

## Genetic Diversity of Maternal Lineage in the Endangered Kiso Horse Based on Polymorphism of the Mitochondrial DNA D-Loop Region

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**ABSTRACT.** To determine genetic characteristics of the maternal lineage of the Kiso horse based on polymorphisms of the mitochondrial DNA D-loop region, we collected blood samples from 136 Kiso horses, 91% of the entire population, and sequenced 411 bp from 15,437 to 15,847 in the region. First of all, we estimated the demographic history; by searching homology between the obtained and known sequences using Basic Local Alignment Search Tool, by mismatch analysis to evaluate the mutation processes using Arlequin, and by building a phylogenetic tree showing the relationship of the mtDNA haplotypes for 24 horse breeds around the world using Molecular Evolutionary Genetics Analysis software. The results suggested that various horses that came to Japan stayed at Kiso region and became ancestors of Kiso horse and also genetically supported the theory that the Kiso horse was historically improved by other Japanese native horse breeds. Next, we analyzed the diversity of current maternal lineage by classifying the resulting sequences, and by calculating the haplotype diversity and nucleotide diversity using Arlequin. Then, we visualized the relationship among haplotypes by a median-joining network using NETWORK 4.6.0.0. The results suggested the diversity of maternal lineage in the Kiso horse was reasonably maintained. Lastly, we predicted future change of the diversity of maternal lineage in Kiso horse by assessing the regional distribution of the acquired haplotypes. The distribution suggested that diversity of maternal lineage would possibly be reducing.

**KEY WORDS:** D-loop region, genetic diversity, Kiso horse, maternal lineage, mitochondria DNA

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Kiso horse, one of 8 breeds of horse native to Japan [12-14, 21, 22, 29], is in danger of extinction, and there are calls for scientific evidence-based conservation of the breed [26, 27]. In the Kiso region, which is a mountainous area of Japan that is by no means affluent, horses have been used not only for transportation and agriculture but also for the manure they produce, which is essential for poor soil of the region, and for the precious cash income via production of foals [12]. These factors resulted in the formation of a unique culture in Kiso that centered on the horse, including a unique style of house built with a stable on the sunniest side of the ground floor. Therefore, the Kiso horse is important not only as a regionally unique genetic resource but also as a living asset that symbolizes the regional culture. Accordingly, conservation of the Kiso horse holds much significance for building a more diverse society and for preserving regional identity.

With the rapid progression of mechanization, the demand for horses disappeared, and by 1976, the number of Kiso horses had dropped to 32. Breeders of the Kiso horse, who had witnessed this decimation, undertook actions to conserve the breed. These efforts resulted in the recovery of the

number of Kiso horses to 150 [12, 26, 27]. However, there are still concerns about the various factors that can reduce diversity, including the harmful effects of inbreeding through backcrossing [12].

Within the concerning diversity issue, the diversity of maternal lineage, which has been preserved through traditional breeding by private owners keeping horses singly or in pairs, is especially worried. The main reason is the collapse of traditional breeding brought by the aging of Kiso horse breeders and the lack of successors. This is because 1) Japan has witnessed an increasingly lower birth rate combined with aging society, together with ever greater concentration of the population in big cities; there is depopulation of the Kiso region with the outflow of young people from the area, and 2) westernization and uniformity of lifestyles has led to the loss of the traditional horse-centered lifestyle, and the emotional distance between horses and people is steadily growing.

Given above, it is necessary to conserve the diversity of maternal lineage of Kiso horse in order to maintain their genetic diversity [6, 24-27]. In evolutionary biology, the diversity of mitochondrial DNA (mtDNA), in particular the D-loop region, is analyzed to assess the close relationships of the maternal lineage between breeds and within the species [3, 8, 10]. In this regard, we analyzed the diversity of the mtDNA D-loop region and attempted to elucidate the genetic characteristics of their maternal lineage, in order to contribute to the retention of Kiso horse, enabling the indigenous genetic resource that bears regional culture to be passed down to future generations.

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Table 1. The accession numbers of the haplotypes in 24 horse breeds analyzed for phylogenetic relationship with the haplotypes in the Kiso horse

Population	No. of haplotypes	Accession Number	Abbreviation	Geographical Region
Akhal-Teke	8	DQ327950, DQ327953, DQ327956-60, DQ327962	AT1-8	West Asia
Arabian	15	AF064628-9, EF437553-62, EF437564-6	ARE1-15	West Asia
Belgian	3	AF064630-2	BEL1-3	Europe
Breton	2	AB329597, AB329617	BRT1-2	Europe
Cheju	7	AF014405-8, AF014410-12	CJ1-7	East Asia
Hokkaido	3	AB329589, AB329596, AB329620	HKD1-3	Japan
Hucul	12	JF951834-7, JF951840-7	HCL1-12	Europe
Icelandic	14	HQ153701, HQ153711-2, HQ153715, HQ153719, HQ153722-6, HQ153728, HQ153753-4, HQ153757	ICL1-14	Europe
Irish Draught	30	DQ327892, DQ327898-900, DQ327902, DQ327904-6, DQ327909-10, DQ327912, DQ327915-6, DQ327918-21, DQ327923, DQ327925-28, DQ327933-36, DQ327938, DQ327944-5, DQ327948	ID1-30	Europe
Kiso*	3	AB329600, AB329626-7	KS1-3	Japan
Misaki	1	AB329624	MSK	Japan
Mongolian	19	AF014413-5, AF056071, DQ327986-7, DQ327989-92, AB329587, AB329592, AB329604, AB329606, AB329618, AB329622-3, AB329625, AB329628	MNG1-19	East Asia
Noma	2	AB329591, AB329609	NM1-2	Japan
Orlov Trotter	9	DQ328002-5, DQ328007, DQ328012-3, DQ328015, DQ328018	OT1-9	North Asia
Percheron	2	AB329603, AB329619	PCR1-2	Europe
Pottoka	10	AY519958-67	POT1-10	Europe
Przewalskii	2	AP012267, AP012269	PRW1-2	Europe
Taishu	2	AB329588, AB329614, AF169009-10	TS1-4	Japan
Thoroughbred	18	AB329590, AB329593-5, AB329599, AB329601-2, AB329605, AB329607-8, AB329610-3, AB329615, AB329621, D14991, D23665	TRB1-18	Europe
Tibetan	3	EF597512-4	TB1-3	East Asia
Tokara	1	AB329598	TKR	Japan
Vyatka	10	DQ328020-4, DQ328026, DQ328028, DQ328034-5, DQ328037	VYT1-10	North Asia
Yakutian	13	DQ328038-40, DQ328042-5, DQ328050, DQ328052-4, DQ328056-7	YK1-13	North Asia
Yunnan	1	AF014416	YN	East Asia

\* From Kakoi, H. *et al.* [14]

## MATERIALS AND METHODS

From September 2008 to September 2011, we collected blood samples from 136 Kiso horses, which represent 91% of all the horses registered with the Kiso Horse Conservation Association. The horses were bred in the mountainous region of central Japan: mainly in Nagano, Gifu and Aichi prefectures. Within the surveyed horses, 17 were males, 102 were females, and 17 were geldings.

Approximately 20 ml of blood was drawn from the jugular vein. After the sample was transferred to an EDTA blood collection tube, it was promptly taken to the laboratory, and the DNA was extracted using the Wizard<sup>®</sup> DNA extraction kit (Promega KK, Tokyo, Japan).

The 411-bp reference sequence of the mtDNA D-loop region between positions 15,437 and 15,847 [33] from each horse was sequenced using the following primers; forward: 5' CTAGCTCCACCATCAACACC-3', reverse: 5'-ATG-GCCCTGAAGAAAGAACC-3'. Using the GeneAmp<sup>®</sup> kit (Applied Biosystems, Lifetechnologies Co., Carlsbad, CA, U.S.A.), a total of 15  $\mu$ l of the reagent containing 20 ng of a DNA template, 15 pmol primer set, 2.5 mM MgCl<sub>2</sub>, 0.33 mM dNTPs, 1.5  $\mu$ l of 10  $\times$  buffer and 1.5 U AmpliTaq Gold<sup>®</sup> was subjected to initial denaturation for 10 min at 95°C, followed

by 30 cycles of PCR (30 sec at 94°C, 1 min at 60°C and 30 sec at 72°C) and final extension for 10 min at 72°C. The PCR products were incubated with exonuclease I and shrimp alkaline phosphatase and used as template DNA. Then, direct sequencing was performed using the Big Dye<sup>®</sup> Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems). The sequence products were analyzed using an automatic DNA sequencer (ABI PRISM3130XL, Applied Biosystems), and the resulting sequences were processed using Sequencing Analysis Software (Applied Biosystems). The accuracy of sequencing was determined using Sequencing Scanner v1.0 (Applied Biosystems). All the samples were amplified and sequenced well. Variation of each haplotype was confirmed using multiple alignments by Clustal X [17].

To estimate the demographic history of the Kiso horse, we used the Basic Local Alignment Search Tool (BLAST) of National Center for Biotechnology Information (NCBI) and searched for homology between the obtained sequences and known sequences. Using Arlequin [4], we also performed mismatch analysis to estimate the mutation processes. We then used Molecular Evolutionary Genetics Analysis (MEGA) software to build a phylogenetic tree [28], which shows the relationship of the mtDNA haplotypes for 24 horse breeds around the world [7, 14, 16, 20, 23, 32], includ-

Table 2. The mitochondrial DNA D-loop region of haplotypes K1-K7 in the Kiso horse compared with the reference sequence X79547

Nucleotide position	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1			
	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5			
haplotype (n)	4	5	5	5	6	6	6	6	6	6	6	7	7	7	7	7	7	7	7	8	8	8	8			
	9	3	8	9	0	0	0	1	1	1	5	5	0	0	2	2	4	7	7	7	7	0	1	2	2	
X79547	T	A	G	T	G	C	G	A	A	T	A	T	T	C	G	C	A	C	C	C	A	C	C	A	A	
K 1 (40)	C	.	A	.	.	T	A	.	.	.	.	.	C	.	A	.	G	.	T	.	G	.	T	.	.	
K 2 (33)	C	.	.	.	.	T	A	.	.	.	.	.	C	.	A	.	G	.	T	.	G	.	T	.	.	
K 3 (28)	C	.	.	C	.	T	.	G	G	.	.	.	C	.	A	.	.	T	.	T	.	T	.	.	G	
K 4 (19)	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.
K 5 (9)	C	G	A	.	A	T	.	.	.	.	G	.	.	T	A	.	.	T	.	.	T	.	.	G	.	
K 6 (6)	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	G	.	
K 7 (1)	C	.	.	.	T	.	.	.	.	C	.	C	.	.	A	.	.	T	.	.	T	.	.	.	G	

\* n: number of the horse.

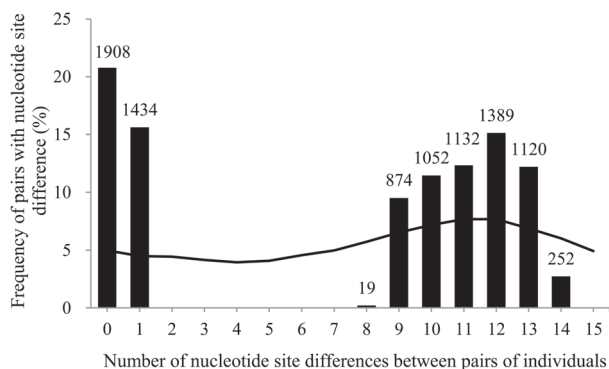


Fig. 1. Mismatch distribution of mtDNA sequences in the Kiso horse. The number of pairs with nucleotide site differences is labeled on the top of each bar. The polygonal line represents the predicted frequency.

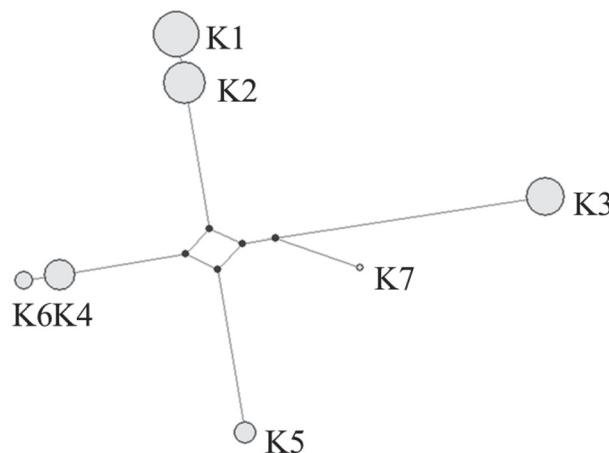


Fig. 2. Median-joining network of 7 haplotypes of the Kiso horse. The circle size represents the size of each haplotype group.

ing Przewalskii (Table 1). The statistical reliability of each node within this phylogenetic tree was confirmed by 1,000 random bootstrap replicates.

To assess the diversity of current maternal lineage in the Kiso horse, we classified the resulting sequences and calculated the haplotype diversity (*h*) and nucleotide diversity ( $\pi$ ) using Arlequin. The relationship among haplotypes was visualized by a median-joining network using NETWORK 4.6.0.0 (<http://www.fluxus-engineering.com>. Fluxus Technology Ltd., Suffolk, U.K.) [1].

To predict future change of the diversity of maternal lineage in Kiso horse, we assessed the regional distribution of the acquired haplotypes. Then, we discussed the efficient management plan for conservation of the horse.

RESULTS

According to the results of analysis of the D-loop region (411 bp of 136 Kiso horses), we confirmed that the Kiso horse has 7 haplotypes (K1–K7), namely haplotypes K1, K2, K3,

K4, K5, K6 and K7. Forty horses (29.4%) had K1, 33 horses (24.3%) had K2, 28 horses (20.6%) had K3, 19 horses (14.0%) had K4, 9 horses (6.6%) had K5, 6 horses (4.4%) had K6, and 1 horse (0.7%) had K7. When we compared these results with the reference standard X79547 [33], there were 25 polymorphic sites in the corresponding genomic region of the Kiso horse (Table 2). These 25 mutations were all point mutations between identical types of bases showing significant deviation that occurs between transversion and transition in the course of evolution of mtDNA in mammals [30], and the analyzed region was A/T rich (A: 30.3%, T: 27.7%, C: 28.5% and G: 13.5%); this finding correlates with the findings of previous report on equine species [11].

According to the BLAST results, the 6 haplotypes, K1, K2, K4, K5, K6 and K7, correlated with AB329626 (Kiso horse-derived H46), AB329627 (Kiso horse-derived H47), AB329588 (Taishu horse-derived H2), AB329600 (Kiso horse-derived H14), AB329589 (Hokkaido horse-derived H3) and AB329604 (Mongolian native horse-derived H18), respectively. There were no sequences, however, that

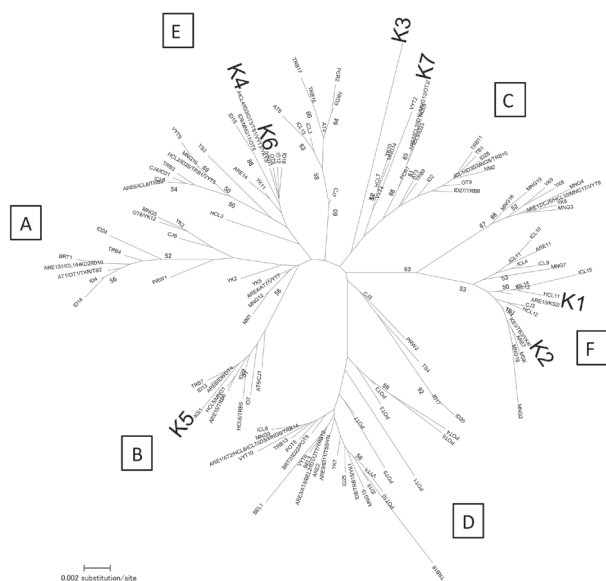


Fig. 3. Neighbor-joining phylogenetic relationships between 7 haplotypes of the Kiso horse and the haplotypes of other 24 breeds. The branched numbers represent bootstrap probability (1,000 replicates), and the letters A–F represent the cluster categories according to Vilà *et al.* [31].

matched K3, suggesting that K3 is a new sequence in terms of evolution. In the mismatch analysis, sequence differences ranged from 1 to 14 bp, showing a bimodal distribution with a major peak at 0 mutational differences and a secondary one at 12 mutational differences (Fig. 1).

The  $h$  in the Kiso horse was  $0.79 \pm 0.01$  (Mean  $\pm$  SD), while  $\pi$  was  $0.017 \pm 0.009$ . The number of nucleotide mutations among the haplotypes ranged from 1 (between K1 and K2 and between K4 and K6) to 14 (between K3 and K5), and the distance between bases ranged from 0.002 to 0.036. According to the median-joining network, which visualizes this information, K1 and K2 as well as K4 and K6 form a group (each pair); this result shows that the mtDNA D-loop haplotypes of the Kiso horse were divided into 4 clusters (Fig. 2).

The relationship between the Kiso horse haplotypes and the haplotypes of horse breeds worldwide was elucidated in the neighbor-joining tree (Fig. 3). The 7 haplotypes of the Kiso horse – K1, K2, K3, K4, K5, K6 and K7 – were distributed in clusters F, F, C, E, B, E and C, respectively [31].

With regard to the regional distribution of each haplotype, the distribution of the haplotype was different in each prefecture. In Nagano prefecture, main breeding region of Kiso horse, there were K1 (28 horses), K3 (24 horses), K2 (15 horses), K4 (7 horses), K5 (4 horses), K6 (3 horses) and K7 (1 horse) in decreasing order. Then, the distribution in Gifu prefecture in decreasing order was K2 (11 horses), K4 (7 horses), K1 (5 horses), K6 (3 horses), K3 (2 horses) and K5 (1 horse). The distribution in Aichi prefecture in decreasing order was K1 (6 horses), K5 (3 horses) and K4 (2 horses) (Fig. 4).

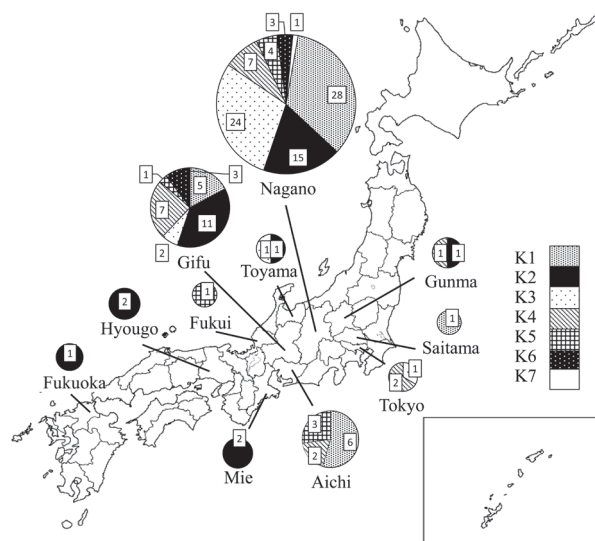


Fig. 4. Distribution of the surveyed horses. The diameter of the circle graph shows the size of each population. The figures within the graph represent the number of horses for each haplotype.

## DISCUSSION

In this study, we attempted to elucidate the genetic characteristics of the Kiso horse by looking at the genetic diversity of the maternal lineage. Our research provided compelling suggestions about the demographic history, the current maternal lineage diversity and prediction about its future changes.

First, we would like to discuss the demographic history of the Kiso horse. The horse has a high degree of mitochondrial diversity. This is because approximately 6,000 years ago, many horses that inhabited Eurasia were caught and domesticated, and the descendants of those horses rapidly spread throughout the world [31]. The fact that K1–K7 are categorized into various clusters shows that the Kiso horse also has various ancestors. In addition, although K3 and K5 are haplotypes confirmed only in the Kiso horse, we were able to confirm that haplotype K1 is present in Arabian horse; haplotype K2 is present in the Mongolian native horse; K4 is present in the Taishu horse (Japanese native), Misaki horse (Japanese native) and Mongolian native horse; K6 is present in the Hokkaido horse (Japanese native); and K7 is present in the Asian and European breeds of horses. Furthermore, mismatch analysis showed bimodal distribution, which suggests reconvergence of the populations [6]. These results may support the information on the demographic history of the Kiso horse obtained from various studies of proteins and nuclear DNA and from archeological researches, that is, a) horses with various roots entered the Japanese archipelago from areas, such as the Mongolian grasslands, and then stayed in Kiso, and these horses became the ancestors of the Kiso horse, and b) in the various eras, mares were brought in Kiso area from other regions of Japan, and the Kiso horse breed was improved in accordance with the region and/or the era [12, 21, 22, 29].

Second, we would like to discuss the current diversity of the maternal lineage in the Kiso horse. The Kiso horse has 7 haplotypes that were divided into 4 clusters. In this study, because we were able to collect samples from 91% of the entire population of this breed, we were able to identify more haplotypes including a new one than those identified in previous report [14]. Unfortunately, the 28-year-old mare with the K7 haplotype died immediately after the survey; therefore, haplotype K7 has disappeared from the Kiso horse population. However, the number of haplotypes in the remaining Kiso horses is smaller than those in the Lusitano (27 haplotypes) [18], Lipizzan (37 haplotypes) [15] and Arabian horses (27 haplotypes) [2], but greater than those in the Iberian, South American and North American horses (2–6 haplotypes; mean 3.8) [19]. Furthermore, if we keep in mind the Sorraia horse, which is also endangered with a similar size of the population as the Kiso horse has only 2 haplotypes [18], the number of haplotypes in the Kiso horse is by no means small. In addition,  $h$  in the Kiso horse is  $0.79 \pm 0.01$ , which is slightly lower than that in the Thoroughbred and Breton horses (0.89 and 0.88, respectively) [9]. Also,  $\pi$  in the Kiso horse is  $0.017 \pm 0.009$ , which is similar to that in the Mongolian native (0.021), Thoroughbred and Breton (0.020) and Percheron (0.014) horses [9]. Given that  $\pi$  ranges from 0.000 to 0.104 in different breeds, with a mean of 0.0104 [14, 18, 19],  $\pi$  in the Kiso horse is considered an average level. These data show that although the Kiso horse has only a small population, the genetic diversity of the maternal lineage is comparatively preserved.

Third, we would like to predict future changes of the maternal lineage in the Kiso horse. Interestingly, although we did not find regional differences in microsatellite DNA distribution in the previous study [27], the mtDNA D-loop region haplotype exhibited different distribution in each prefecture. This may be because of recent history of the Kiso horse. The Kiso horse, which experienced a bottleneck in 1970s, was almost exclusively bred within Nagano prefecture until the 1980s, and later, Kiso horse enthusiasts in various regions began breeding this horse until the present day. Therefore, it is thought that there was a founder for the maternal lineage in each region, and colonies were formed in each region mainly with that founder. In addition to this regional isolation and artificial factors [15, 34], there has been a decline in the number of private owners, because of aging, together with the increasing number of zoological parks and equestrian clubs keeping the Kiso horses in herds of several animals. This trend, bringing a biased-breeding for a specific use of the horse and/or the preference of a limited number of breeders keeping horses in herds, is suggested to accelerate the deviation of haplotypes of each region and the decline in the diversity of the maternal lineage.

In this study, we attempted to understand the Kiso horse population on the basis of the diversity of the maternal lineage. The results clearly show that the genetic diversity of the maternal lineage in the Kiso horse has been maintained reasonably well. Nevertheless, the population is still small, and thus, the Kiso horse may be in the extinction vortex: when the population becomes small, and that

such decline could accelerate loss of their genetic diversity, while inbreeding will progress further [6]. Moreover, various genes of the Kiso horse have been fixed because of the past population bottleneck, and those genes are considered to include the genes that bring about inbreeding depression [5]. Furthermore, as suggested in this study, it is possible that diversity of maternal lineage of Kiso horse which was preserved by traditional manner of breeding will be decreasing. Consequently, to preserve the Kiso horse for our next generation, the following key vital measures should be taken, that is, i) clarify the genetic characteristics of the Kiso horse from various perspectives, ii) use reproductive technologies to preserve sperm and zygotes, iii) search for new ways to utilize the horses, thereby inspiring demand to breed the Kiso horse and iv) take action aimed at gathering national support for conserving our unique genetic resource and living cultural asset of the region.

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