NOTE
Wildlife Science

Genetic characteristics of feral Misaki horses based on polymorphisms of microsatellites and mitochondrial DNA

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ABSTRACT. The Misaki horse is a Japanese native horse, known as the "feral horse of Cape Toi". In this study, we acquired the genetic information to establish their studbook, and analyzed their genetic characteristics for conservation. We genotyped 32 microsatellites and a mitochondrial D-loop region in 77 Misaki horses (80.2% of the population). The average number of alleles, observed heterozygosity, and expected heterozygosity were 3.4, 0.509, and 0.497, respectively. A neighbor-joining phylogenetic tree of individuals was constructed. Moreover, the results suggested that Misaki horses experienced a bottleneck, but it was neither severe nor recent. In addition, three mitochondrial haplotypes were confirmed. Consequently, we clarified the genetic background of Misaki horses that have been resident at Cape Toi for a long time.

KEY WORDS: conservation, genetic characteristics, Misaki horse

The Misaki horse is a native horse in Japan, known as the "feral horse of Cape Toi" (Fig. 1), and is designated as a natural monument of Japan for unique livestock husbandry in the natural environment. The horses are confined to the Cape throughout the year but graze freely with minimum human intervention; only the Council for the Protection of Misaki Horses maintains the environment of the Cape and observes the horse population (Fig. 2).

Since the Akitsuki clan, when the Lord of the Takanabe Domain established it in 1697, Cape Toi has been enclosed as a ranch to produce Misaki riding horses for the ruling Samurai class [8]. At the ranch, the horses grazed yearlong in a pastoral setting without human intervention, and the appearance of the horses grazing on the Cape is the same now as in years past. The importance of the biological and cultural heritage of these horses and their habitat was recently recognized, and the area was designated as a natural monument of Japan in 1953 [15].

Misaki horses are maintained along with the environment of Cape Toi. From Taisho (1912–1926) to the beginning of the Showa period (1926–1989), 150 to 160 horses existed in approximately 130 ha of grassland, where zoysia and cogon grasses grew [8]. Later, the number of Misaki horses decreased with the grassland area, due to an increase in thicket from poor maintenance during and after the Second World War. Furthermore, the promotion of cedar plantations after the war accelerated the loss of grassland. In 1964, the grassland area decreased to 34 ha and the number of Misaki horses decreased sharply to 48. Thereafter, the council for the protection of Misaki horses was established in 1968 to conserve Misaki horses and the environment at Cape Toi, and to protect foals, and pregnant, sick, and invalid horses, and to implement the control of parasites, thickets, and inedible grasses. As a result, the number of Misaki horses recovered to 123 (19 yearlings) in 2006, and there are currently 102 horses.

An objective understanding of the current condition of the population is important to formulate sustainable conservation of rare

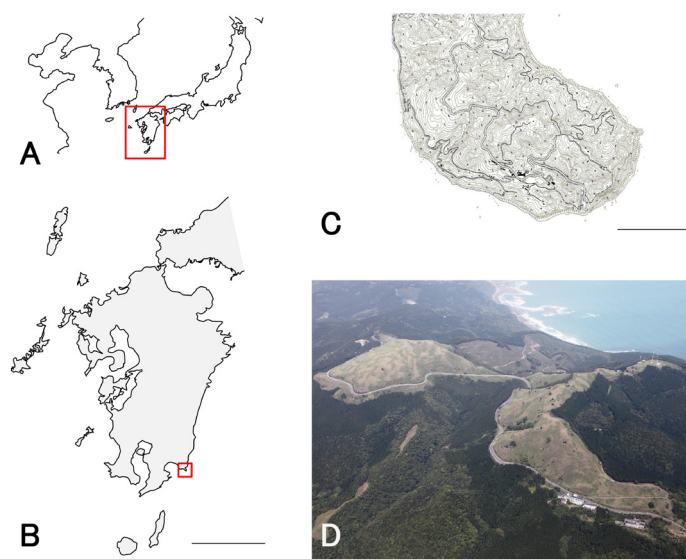**Fig. 1.** Misaki horses grazing at Cape Toi.

Fig. 2. Cape Toi. A wide area map (A), a map of Kyushu: southernmost of the four main islands of Japan, bar=100 km (B), Digital Map 25000 (Map Image) of the cape, published by Geospatial Information Authority of Japan, bar=1 km (C), and an aerial photograph of the cape (D).

animals. Conservation genetics is essential to understand the genetic diversity of an endangered species, and various genetic indexes are used to assess the genetic diversity of a population, including microsatellites and mitochondrial DNA [5]. Microsatellites, short repetitive sequences with a high mutation rate, are useful markers for determining the genetic structure of a population and the relationships between individuals [4, 6]. Microsatellites are also used to test parentage, and can help to form a science-based studbook. In addition, mitochondrial DNA is a maternally inherited marker that is widely used to evaluate inter- and intra-species maternal relationships, because mitochondrial DNA is prone to mutation and its rate of evolution is 5- to 10-fold greater than that of nuclear DNA [2, 7].

In this study, we acquired basic genetic information to establish a studbook for Misaki horses, and to analyze their genetic characteristics for their conservation. To accomplish this, we genotyped 32 microsatellites and a mitochondrial DNA D-loop region in 77 Misaki horses (80.2% of the population).

Blood samples were collected from 77 (MS001-MS077; 38 males and 39 females) of the 96 Misaki horses available (46 males and 50 females) in May 2015. The average age of the horses was 5.6 years, ranging from 2 months to 15 years. Blood sampling was performed in accordance with ethical guidelines by the Animal Care and Use Committee of Gifu University, ensuring the welfare of the horses. Genomic DNA was extracted according to the manufacturer's protocols (QIAamp DNA Mini Kit, QIAGEN K.K. Japan, Tokyo, Japan).

A total of 32 microsatellite markers used for the Japanese Thoroughbred Registration as routine parentage testing were used in this study. They were *AHT4*, *AHT5*, *ASB2*, *ASB17*, *ASB23*, *CA425*, *HMS2*, *HMS3*, *HMS6*, *HMS7*, *HTG4*, *HTG10*, *LEX3*, *LEX33*, *TKY19*, *TKY28*, *TKY321*, *VHL20*, *TKY279*, *TKY287*, *TKY294*, *TKY297*, *TKY301*, *TKY312*, *TKY325*, *TKY333*, *TKY337*, *TKY341*, *TKY343*, *TKY344*, *TKY374*, and *TKY394*. Their corresponding NCBI accession numbers are Y07733, Y07732, X93516, X93531, X93537, U67406, X74631, X74632, X74635, X74636, AF169165, AF169294, AF075607, AF075635, AB048330,

AB048335, AB034629, X75970, AB033930, AB033938, AB034603, AB034606, AB034610, AB034621, AB044826, AB044834, AB044838, AB044842, AB044844, AB044845, AB044874, and AB048299. Allele discriminations were based on the consensus of the Equine Genetics and Thoroughbred Parentage Testing Standardization Workshop, International Society for Animal Genetics (ISAG). Microsatellites were genotyped as previously described by Kakoi et al. [9] and Tozaki et al. [22] with minor modifications. Specifically, all previously published procedures were followed, but amplification with the original primers for TKY337 was low. Thus, this marker was analyzed using the following primers instead: forward primer, 5′-TAAGACTCAAGAGGTCAATC-3′ and reverse primer, 5′-TACTCTCCAACTCTTCCACT-3′. The coefficient of disequilibrium (D') was calculated using SNPAlyze ver 8.0 (Dynacom, Chiba, Japan) to confirm the level of linkage disequilibrium between markers. No strong linkage disequilibrium was observed between any pair of markers and all markers were used for subsequent analyses.

The 411-bp reference sequence of the mitochondrial DNA D-loop region between positions 15,437 and 15,847 was directly sequenced using the forward primer 5′-CTAGCTCCACCATCAACACC-3′ and the reverse primer 5′-ATGGCCCTGAAGAAAGAACC-3′ [16, 17, 19]. The amplicon was sequenced using a BigDye® Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems, Thermo Fisher Science, Waltham, MA, U.S.A.). Haplotype searches were performed as previously described [10, 16, 17, 19] using the Basic Local Alignment Search Tool of the National Center for Biotechnology Information.

Using GENEPOP version 4.2 [14], the number of alleles (Na), observed heterozygosity (Ho), expected heterozygosity (He), and fixation index (F_{IS}), [13, 23] were determined, and deviations from the Hardy-Weinberg Equilibrium and linkage disequilibrium between the marker pairs were confirmed. LEX3 was excluded from the remaining analyses, because it is on the X chromosome.

The presence or absence of a past genetic bottleneck was assessed using BOTTLENECK version 1.2.02. For the assessment, we confirmed three assumption models: infinite allele model (IAM), stepwise mutation model (SMM), and two-phase model (TPM) [3, 20].

The genetic distance between each pair of individuals was calculated based on the proportion of shared alleles (D_{SA}) using Populations 1.2.30 [11]. To visualize the relationships between individuals [1], a neighbor-joining (NJ) phylogenetic tree was created using MEGA 6.06 [21]. In NJ phylogenetic trees, individuals that are closely related, such as parent-offspring pairs and siblings, are placed close together. Therefore, NJ phylogenetic trees are useful for visually representing the genetic relationships between individuals.

No deviation from the Hardy-Weinberg Equilibrium was found by Hardy Weinberg Exact Tests for each marker in GENOPOP ($P>0.05$), suggesting that there was no null allele within the markers. Moreover, GENOPOP revealed that the average Na of Misaki horses was 3.4 and ranged from 1 to 6, the average Ho was 0.509 and ranged from 0.000 for HMS3 and LEX33 to 0.883 for TKY341, the average He was 0.497 and ranged from 0.000 for HMS3 and LEX33 to 0.762 for TKY28, and the mean F_{IS} was −0.021 (Table 1).

The probability values obtained in the assessment for the excess of genetic diversity tested using the Wilcoxon signed-rank test under the 3 models, IAM, SMM, and TPM, were <0.0010, 0.038, and <0.001, respectively. The distribution of allele frequencies across all loci formed a normal "L-shaped" distribution, indicating the existence of a relatively large number of rare alleles [12]. Thus, it was concluded that Misaki horses may have experienced a bottleneck, but it was neither a severe nor recent one.

The D_{SA} showing the genetic distance between individuals was determined. Using D_{SA}, an NJ phylogenetic tree of individuals was successfully constructed (Fig. 3), and the genetic distance between individuals was visualized.

The mitochondrial DNA sequencing showed that the Misaki horse population had three mitochondrial DNA haplotypes. These haplotypes were consistent with the results reported by Kakoi et al.: AB329588 (H2), AB329624 (H10), and AB329596 (H44) [9]. The number of horses with mitochondrial haplotypes AB329588 (H2), AB329624 (H10), and AB329596 (H44) was 15, 31, and 31, respectively.

In this study, we successfully genotyped all 77 Misaki horses, which enabled us to identify individuals and provided basic information to establish a Misaki horse studbook. Moreover, the data characterized the genetic background of the Misaki horse population that has been isolated and resident at Cape Toi for many years.

Identification of individuals had previously been conducted by the Council for the Protection of Misaki Horses. However,

Table 1. The number of alleles (Na), observed heterozygosity (Ho), expected heterozygosity (He), and fixation index (F_{IS}) in each locus

| Locus	Na	Ho	He	F_{IS}
AHT4	4	0.247	0.267	0.075
AHT5	4	0.481	0.414	−0.162
ASB17	6	0.701	0.726	0.033
ASB2	4	0.714	0.679	−0.052
ASB23	4	0.584	0.565	−0.034
CA425	3	0.532	0.482	−0.104
HMS2	3	0.545	0.527	−0.035
HMS3	1	0.000	0.000	-
HMS6	3	0.455	0.467	0.026
HMS7	5	0.805	0.739	−0.090
HTG10	4	0.740	0.691	−0.072
HTG4	3	0.273	0.264	−0.035
LEX33	1	0.000	0.000	-
TKY19	3	0.403	0.419	0.038
TKY279	5	0.532	0.523	−0.018
TKY28	5	0.727	0.762	0.045
TKY287	2	0.468	0.490	0.045
TKY294	4	0.558	0.543	−0.029
TKY297	3	0.545	0.500	−0.092
TKY301	2	0.312	0.281	−0.111
TKY312	4	0.649	0.601	−0.081
TKY321	3	0.519	0.572	0.091
TKY325	3	0.545	0.589	0.074
TKY333	5	0.662	0.664	0.002
TKY337	2	0.195	0.196	0.008
TKY341	5	0.883	0.724	−0.220
TKY343	3	0.545	0.517	−0.056
TKY344	3	0.610	0.660	0.075
TKY374	2	0.377	0.451	0.166
TKY394	3	0.688	0.625	−0.101
VHL20	4	0.468	0.468	0.001
Mean	3	0.509	0.497	−0.021
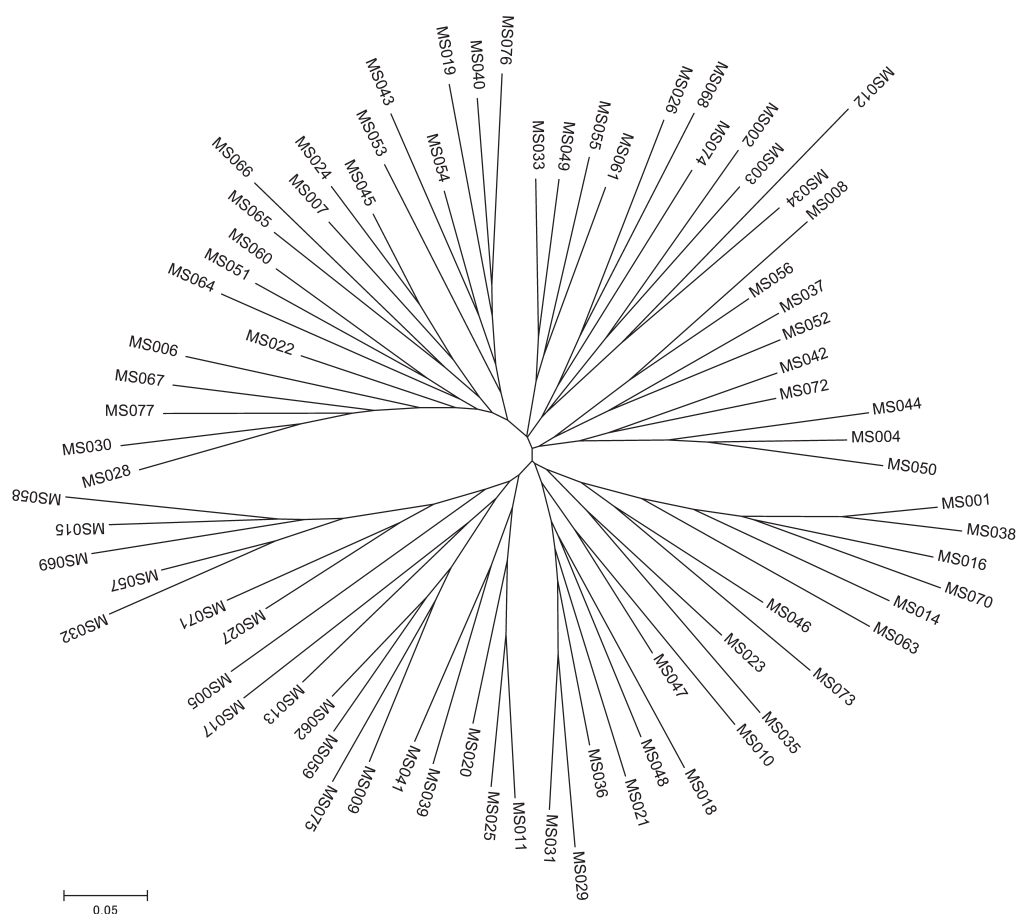
Fig. 3. A neighbor-joining phylogenetic tree of the genetic distances (D_{SA}) among individual Misaki horses. The numbers in the tree are the identification numbers of individual horses.

because Misaki horses are feral, the determination of paternal lines was difficult, and a studbook was not established. In this study, we acquired genetic data from almost all of the horses, including stallions, so that determination of the parentage using microsatellites was possible from blood or hair samples of foals and their dams. Consequently, establishing a studbook authorized by the Japan Equine Affairs Association, which requires clarification of ancestors back to three generations, is possible if we continue to monitor the Misaki horse population using microsatellites.

Although a stallion named Komatsu, half Standard bred and half Japanese breed (Hokkaido × extinct Nanbu), had been introduced for a year in 1913, Misaki horses have been a closed population for more than 300 years [8]. Therefore, the Na, Ho, and He of Misaki horses were as low as 3.3, 0.509, and 0.497, respectively; These indicators were lower than those for Yonaguni horses (Na=4.4, Ho=0.591, and He=0.601; N=78) that had been isolated on Yonaguni Island for a long time [18], and were much lower than those for other Japanese native horses that were used to reconstruct the population: Miyako horses (Na=4.2, Ho=0.701, and He=0.649; N=35) [16] and Kiso horses (Na=6.3, Ho=0.674, and He=0.662; N=125) [18]. In addition, only three maternal lineages were observed in the Misaki horses, including one haplotype that has not been confirmed in other horses, suggesting isolation of the population at Cape Toi. Consequently, our genetic data support the history of the horses, being isolated for years and inbred within the environment, and suggest that the Misaki horses have historical value.

Although management of the Misaki horses is undertaken without direct human intervention, the number of horses has changed in accordance with human management of the Cape environment. Around the time of the Second World War, poor maintenance of the environment decreased the grassland area, and the number of Misaki horses decreased sharply [8]. In this study, the results suggest that the Misaki horses had experienced a bottleneck, but the allele frequency across all loci formed a normal "L-shaped" distribution. These indeterminate results suggest that the Misaki horse population was small and slightly reduced in the past, reflecting the history of Misaki horses after the war: the population of Misaki horses was reduced concurrently with less direct human management of the environment after the war. Therefore, maintenance of the Cape Toi environment plays a crucial role in the conservation of Misaki horses.

The genetic relationships among individual horses were visualized using an NJ phylogenetic tree. This visible genetic information could be a tool for the Council for the Protection of Misaki Horses to carry out scientific management of individual horse numbers in the Cape. To date, the council has released some horses from the Cape, but candidates are selected based on the

subjective opinions of the council. However, using our D_{SA} data, this decision will be more objective. Referral to the raw D_{SA} data is needed for an accurate assessment, but such complicated data are not straightforward for the council members to interpret. Thus, we have presented NJ data that clearly distinguish the D_{SA} of the horses at one glance.

In addition, Misaki horses had three mitochondrial haplotypes revealing their maternal lineages, but it was difficult to retrospectively explain the distribution of these mitochondrial haplotypes within the population. Interestingly, one of the mitochondrial haplotypes was not seen in other native Japanese horses. As Misaki horses might not have experienced a severe bottleneck, this original haplotype may indicate ancestry in the horses and suggests that Misaki horses are unique genetic resources of the region.

For the sustainable conservation of Misaki horses, we should carefully consider our approach to their conservation to avoid unintended consequences [5]. Even if the number of horses increases, which is favorable for stakeholders, we need to discuss interactions between the horses and the environment that nurtures them. Increases in the number of horses also influence the environment, which will continue to change in the future. While such an increase might seem positive at first, it may bring unintended consequences. Thus, we should thoroughly and carefully consider sequential and balanced interactions between the number of feral horses and the environmental load.

REFERENCES

1. Bowcock, A. M., Ruiz-Linares, A., Tomfohrde, J., Minch, E., Kidd, J. R. and Cavalli-Sforza, L. L. 1994. High resolution of human evolutionary trees with polymorphic microsatellites. *Nature* **368**: 455–457. [Medline] [CrossRef]
2. Brown, W. M., George, M. Jr. and Wilson, A. C. 1979. Rapid evolution of animal mitochondrial DNA. *Proc. Natl. Acad. Sci. U.S.A.* **76**: 1967–1971. [Medline] [CrossRef]
3. Cornuet, J. M. and Luikart, G. 1996. Description and power analysis of two tests for detecting recent population bottlenecks from allele frequency data. *Genetics* **144**: 2001–2014. [Medline]
4. Fernández, J., Villanueva, B., Pong-Wong, R. and Toro, M. Á. 2005. Efficiency of the use of pedigree and molecular marker information in conservation programs. *Genetics* **170**: 1313–1321. [Medline] [CrossRef]
5. Frankham, R., Ballou, J. D. and Briscoe, D. A. 2009. Genetic diversity. pp. 41–65. *In*: Introduction to Conservation Genetics, 2nd ed., Cambridge University Press, Cambridge.
6. Goldstein, D. B., Ruiz Linares, A., Cavalli-Sforza, L. L. and Feldman, M. W. 1995. An evaluation of genetic distances for use with microsatellite loci. *Genetics* **139**: 463–471. [Medline]
7. Hutchison, C. A. 3rd., Newbold, J. E., Potter, S. S. and Edgell, M. H. 1974. Maternal inheritance of mammalian mitochondrial DNA. *Nature* **251**: 536–538. [Medline] [CrossRef]
8. Japan Equine Association. 1984. Japanese Native Horses—Its Conservation and Usage. pp. 1–243. Japan Equine Association, Tokyo (in Japanese).
9. Kakoi, H., Nagata, S. and Kurosawa, M. 2001. DNA typing with 17 microsatellites for parentage verification of racehorses in Japan. *Anim. Sci. J.* **72**: 453–460.
10. Kakoi, H., Tozaki, T. and Gawahara, H. 2007. Molecular analysis using mitochondrial DNA and microsatellites to infer the formation process of Japanese native horse populations. *Biochem. Genet.* **45**: 375–395. [Medline] [CrossRef]
11. Langella, O. 2002. POPULATIONS 1.2.31. Population genetic software CNRS UPR9034. http://bioinformatics.org/~tryphon/populations/ [accessed on September 11, 2018].
12. Luikart, G., Sherwin, W. B., Steele, B. M. and Allendorf, F. W. 1998. Usefulness of molecular markers for detecting population bottlenecks via monitoring genetic change. *Mol. Ecol.* **7**: 963–974. [Medline] [CrossRef]
13. Nei, M. and Chesser, R. K. 1983. Estimation of fixation indices and gene diversities. *Ann. Hum. Genet.* **47**: 253–259. [Medline] [CrossRef]
14. Raymond, M. and Rousset, F. 1995. GENEPOP (version 1.2): population genetics software for exact tests and ecumenicism. *J. Hered.* **86**: 248–249. [CrossRef]
15. Scherf, B. D. 2000. Farm animal genetic resources. pp. 37–646. *In*: World Watch List of Domestic Animal Diversity, 3rd ed., Food and Agriculture Organization of the United Nations, Rome.
16. Senju, N., Tozaki, T., Kakoi, H., Almunia, J., Maeda, M., Matsuyama, R. and Takasu, M. 2017. Genetic characterization of the Miyako horse based on polymorphisms of microsatellites and mitochondrial DNA. *J. Vet. Med. Sci.* **79**: 218–223. [Medline] [CrossRef]
17. Senju, N., Tozaki, T., Kakoi, H., Shinjo, A., Matsuyama, R., Almunia, J. and Takasu, M. 2017. Genetic diversity of the Yonaguni horse based on polymorphisms in microsatellites and mitochondrial DNA. *J. Vet. Med. Sci.* **79**: 425–431. [Medline] [CrossRef]
18. Takasu, M., Hiramatsu, N., Tozaki, T., Kakoi, H., Nakagawa, T., Hasegawa, T., Huricha., Maeda, M., Murase, T. and Mukoyama, H. 2012. Genetic characterization of the endangered Kiso horse using 31 microsatellite DNAs. *J. Vet. Med. Sci.* **74**: 161–166. [Medline] [CrossRef]
19. Takasu, M., Ishihara, N., Tozaki, T., Kakoi, H., Maeda, M. and Mukoyama, H. 2014. Genetic diversity of maternal lineage in the endangered Kiso horse based on polymorphism of the mitochondrial DNA D-loop region. *J. Vet. Med. Sci.* **76**: 1451–1456. [Medline] [CrossRef]
20. Takezaki, N. and Nei, M. 1996. Genetic distances and reconstruction of phylogenetic trees from microsatellite DNA. *Genetics* **144**: 389–399. [Medline]
21. Tamura, K., Stecher, G., Peterson, D., Filipski, A. and Kumar, S. 2013. MEGA6: Molecular Evolutionary Genetics Analysis version 6.0. *Mol. Biol. Evol.* **30**: 2725–2729. [Medline] [CrossRef]
22. Tozaki, T., Kakoi, H., Mashima, S., Hirota, K., Hasegawa, T., Ishida, N., Miura, N., Choi-Miura, N. H. and Tomita, M. 2001. Population study and validation of paternity testing for Thoroughbred horses by 15 microsatellite loci. *J. Vet. Med. Sci.* **63**: 1191–1197. [Medline] [CrossRef]
23. Weir, B. S. and Cockerham, C. C. 1984. Estimating F-statistics for the analysis of population structure. *Evolution* **38**: 1358–1370. [Medline]