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Original paper

Special issue **"The Frontline of the Researches on Conservation and Management of Japanese Macaques"**

Genetic assessment on the origin of alien macaques in the Boso Peninsula in Japan

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Abstract. Japanese macaques and alien macaques have hybridized in the Boso Peninsula, Chiba Prefecture, Japan. In this study, the origin of the alien species was investigated by molecular assessments with mitochondrial DNA (mtDNA) and Y-chromosome genes. Maternal origin was assessed by comparing mtDNA sequence records. The results suggested that the alien species in the southern part of peninsula originated from the rhesus macaques in eastern China. Y-chromosome assessments with three microsatellite (Y-STR) loci detected a unique haplotype that is distributed in Japanese macaque habitats. Its origin was assessed by the TSPY (testis-specific protein on Y-chromosome) gene, suggesting the possibility of the involvement of long-tailed macaques in the Indochina region or rhesus macaques different from known source. Further investigation of historical documents and interviews disclosed the existence of a facility of long-tailed macaques planned for vaccine production in the past. This study presented novel evidence that the hybridization of Japanese macaques in the Boso Peninsula has the possibility to associate not only with rhesus macaques, but also with long-tailed macaques from the Indochina region. It is important to further monitor the status of Japanese macaques and changes in their hybridization in the peninsula for future conservation purposes.

Key words: hybridization, molecular marker, mtDNA, TSPY, Y-STR.

In accordance with international trends aimed at comprehensively conserving biodiversity and using biological resources sustainably, the Japanese government enacted the Invasive Alien Species Act in 2005 to prevent damages from invasive alien species (Mito and Uesugi 2004). The objectives of this act are to regulate various actions, such as raising, planting, storing, carrying, and importing invasive alien species (IAS) in addition to mitigating the IAS that already exist in Japan, and prevent damages against biodiversity, human safety, or agriculture in Japan (Japanese Ministry of the Environments 2004). The act has been expanded to cover hybrids by an amendment in 2013. The most serious concern of the alien species issue on Japanese macaques (*Macaca fuscata*), an endemic non-human primate in Japan, is impacted by hybridization, which will destroy species biodiversity. The primates designated as specific alien species so far in the act are three macaques (rhesus macaques *M. mulatta*, Taiwanese macaques *M. cyclopis*, and long-tailed macaques *M. fascicularis*) and two of their hybrids (hybrids with *M. mulatta* and hybrids with *M. cyclopis*).

Rhesus macaques, which have become feral and hybridized with Japanese macaques, live in the southern tip of the Boso Peninsula in Honshu, Japan (Fig. 1). This species is naturally distributed across the Asian continent, from Afghanistan in the west, to China in the east (Fooden 2000). Although the history of their introduction to Boso peninsula is less known, it likely happened in the 1950s when monkeys became popular in the tourism industry and were later released when the business was abandoned (Hagihara et al. 2003). Based on the national policy, Chiba Prefecture began their eradication plan from 2007 (Chiba Prefecture 2012). The plan was revised in 2012 and the hybrid population is being reduced by euthanasia. Their population size was estimated to be 600–700 in 2012 (Chiba Prefecture 2012).

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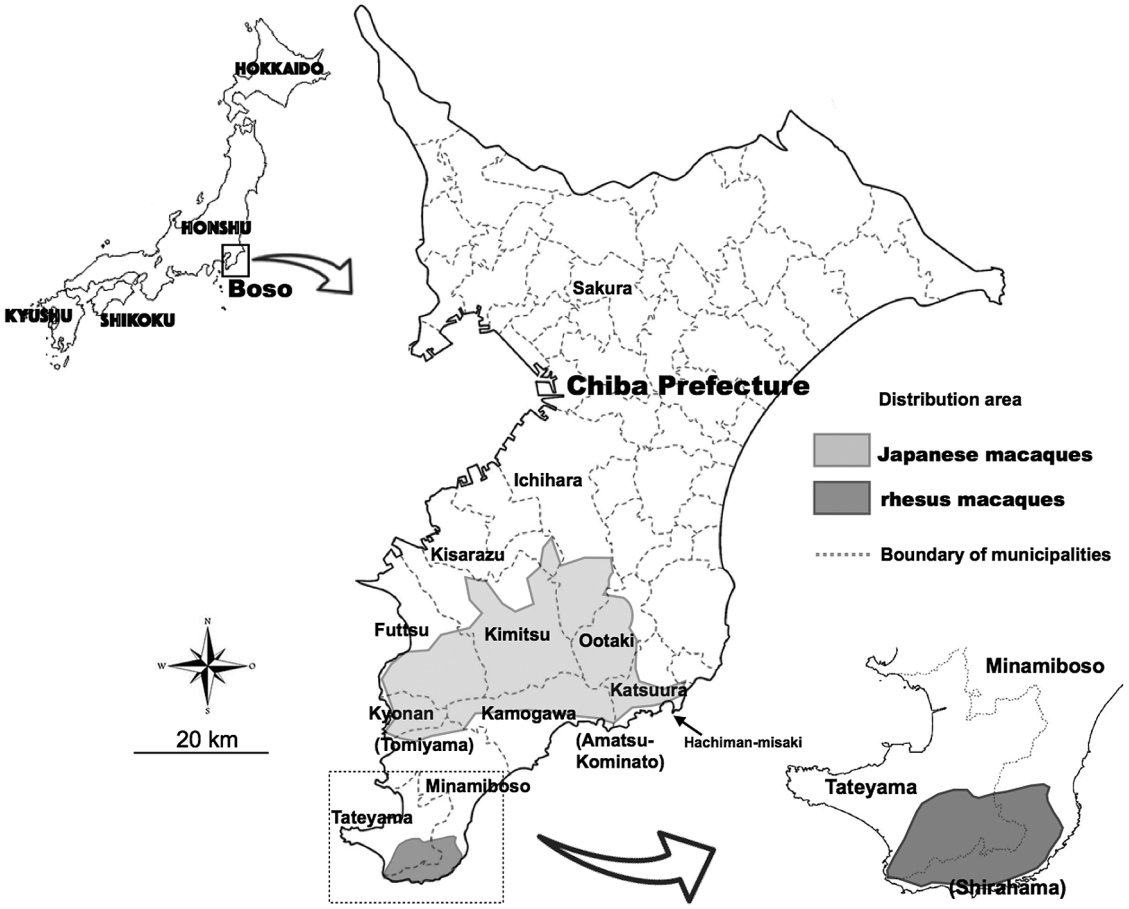

Fig. 1. Map showing the location of habitats of indigenous Japanese macaques (in 2015, Chiba Prefecture 2017) and introduced rhesus macaques (year unspecified, Chiba Prefecture 2012) in the Boso Peninsula, Chiba Prefecture, Japan. Approximate distribution areas of groups are shown in color. The town name in parentheses indicates the town that existed before the merger of municipalities.

The native Japanese macaque is distributed in the central hilly area, and there is an empty area of approximately 20 km, separating them from the habitat of rhesus macaques (Fig. 1). The geographical distribution of Japanese macaque groups was confirmed for the first time in 1923 (Iwano 1974). The existence of alien macaque groups and their habitat information were firstly reported in 1996 (Chiba Prefecture and Boso Monkey Management Study Group 1996). Migrations of solitary males were reported between these two species habitats (Hagihara et al. 2003). The Japanese macaque habitat extends to the south, north, and east due to recent population increases. Their habitat spans ten municipalities and was estimated to be 747 square kilometers in 2015 (Chiba Prefecture 2017).

Macaques form a population of matrilineal groups that make up social units, and the contrast between female philopatry and male dispersal is known in the life history of males and females (Pusey and Packer 1987; Melnick et al. 1992). It is known that sex-specific genes of mitochondrial DNA (mtDNA) and Y-chromosome DNA, which reflect this socio-ecological characteristic, are effective for population surveys as molecular markers for interspecific hybridization (Tosi et al. 2002).

Government officials and researchers have reported hybridization caused by the alien macaques on the Boso Peninsula (Kawamoto et al. 2004, 2007, 2017). Kawamoto et al. (2004) firstly reported hybridization in the feral rhesus monkey group and hybridization in the Japanese macaque group. After that, the hybridization status of the rhesus group was further investigated, and the results forewarned that the impact of the alien species could spread broadly to Japanese macaque population on the peninsula (Kawamoto et al. 2007). In the population monitoring of these surveys, hybrid individuals were identified by inspection of morphological characteristics such as relative tail length and coat color (Hamada 2013), and genetic examination using nuclear markers such as

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blood protein electromorphs (adenosine deaminase and NADH-dependent diaphorase) that specifically distinguish Japanese macaques from other Asian macaques (Nozawa et al. 1977; Kawamoto et al. 2004, 2007). Maternally inherited mtDNA has been used in this monitoring as a molecular marker for determining the natal place of monkeys, where the difference between Japanese macaques on the peninsula and the feral rhesus macaques in southern Boso area were examined from the difference in PCR product sizes or their sequence differences (Hagihara et al. 2003). It is confirmed that macaque populations on the peninsula are hybridizing in their habitats. However, information is limited regarding their place of origin and sufficient consideration has not been given to the possibility of the involvement of species other than rhesus macaques.

In this study, the origin of alien macaques was investigated with sex-specific markers of mtDNA and Y-chromosome DNA. DNA sequencing and fragment analyses were performed for species diagnostics. The possibility of alien species and its origin was finally assessed from molecular phylogenetic analysis using the available sequence information of macaques registered in the DNA database. For alien species newly hypothesized based on the obtained results, written records, and interviews with locals further examined the potential involvement in hybridization with Japanese macaque populations in the suspicious areas.

Materials and methods

Molecular markers

Mitochondrial DNA (mtDNA) and Y-chromosome genes were used to identify the species involved in hybridization. Genetic markers were transmitted in a sex-specific manner for females and males, and lots of sequence information were available for them. Due to its high evolutionary rate, mtDNA is the most popular marker in studies on evolution, phylogeny, and ecology. Information on rhesus macaques initially increased for monkeys used in the biomedical sciences and has lately been augmented for wild populations. On the other hand, due to its low evolutionary rate, the Y-chromosome gene is generally substandard as a marker. In evolutionary research on primates, using variations of the TSPY (testis-specific protein on Y-chromosome) gene (Kim et al. 1996; Tosi et al. 2000) has gradually increased and related information has recently been enriched. In addition, microsatellite DNA markers for Y-chromosomes (Y-STR) initially developed in human genetics have been applied to non-human primates (for example, Erler et al. 2004; Kawamoto et al. 2008).

Two kinds of molecular markers, Y-STR and TSPY, were adopted to evaluate the origin of Y-chromosome DNA in this study. At first, haplotypes of Y-chromosome were classified by fragment analyses of three Y-STR loci. From this haplotyping, the distribution area and haplotype frequencies were summarized to see fundamental differences of Y-chromosome composition between Japanese macaque populations in the central hilly area and rhesus population in southern Boso area. Two different sampling periods (before May 2009 and during March 2011–January 2018) were compared to evaluate changes in Y haplotype composition. For the rhesus habitats in Tateyama city and Minamiboso city (separated into Shirahama and Tomiyama towns before the merger of municipalities in March 2006), male migrants from Japanese macaque groups were judged based on features of morphology and mtDNA types. For the Japanese macaque habitats, the Y haplotypes of hybrid individuals were evaluated based on morphological features of relative tail length and coat color and genotyping results of protein-coding loci of adenosine deaminase and NADH-diaphorase. From the screening of Y haplotypes, three different representatives of Y-chromosomes, each for the Japanese macaque, the rhesus macaque, and the other one that could be assigned to neither species, were selected, then they were subjected to the sequencing of the TSPY gene to assess species origin.

Study area and samples

Samples of blood, tissue, or fecal DNA were collected from the Boso Peninsula (Fig. 1, Table 1, Supplementary Table S1). Blood samples were taken from captured individuals within the scope of the prefecture's program. Clotting was prevented with sodium heparin or EDTA, and cryopreserved blood cells were used in experiments. Tissue samples of ear skin collected from monkeys exterminated by the government's pest control project were also used in this study. Tissue DNA was extracted using conventional Phenol/Chloroform/Isoamyl alcohol and ethanol precipitation methods (Sambrook et al. 1989 with slight modifications). Fecal samples were collected in the Japanese macaque habitat following the procedure of a previous study (Hayaishi and Kawamoto 2006). Fecal DNA samples were prepared following the procedure of Kawamoto et al. (2013).

Sampling was performed with official permission

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Table 1. List of samples examined

Marker	Analysis	Sample size	Source	Sampling area (sample size)	Note
Mitochodrial DNA	Sequencing of non-coding region	36	blood	RMH (8), JMH (28*)	See Supplementary Table S1 for details
		2	tissue	JMH (2)	See Supplementary Table S1 for details
		17	feces	JMH (17)	See Supplementary Table S1 for details
		409	database		See Table 2 and Supplementary Table S2 for details
	Subtotal	464			
Y chromosome	Fragment analysis of 3 Y-STRs	262	blood	RMH (90), JMH (172*)	See Table 4 for details
	Subtotal	262			
Y chromosome	Sequencing of TSPY gene	15	blood	RMH (1), JMH (14*)	
		13	feces	JMH (13)	
		14	database		See Table 3 for details
	Subtotal	42			
	Total	768			

RMH = rhesus macaque habitat in Boso Peninsula, JMH = Japanese macaque habitat in Boso Peninsula (See Fig. 1 for details)
* Blood samples for which diagnostic blood proteins were examined to judge interspecific hybridization.

given in the prefectural plan, and the handling of animals and sampling procedures followed the guidelines of the Primate Research Institute, Kyoto University (Guideline for Field Research of Non-human Primates) and that of the Mammal Society of Japan (Guidelines for the Procedure of Obtaining Mammal Specimens).

DNA analysis

Direct sequencing of the non-coding region was done with mtDNA typing. An amplicon from blood, tissue DNA, or fecal DNA was subjected to direct sequencing, where the PCR reaction mixture (25 µl) contained 1 µl of template DNA, 12.5 µl of 2 × buffer, 0.4 mM of each dNTP, 300 nM of each of the primers, LqqF (forward) 5'-TCCTAGGGCAATCAGAAAGAAAG-3' (Li and Zhang 2004) (corresponding to nucleotides 15936–15958 of a complete mtDNA sequence of Japanese macaques, accession no. NC_025513 in DDBJ/ENA/GenBank databases) and Saru5 (reverse) 5'-GGCCAGGACCAA GCCTATTT-3' (Hayasaka et al. 1991) (nucleotides 609–628, NC_025513), and 0.5 U of DNA polymerase KOD-FX (Toyobo, Osaka, Japan). The thermal cycling condition involved initial heat denaturation at 94°C for 2 min, followed by 35–45 cycles of denaturation at 98°C for 10 sec, annealing at 58°C for 30 sec and extension at 68°C for 30 sec. Four additional internal primers were also used for sequencing; 53F (forward) 5'-CTCACCA TCCTCCGTGAAAT-3' (nucleotides 16393–16412, NC_025513), Saru4 (forward) 5'-ATCACGGGTCTATCAC CCTA-3' (nucleotides 2–21, NC_025513), 51R (reverse) 5'-CATGGAAAGCTCCCGTGACT-3' (nucleotides 28–47, NC_025513), and mdl341 (reverse) 5'-GTTTGGA TGAAGGTCGGAGA-3' (nucleotides 315–324, NC_025513). Sequencing was performed with an ABI 3130xl Genetic Analyzer (Applied Biosystems, CA, USA).

TSPY direct sequencing was conducted similarly to the method of mtDNA sequencing, where primers TSPY-A (forward) and TSPY5R (reverse) were used to obtain a PCR amplicon, and internal primers 470F (forward), 485R (reverse), and 740R (reverse) were used to obtain sequence reads (Tosi et al. 2000). Y-STR analysis was done with three STR markers on Y-chromosomes, *DYS472*, *DYS569*, and *DYS645* (Kawamoto et al. 2008). Haplotypes were classified from combinations of allele types. The fragment analysis condition followed the procedure of Kawamoto et al. (2008). Allele sizes were determined using GeneMapper v. 4.1 (Applied Biosystems).

The DNA sequences obtained in this study were registered in DDBJ/ENA/GenBank databases under accession numbers LC585811–LC585868.

Data analysis

DNA sequences were verified with Sequence Navigator (Applied Biosystems). Sequences of mtDNA or TSPY genes were compared to reference sequences in the data-

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Table 2. List of reference rhesus sequences and their accession numbers for mtDNA assessment

Locality	Abbreviation	Population type	Reference	Accession Nos.	*n*
Anhui	China E	Wild	unpublished data in the DNA database	AF135271	1
Bangladesh		Wild	Hasan et al. 2014	KJ767675–KJ767715	41
China East	China E	Breeding center	Smith and McDonough 2005	AY646930–AY646966	37
China West1	China W	Breeding center	Smith and McDonough 2005	AY646967–AY646996	30
China West2	China W	Breeding center	Smith and McDonough 2005	AY646997–AY647010	14
China West3	China W	Breeding center	Smith and McDonough 2005	AY647011–AY647017	7
Fujian	Chine E	Wild	unpublished data in the DNA database	AF135272–AF135284	13
		Wild	unpublished data in the DNA database	AY682599–AY682602	4
				Subtotal	(17)
Guangdong	China W/S/E	Breeding center	Smith et al. 2006; Li et al. 2011	DQ373083–DQ373113	31
Guangxi	China W/S/E	Breeding center	unpublished data in the DNA database	AF135285–AF135304	20
Guizhou	China E/W	Wild	unpublished data in the DNA database	AF135305	1
		Unknown	Li and Zhang 2004	AY682604	1
				Subtotal	(2)
Henan	China E	Wild	unpublished data in the DNA database	AF135306–AF135313	8
Hubei	China E	Wild	unpublished data in the DNA database	AF135316	1
Hunan	China W	Wild	unpublished data in the DNA database	AF135314–AF135315	2
India 1		Breeding center	Smith and McDonough 2005	AY647018–AY647121	104
India 2		Breeding center	Smith and McDonough 2005	AY647122–AY647126	5
Myanmar		Breeding center	Smith and McDonough 2005	AY647127–AY647139	13
Nepal		Wild	Kyes et al. 2006	AY823276–AY823296	1
Sichuan	China NW	Breeding center	unpublished data in the DNA database	AF135335–AF135338	4
		Breeding center	Smith et al. 2006; Li et al. 2011	DQ373252, DQ373254, DQ373257, DQ373258, DQ373262, DQ373263, DQ373269, DQ373272, DQ373276–DQ373284	17
				Subtotal	(21)
Thailand		Wild	Bunlungsup et al. 2017a	LC167033–LC167044	12
Vietnam		Breeding center	Smith et al. 2006	DQ373351–DQ373361	11
		Breeding center	unpublished data in the DNA database	AF135354–AF135360	7
				Subtotal	(18)
Yunnan	China SW	Breeding center	unpublished data in the DNA database	AF135339–AF135353	15
		Unknown	Li and Zhang 2004	AY682603	1
				Subtotal	(16)
Zhejiang	China E	Wild	unpublished data in the DNA database	AF135361–AF135368	8
				Total	409

base as listed in Tables 2 and 3. Multiple sequence alignments were taken with ClustalX (ver. 2.0) (Thompson et al. 1997) after selecting conserved blocks using Gblocks version 0.91b (Castresana 2000). Species origin was verified by evaluating the phylogenetic relationships of sequences using programs in MEGA6 (Tamura et al. 2013).

Unique Y haplotypes were classified in the Y-STR analysis. A total of 261 males were grouped in the municipality origin. Then the distribution of haplotypes was compared to explore which species were involved. In order to evaluate the relationship among Y haplotypes classified by the STR analyses, an unrooted tree diagram was constructed assuming a stepwise mutation model (Kimura and Ohta 1978). Here, the genetic distance between Y haplotypes was simply defined as the sum of the differences in the number of repetitive units over the three Y-STR loci. The tree diagram was generated

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Table 3. List of reference macaque sequences and their GenBank accession numbers for TSPY gene assessment

Common name	Scientific name	Locality	Reference	Accession number
Japanese macaques	*Macaca fuscata*	Boso, Chiba	present study	LC585866
		Unknown	Tosi et al. 2000	AF284254
		Unknown	Tosi et al. 2000	AF284255
Rhesus macaques	*M. mulatta*	Boso, Chiba	present study	LC585867
		China	Tosi et al. 2000	AF284259
		India	Tosi et al. 2000	AF284260
Taiwanese macaques	*M. cyclopis*	Taiwan	Tosi et al. 2000	AF284245
Long-tailed macaques	*M. fasicuclaris*	Unknown	Tosi et al. 2000	AF284246
		Philippine	Tosi et al. 2000	AF284247
		Salawak	Tosi et al. 2000	AF284248
		Sepilok	Tosi et al. 2000	AF284249
		Thailand	Tosi et al. 2000	AF284250
		Borneo	Tosi et al. 2000	AF284251
		Java	Tosi et al. 2000	AF284252
		Vietnam	Tosi et al. 2000	AF284253
Pig-tailed macaques	*M. nemestrina*	NE Borneo	Tosi et al. 2000	AF284238
U haplotype	Unknown	Boso, Chiba	present study	LC585868

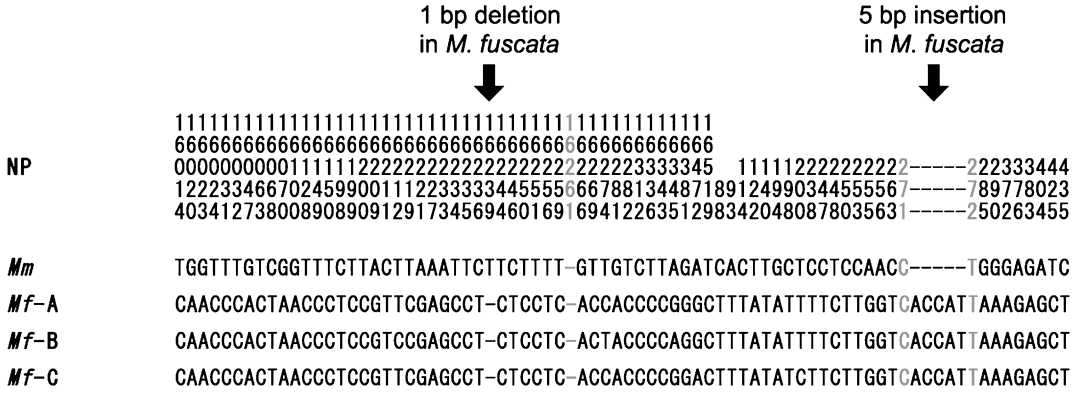

Fig. 2. Comparison of mtDNA sequences by alignments of three Japanese types (*Mf*-A, *Mf*-B, and *Mf*-C) and one rhesus type (*Mm*) detected in Boso, Chiba. Nucleotide location in this figure refers to the nucleotide position (NP) number of a complete mtDNA sequence of rhesus macaques (GenBank accession no. KF830702). Gray letters indicate corresponding sites in the reference KF830702 without variation in this study.

from the obtained distance matrix using the programs Neighbor and Drawtree implemented in the software package PHYLIP (Felsenstein 1989). The TSPY sequences of three unique Y-STR haplotypes were finally compared with reference TSPY data to find relevant species and their geographic origin.

Results

Assessment using mtDNA

Differences among species were clearly found in mtDNA by sequencing 1007–1011 bps. There were size differences in the amplicons. An insertion of five bases and a deletion of a single base were detected in Japanese macaques compared to the exotic sequence (Fig. 2). In 47 samples (28 blood samples, two tissue samples, and 17 feces) typed as Japanese macaques, three mtDNA haplotypes (*Mf*-A, *Mf*-B, and *Mf*-C types in Fig. 2) were distinguished due to base substitutions. On the other hand, all of the examined samples from the alien habitat in southern Boso showed the same sequence (*Mm* type in Fig. 2).

The exotic mtDNA sequence in southern Boso was preliminarily evaluated to be a rhesus type by BLAST (Basic Local Alignment Search, a sequence homology

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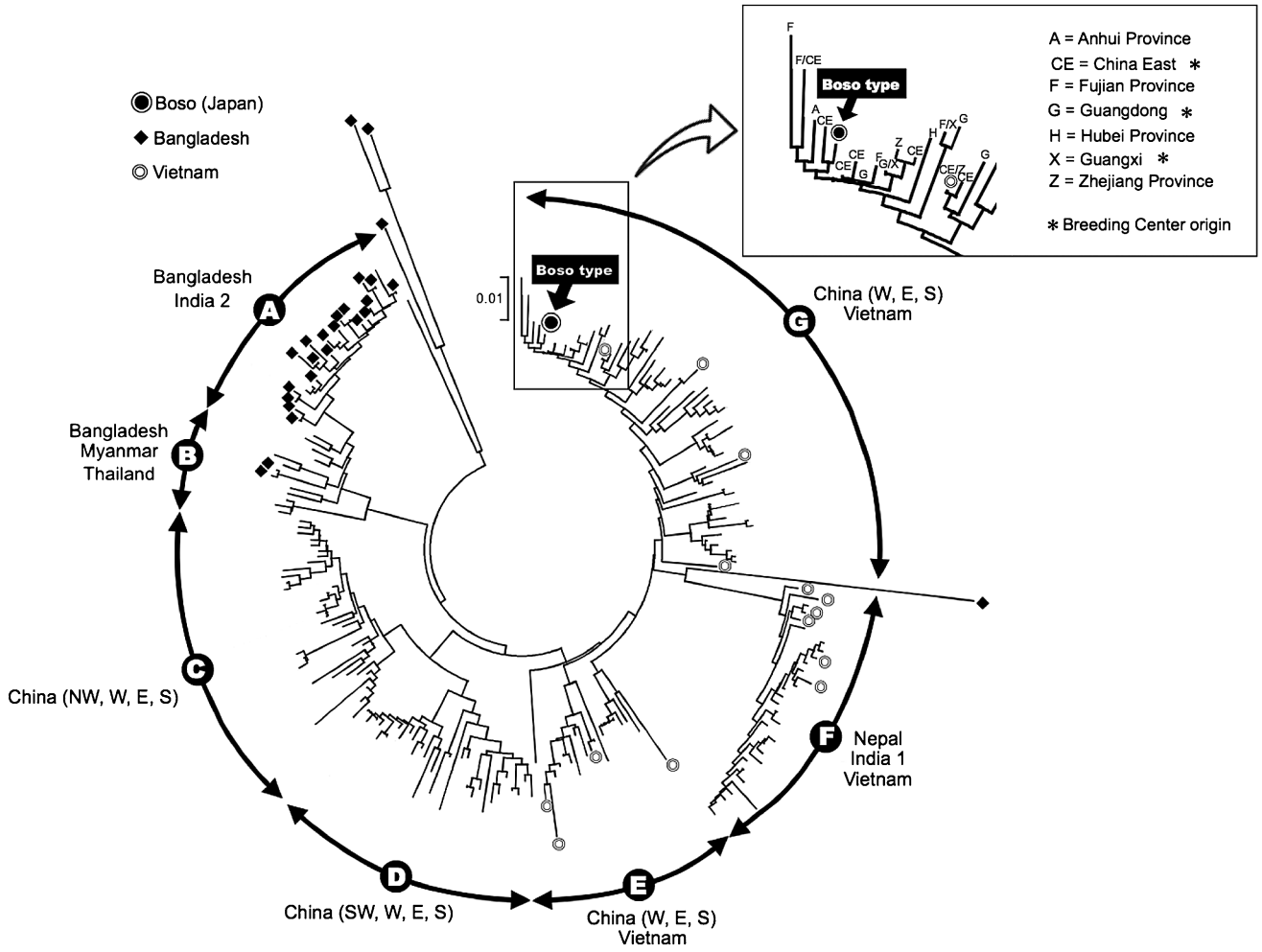

Fig. 3. A neighbor-joining tree drawn to find the origin of rhesus macaque type in Boso, where 223 haplotypes defined from 410 records in the database by matching sorted 445 bps in mtDNA D-loop were compared to the Boso type. The default parameters were set when the tree was constructed using programs in MEGA6 (Tamura et al. 2013). The reference haplotype data were categorized by locality origin. The haplotypes of Bangladesh and Vietnam, which showed diversity and were scattered in the tree, are represented by separate symbols. For closely related haplotypes, the Boso type was compared in a separate box. The haplotypes that make up the haplogroups A to G are summarized in Supplementary Information. Abbreviations for Chinese locality are given as NW = northwest, SW = southwest, W = west, E = east, and S = south by taking descriptions of Smith and McDonough (2005) and Satkoski et al. (2008) into consideration.

search tool supported by NCBI, USA). It was further compared to 409 reference sequences (Table 2) to investigate the place of origin. The final length of the compared sequence was only 445 bps after Gblocks sorting due to available information. As a result, 223 mtDNA haplotypes were distinguished. There was one registered record (accession number AY646959) completely identical to the exotic type. It was a record on a rhesus monkey imported from China to USA (Smith and McDonough 2005). Figure 3 is a neighbor-joining tree (Saitou and Nei 1987) drawn from the 223 haplotypes. The contents of those haplotypes are summarized in Supplementary Table S2. From this result, it was concluded that the rhesus macaques in Boso did not originate from South Asia (India, Bangladesh, and Nepal) and Southeast Asia (Myanmar and Thailand). When inspecting their origin and vicinity in China, it was not easy to point a specific place. Composed of sub-clusters, the Boso rhesus type was clustered together with those from Anhui, Fujian, Guangdong, Guangxi, Hubei, and Zhejiang Provinces and an unknown area in eastern China (Fig. 3).

Assessment using Y-chromosome DNA

Table 4 shows the results of the Y-STR analysis. A total of eight Y haplotypes were distinguished by Y-STR polymorphisms. The repetitive units of the *DYS472*, *DYS569*,

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Table 4. Summary of Y chromosome haplotypes defined by Y-STR markers (*DYS472*, *DYS569*, and *DYS645*)

Sampling period: Before May 2009 Area: Whole habitat area

Name of haplotype	Species assignment	Allele size			Tateyama Shirahama*	Tomiyama*	Kyonan	Futtsu	Kimitsu	Ootaki	Kisarazu	Ichihara	Kamogawa	Amatsu-Kominato**	Katsuura	Total
		DYS472	*DYS569*	*DYS645*												
J1	*M. fuscata*	120	282	278	31	4	7	8	6	0	0	2	5	0	—	63
J2	*M. fuscata*	120	282	279	13	4	3	3	7	1	0	0	4	2	—	37
J3	*M. fuscata*	120	286	279	0	0	0	0	1	0	0	0	0	0	—	1
R1	*M. mulatta*	105	278	300	7	0	0	0	0	0	0	0	0	0	—	7
R2	*M. mulatta*	108	274	274	4	0	0	0	0	0	0	0	0	0	—	4
R3	*M. mulatta*	117	274	292	1	0	0	0	0	0	0	0	0	0	—	1
R4	*M. mulatta*	123	274	274	34	0	2	0	0	0	0	0	0	0	—	36
U	Unassigned	111	274	274	0	0	0	0	0	0	1	1	0	0	—	2
				Total	90	8	12	11	14	1	1	3	9	2	—	151

Sampling period: March 2013–January 2018 Area: Japanese macaque habitat only

Name of haplotype	Species assignment	Allele size			Tateyama	Minamiboso	Kyonan	Futtsu	Kimitsu	Ootaki	Kisarazu	Ichihara	Kamogawa	Amatsu-Kominato**	Katsuura	Total
		DYS472	*DYS569*	*DYS645*												
J1	*M. fuscata*	120	282	278	—	—	14	3	16	—	—	—	—	—	21	54
J2	*M. fuscata*	120	282	279	—	—	4	2	13	—	—	—	—	—	28	47
J3	*M. fuscata*	120	286	279	—	—	0	0	0	—	—	—	—	—	2	2
R1	*M. mulatta*	105	278	300	—	—	1	0	1	—	—	—	—	—	0	2
R2	*M. mulatta*	108	274	274	—	—	0	0	0	—	—	—	—	—	0	0
R3	*M. mulatta*	117	274	292	—	—	0	0	0	—	—	—	—	—	0	0
R4	*M. mulatta*	123	274	274	—	—	0	0	0	—	—	—	—	—	0	0
U	Unassigned	111	274	274	—	—	0	0	0	—	—	—	—	—	8	8
				Total	—	—	19	5	30	—	—	—	—	—	59	113

* Tomiyama and Shirahama towns entered Minamiboso City due to the merger of municipalities in March 2006.
** Amatsu Kominato Town entered Kamogawa City due to the merger of municipalities in February 2005.
—: not tested.
The counts of unique haplotypes were summrized.
Each haplotype was assigned to species judging from the genetic profile of sample individual given by other dianostic markers.

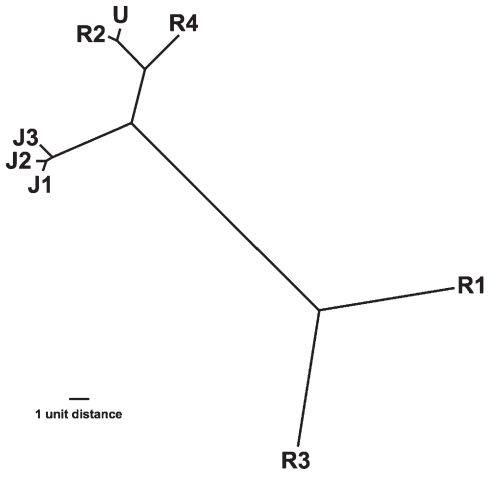

Fig. 4. An unrooted tree diagram showing the relationship among eight Y haplotypes distinguished by Y-STR analyses. Abbreviation name and allelic profile of each Y haplotype are summarized in Table 4. The genetic distances between Y haplotypes were measured with the total number of repetitive units (unit distance). The tree diagram was drawn from an outfile of UPGMA tree given by Neighbor program using Drawtree program in PHYLIP package (Felsenstein 1989).

and *DYS645* markers were three bases, four bases, and one base, respectively, and the number of alleles detected for *DYS472*, *DYS569*, and *DYS645* was six, four, and five, respectively.

The relationship among the Y haplotypes was evaluated with an unrooted tree diagram as shown in Fig. 4. The three Japanese haplotypes (J1, J2, and J3) were closely clustered, but the other haplotypes were distantly related to the Japanese group. Two groups, one consisting of R2, R4, and U and the other of R1 and R3, were observed in the tree diagram as non-Japanese Y haplogroups.

Six haplotypes; J1, J2, J3, R1, R4, and U were detected in the Japanese macaque habitat. Six haplotypes of R1, R2, R3, R4, J1, and J2 were detected in the rhesus macaque habitat. J1, J2, and J3 were judged to be the Y-chromosome types derived from Japanese macaques, and R1, R2, R3, and R4 were judged to be the Y-chromosome types derived from rhesus macaques, based on the area of occurrence, frequency, and the STR allele types constituting the Y haplotypes (Table 4 and Fig. 4). The U type was detected in a small part of the eastern and northern area of the Japanese macaque habitat, but not in the rhesus macaque area. However, the ten individuals having this type showed more or less different characteristics in the relative tail length and coat color morphology, and many of them had blood protein variations of macaques other than Japanese macaques (Supplementary Table S3). Therefore, this Y haplotype was separately denoted as an un-assignable type (abbreviated as U type) to distinguish it from the Japanese macaque type, and its carrier was regarded as a hybrid with an unknown alien macaque species.

Upon comparing samples that had been collected until May 2009 when the hybridization was less advanced, a total of eight unique Y haplotypes, three Japanese types, four rhesus types, and one un-assignable type (U type), were distinguished from an examination of 151 male blood samples collected in 11 administrative districts (the municipalities at the time) (Table 4). Two Japanese types and four rhesus types were detected in Tateyama city and Shirahama town (currently Minamiboso city) in the rhesus habitat. On the other hand, only the Japanese macaque types, excluding the two cases of R4 type (Kyonan town), were found in all eight administrative districts of the Japanese macaque habitat. The U type was detected only in Kisarazu city and Ichihara city at that period. When comparing the results of the 111 males collected in the Japanese macaque habitat from March 2013 to January 2018, a total of eight U type individuals were detected in multiple groups in Katsuura city (Table 4).

The TSPY gene sequences were compared to ascertain the origin of the U type, which was uncertain from the Y-STR analysis. For a representative comparison, samples of Japanese macaques (Kyonan town), rhesus macaques (Minamiboso city), and the U type (Katsuura city) were sequenced for 1491 bps and compared with deposited database of rhesus macaques, Taiwanese macaques, long-tailed macaques, and pig-tailed macaques (*M. nemestrina*) (Fig. 5). In the constructed tree, the U type was clustered together with rhesus macaque types from India and China and a long-tailed macaque type from Vietnam. The rhesus type in Boso was also placed in the same cluster. The Japanese type in Boso formed a cluster together with the registered types of Japanese macaques. No sequence differences were detected among eight individuals of the U type from Katsuura city in Table 4.

Discussion

The DNA markers adopted in this study contrast in the practices of female philopatry and male dispersal (Pusey and Packer 1987; Melnick et al. 1992). The strong philopatric features of females make mtDNA dispersal restricted from natal groups. Males can geographically disperse mtDNA but cannot transmit it due to its maternal

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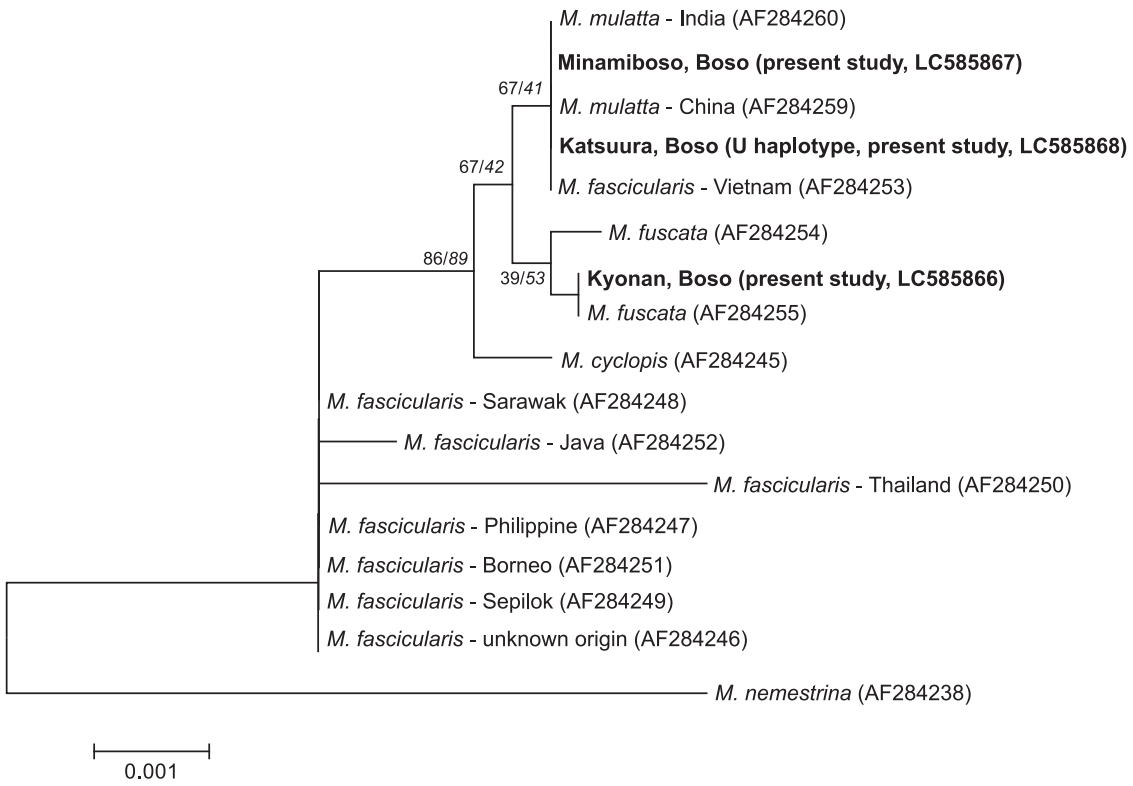

Fig. 5. A neighbor-joining tree constructed from representative sequences of TSPY gene by matching sorted 1491 bps (Table 3). The default parameters were set when the tree was constructed using programs in MEGA6 (Tamura et al. 2013). Three types of TSPY sequences detected in the present study, including the U type defined by Y-STR haplotyping, were compared with reference sequences of macaque species. The values above the branch node indicate the results of interior-branch test (in non-italic) and the percentage bootstrap values (in italic) obtained from assessments with the NJ algorithm (1000 replications) (Sitnikova et al. 1995).

mode of inheritance. Therefore, the dispersal lifetime of mtDNA by males is limited within one generation. Meanwhile, chromosomal pairing is cytogenetically restricted, and the recombination portion is small between the Y-chromosome and X-chromosome in macaques (Hirai et al. 1991). Although its mutation rate is not as high as mtDNA, the non-recombined part of Y-chromosome can simply accumulate mutations like mtDNA. Due to these characteristics, both mtDNA and Y-chromosomes were regarded as suitable tools to assess the origin of alien species in this study. Like autosomal chromosomes, Y-chromosomes are transmitted across generations. Thus, interspecific introgression can be thought to begin through male migration, and the impacts of alien species can spread geographically by succeeding male dispersal in later generations.

In the rhesus population of southern Boso, there was only one type of mtDNA, but multiple Y-haplotypes were detected. High maternal homogeneity suggests a monophyletic origin of the population, and the diversity of the Y haplotypes implies involvement of multiple males in founding.

Regarding the results of the mtDNA assessment, a preliminary study by Hagihara et al. (2003) speculated its origin from China or its vicinity by the examination of captive rhesus specimens collected at the Primate Research Institute, Kyoto University in Japan. The present study further compared their origin with data on captive rhesus macaques imported to the Regional Primate Centers in the USA, as well as data on wild individuals in the source countries. An earlier investigation by Smith and McDonough (2005) classified Chinese rhesus types into four groups, consisting of one group from the eastern region (denoted as “ChiE haplogroup” in Smith and McDonough (2005), corresponding to “China E” in Fig. 3) and three groups from the western region (denoted as “ChiW1, ChiW2, and ChiW3 haplogroups” in Smith and McDonough (2005), corresponding to “China W” in Fig. 3), where the Boso rhesus type matched completely with a haplotype in the “ChiE haplogroup” (accession number AY646959; Haplotype 47 in Supplementary Table S2). There were regional variations

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in mtDNA haplotypes that were close to the rhesus type of Boso in Fig. 3. Most of them were wild or captive rhesus macaques from provinces in eastern China. However, the cluster also contained haplotypes from breeding centers in south-central or southeast China. Satkoski et al. (2008) pointed out that there was anthropogenic activity in Chinese breeding colonies that resulted in the export of hybrids. Considering this influence, it may be reasonable to conclude that the origin of rhesus populations in southern Boso is somewhere in east China, probably Anhui, Fujian, Zhejiang, or Jiangsu Province. If hybridization in the Boso Peninsula cannot be prevented, the species biodiversity of the Japanese macaque will be significantly affected by mixing with the rhesus macaque from east China.

This study presented new results for understanding the history of the macaque hybridization on the Boso peninsula. It is noteworthy that the U type of the Y-chromosome haplotype was found only in a restricted habitat of Japanese macaques and was not observed in the rhesus area of southern Boso. There were no groups of Japanese macaques in the U type area in 1973 before their habitat expansion (Boso Japanese Monkey Research Group 1979). The individuals carrying the U type contained many hybrids in the inspection of morphological traits and diagnostic autosomal protein markers (Supplementary Table S3). The Japanese macaque groups in Katsuura city has been recently established by population expansion, and their habitat is located farther from the rhesus distribution area than the Japanese macaques in the western part of the peninsula. As the rhesus specific Y-chromosome types R1–R4 have not been observed in Katsuura (Table 4), it is also inferable that the influence of gene flow from the rhesus population in southern Boso may be low in this area. These circumstantial findings imply the involvement of alien macaques other than the rhesus macaques in southern Boso. Unfortunately, the details of morphological features of the U type carrier are unknown due to the paucity of records on captured individuals. It has been discussed that rhesus population in southern Boso is the cause of hybridization with Japanese macaques on the Boso Peninsula, but based on the obtained results in this study, it is now necessary to investigate further the cause(s) of hybridization. Hagihara et al. (2003) reported alien macaques listed in the administrative statistics and interviewing records to leisure facilities at that moment, but there were no records of alien species that seemed to be related to the distribution of the U carrier.

Three reference data of rhesus and long-tailed macaques showed the same sequence with the TSPY sequence of U type (Fig. 5), as well as the sequence of rhesus in southern Boso. The U sequence matched with one of the two clusters of long-tailed macaques of Indochina origin (Vietnam), but not a Sundaic origin (Malaysia, Indonesia, and the Philippines). Though this result could not exclusively conclude the origin of the U type from long-tailed macaques, its possibility was supported as well as that of rhesus origin. Studies on the evolution and phylogeny of macaques in Southeast Asia revealed ancient introgression between rhesus macaques and long-tailed macaques in the region around 15 degrees north latitude of the Indochina Peninsula (Tosi et al. 2002; Hamada et al. 2006; Street et al. 2007; Bonhomme et al. 2009; Barr et al. 2011, Jadejaroen et al. 2015; Bunlungsup et al. 2017b). The populations of long-tailed macaques in the region share the Y-chromosomes with rhesus macaques due to this evolutionary history. Thus, the result of the Y-chromosome assessment in this study suggests that long-tailed macaques from Indochina may have been involved in hybridization. As an alternative case, if long-tailed macaques are not involved, a rhesus population different from that in southern Boso could be considered as the cause of hybridization because of absence of the U haplotype in southern Boso.

As the males with the U type had a Japanese mtDNA haplotype (for males in Katsuura city, see note in Supplementary Table S1), it may be reasonable to consider the Y gene flow by immigrant males. To explain this contrast between Y DNA type and mtDNA type, a hypothesis was considered that there had been extinction or removal of source alien macaque population(s) in the survey area of this study. One way to further test this hypothesis was to discover records of foreign macaque introduction or find persons who knew about the history of introduction in the study area.

The incident of anthropogenic introduction was further investigated through a survey of historical documents and interviews with people who knew the past. Owing to many collaborators, it became clear that there was a national project for the production of the polio vaccine in the Boso Peninsula around 1960. The project, which began in 1958, aimed at developing the Salk vaccine (Francis et al. 1955; Meldrum 1998) that could be produced by inactivating the virus propagated in a culture of monkey kidney cells. It was confirmed that a facility for breeding long-tailed macaques was established in the project, firstly at Sakura city in 1959, and was then relo-

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Fig. 6. Photographic record of a group of long-tailed macaques provisioned at the tip of Hachiman-misaki, Katsuura city in April 1981 (courtesy of Mr. Satoshi Inoue).

cated to Katsuura city in 1960 (Chiba Serum Institute 1977, 1997; Katsuura city 1961) (Fig. 1). The breeding colony was converted into a tourist facility in the late 1960s when changes in vaccine production made it unnecessary to maintain it (Chiba Serum Institute 1997). Finally, the facility was closed in 1984 by removing all the monkeys from the place. The population of long-tailed macaques had been kept at the tip of Hachiman-misaki (Fig. 1) in free-ranging conditions by the city's provisioning efforts (Fig. 6), and there were 230 individuals at most in 1964 (Katsuura city 1964). The signboard at that time stated that the origin of the monkeys was the Malay Peninsula (Chiba Serum Institute 1997). However, when we checked a report on trade statistics (Kawanishi and Honjo 1971), little evidence was given to support the number of imports from the Malay Peninsula at that time. In addition, the sequence of U individuals did not match the TSPY gene sequences of long-tailed macaques in the Malay Peninsula provided by the Wildlife Genetic Resource Bank (WGRB) Laboratory of the Malaysian government (GenBank accession numbers KJ690361–KJ690376). Therefore, given the impact of imported long-tailed macaques from the polio vaccine project, it can be inferred that their origin was not in the Malay Peninsula.

Long-tailed macaques may have dispersed into the surrounding area over the past 25 years. In the early stage, there was no group of Japanese macaques in the surrounding area, but subsequent increased encounters between the two species might have triggered hybridization. Since the transplanted long-tailed group finally was removed from the leisure facility, this hybridization impact might have remained in the form of unidirectional gene flow to Japanese macaques. As there is no available specimen or sample of the long-tailed macaque at present, it is hard to provide more scientific evidence to support this speculation.

In conclusion, this study suggests that the ongoing hybridization of Japanese macaques on the Boso Peninsula may be associated not only with rhesus macaques, which came from eastern China, but also with long-tailed macaques from the Indochina region. Keeping the findings of this assessment in mind, it is important to further monitor the status and change of hybridization on the Boso Peninsula and use the outcomes to establish countermeasures to conserve Japanese macaques in the future.

Supplementary data

Supplementary data are available at *Mammal Study* online.
Supplementary Table S1. Information on the samples used in mtDNA examination.
Supplementary Table S2. List of mtDNA 223 haplotypes classified by multiple alignments of 409 D-loop reference data deposited in the GenBank database.
Supplementary Table S3. Results of hybrid judgement for males that carried the U type of Y chromosome in Table 4.

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