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Genetic variation of the native Rusa deer (*Rusa timorensis*) in Java and Bali (Indonesia) as revealed using non-invasive sampling

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Abstract. *Iman MH, Kuswandi PC, Subrata SA. 2024. Genetic variation of the native Rusa deer* (Rusa timorensis) *in Java and Bali (Indonesia) as revealed using non-invasive sampling. Biodiversitas 25: 355-360.* Rusa deer is a vulnerable species with a large geographic range but natively inhabits Java and Bali. Despite the wide distribution, its native population is declining, raising a concern about a small population's adverse genetic effect. It encourages genetic studies to provide baseline data that has been vacant recently. This research aimed to demonstrate an application of non-invasive sampling to collect DNA samples and a simple procedure to obtain and analyze genetic data for the Rusa deer. This research also aimed to provide genetic variation of the native deer population as baseline data. The research sites were Baluran, Alas Purwo in East Java, and Bali Barat national parks from which fecal samples were collected. Moreover, 20 DNA samples were isolated from the feces using a kit (Dneasy PowerSoil Pro from Qiagen) and amplified at the control region gene using a forward: AAACCAGAAAAGGAGAGCAAC and a reverse: TCATCTAGGCATTTTCAGTGCC primer. The amplicons were sequenced, and the number of Haplotypes (Hn), Haplotype diversity (Hd), nucleotide diversity (π), site polymorphism, and phylogeographic tree were determined. The result showed that all the sequences had coverage of 100% and identity >98% with the Rusa timorensis sequence available in the GenBank. Furthermore, we found Hn = 11, Hd = 0.88, π = 0.005 and 30 site polymorphisms. Therefore, compared to an introduced population, the Rusa deer has a richer Hd and higher site polymorphism but a poorer π. Furthermore, we found that the Baluran population had high Hd, π, and is possibly forming a distinct clade.

Keywords: D-loop, diversity, feces, mtDNA, threatened

INTRODUCTION

Rusa deer (*Rusa timorensis* Blainville, 1822) is a protected and vulnerable species with a large geographic range but natively inhabits only Java and Bali (Corbet and Hill 1992). Limited range and declining population are the main reasons for its vulnerability. This species has been widely introduced and thrived in many areas outside its native range, including Australia, Brazil, Papua, Kalimantan, Sulawesi, Lesser Sunda Islands, Maluku, Malaysia, Mauritius, New Caledonia, New Zealand, Thailand, and Timor-Leste (Hedges et al. 2015). Highly adaptable traits and economic value are likely the motive of the introductions (Ali et al. 2021). Meanwhile, in the native range, the deer populations are not evenly distributed, but condensed into sparsely distributed conservation areas of Java and Bali (Rahman et al. 2020). Despite the large size of introduced populations, its native population is declining due to habitat degradation, fragmentation, and illegal hunting in those conservation areas (Hedges et al. 2015). The recent population size is estimated not to exceed 10,000 individuals and is spread over no more than ten populations in Java and Bali. The population size is likely still declining despite various conservation, such as cooperative programs and partnerships with local communities to prevent poaching and protect the areas, have been conducted (Hedges et al. 2015).

The consequence of those population dynamics on the genetic variation is unclear because further information about it is unavailable. This raises concerns about the adverse genetic effects of small populations, including inbreeding depression and low genetic diversity (Fitzpatrick and Funk 2019). The effects may decrease the deer reproductive fitness, as has been observed in other species of deer, such as Eld's deer (Angom et al. 2017) and red deer (Edelhoff et al. 2020). Furthermore, it was supposed that there is a bottleneck (Hedges et al. 2015), and founder effects may impede genetic recovery in regrowth populations. Consequently, evaluating genetic variation in conjunction with routine population monitoring is crucial to anticipate and mitigate these unfavorable outcomes.

Genetic assessment and monitoring necessitate a baseline to compare data and an established practical technique enabling repetitive studies. Unfortunately, there is no genetic information available from the native deer population. Although the information has been provided in several reports of the introduced population, such as the works of Zein (2007) and de Garine-Wichatitsky et al. (2009), no data is available from the Java and Bali populations. This lack of basic information disallows data comparison to assess the level of genetic variation. It hampers the understanding of the effect of population management, such as introduction and re-stocking, and illegal hunting on the genetic variation of the deer. Likewise, phylogenetic

analysis to gain insights into the process of local adaptation is currently unavailable. Moreover, technical difficulties likely hamper genetic data collection, as Khan and Tyagi (2021) indicated. This challenge demands establishing inexpensive and practical techniques to obtain genetic data. Non-invasive genetic sampling combined with simple data analysis may be adequate to provide basic genetic data. This technique has been commonly applied to assess the genetic variation of several deer species, including hog deer (Gupta et al. 2022) and pampas deer (*Ozotoceros bezoarticus*) (de Fátima Sallum Leandro et al. 2022). It has provided supportive information for its population management.

This research aimed to demonstrate the application of non-invasive sampling to collect DNA samples and a simple procedure to obtain and analyze genetic data for the Rusa deer. This research also aimed to assess the genetic variation of the native deer population as baseline data. We used fecal samples as a DNA source, amplified and sequenced them at the control region, and analyzed the sequence data to assess genetic variation. This simple procedure sufficiently provides basic genetic information useful for conservation measures of the deer.

MATERIALS AND METHODS

Study area

The research was conducted in the Baluran, Alas Purwo, and Bali Barat National Parks (114°20'-114°30′E and 7°45′S-8°45′S; Figure 1). In these parks, the Rusa deer mostly inhabits the savannahs, tropical monsoon, and lowland forests. Community pressure on these parks has recently been high, threatening their habitat and population (Hedges et al. 2015).

Procedures

Feces collection and preservation

In those sites, ten to twenty fresh feces samples of the deer per stool were collected during fieldwork from March to April 2022. The freshness of the samples was indicated by the moist surface. The sample collections were conducted in the early morning at the spots where the deer population was observed on the previous day, and the samples were collected systematically following north-direction line transects. A point (1-meter radius) was laid every 5 meters along the transect and spaced 10 m. During the fieldwork, the fecal sample of the deer was examined and identified based on its physical characteristics. Only pellets with uniform size and appearance were selected and carefully collected. Subsequently, these samples were pooled and preserved in 50 mL conical tubes filled with 96% ethanol. Additional information regarding geographic coordinates, time of collection, the freshness of the feces, and the picture of each sample was recorded.

DNA isolation, amplification, and sequencing

The DNA from the feces samples was isolated using a DNA extraction kit (Dneasy PowerSoil Pro®, Qiagen GmbH, Hilden, Germany) following the manufacture protocol with a minor adjustment of an overnight incubation. Meanwhile, the DNA concentration was measured using a fluorometer (Quantus®, Promega Corp., US). Isolated DNA was amplified at the control region gene using a specific primer for Rusa D-Loop mtDNA with forward primer: AAACCAGAAAAGGAGAGCAAC and reverse primer: TCATCTAGGCATTTTCAGTGCC (Zein 2007). The Polymerase Chain Reaction (PCR) was conducted using a thermocycler (Multigene Optimax, Labnet International, US) involving 12.5 µL pre-mixed PCR reagent (PowerPol 2X PCR Mix; ABClonal Technology, US), 8.5 µL ddH_2O, 0.5 µL each of forward and reverse primer (0.2 µ M), and 3 µL DNA template, with a total volume of 25 µL. Furthermore, the PCR was conducted in 35 cycles, including pre-denaturation (95°C; 5m), denaturation (95°C; 30s), annealing 60°C for the 30s, elongation (72°C; 1m), and post elongation (72°C; 10m). Amplicons were visualized in the 2% agarose gel at 100 volts within 30 minutes using an electrophoresis apparatus (RunView®, Cleaver Scientific Ltd., UK). After electrophoresis, the sequence of the amplicons was determined using the Sanger sequencer (The Applied Biosystem™ 3500 Genetic Analyzer; Thermo Fisher, US). Subsequently, the sequence data was used to identify alleles for further genetic variation analysis.

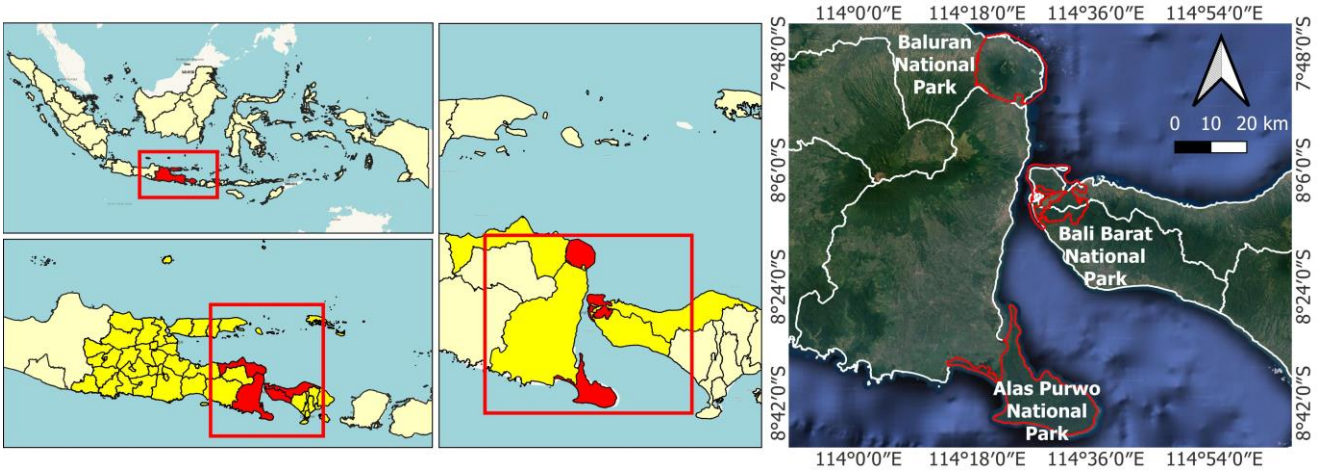

Figure 1. Research sites include Baluran, Alas Purwo, and Bali Barat National Park in Java and Bali Island, Indonesia

Data analysis

Sequence data were edited and aligned using BioEdit (Hall 2004) version 7.2.5. Genetic variation, including polymorphic sites, number of Haplotypes (Hn), Haplotype (Hd), and nucleotide diversity (π), was calculated using DnaSP (Rozas et al. 2017) version 6.12.03. Meanwhile, the phylogeographic tree was reconstructed using MEGA-X (Kumar et al. 2018) version 10.2.5 involving Rusa deer sequence (AF291883), Sambar deer (MF177004, MF177003, MF177009), Sika deer (AF291878, AF291881, AF291879) sequences available in the GenBank of The National Center for Biotechnology Information (NCBI) database.

RESULTS AND DISCUSSION

Sample identification using PCR

We successfully amplified the DNA of the control region gene from feces samples collected in the field. Of 210 feces collected from the field, we sorted fresh feces and randomly selected 20 representing three populations. The feces yielded genomic DNA with varying concentrations from 3.48-281.00 ng/uL (average = 58.14 ng/uL). All sample were successfully amplified at the control region gene resulting in amplicons of 1,200 base pairs. Although the amplicon concentration was varied, the length was consistent as expected, as shown by gel visualization in Figure 2. Furthermore, after data preparations, we found that only 769 of the 1,200 base pairs of the sequence were appropriate for further analysis. The first analysis was to check the identity of the sequence against the NCBI database. The result showed that all the amplified sequence has coverage of 100% and identity >98% with *Rusa timorensis* (JN6326990).

Genetic variation

Further analysis showed that the overall population has a high level of haplotype (Hd) but a low level of nucleotide diversity (π). All samples (n=20) can be assigned to 11 haplotypes and had a large Hd but small π (Table 1). Polymorphism was observed in 30 sites. Furthermore, we found no haplotype sharing among populations (Table 2).

We observed each population and found different co-occurrence patterns of Hd, π, and Hn. Baluran had a large Hd and π. Alas Purwo population had a large Hd but small π, meanwhile Bali Barat had a small Hd, and π. Regarding haplotype number, Baluran was the largest compared to other populations (Table 1).

Based on the phylogeographic tree, we found that all the sequences diverged from a branch point, indicating that all individuals had the same ancestral root. Furthermore, although we found no shared haplotype among populations, the haplotypes can be arranged into three groups (clades) based on their relatedness (Figure 3). Each group consisted of haplotypes originating from different populations. The first group consisted mainly of Alas Purwo (haplotypes 1, 2, and 3) and only a minority from Baluran (haplotype 4) population. The second group was composed mostly of Bali Barat (haplotypes 10 and 11) and only a haplotype from Baluran (haplotype 5) populations. Both groups may represent a clade. Meanwhile, the last group was a distinct clade comprised mostly of Baluran (haplotypes 6 and 8) and a minority from Bali Barat (haplotype 9) populations. Haplotypes from the Baluran population were common in all groups.

Discussion

Our finding indicates the suitability of a non-invasive sampling strategy for the genetic assessment of the Rusa deer, particularly at the mitochondrial genes. These genes follow a maternal line heredity pattern and are not recombined. Therefore, they are suitable for phylogenetic analysis. Additionally, the high abundance of mitochondrial DNA within cells facilitates the amplification process considerably. Besides convenience, the strategy has practical advantages in less cost and time consuming (Ferreira et al. 2018) and minimal disturbance (Banks and Piggott 2022) to obtain DNA from wild animals. However, despite the advantages, a previous study showed concern about the issue of low DNA quality and quantity obtained using non-invasive sampling (Ando et al. 2020). Particularly when applying the strategy in a tropical environment, rapid DNA degradation due to high UV and humidity exposure complicated these challenges (Goossens and Salgado-Lynn 2013). However, those issues only moderately affect mitochondrial DNA analysis because it has a higher DNA copy within a cell than nuclear DNA (Linacre and Tobe 2013). Furthermore, the low DNA quality and quantity issue is mitigated by processing only fresh samples (about <24 hours), as indicated by the moist or shiny surfaces of the feces. A previous study suggested collecting feces that age less than 24 hours will get the optimum deer species DNA (Nugroho et al. 2022). We also used a commercially silica-based isolation kit to extract DNA from feces. The kit maximizes the quality and quantity of the yield meanwhile minimizing PCR inhibitor. Furthermore, in this recent study, we reduce the feces misidentification opportunity by carefully selecting feces samples that represent individual Rusa deer feces based on visual recognition. Before collecting the samples in the field, we visited a local deer farm to train in obtaining appearance records (size and color) relevant to species and feces age. Based on these records, we were able to select appropriate samples. Not only does avoiding feces misidentification, but this step also results in better recognition of the freshness of the feces. Our anticipation resulted in optimal DNA sequences adequate for mitochondrial genetic analysis of the Rusa deer. We suggest applying this technique for a larger sample size representing most of Indonesia's geographic deer distribution. The genetic information resulting from such a study is invaluable to determining the level of genetic variation, setting up a conservation management unit for the deer, and resolving the issue of the under-species taxonomy of the deer (Hedges et al. 2015).

Moreover, compared to the introduced populations, the sampled Rusa deer has a richer Hd and higher site polymorphism but a poorer π. Using the same genetic marker and sample size, a previous study on the introduced population of Rusa deer in east Nusa Tenggara, Indonesia, reported 8 haplotypes (Hn), 16 sites of nucleotide

polymorphism, Hd = 0.056, and π = 0.039 (Zein 2007). A larger population size of the native population likely causes a richer genetic variation as measured by Hn, Hd, and site polymorphism. Meanwhile, π likely uncorrelated with the population size, as Grant and Bowen (1998) reported. The finding also shows that although the introduced population has a lower population size, they do not experience the founder effect. It is likely that their parent populations already have a high genetic variation.

Furthermore, compared to other wild populations of Cervidae in Asia, the number of haplotypes (Hn) of the Rusa deer is higher than Hog and Red deer, but lower than other deer species. Meanwhile, the Hd of the Rusa deer is higher than Eld's, Hog, and Swamp deer. The noticeable finding is that π the Rusa deer is the smallest (Table 3). It reveals that the Rusa deer population might have declined severely in the past causing a bottleneck effect and substantial reduction of genetic variation, but the population is growing recently. It suggests a strong capability of the deer to adapt to severe population disturbances in Java. However, the lower level of genetic variation may limit the adaptation.

Phylogeographic analysis shows that historically, all Rusa deer originate from the same ancestor and then diverge into three genetic groups. The haplotypes from the Baluran population in all groups denote those genetic lineages. Furthermore, we found a few haplotype sharing among populations, demonstrating a low possibility of gene flow of female Rusa deer after the divergence. It seems that the male-biased dispersal trait, as Spaggiari and de Garine-Wichatitsky (2006) reported in the New Caledonia populations, causes the retention of mitochondrial genes within their natal populations. As our finding is based only on a maternally inherited marker, it may well represent a historical evolution of the deer but conveys an incomplete assessment of the genetic variation of the deer. Further studies based on biallelic markers such as microsatellites and SNP are suggested to discover a finer resolution of genetic diversity, population structure, and biased gene flow. Additionally, natural dispersal is unlikely the sole determinant of gene flow as the introduction has been common for this species since long ago (Martins et al. 2018). Particularly in our research sites, the national parks recently implemented an introduction as part of their restocking programs (pers. comm). It is plausible that the introduction may have an impact on the observed genetic variation, which could potentially distort the effect of natural dispersals.

Table 1. Haplotype and nucleotide diversity of the Rusa deer.

Population	Genetic diversity			
	n	Hn	Hd	π
Alas Purwo	4	3	0.83	0.002
Baluran	8	5	0.78	0.009
Bali Barat	8	3	0.46	0.002
All	20	11	0.88	0.005

Note: n: Number of sequences, Hn: Number of haplotypes, Hd: Haplotype diversity, π: Nucleotide diversity

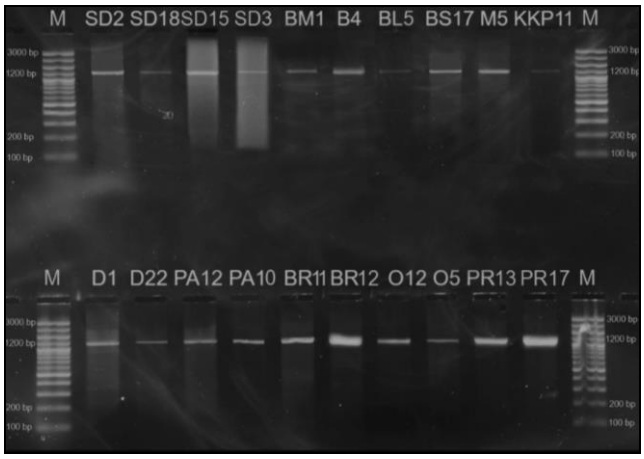

Figure 2. Gel visualization showing amplicon length as expected (1200 bp). Figures above the lanes represent sample identity

Table 2. Nucleotide sites polymorphism

Haplotype	Nucleotide position																											Individual	Population
						1	1	1	1	1	1	2	3	4	5	6	6	6	6	6	6	7	7	7	7	7	7	7		
			1	2	7	7	2	2	2	5	5	5	8	5	4	7	6	4	4	5	6	7	9	0	1	2	3	4	5	6	
	6	7	2	9	4	5	6	7	8	4	5	8	7	1	3	4	8	2	5	5	5	8	5	0	0	4	4	9	6	3	
AF291883*	A	G	T	G	G	C	A	G	T	C	C	C	T	T	C	C	T	C	A	T	C	T	A	C	T	A	T	G	C	C	
1	G	.	C	T	.	.	G	A	C	.	G	.	.	.	T	T	.	T	G	.	.	.	G	.	.	.	G	.	.	.	SD2, SD15	Alas Purwo
2	.	.	C	T	.	.	G	A	C	.	G	.	.	.	T	T	.	T	G	.	.	.	G	.	.	.	G	.	.	.	SD3	Alas Purwo
3	.	.	C	T	T	T	G	A	C	.	G	.	.	.	T	T	.	T	G	.	.	.	.	.	.	.	G	.	.	.	SD18	Alas Purwo
4	.	.	C	T	.	.	G	A	.	.	G	.	.	.	T	T	.	T	G	.	.	.	G	.	.	.	G	.	T	.	BM1, BL5, BS17, M5	Baluran
5	.	.	C	T	.	.	G	A	C	.	G	.	.	C	T	T	.	T	G	.	.	.	G	.	.	.	G	.	.	.	B4	Baluran
6	.	A	C	T	.	.	.	A	.	T	.	.	C	.	T	T	.	.	G	.	.	.	.	.	.	.	G	.	.	.	KKP11	Baluran
7	.	.	C	T	.	.	.	A	.	T	.	.	C	.	T	T	G	.	G	.	.	.	.	.	.	.	G	.	T	.	D1	Baluran
8	.	.	C	T	.	.	.	A	.	T	.	.	C	.	T	T	.	.	G	G	T	G	.	G	G	C	G	T	.	T	D22	Baluran
9	.	.	C	T	.	.	.	A	.	T	.	.	C	.	T	T	.	.	G	.	.	.	.	.	.	.	G	.	.	.	PA10	Bali Barat
10	.	.	C	T	.	.	G	A	.	.	G	T	.	.	T	T	.	T	G	.	.	.	G	.	.	.	G	.	.	.	PA12, BR11, O5, O12, PR13, PR17	Bali Barat
11	.	.	C	T	.	.	G	A	.	.	G	.	.	.	T	T	.	T	G	.	.	.	G	.	.	.	G	.	.	.	BR12	Bali Barat

Note: *) reference sequence retrieved from NCBI

Table 3. Genetic diversity of several wild species of Cervidae in Asia assessed at the mt-DNA control region

Species	Origin	*n*	*Hn*	*Hd*	π	Reference
Eld's deer (*Rucervus eldii* M'Clelland, 1842)	India	113	23	0.80	0.023	(Ghazi et al. 2021a)
Hog deer (*Axis porcinus* Zimmermann, 1780)	Katie Province, Cambodia	28	4	0.61	0.016	(Gupta et al. 2022)
Red deer (*Cervus elaphus* Linnaeus, 1758)	Baishitou, China	32	7	0.90	0.009	(Zhou et al. 2015)
Red muntjac (*Muntiacus vaginalis* Boddaert, 1785)	Indian Himalayan Region	107	17	0.90	0.016	(Singh et al. 2022)
Sambar deer (*Rusa unicolor* (Kerr, 1792))	India	168	56	0.94	0.029	(Ghazi et al. 2021b)
Swamp deer (*Rucervus duvaucelii* G.Cuvier, 1823)	India	90	13	0.81	0.021	(Kumar et al. 2017)

Note: n: Number of sequences, Hn: Number of haplotypes, Hd: Haplotype diversity, π: Nucleotide diversity

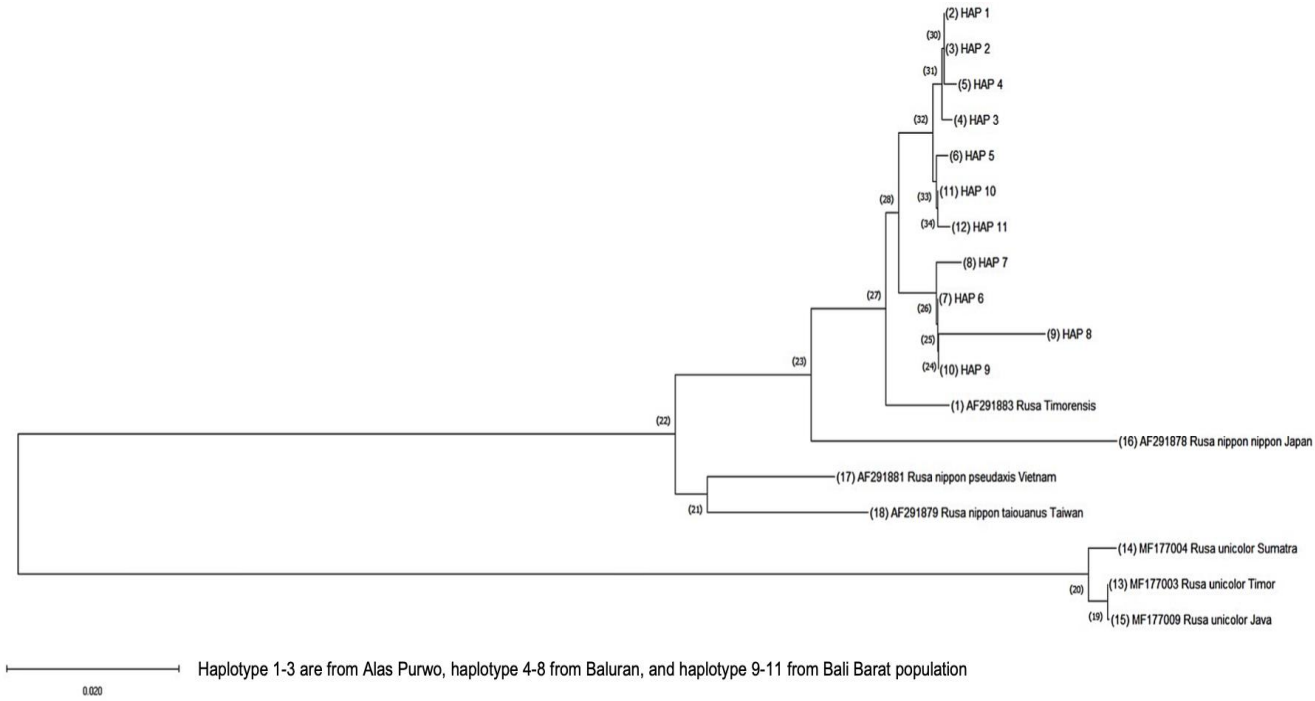

Figure 3. Phylogeographic tree reconstructed from sequences of the mt-DNA control region of the Rusa deer. Haplotypes with accession number were retrieved from the NCBI database

It is likely that the mt-DNA genetic variation of each population, as observed in Hn, Hd, and π, reflects population dynamics in the past. Hedges et al. (2015) reported that the Baluran deer population is estimated to be the largest population in Java. It has consequences on a higher Hn and Hd and possibly forms a distinct clade. Although it might be affected by population decline, those large genetic variation reflects a stable population with a long evolutionary history. Furthermore, this population might serve as individuals' main source, as shown by shared haplotypes in all other groups. Meanwhile, small Hd in Alas Purwo and Bali Barat reflect the possibility of population decline in the past. Likely, the Alas Purwo population recovered successfully, but Bali Barat did not, as reflected in large and small π respectively. These findings align with a report by Hedges et al. (2015).

Furthermore, we suggest managing the deer as a large population consisting of sub-populations. The Baluran population should be preserved as a genetic source, and corridors should be established to connect the surrounding sub-populations. The introduction of species by humans should only be considered as a secondary option after natural dispersal has been proven ineffective, for example, in the Bali Barat population. We conclude that non-invasive sampling is prospective for collecting genetic data practically and inexpensively. This technique reveals that the overall genetic variation of the native Rusa deer is still at a common level.

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