Research Article

Genetic Variation Within Four Captive Chital (*Axis axis*) Populations in Indonesia

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ABSTRACT

Chital is a native animal from South Asia. Chital had been introduced to many countries, including Indonesia. Chital was first introduced to Indonesia in 1811 at Bogor Palace and since had been kept captive around Indonesia. Currently, no research had been done concerning the genetic variation of Indonesian chital. Therefore, the purpose of this research was to analyze genetic variation and phylogenetic relationship of chital from Pusat Inovasi Agroteknologi Universitas Gadjah Mada (PIAT UGM), Prambanan Temple, Gembira Loka Zoo, and Bogor Palace, based on mitochondrial *D-loop* fragment. This study used a Polymerase Chain Reaction (PCR) method. DNA was extracted from faecal samples and amplified with L15995 and H16498 primers. The analysis used for this research were genetic variations, haplotype networking, and phylogenetic relationships between populations. This study detected 5 haplotypes out of 20 sequences with 10 polymorphic sites and 2 indels. The haplotype diversity and the nucleotide diversity were 0.443 and 0.002 respectively, and the genetic distance was between 0 and 2.03% (average 0.55%). This research also showed one main haplotype, labelled as haplotype 1, which consisted of all individuals from PIAT and Prambanan Temple, four individuals from Bogor Palace, and one individual from Gembira Loka. This grouping proves that the majority of chital population in Indonesia came from Bogor Palace. One individual from Gembira Loka has a considerable genetic divergence from the rest of the samples, which might indicate it originated from a different source population.

INTRODUCTION

Chital or also called spotted deer (*Axis axis*) is a native animal in South Asia. Even so, this animal has been introduced to many countries (Sankar & Acharya 2004). Chital in Indonesia was originally introduced by the British colonials to Bogor Palace in 1811 (Garsetiasih & Herlina 2005). In the present, chital can be seen around Indonesia, kept in zoos or as livestock (Suharto et al. 2019; Gembira Loka Zoo 2022)

As time goes by, a lot of changes occurred to the policies for captive animals. Animals were kept in captivity originally as a fulfilment of human needs, such as for recreational purposes or kept as livestock. Nowadays, animals are also kept in captivity as a conservation effort, including the animals in zoos (Keulartz 2015). Some animals are deliberately kept

to increase their population size to then be released into the wild. Even though this action is a good effort, the release of unfit animals into the wild could cause some problems. Without proper preparation, released animals cannot survive in the wild. This low survivability is caused by several factors, which could be different across species (Farquharson et al. 2021). The release of individuals from a captive population with high homozygosity can also reduce the genetic variation of the species in the wild. Therefore, genetic variation of captive animals should also be concerned (Purohit et al. 2021). Reduction in genetic diversity has been associated with an increased risk of extinction (Saccheri et al. 1998). To minimize loss of genetic diversity, several zoos have strategies and different methods for genetic conservations. Some of those are to prevent inbreeding, maintaining a high and constant population size in all generations, and population fragmentations, while also keeping track of the DNA of captive animals (Leus et al. 2011).

Genetic variation of chital using Mitochondrial DNA control region (*D-loop*) fragment target has been investigated in Pakistan, Australia, and Croatia, which they were also introduced (Abbas et al. 2016; Hill et al. 2019; Šprem et al. 2021). Meanwhile, no research has been done concerning the genetic variation of chital population in Indonesia. In addition, some captive areas in Indonesia often transfer their animals to and from other places, which can cause uncertainty about the population origin of those animals. Therefore, genetic characterization and genetic variation research need to be done for Indonesian chital to understand the genetic structure of those populations and to determine the origin of the animals. This study aimed to analyze the genetic variation and phylogenetic relationship of chital from Bogor Palace, Gembira Loka Zoo, PIAT UGM, and Prambanan Temple, based on mitochondrial *D-loop* fragment.

MATERIALS AND METHODS
Sample collection
Faecal samples from five individuals were collected each from Bogor Palace (BP2D, BP3D, BP4D, BP7D, and BP8D), Gembira Loka Zoo (GL3D, GL4D, GL6D, GL7D, GL11D), PIAT UGM (PI2D, PI3D, PI4D, PI5D, PI7D), and Prambanan Temple (PT1D, PT2D, PT4D, PT5D, PT10D), with the total of 20 samples. Each of these locations held its own captive chital in a closed enclosure. Three fresh faecal pellets were collected and stored in a stool collection tube with 96% ethanol as the preservative. The tubes were then stored inside a cooler box for transportation. Samples were then transported to the Laboratory of Genetics and Breeding (Faculty of Biology, Universitas Gadjah Mada) and kept inside a box at room temperature until the following process.

DNA extraction
Each faecal sample was removed from the stool collection tube and the surface was scraped using a sterile scalpel. The scraps were then collected in a total of about 160-240mg of materials for DNA extraction. The DNA extraction was done using QIAamp Fast DNA Stool Mini Kit (QIAGEN, Valencia, CA, USA) according to the manufacturer's protocol, with a modification of using 100 µL instead of 200 µL ATE buffer.

D-loop Amplification
The partial *D-loop* fragment was amplified using primers: L15995 (5'-CTCCACTATCAGCACCCAAAG-3') (Taberlet & Bouvet 1994) and H16498 (5'-CCTGAAGTAAGAACCAGATG-3') (Fumagalli et al.

1996), which are universal *D-loop* primers used for mammals (Harsini et al. 2017). PCR amplifications were performed using T100 Thermal Cycler (Biorad) with 25µL reaction volume consisting of 12.5 µL of MyTaq™ HS Red Mix (Bioline), 1 mM $MgCl_2$, 0.4 µM each of forward and reverse primer, 4.5 µL ddH_2O, and 5 µL template DNA (11.09-27.34 ng/ µL). The DNA amplification PCR profile following Arisuryanti et al. (2020) consisted of pre-denaturation of the template at 95°C for 1 minute, denaturation at 95°C for 15 seconds, annealing at 50°C for 30 seconds, extension at 72°C for 30 seconds, repeated for 35 cycles, and final extension at 72°C for 10 minutes. PCR product yields 410-455bp of amplicons.

Electrophoresis and Sequencing
Electrophoresis of the PCR products was run on 1% agarose gel stained with FloroSafe (Bioline) and buffered with Tris-Acetate EDTA (TAE) buffer at 100 volts for 25 minutes. Visualization was conducted under UV light. All amplified amplicons were sent to Apical Scientific Sdn. Bhd. (1st BASE, Malaysia) via P.T. Genetika Science Indonesia (Jakarta) for purification and sequencing using the Big Dye Terminator (Applied Biosystems) and the ABI 3730xl Genetic Analyzer (Applied Biosystems). Amplicons were sequenced through bi-direction using primers used for PCR amplification.

Sequence Editing
Sequences obtained were edited and the consensus sequences were made with GeneStudio program and validated with SeqMan and EditSeq on DNASTAR program (DNASTAR Inc., Madison, USA). Chromatograms were inspected manually to check ambiguous nucleotides.

Sequence Alignment
The consensus sequences were then analysed using Opal package (Wheeler & Kececioglu 2007) in MESQUITE ver. 3.6.1 program (Maddison & Maddison 2019) and ClustalW in MEGAX (Kumar et al. 2018).

Substitution Model Selection
An analysis of the most fitting substitution model was done using jModelTest2.1.10 (Darriba et al. 2012) based on the Bayesian Information Criterion (BIC).

Nucleotide Composition and Genetic Distance
Nucleotide composition and genetic distance were analysed using an already integrated function in MEGAX. Genetic distance was analysed using Tamura 3-Parameter with Gamma Distribution model (T92+G) with 1,000 bootstrap replicates.

Genetic Variation Analysis
Genetic variation analysis was done using DnaSP ver.6 program (Rozas et al. 2017). Parameters analysed in this research include haplotype number, number of polymorphic sites, haplotype diversity, and nucleotide diversity.

Haplotype Network and Principal Coordinate Analysis (PCoA)
Haplotype network and PCoA analysis were done using additional sequences from GenBank. The sequences used are shown in Table 1. Haplotype network was constructed using median joining network method in NETWORK ver 10.2. Principal Coordinat Analysis (PCoA) was done

using GenAlEx ver. 6.51b2 plugin for Microsoft Excel (Peakall & Smouse 2012).

Phylogenetic Analysis

Phylogenetic analyses were done using additional sequences from Gen-Bank. The sequences used are shown in Table 1. The phylogenetic tree was constructed using Maximum Likelihood (ML) and Bayesian Inference (BI) methods. Phylogenetic analysis with Maximum Likelihood was done in MEGAX program using Tamura 3-Parameter + Gamma Distribution with invariable sites (T92+G+1) with 1,000 bootstrap. Analysis with Bayesian Inference was done using BEAST v1.10.4 program (Suchard et al. 2018). Bayesian Inference analysis was done using Hasegawa-Kishino-Yano + Gamma Distribution model (HKY+G). The Markov Chain Monte Carlo (MCMC) was run for 10^7 generations with a sampling frequency set to every 1,000 generations. Phylogenetic tree visualization was done using FigTree v.1.4.4 program (Rambaut 2019).

RESULTS AND DISCUSSION

Genetic Variability

Analysis was done using 411 bp of *D-loop* fragment. The average nucleotide composition (Table 2) revealed a relatively similar composition between each population. This indicates no major mutation had occurred. Based on the genetic distance (Table 3), the chital from PIAT UGM and Prambanan Temple have identic sequences. Those populations had a relatively lower genetic distance compared to the population from Bogor Palace. Chital was first introduced to Indonesia in the Bogor Palace. Chital population in Bogor Palace had grown significantly and became difficult to be sustained inside the Bogor Palace area. Since then, some individuals had been transferred to several places in Indonesia to maintain the population size in Bogor Palace (Garsetiasih & Herlina 2005). This low genetic distance between population in Bogor Palace and both PIAT UGM and Prambanan Temple could indicate that the population in PIAT UGM and Prambanan Temple originated from Bogor Palace.

Based on the genetic variation analysis (Table 4), chital population in Bogor Palace has 2 haplotypes with 1 variable site, which is an indel. This population has a low haplotype diversity (Hd) value of 0.400. This finding is in line with previous research done in Australia (Hill et al. 2019) and Croatia (Šprem et al. 2021). Analysis using 576 bp of *D-loop* fragment on 35 individuals from Queensland, Australia found 2 haplotypes with 4 polymorphic sites, with Hd of 0.461±0.07 and nucleotide

Table 1. Sequences obtained from GenBank.

No	Accession Number	Species	Location	Author
1	MN226865	*Axis axis*	Australia	Hill et al. 2019
2	MN226866	*Axis axis*	Australia	Hill et al. 2020
3	MZ421332	*Axis axis*	Croatia	Šprem et al. 2021
4	MZ421333	*Axis axis*	Croatia	Šprem et al. 2021
5	JN596141	*Axis axis*	India	Kumar et al. (direct submission)
6	JN596142	*Axis axis*	India	Kumar et al. (direct submission)
7	MT998906	*Axis axis*	Island of Lanai, Hawaii	Buchholz et al. (unpublished)
8	MT998894	*Axis axis*	Texas	Buchholz et al. (unpublished)
9	MW348981*	*Rucervus duvaucelii*	India	Kumar et al. (direct submission)
10	MH392156*	*Axis porcinus*	India	Gupta et al. (direct submission

Note: *Used only for phylogenetic analysis as outgroup

diversity (π) value of 0.0023±0.0004 (Hill et al. 2019). Analysis using 576bp of *D-loop* fragment on 32 individuals from the Island of Rab, Croatia found 2 haplotypes with 7 polymorphic sites, with Hd value of 0.514 and π value of 0.006. Meanwhile, the analysis of 7 individuals from Dugi Otok, Croatia found 2 haplotypes with 7 polymorphic sites, with Hd value of 0.286 and π value of 0.004. Both of these populations from Croatia shared the same haplotypes (Šprem et al. 2021).

Table 2. Average nucleotide composition of 411 bp *D-loop* fragment of chital from Bogor Palace, Gembira Loka, PIAT UGM, and Prambanan Temple.

Population	T	C	A	G	A+T	G+C
Bogor Palace (n=5)	29.52	24.64	32.17	13.67	61.69	38.31
Gembira Loka (n=5)	29.45	24.62	32.28	13.65	61.73	38.27
PIAT UGM (n=5)	29.51	24.63	32.20	13.66	61.71	38.29
Prambanan Temple (n=5)	29.51	24.63	32.20	13.66	61.71	38.29

Table 3. Average genetic distance of chital from Bogor Palace, Gembira Loka, PIAT UGM, and Prambanan Temple.

	Bogor Palace	Gembira Loka	PIAT	Prambanan Temple
Bogor Palace				
Gembira Loka	0.55% (0-2.03%)			
PIAT UGM	0.05% (0-0.24%)	0.50% (0-1.77%)		
Prambanan Temple	0.05% (0-0.24%)	0.50% (0-1.77%)	0.00%	

Notes: The number inside the bracket represent the range of each samples genetic distance.

According to an article in Trubus magazine in 1996 (as cited in Garsetiasih & Herlina 2005), chital was introduced to Bogor Palace, Indonesia in 1811 with 6 pairs of individuals. This could cause a founder effect which could significantly reduce the genetic variability of the population. Those individuals were then allowed to breed with each other freely, and for 210 years, no additional individual was brought in from other populations (R.Y. Andini, personal communication, November 11, 2021). This process could then further reduce the genetic variability of the population, which led to the low diversity in the present. Bogor Palace has a relatively small area for the present chital population, which strictly limits the carrying capacity. According to Garsetiasih and Herlina (2005), Bogor Palace could ideally only support 169-286 individuals of chital, but the carrying capacity was fluctuating. Individuals from Bogor Palace were from time to time needed to be translocated to other places because the low carrying capacity could not support the population. The population is deliberately kept being around 400 to 600 individuals. Sometimes, a reduction of hundreds of individuals happened to establish this constant number (R.Y. Andini, personal communication, November 11, 2021). This could potentially become a genetic drift which further reduces the already low genetic variation. As most chitals from around Indonesia were translocated from Bogor Palace (R.Y. Andini, personal communication, November 11, 2021), the low genetic variation of this source population could further cause the low genetic variation of chital in Indonesia.

Compared to the population from Australia and Croatia, the Bogor

Palace population have a lower genetic variability. Chital in Indonesia shared a similar introduction history with the population from Australia and Croatia. Chital was introduced at two different times to Queensland in the 19th century (Hill et al. 2019). As Queensland received two founder populations two different times, this original population in the past might had more genetic diversity with more haplotypes compared to the population in Bogor Palace. As the founder population could have lower genetic diversity, this could explain why the same condition can be applied to the present. In Croatia, chital was first introduced to Brijuni Island in 1911. Eight individuals from Brijuni Island were then translocated to Rab in 1974. In 2012, 13 individuals from Brijuni Island escaped and were established on the island of Dugi Otok (Šprem et al. 2021). Since the population established in Croatia was introduced a century later than the one in Queensland and Bogor Palace, fewer generations had passed which means fewer genetic diversity loss probability. This could explain why the genetic diversity of Rab Island chital population is higher than the one from Bogor Palace and Queensland.

Based on the genetic diversity analysis (Table 4), the chital population in Gembira Loka Zoo has 4 haplotypes out of 5 samples with 9 variable sites. This population has the Hd value of 0.9 and π value of 0.00829. This Hd value is high, especially compared to the value from other populations in this study. Gembira Loka Zoo often received animals from BKSDA (Indonesian Natural Resources Conservation Center), which were confiscated from illegal keepers. Gembira Loka had received new chital individuals from BKSDA every couple of years for the past several years (B.R. Samuels, personal communications, April 21, 2022). These confiscated individuals were from unknown origins, hence it could be possible that some of these individuals did not originate from Bogor Palace population. This varied source of individuals could cause the high genetic diversity of this population.

Based on the genetic variability analysis (Table 4), the chital population in both PIAT UGM and Prambanan Temple shared similar haplotypes (haplotype 1) and were the only haplotype observed in both populations. This haplotype was also shared with the Bogor Palace population. PIAT UGM and Prambanan Temple both received the first few individuals from Bogor Palace around the late 2000s to early 2010s. Both populations then grew to around 50 to 60 individuals around mid 2020 to early 2021 (Adji & Astuti 2020; D. Sutanto, personal communication, November 1, 2021). The chital population from Bogor Palace already have a low genetic variation. Since Bogor Palace is the source population and the transfer was relatively recent, the few individuals which were transferred to Prambanan Temple and PIAT UGM might already have a very low genetic variability. This could explain why only 1 haplotype exists in Prambanan Temple and PIAT UGM in the present and how it shares the

Table 4. Genetic diversity indices based on 411 bp *D-loop* fragment of chital populations from Bogor Palace, Gembira Loka, PIAT UGM, and Prambanan Temple.

Population	Bogor Palace	Gembira Loka	PIAT UGM	Prambanan Temple	All Samples
n	5	5	5	5	20
Nh	2	4	1	1	5
S	1	9	0	0	10
Hd	0.400	0.900	0.000	0.000	0.442
π	0.000	0.008	0.000	0.000	0.002

Notes: n. number of samples; Nh. Number of haplotypes; S. number of polymorphic sites; Hd. Haplotype diversity; π. Nucleotide diversity.
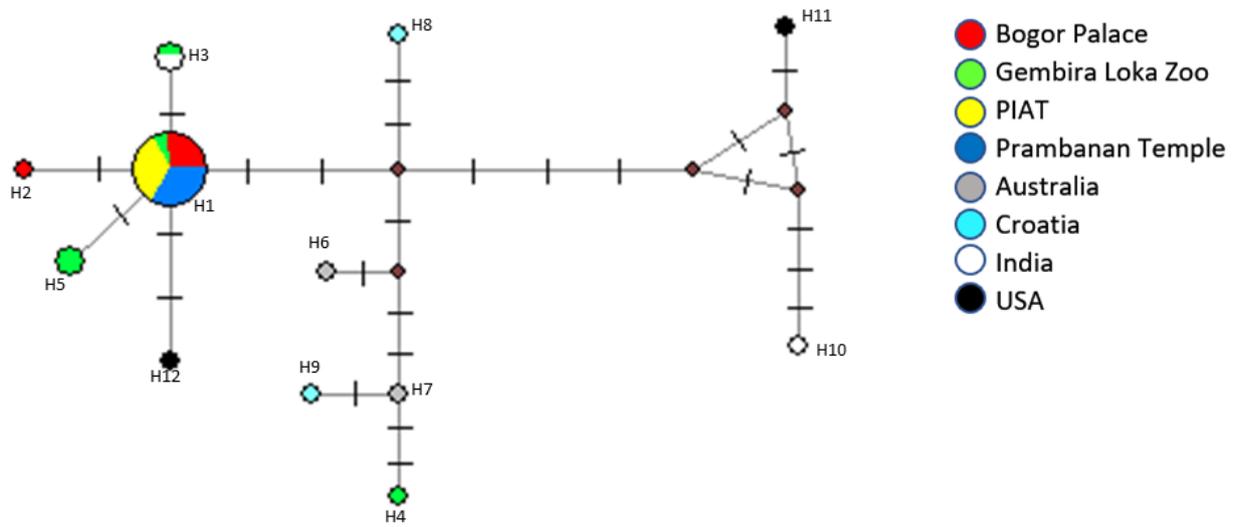
Figure 1. Haplotype network based on 401bp *D-loop* fragment of chital from this study and sequences obtained from NCBI GenBank.

haplotype with Bogor Palace population. Furthermore, both of these populations had undergone mass mortality events. The population from PIAT UGM recently underwent mass mortality of more than 30 individuals which was caused by the disease. A large number of fawns from Prambanan Temple often died by drowning in the pool. Both of these events could become a bottleneck effect that eliminates several haplotypes from these populations if ever existed.

Phylogenetic Relationship
Phylogenetic relationships were analysed using 401 bp of *D-loop* fragment. The haplotype network (Figure 1) reveals the main haplogroup which consists of most samples from this study. Haplotype 1 is shared between all study populations. Haplotype 2, 3, and 5 only have 1 mutation step from haplotype 1. Haplotype 3 is shared between population from Gembira Loka Zoo and from India. Haplotype 4 which consists of 1 individual from Gembira Loka Zoo (GL6D) is relatively far from the main group, where it is closer to a population from Australia. Principal Coordinate Analysis (Figure 2) shows a similar result with the haplotype network.
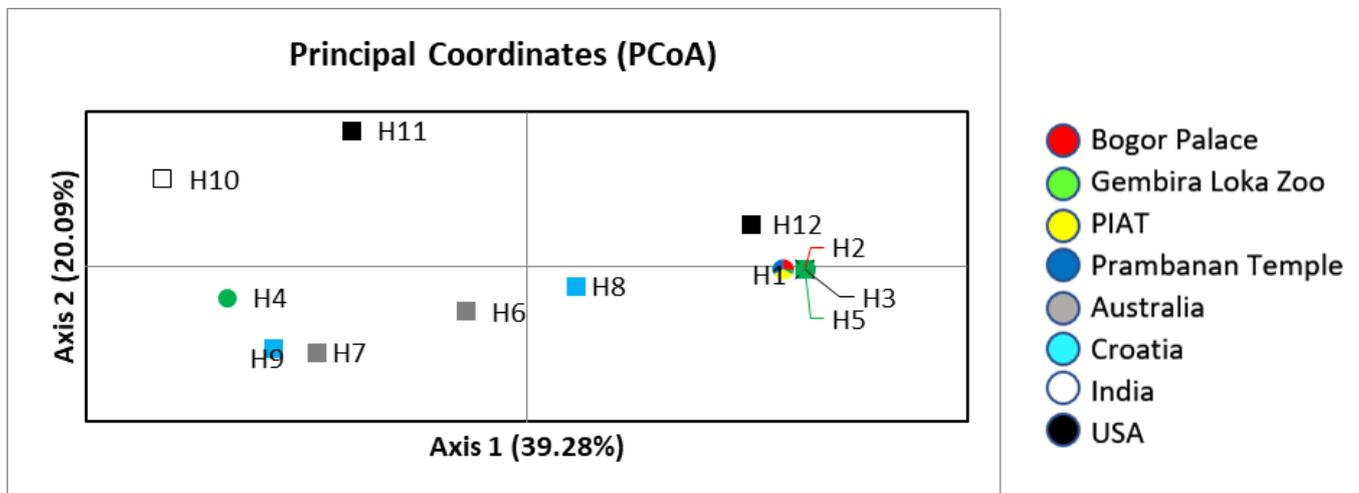
Figure 2. PCoA between haplotypes based on 401 bp *D-loop* fragment of chital population from this study and sequences obtained from NCBI GenBank.
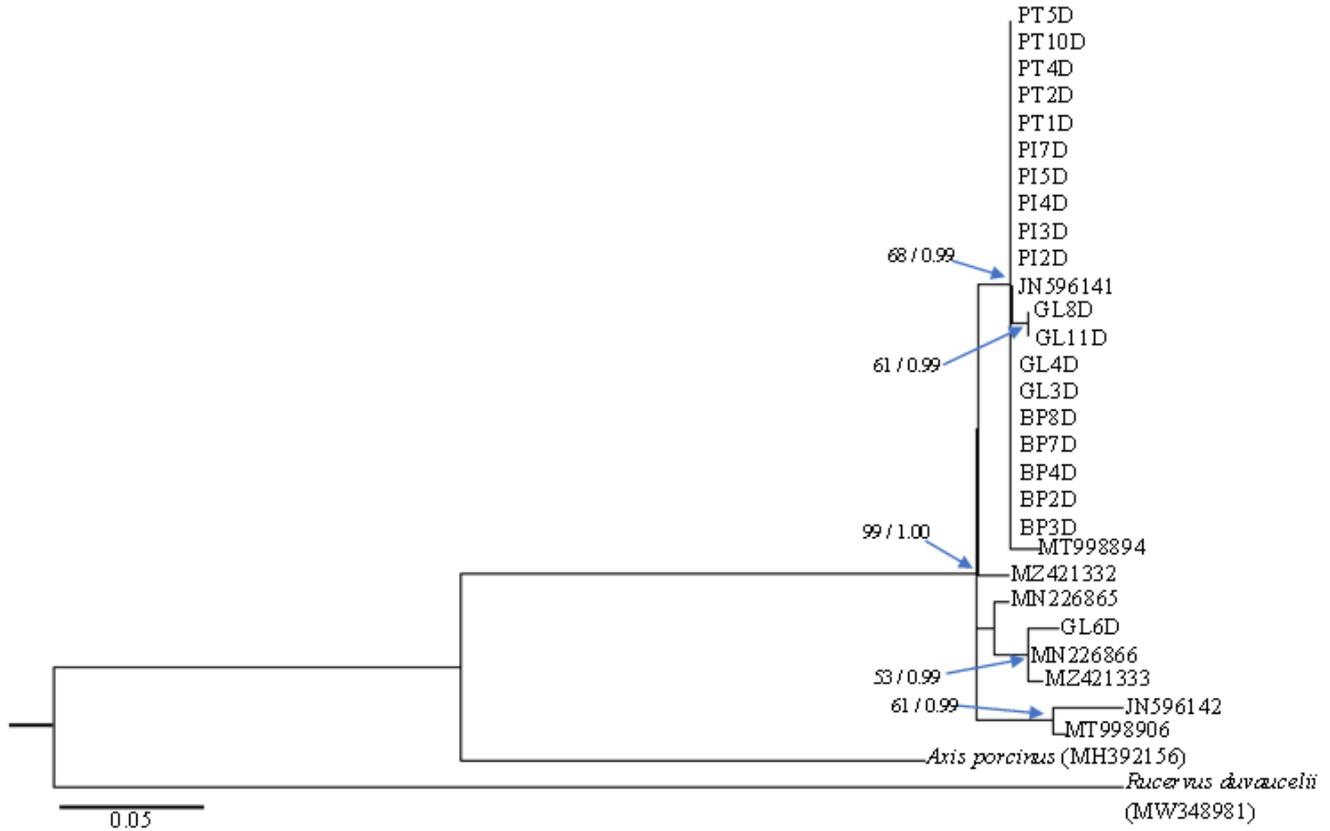
Figure 3. Phylogenetic tree of chital based on 401bp *D-loop* fragment of chital population from this study and sequences obtained from NCBI GenBank. Number on the node shows bootstrap value (ML) and posterior probability (BI) respectively

The phylogenetic tree (Figure 3) shows a grouping of most samples from this study with one sequence from India (JN596141). A sequence of chital from Texas, USA is also closely related to this group. This result is consistent with the haplotype network (Figure 1) and PCoA (Figure 2) results. As the population from India is native, this close relationship could indicate that the population which was first introduced to Indonesia originated from India. Nevertheless, a certain claim could not be made considering the few numbers of *D-loop* sequence data of chital available in the present. Sample GL6D, which is the haplotype 4, formed a clade with a sample from Australia (MN226866) and Croatia (MZ421333). This result is also consistent with the haplotype network (Figure 1) and PCoA (Figure 2) results.

As sample GL6D is closely related to the population from Queensland, Australia, a possible explanation would be that this individual originated from Australia. As it is also closely related to the population from Croatia, another possible explanation would be that these three haplotypes originated from closely related populations. The population from Queensland, Australia originated from Sri Lanka (Hill et al. 2019), while the origin of the population from Croatia is unknown (Šprem et al. 2021). This GL6D individual could also be originated from Sri Lanka, or another population closely related to the population in Sri Lanka. Nevertheless, this possibility could not be proven in the present as no chital *D-loop* sequence data from Sri Lanka is available as of now.

Both theories could be considered as a possibility considering the increasing trend of animal import and smuggling in Indonesia. These include exotic and wild animals and are often will be kept as a pet or a collection. A lot of animals kept in Gembira Loka are obtained from confiscated animals by BKSDA. The GL6D individual might be imported

from outside Indonesia quite recently by smugglers or certified traders and was kept by an Indonesian citizen, which was then confiscated by BKSDA and donated to Gembira Loka Zoo.

CONCLUSION

From this research, two haplotypes (haplotype 1&2) were found in Bogor Palace with 0.400 haplotype diversity value, one haplotype (haplotype 1) was found in both PIAT UGM and Prambanan Temple with 0 haplotype diversity value, and four haplotypes (haplotype 1,3,4,5) were found in Gembira Loka Zoo with 0.900 haplotype diversity value. Haplotype 1,2,3, and 5 were closely related to each other and one haplotype from India, while haplotype 4 was more closely related to Australian and Croatian populations.

AUTHORS CONTRIBUTION

M.Z.M.P. performed sample collection, laboratory work, data analysis, and writing of manuscript. T.A. designed the data analyses and supervised all the processes. Z.R. gave input on the research planning and co-supervised the research.

CONFLICT OF INTEREST

The authors state that they do not have any conflicts of interest. The authors are responsible for the article's content and writing.

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