture (data not shown). Therefore, this PCR method could be satisfactorily applied to our epidemiological survey.

From the PCR screening, 23/39 samples (ID 1–15, 18–20, 26, 29, 33, 35 and 36) were positive (Fig. 1B). However, 18 samples (ID 1–10, 12–15, 18, 19, 33, and 35) showed faint bands following electrophoresis. We attempted to clone the 23 PCR-amplified DNA fragments and determine their nucleotide sequences. BLAST analysis showed that 6/23 DNA fragments (ID 11, 20, 26, 29, 33 and 36) were derived from bacteria. The origin of the remaining samples could not be determined. The nucleotide sequences for 3/6 samples (ID 11, 26 and 33) showed high similarities with 16S rRNA genes from Mhf. In addition, the sequence of sample ID 20 revealed homology with that of CMt. The remaining two samples (ID 29 and 36) seemed to be derived from '*Candidatus* Phytoplasma mali' and *Synechococcus* sp., respectively. Therefore, it was shown that the hemoplasma infection rate

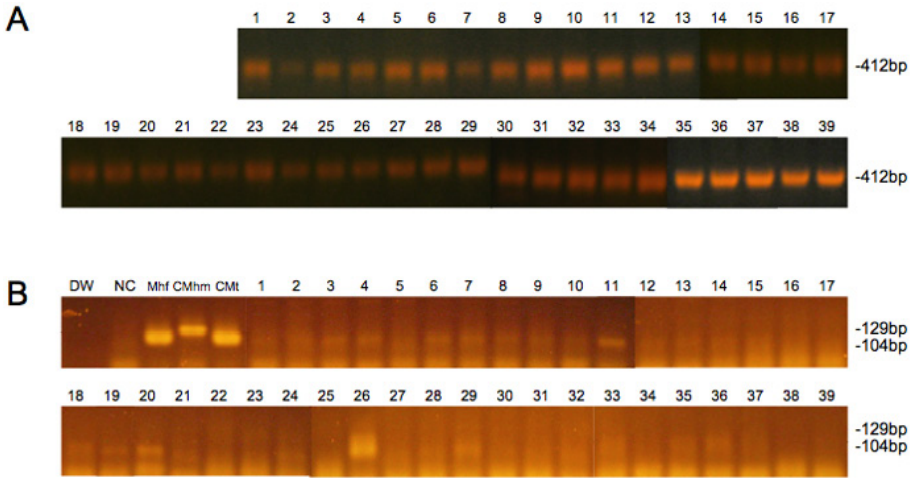

Fig. 1. PCR amplification of hemoplasma derived-DNA. (A) Agarose gel electrophoresis of PCR-amplified GAPDH from blood samples of Iriomote cats. (B) Agarose gel electrophoresis of PCR-amplified hemoplasma-derived DNA. Numbers on each lane represent sample IDs. DW, distilled water; NC, negative control (uninfected genomic DNA from domestic cat); Mhf, *M.haemofelis*-derived 16S rRNA gene (positive control); CMhm, '*C.* M. haemominatum'-derived 16S rRNA gene (positive control); CMt, '*C.* M. turicensis'-derived 16S rRNA gene (positive control).

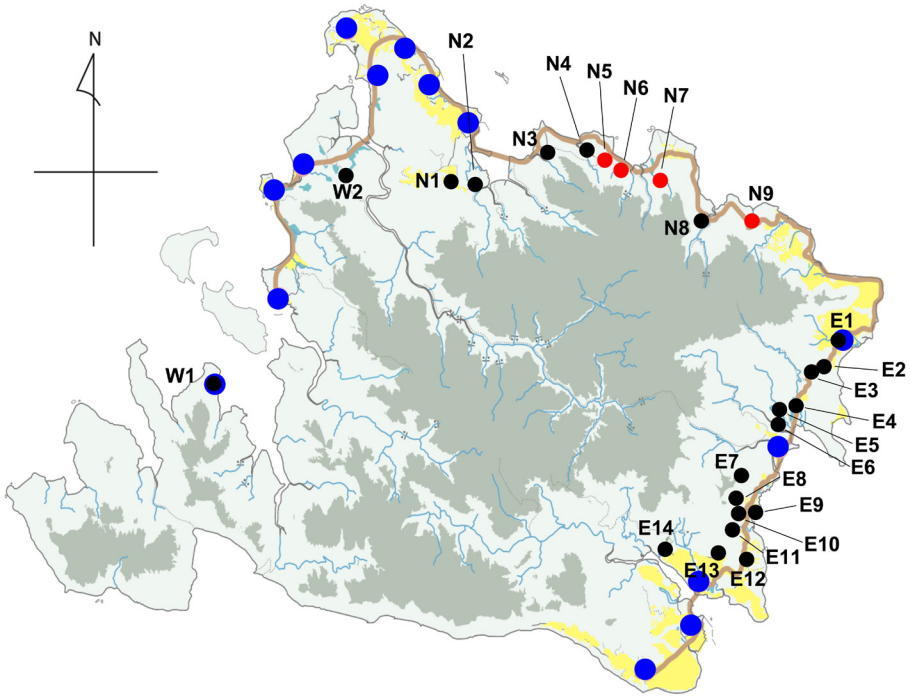

Fig. 2. Map of Iriomote-jima and location of the capture/rescue points for the Iriomote cats investigated in this study. Black and red dots indicate the capture and rescue points, respectively, of Iriomote cats. Red dots represent the capture/rescue point of hemoplasma-positive Iriomote cats. Blue dots show locations of human habitation. Croplands are shown in yellow.

was 12.9% (4/31 cases) in the present study.

Location of hemoplasma-positive Iriomote cats and phylogenic analysis: Figure 2 shows the capture or rescue points of the cats examined in this study. The hemoplasma-positive Iriomote cats seemed to be localized to the northern district of the island.

A phylogenic analysis of hemoplasmas was conducted based on the obtained sequence data, and relationships

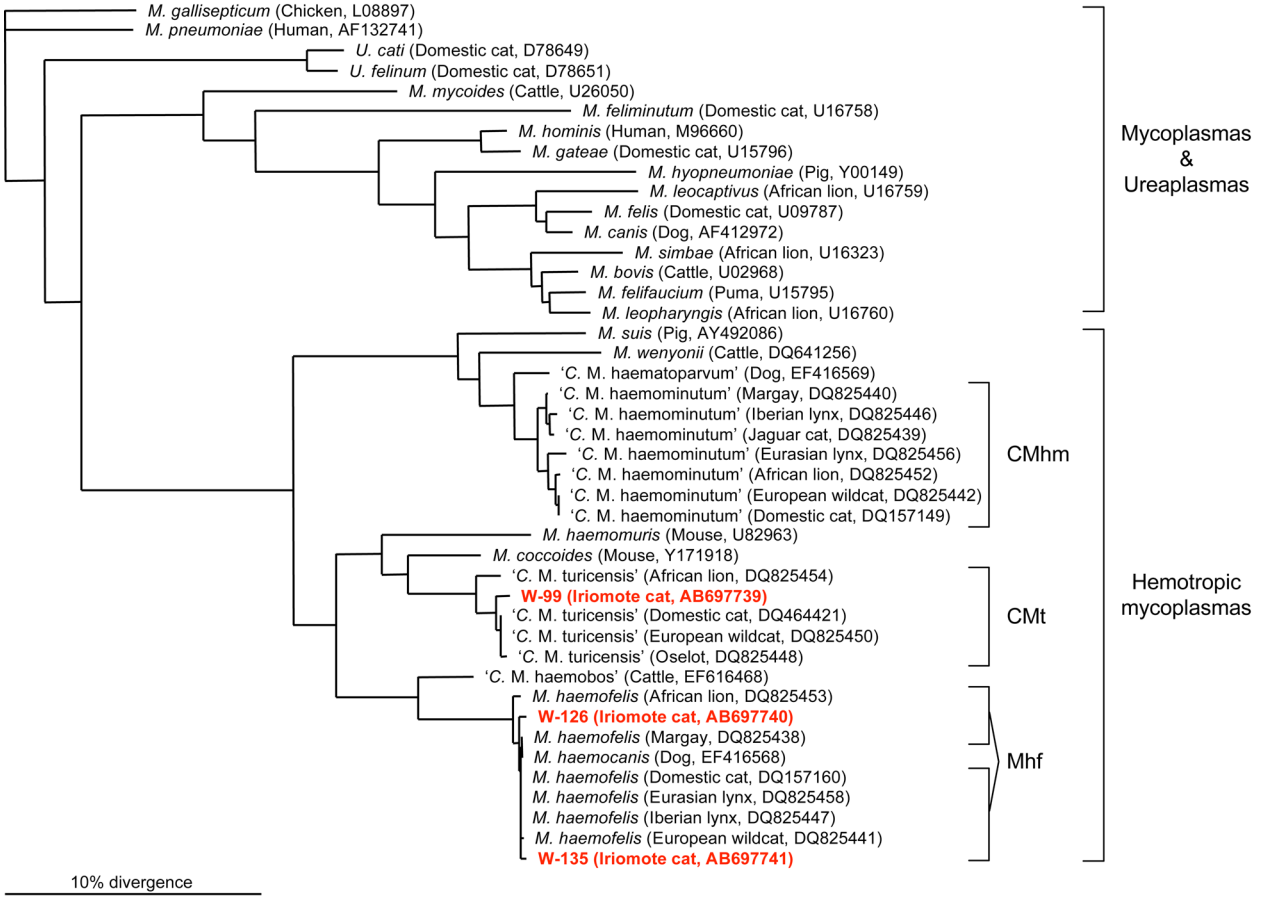

Fig. 3. Phylogenetic analysis of hemoplasmas and related mycoplasmas. Partial DNA sequences of approximately 930-bp 16S rRNA genes were applied in this analysis. The representative clones from Iriomote cats are shown as cat ID in red (GenBank/EBML/DDBJ accession number, AB697739-AB697741). Hemoplasmas compared were: domestic cat Mhf (DQ157160) [28]; African lion Mhf (DQ825453) [30]; Iberian lynx Mhf (DQ825447) [30]; Eurasian lynx Mhf (DQ825458) [30]; Margay Mhf (DQ825438); European wildcat Mhf (DQ825441); domestic cat CMhm (DQ157149) [28]; Iberian lynx CMhm (DQ825446) [30]; Jaguar cat CMhm (DQ825439) [30]; Margay CMhm (DQ825440) [30]; Eurasian lynx CMhm (DQ825456) [30]; African lion CMhm (DQ825452) [30]; European wildcat (DQ825442) [30]; domestic cat CMt (DQ464421) [32]; African lion CMt (DQ825454) [30]; Oselot CMt (DQ825448); and European wildcat CMt (DQ825450); '*C.* M. haematoparvum' (Dog, EF416569) [26]; *M. haemocanis* (Dog, EF416568); *M. coccoides* (Mouse, AY171918) [14]; *M. haemomuris* (Mouse, U82963) [19]; '*C.* M. haemobos' (Cattle, EF616468) [12]; *M. wenyonii* (Cattle, DQ641256) [11]; and *M. suis* (Pig, AY492086). Mycoplasmas and ureaplasmas examined were: *M. feliminutum* (Domestic cat, U16758) [2]; *M. felis* (domestic cat, U09787) [2]; *M. gatae* (domestic cat, U15796) [2]; *M. leocaptivus* (African lion, U16759) [2]; *M. leopharyngis* (African lion, U16760) [2]; *M. simbae* (African lion, U16323) [2]; *M. felifaucium* (Puma, U15795) [2]; *M. canis* (Dog, AF412972); *M. hominis* (Human, M96660) [1]; *M. pneumonia* (Human, AF132741); *M. bovis* (Cattle, U02968) [10]; *M. mycoides* (Cattle, U26050) [18]; *M. hyopneumoniae* (Pig, Y00149) [24]; *M. gallisepticum* (Chicken, L08897) [21]; *U. cati* (domestic cat, D78649) [7]; and *U. felinum* (domestic cat, D78651) [7].

analyzed by comparing with sequences of other known mycoplasmas. Longer DNA fragments were successfully amplified from only three (ID 20, W-99; 26, W-126; 33, W-135) of four positive samples. The phylogenetic analysis revealed that samples ID 26 (W-126) and ID 33 (W-135) could be classified as Mhf, and ID 20 (W-99) was categorized into CMt (Fig. 3). Similar results were observed with the BLAST analysis. These clones were very closely related to Mhfs and CMts isolated from domestic cats and other wild felids. The divergence among hemoplasmas was too small to identify or to assume the origin of hemoplasmas in Iriomote cats.

DISCUSSION

A limited number of previous papers describe infectious diseases in Iriomote cats, especially viral and protozoan diseases [13, 20]. Fortunately, no Iriomote cats have been identified to be infected with highly pathogenic viruses such as feline parvovirus, feline herpesvirus-1, FIV or feline leukemia virus. These occasionally induce severe and lethal diseases in domestic cats. Additionally, hepatozoon-infected Iriomote cats seem to rarely display clinical signs of disease [20]. However, infections with several feline pathogens, including feline calicivirus, feline coronavirus and feline

foamy virus, have been identified in Iriomote cats [13]. Therefore, it is very important for the conservation of this endangered species to survey and prevent the introduction of novel and/or possibly highly pathogenic infectious agents.

In this study, we focused on hemoplasma infection and analyzed its epidemiology in Iriomote cats. Our analysis showed that 4/31 cases (12.9%) were PCR-positive for Mhf or CMt. This infection rate is lower than that of domestic cats (26.3%) on mainland Japan [23]. One of the risk factors for hemoplasma infection in domestic cats is direct blood-to-blood transmission from a fight wound. One of the reasons the infection rate in Iriomote cats is lower might be related to the different frequency of contact opportunities between domestic and Iriomote cats. We expect that the frequency of contact is lower among Iriomote cats than domestic cats because of their smaller number. A previous study has shown that the hemoplasma infection rate among African lions that form a pride was higher than that of solitary wild felids in Europe and South America [30].

We found that the prevalence of *Hemoplasma* species in Iriomote cats was different from that in domestic cats and other wild felids. The majority of previous studies revealed that the most common species of hemoplasma in felids was CMhm followed by Mhf and CMt [17, 23, 30]. However, we were unable to identify CMhm infection in Iriomote cats. It is unlikely that Iriomote cats do not have a susceptibility to CMhm, based on the findings in other wild felids [30]. An alternative possibility is that CMhm has not been introduced or is not prevalent on Iriomote-jima. Surveillance of domestic cats or other animals on Iriomote-jima for hemoplasma infections may help clarify their origin in these particular animals. In addition, we did not detect co-infection of hemoplasma species in this study. This is another different feature of hemoplasma infection in Iriomote cats compared with domestic cats. Our previous molecular epidemiological study in domestic cats showed that 5.5% of cats had been infected with two or three species of feline hemoplasmas [23]. A co-infection of hemoplasma species might have not been detected because of the limited number of examined cases. A co-infection may be identified in a future study, if a large number of Iriomote cats are included. However, the frequency of co-infections in Iriomote cats would likely be lower than that in domestic cats, because this study showed that the prevalence of hemoplasma infection itself was lower in Iriomote cats, even though it was a single-species infection.

The findings presented in this study are related to the locations of the hemoplasma-positive Iriomote cats. Hemoplasma-positive cats were captured or found at points N5 (W-119J), N6 (W-135), N7 (W-126) and N9 (W-99), suggesting a limited area of habitation that was non-residential. However, if the source of infection was domestic cats, it does not follow that there is a cluster of infected Iriomote cats in this northern area, because it is not inhabited by humans. Therefore, the number of domestic cats is expected to be much lower than in other areas. Although domestic cats have never accidentally been captured, at least during our ecological surveys [9], it might also be necessary to investigate the activities of domestic cats.

Hemoplasmas found in Iriomote cats, especially Mhf, might be a threat to their conservation based on findings in domestic cats [5, 17]. Among three species of feline hemoplasmas, Mhf is believed to be the most virulent species. A single infection of CMhm or CMt itself rarely induces disease, and underlying diseases and/or Mhf infection is required for the development of acute disease onset by CMhm or CMt infection [5, 17]. It is notable that three of four hemoplasma-positive Iriomote cats possessed Mhf. Hematological data were available only for cat W-126; however, no obvious clinical symptoms were observed at the time of capture. Although the direct cause of death in the other two Iriomote cats (W-119J and W-135) was trauma, the presence of acute hemoplasmosis cannot be excluded. There is further scope to discuss pathogenicity; however, it is plausible that hemoplasma does not establish the acute onset of hemoplasmosis immediately after infection.

In the present study, we identified two species of hemoplasma infecting Iriomote cats. The number and location of hemoplasma-positive cats were limited; however, continuous surveillance of hemoplasma infection in Iriomote and domestic cats is necessary for the conservation of this endangered species.

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