Phylogenetic relationship of the southern Japan lineages of the sika deer \textit{(Cervus nippon)} in Shikoku and Kyushu Islands, Japan

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Abstract. Samples of the sika deer \textit{(Cervus nippon)} were collected from Kyushu and Shikoku Islands of Japan and surrounding areas, and their nucleotide sequences were analyzed. Sequences of the whole control region of the mitochondrial DNA were determined and phylogenetic trees were constructed using the neighbor-joining method and the maximum likelihood method. We also investigated gene genealogies for the sequences using the statistical parsimony network approach. Phylogenetic trees showed that only the Yakushima/Tanegashima populations were genetically distant from other populations. The statistical parsimony network, however, indicated a close relationship of the Miyazaki populations to some of the Shikoku populations. It was suggested that Shikoku Island played an important role in the divergence from the southern Japan lineage of \textit{C. nippon}.

Key words: \textit{Cervus nippon}, Kyushu, mitochondrial control region, phylogeny, Shikoku.

The sika deer \textit{(Cervus nippon)} in the Japanese Archipelago can be divided into two distinct lineages: the northern Japan group and the southern Japan group according to the analyses of mitochondrial DNA (Nagata et al. 1995, 1999; Tamate and Tsuchiya 1995; Tamate et al. 1998; Yamada et al. 2006). This species is considered to have diverged into the two groups in the Chinese Continent about 0.3 to 0.5 million years ago (Nagata et al. 1999).

The northern Japan group is distributed in Hokkaido Island \textit{(C. n. yesoensis)} and most parts of Honshu Island \textit{(C. n. centralis)} except for western Honshu (the Chugoku district). The southern Japan group is distributed in Yamaguchi of western Honshu \textit{(C. n. centralis)}, Kyushu and Shikoku Islands \textit{(C. n. nippon)} and surrounding smaller islands \textit{(C. n. centralis, C. n. nippon, C. n. yakushimae, C. n. mageshimae)}, and Ryukyu Islands \textit{(C. n. keramae)}. In eastern Shikoku Island (Tokushima and Kochi) both the northern and the southern groups inhabit and the two groups are intermingled in some areas (Yamada et al. 2006).

\textit{C. n. nippon} is distributed in Kyushu, Shikoku and Goto Islands (Ohtaiishi 1986; Whitehead 1993). There are major populations in the eastern (Tokushima and Kochi) and southwestern (Ehime and Kochi) parts of Shikoku Island, and minor populations inhabit the central (Kochi) and northwestern (Ehime) areas. Its distribution in Kyushu Island ranges widely from the north (Fukuoka) to the south (Kagoshima).

Phylogenetic information on the control region of \textit{C. n. nippon} on Kyushu and Shikoku Islands is limited to a few samples from Miyazaki (Kyushu Island) collected by Nagata et al. (1999) and from the eastern populations on Shikoku Island collected by Yamada et al. (2006). The phylogenetic relationship of the populations in the whole range of \textit{C. n. nippon} has not been investigated.

As for the distinct lineages of the sika deer, although haplotypes of the northern Japan group (northern Japan

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haplotypes) were found in eastern Shikoku, their distribution in other parts of the range of *C. n. nippon*, such as western Shikoku and the whole Kyushu Island, has not been elucidated. In the control region of mitochondrial DNA of the sika deer there is a tandemly repeated domain, with similar sequence pattern of 37–40 bp. The southern Japan lineage has 4 or 5 repeated units and the northern Japan lineage has 6 to 9 units (Nagata et al. 1999; Yamada et al. 2006). In the present study the number of tandem repeats was determined from samples collected widely from the *C. n. nippon* range in order to check the presence of the northern Japan lineage in its range. Then the phylogenetic relationship among the haplotypes of the southern Japan group (southern Japan haplotypes) including other neighboring subspecies was investigated.

Materials and methods

Samples of muscle tissues from sika deer which were killed either as a game or a pest were collected from 1995 to 2002 from western Shikoku Island, Kyushu Island, the small islands surrounding Kyushu Island and Yamaguchi (the westernmost part of Honshu Island). We collected samples from 95 individual sika deer from localities noted in Table 1. As for Yamaguchi Prefecture we used the same samples as those used in Nagata et al. (1999). As an outgroup we used data obtained from two individual Taiwan sika deer (*C. n. taiouanus*) kept at the Tokuyama Zoo (Shunan City, Yamaguchi, Japan). Sampling localities are shown in Table 1 and Fig. 1. Samples were cut to about 4–6 g pieces and they were stored in a 70% ethanol solution.

### Table 1. Sampling localities and haplotypes found in Kyushu and Shikoku Islands and surrounding areas

<table>
<thead>
<tr>
<th>Subspecies</th>
<th>Region</th>
<th>Number of repeat units</th>
<th>Haplotype</th>
<th>Prefecture</th>
<th>Locality</th>
<th>N</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>C. n. nippon</em></td>
<td>Shikoku Island</td>
<td>4</td>
<td>4Wsk1</td>
<td>Ehime [1]</td>
<td>Uwajima City and Matsuno Town</td>
<td>12</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>4Wsk2</td>
<td>Ehime [2]</td>
<td>Sajyo City</td>
<td>7</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>4Wsk3</td>
<td>Ehime [1]</td>
<td>Uwajima City</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>4Wsk4</td>
<td>Ehime [2]</td>
<td>Sajyo City</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>4Wsk5</td>
<td>Ehime [1]</td>
<td>Uwajima City</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>4Wsk6</td>
<td>Ehime [1]</td>
<td>Matsuno Town</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>4Wsk7</td>
<td>Ehime [1]</td>
<td>Uwajima City</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Kochi [3]</td>
<td>Umaji Village</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>4Esk3*</td>
<td>Tokushima [3]</td>
<td>Naka Town</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>4Esk4*</td>
<td>Tokushima [3]</td>
<td>Naka Town</td>
<td>1</td>
</tr>
<tr>
<td>Kyushu Island</td>
<td></td>
<td>4</td>
<td>4Fko1</td>
<td>Fukuoka [5]</td>
<td>Buzen City, Amagi City, Asakura City and Toho Village</td>
<td>32</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>5Myz1</td>
<td>Miyazaki [6]</td>
<td>Kirishimayama Mountain area</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>5Myz2</td>
<td>Miyazaki [7]</td>
<td>Suito City and Nishimera Village</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>5Myz3</td>
<td>Miyazaki [7]</td>
<td>Nishimera Village</td>
<td>1</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>5Myz4</td>
<td>Miyazaki [7]</td>
<td>Nishimera Village</td>
<td>1</td>
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<td></td>
<td></td>
<td></td>
<td>4Stm1</td>
<td>Kagoshima [8]</td>
<td>Minamiosumi Town (Cape Satamisaki)</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>Goto Islands</td>
<td>4</td>
<td>4Gto1</td>
<td>Nagasaki [10]</td>
<td>Goto Islands</td>
<td>3</td>
</tr>
<tr>
<td><em>C. n. centralis</em></td>
<td>Honshu Island</td>
<td>4</td>
<td>4Ymg1</td>
<td>Yamaguchi [4]</td>
<td>Shimonoseki City</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>Tsushima Islands</td>
<td>4</td>
<td>4Tsm1</td>
<td>Nagasaki [9]</td>
<td>Tsushima Islands</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>4Tng2</td>
<td>Kagoshima [11]</td>
<td>Tanegashima Island</td>
<td>1</td>
</tr>
<tr>
<td><em>C. n. yakushimae</em></td>
<td>Yakushima Island</td>
<td>4</td>
<td>4Yks1</td>
<td>Kagoshima [12]</td>
<td>Yakushima Island</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>4Yks2</td>
<td>Kagoshima [12]</td>
<td>Yakushima Island</td>
<td>1</td>
</tr>
</tbody>
</table>

Numbers in the brackets at the head of locality name correspond to those in Fig. 1. Haplotypes with an asterisk are cited from Yamada et al. (2006).
DNA was extracted from the ethanol-preserved tissue following phenol-chloroform protocols (Sambrook et al. 1989). Extracted DNA was dissolved in TE buffer (10 µl) and stocked at –20°C.

Polymerase Chain Reaction (PCR) amplifications were performed in Gene Amp PCR System 2400 (Applera Corporation: Perkin-Elmer) in a 50 µl total volume containing 1.25 unit Taq DNA polymerase (Applied Biosystems), 5 µl of 10 × PCR buffer, 0.2 mM each dNTP, 1.5 mM MgCl₂, 0.5 µM each primer, and 0.05 µg of DNA template. PCR cycle parameters were as follows: forty cycles each consisted of 95°C for 1 minute, 55°C for 1 minute, and 72°C for 1 minute, with an initial hot start at 95°C for 5 minutes and a final extension at 72°C for 10 minutes. To amplify and sequence the control region of the sika deer, five primers (LD5, LD7, HD2, HD6, and HD8) designed by Nagata et al. (1998) and three primers (CervL1, CervL3 and CervH1) designed by Charles E. Cook (DDBJ accession nos.: AB295410, AB295414, and AB295418, respectively) were used. PCR products were run on 2% agarose (Type II medium EEO; Sigma chemical) gels with Mupid-2 (Advance). Primer and unincorporated dNTPs were removed from PCR products using Micro Spin S-400HR Columns (Amersham Pharmacia Biotech). Dye terminator cycle sequencing was performed with BigDye Terminator Cycle Sequencing Ready Reaction Kit, 3.2 pmol primer and 30–90 ng PCR products. Cycle sequencing parameters were as follows: twenty-five cycles each consisted of 96°C for 10 seconds, 50°C for 5 seconds, and 60°C for 4 minute, with an initial hot start at 96°C for 5 minutes. Products of sequencing reactions were purified with CENTRI-SEP Columns (Applied Biosystems).

Sequence analysis was performed using the computer program Gene Works (Intelligenetics). All alignment were checked and edited manually. The sequence of the whole control region was determined with reference to Anderson et al. (1982). We examined the phylogenetic relationships among the haplotypes using the neighbor-joining (NJ) method (Saitou and Nei 1987) in the computer program ClustalW (Thompson et al. 1994) and maximum likelihood (ML) method (Felsenstein 1981) in the computer program PAUP* version 4.0 beta version (Swofford 2002). Tandem repeats and gaps were deleted for the phylogenetic tree analysis. The genetic distances were estimated using Kimura’s two parameter method (Kimura 1980). ML heuristic searches were performed using the HKY+I+G model (Hasegawa et al. 1985),
which was selected by both hLRTs and AIC in the Modeltest 3.7 (Posada and Crandall 1998). The bootstrap analysis (Felsenstein 1985) consisted of 1,000 replications for the NJ and ML trees. During sequencing analysis, we added the sequence data of the eastern Shikoku sika deer published by Yamada et al. (2006) such as 4Esk1–4Esk4 (DNA Data Bank of Japan AB186349–AB186352). We investigated gene genealogies for control region sequences using the statistical parsimony network approach in the TCS computer program (Clement et al. 2000). In this analysis, gaps were treated as missing data.

Results

The control region of the samples analyzed in the present study ranged in size from 995 to 1037 bp. Their tandem repeats had either 4 or 5 units. They were similar to those of the southern Japan group reported previously (Nagata et al. 1999; Yamada et al. 2006). They are shown in Table 1.

All four haplotypes from Miyazaki had 5 units of tandem repeats. The nucleotide sequences were longer than those from other localities by 32–42 bp. Haplotypes from Yakushima Island (4Yks1 and 4Yks2) had a unique sequence of 7 bases AGGGGGG at 220 bp downstream from the end of the tandem repeats.

By deleting tandem repeats and gaps sequences, 4Esk4, 4Wsk3, 4Wsk4, 4Tng2 and 5Myz2 became identical to 4Esk1, 4Wsk1, 4Wsk2, 4Tng1 and 5Myz1, respectively. The sequence divergence based on comparison of the sequences without tandem repeats and gaps ranged from 0.2 to 2.2% among the haplotypes, where identical haplotypes listed above were treated as a single haplotype. The average divergence from C. n. taiouanus (4Twn1) was 3.5%. Most of the nucleotide substitutions were transitions. Transversion occurred at only one site.

NJ and ML trees are shown in Fig. 2. Haplotypes of C. n. yakushimae (4Yks1 and 4Yks2) and C. n. mageshimae (4Tng1 and 4Tng2) formed a single clade with a high bootstrap probability. In the ML tree, multifurcation occurred.

The statistical parsimony network is shown in Fig. 3. It is indicated that Miyazaki haplotypes were more closely related to Shikoku haplotypes than others.

Each haplotype had tandem repeats, and the sequence of the four (five in the Miyazaki haplotypes) units were different from each other except for the fifth unit in the Miyazaki haplotypes, which was identical to the fourth sequence of 7 bases AGGGGGG at 220 bp downstream from the end of the tandem repeats.

![Fig. 2. Phylogenetic tree of the control region of the sika deer reconstructed by (a) the neighbor-joining method based on Kimura’s two parameter genetic distance and (b) the maximum likelihood method with HKY+I+G model. Tandem repeats and gaps were excluded from the analysis, which made 4Esk4, 4Wsk3, 4Wsk4, 4Tng2 and 5Myz2 identical to 4Esk1, 4Wsk1, 4Wsk2, 4Tng1 and 5Myz1, respectively. C. n. taiouanus (4Twn1) was used as an outgroup. Numbers near the internal branches are bootstrap probability values derived from 1,000 replications. Bootstrap values less than 50% were omitted.](image-url)
These sequences were coded as shown in Fig. 4 and the patterns are shown in Table 2. There were 12 different tandem repeat sequences. A haplotype of Miyazaki, 5Myz1, had the same sequence as some haplotypes of Shikoku in the first four units of tandem repeats (sequence 1). Other Miyazaki haplotypes had a difference of only one base from sequence 1.

Discussion

In the present study only haplotypes that belong to the southern Japan lineage were found. Therefore, in Kyushu and Shikoku, the area where the two distinct lineages exist together is considered to be limited to the eastern part of Shikoku Island.

As for genetic diversity on Shikoku Island, 7 southern Japan haplotypes were found in western Shikoku, which was larger than the 4 haplotypes from eastern Shikoku. The eastern and western populations of Shikoku Island did not share the same haplotype (Table 1). Even in western Shikoku, the northwestern and southwestern populations had no common haplotype. The whole Shikoku Island, therefore, is a unique place, having as many as 15 haplotypes including 4 northern Japan haplotypes and also having areas where two distinct lineages have intermingled (Yamada et al. 2006).

Nagata et al. (1999) revealed that the population of Yakushima Island (C. n. yakushimae) is genetically distant from other populations of the southern Japan group. Present results show that the population of Tanegashima Island (C. n. mageshimae) is closer to that of Yakushima Island. It can be explained by the strait formation history: between 0.15 and 0.1 million years ago Tanegashima Island (present Tanegashima and Yakushima Islands) was separated from the Honshu landmass (present Honshu, Shikoku and Kyushu Islands), and Tanegashima Island and Yakushima Island were separated from each other between 12,000 and 8,500 years ago (Ohshima 1990).

However, the nonbifurcating tree obtained by the ML method may indicate that the assumption about ancestral haplotypes being no longer in the population is invalid and that haplotypes found in the present study have diverged in a radial manner from one common ancestor. In the present study, therefore, genealogical relationships among the haplotypes were further analyzed by the statistical parsimony network. It has been argued that this approach outperforms tree-constructing phylogenetic methods when applied to intraspecific data sets because...
the low number of mutational changes often caused unsolved trees. The statistical parsimony network (Fig. 3) suggests that the southern Japan haplotypes diverged from an eastern Shikoku haplotype. It is notable that Miyazaki haplotypes are closely related to Shikoku haplotypes more than to other Kyushu haplotypes.

The close relationship between the Miyazaki and Shikoku haplotypes was also suggested in the patterns of the tandem repeats (Table 2). Miyazaki haplotypes had a tandem pattern same as or similar to some Shikoku haplotypes although the number of units was different. The close relationship between Miyazaki and western Shikoku populations was also supported by the analysis of the cytochrome b gene (422 bp), in which their sequences were identical (Yamada et al. unpublished). The fifth unit of the tandem repeats in the Miyazaki haplotypes might be the result of the recent duplication of the fourth unit since their sequences are the same (code k).

If the southern Japan lineage is an older group than the northern Japan lineage as argued by Tamate et al. (1998) and Goodman et al. (2001), a haplotype that diverged more recently may have larger tandem repeats because the northern Japan lineage has more units in the tandem repeats than the southern Japan lineage. Therefore, it is possible that the Miyazaki population shares a common ancestor with some of the west Shikoku groups and diverged recently, i.e. about 7,000 years ago when Shikoku was separated from the Honshu landmass from which the present Kyushu Island was isolated about 2,000 years later (Ohshima 1990).

In the present study, the sample size was not large enough to resolve the history of divergence of the southern Japan lineage of C. nippon using a procedure such as nested clade analysis (Templeton 1998). Besides, we could not obtain samples from Oita, Kyushu, although it is geographically closest to Shikoku and the deer population there is suspected to hold important information concerning the relationship of the two Islands in terms of the divergence of the species. Therefore, expansion of the samples in both locality and quantity is necessary for further study.

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References


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Appendix

We registered the following sequence data with DNA Data Bank of Japan.

4Wsk1:AB279706, 4Wsk2:AB279707, 4Wsk3:AB279708, 4Wsk4:AB279709, 4Wsk5:AB279710, 4Wsk6:AB279711, 4Wsk7:AB279712, 4Ymg1:AB279713, 4Fko1:AB279714, 4Stm1:AB279715, 4Tsm1:AB279716, 4Gto1:AB279717, 4Tng1:AB279718, 4Tng2:AB279719, 4Yks1:AB279720, 4Yks2:AB279721, 4Twn1:AB279722, 5Myz2:AB279723, 5Myz3:AB279724, 5Myz4:AB279725