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Primer design for extremely damaged DNA specimens of Asian rhinoceros species

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Abstract

Importance of the work: Small and highly damaged rhinoceros (rhino) specimens have been found; however, there are no primers for amplifying extremely damaged DNA remains of rhinos.

Objectives: To design specific primers for tracking and identifying rhino species from extremely damaged DNA specimens.

Materials & Methods: In total, 40 complete D-loop sequences of three Asian rhinos (Sumatran, Javan and Greater one-horned) were scanned for a region containing both intra- and inter-species variation. Phylogenetic trees were constructed and compared of the complete D-loop and selected fragments. Primer pairs covering the selected region were designed and tested for species specificity and sensitivity.

Results: Most polymorphic sites selected were located between the 100–400 positions of the D-loop and the 257 bp fragment. The phylogenetic trees of the complete D-loop and the 257 bp sequences were constructed and compared to determine whether the region was a good representative of the complete D-loop. These trees showed similar topology, indicating that the 257 bp region was a good representative of the complete D-loop. For the future use of extremely damaged DNA specimens, three overlapping primer sets (Primers I, II and III) were designed covering the region. Phylogenetic trees were constructed of the DNA sequences within each primer set. The tree from the Primer II sequences had the greatest similarity to that of the complete D-loop. In addition, Primer II had high sensitivity, with a detection limit at 1 fg.

Main finding: There were similar topologies between the phylogenetic tree of the complete D-loop and sequences within Primer II. Primer II could be used for rhino genetic studies of extremely damaged DNA specimens.

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Introduction

The rhinoceros or rhino, the fourth largest terrestrial animal, emerged approximately 60 million years ago (Xu et al., 1996). In recent times, numbers of rhinos have been decreasing substantially, mainly due to hunting for rhino horns. Even though the international trade in rhino horns has been banned since 1977, there has been ongoing poaching (Zschokke et al., 2011). Nowadays, there are five extant rhino species that are classified as near threatened, vulnerable or critically endangered species. The White rhino (*Ceratotherium simum*) dispersed in Africa is a near threatened species with approximately 15,000 individuals still alive (International Rhino Foundation, 2022). The Black (*Diceros bicornis*) and the Greater one-horned (*Rhinoceros unicornis*) rhinos, are African and Asian rhino species, respectively, are vulnerable species, with recent reports of approximately 6,000 and 4,000 individuals, respectively (International Rhino Foundation, 2022; International Union for Conservation of Nature, 2022). Sumatran (*Dicerorhinus sumatrensis*) and Javan (*Rhinoceros sondaicus*) rhinos live mainly in Indonesia and are listed as critically endangered with less than 80 of each species (Zafir et al., 2011; Zschokke et al., 2011; Ewart et al., 2018a; Margaryan et al., 2020; International Rhino Foundation, 2022). Notably, in the past, Javan rhinos had a much wider range habitat, with recordings in Bangladesh and Southeast Asian countries, such as Myanmar, Thailand, Laos, Malaysia and Indonesia (Margaryan et al., 2020), indicating that the Asian rhino has already become extinct in many countries including Thailand. The last footprint trace of a rhino in Thailand was found in 1997 in the Hala-Bala Wildlife Sanctuary (Lynam, 1999).

Today, many Asian people have a strong belief that rhino horns can cure many life-threatening diseases, including cancer, without any scientific evidence support (La-Ong, 2012; Gao et al., 2016; Ewart et al., 2018b; Saengas, 2022). In the past, rhino horns were also used for traditional medicines and carved horns ornaments, such as cups and jewelry, were used to show high social status and as a symbol of prosperity, especially by Chinese people (Saengas, 2022). During the Ming dynasty, during the 15th and 16th centuries, China expanded both economic and cultural exchanges with Southeast Asia, with rhino horns being used as tribute from Southeast Asia countries to China (Saengas, 2022), indicating that rhino horns have been very important in Asia since ancient times.

In Thailand, rhino remains have been found at certain archaeological sites, such as the Ban Chiang World Heritage site (dated 3,000 years before the present (YBP)) at the Udonthani Province, Sribuathong archaeological site (dated 2,500–3,000 YBP) in Angthong province and at the Wat Phra Si Rattana Mahathat archaeological site (dated 1,000 YBP) in Lopburi province (Thai Fine Art, 2023). However, no rhino horn has been recovered from these sites—only bones and teeth were found. These ancient remains were identified as coming from rhinos based on morphology and most of them cannot be identified at species level. Furthermore, the morphological method cannot be used to trace the genetic relationship, nor to determine the origin and distribution of these rhino specimens. Hence, DNA examination of ancient rhino specimens is very important to clarify the rhino species and uncover the origin and distribution of such ancient specimens.

Several primers have been designed for genetic studies of rhinos. Among these, the *cyt b* gene and D-loop in mitochondrial DNA (mtDNA) have been selected as the main targets for species identification (Hsieh et al., 2003; Fernando et al., 2006). However, as the *cyt b* is very conserved within species, the D-loop is a better choice because it contains higher genetic diversity that can be used for both species identification and genetic diversity examination. Other studies have selected different locations and sizes (392–970 bp) of D-loop regions (Fernando et al., 2006; Zschokke et al., 2011; Margaryan et al., 2020; Das and Choudhury, 2021; Ghosh et al., 2022). However, the sizes of these amplicons are too long for extremely damaged DNA specimens, including ancient remains for which the sizes of DNA presented in ancient specimens, especially in tropical areas, are in the range 50–200 bp. In addition, the successful amplification of ancient DNA (aDNA) in Thailand was in the range 131–179 bp which is shorter than for all the primer sets presented in other studies (Wannajuk et al., 2013; Siripan et al., 2019; Chittavichai et al., 2021). Hence, new primers need to be designed that can produce shorter D-loop fragments.

In the present study, primers were designed for identifying three Asian rhino species: Sumatran, Javan and Greater one-horned rhinos. These primers can produce polymerase chain reaction (PCR) products that are not longer than

179 bp that are located in the D-loop region. These primers could be further used for species identification, forensic tracking and evolutionary and archaeological studies of rhino specimens.

Materials and Methods

Target selection for primer designing region

Inter- and intra-species nucleotide positions within the D-loop sequences needed to be detected to find the target of the new primers used for identifying species and examining genetic diversity. In total, 40 complete mitochondrial D-loop sequences of three Asian rhino species—Javan (5 sequences), Great one-horned (17 sequences) and Sumatran (18 sequences)—from the GenBank database (Table S1) were aligned using the Clustal Omega program from the European Bioinformatics Institute (Madeira et al., 2022). The complete D-loop sequences of each rhino species were separately analyzed for nucleotide diversity using the DnaSP version 6.12.23 program (Rozas et al., 2017). Then, the distribution of nucleotide diversity of each species was visualized graphically. Next, all three graphs were combined before selecting the target of the new primers for all three rhino species. The region, 257 bp in length containing the most genetic diversity was selected for the phylogenetic analysis to determine whether it was a proper representative of the complete D-loop.

Phylogenetic analysis of complete D-loop and selected region

The complete D-loops of all three rhino species were aligned and trimmed using the BioEdit program (Hall, 1999). The phylogenetic trees based on the 257 bp region and the complete D-loop sequences of all three rhino species were constructed using the neighbor-joining (NJ) method in the MEGA11 software (Tamura et al., 2021) with a Kimura 2-parameter. *Equus caballus* was used as an outgroup. The topologies of these two trees were compared.

Primer design, phylogenetic analysis and polymorphism detection of sequences within the primer set

The three primer sets located within the selected region were manually designed for the amplicons with an expected size less than 179 bp. Candidate primers were checked for physical parameters, including the guanine and cytosine (GC) content, melting temperature, self-complementary and cross-species binding using the Primer-BLAST program (Ye et al., 2012).

To examine if the region of each primer set was a suitable representative of the selected region and the complete D-loop, the NJ trees based on the 40 D-loop sequences within each primer set were constructed using MEGA 11, with *Equus caballus* used as an outgroup. Then, the topologies of all the trees based on the sequences within the new primer sets were compared to those from the chosen region and the complete-D-loop. The polymorphic sites within each primer set were detected based on DnaSP to provide the pattern of nucleotide diversity for each rhino species.

Primer assay validation

Without the DNA sources of extant rhinos in Thailand, the 550 bp fragments of the partial D-loop sequence of Javan (*R. sondaicus*) and Sumatran (*D. sumatrensis*) rhinos, originating from GenBank (accession numbers: KY117574 and MF066643, respectively) were synthesized and used as DNA templates for the primer assay. The positions of the Javan and Sumatran DNA templates were located at 15,319–15,868 and 15,326–15,875, respectively.

PCR amplification was accomplished using Platinum™ *Taq* polymerase (Invitrogen; USA) in 50 µL total volume containing 5 µL 10X PCR buffer, 1.5 µL 50mM $MgCl_2$, 0.2 µL of Platinum™ *Taq* polymerase, 1 µL 10mM dNTP mixture (Invitrogen; USA), 1 µL DNA template (1 ng of synthesized DNA), 0.5 µL of each 10 µM primer, 0.5 µL BSA and distilled water. The PCR conditions were: initial denaturation at 94°C for 1 min; 40 cycles of denaturation at 94°C for 30 s; annealing at temperatures depending on the set of primers (Table 1) for 30 s and extension at 72°C for 1 min; and a final extension at 72°C for 2 min. A negative control was included in all sets of reactions. Then, the sizes of the PCR products were visualized based on gel electrophoresis on 2% agarose gel, stained with ethidium bromide.

Table 1 Details of primer sequences of Primers I, II and III in this study

Set	Species	Primer	Sequence (5'- 3')	%GC	Tm	Self-complementarity			Ta	PCR Size
						IUPAC	5'	3'		
I	*R. sondaicus*	RDL-F1	Forward:	42	60.0°C	W=A	8.00	3.00	54°C	169 bp
	R. unicornis		TGCATTAAATTGTWTGCCCCATGC			W=T	5.00	2.00		169 bp
	D. sumatrensis	RDL-R1	Reverse:	38	58.9°C	H=A, T	7.00	3.00		170 bp
			GGCCCGATCAATAATAHAATGTACTATGC			H=C	11.00	7.00		
II	*R. sondaicus*	RDL-F2-R	Forward:	35	54.8°C	Y=C	4.00	1.00	51°C	158 bp
	R. unicornis		GAGGAGATATTACATAAGACATYAGG			Y=T	4.00	3.00		158 bp
		RDL-R2	Reverse:	33	53.5°C	W=T	7.00	0.00		
			GTTGWCTAGAAATGATTTGACTTG							
	D. sumatrensis	RDL-F2-D	Forward:	24	52.3°C	R_1=A, R_2=G	5.00	2.00	49°C	162 bp
			GAGGAGTTAATTTTR$_1$CATAAR$_2$ACAT			R_1=G, R_2=A	6.00	4.00		
						R_1=A, R_2=A	8.00	4.00		
		RDL-R2	Reverse:	33	53.5°C	W=A	7.00	0.00		
			GTTGWCTAGAAATGATTTGACTTG							
III	*R. sondaicus*	RDL-F3	Forward:	38	58.7°C	H=A, T	7.00	2.00	54°C	164 bp
	R. unicornis		GGCCGCATAGTACATTHTATTATTGATCG			H = C	7.00	3.00		164 bp
	D. sumatrensis	RDL-R3	Reverse: ATGGGCCCGGAGCGAGAAC	68	69.1°C	–	6.00	0.00		162 bp

PCR = polymerase chain reaction; Tm = melting temperature; Ta = annealing temperature; GGCC = restriction site of *HaeIII*; GAGGA = restriction site of *BseRI*; IUPAC = ambiguous bases: W = adenine (A) and thymine (T); H = adenine (A), cytosine (C) and thymine (T); Y = cytosine (C) and thymine (T); R = guanine (G) and adenine (A). R_1 and R_2 = the ambiguous base, R at first and second positions of Primer set II (RDL-F2-D).

For the specificity test, the DNA template of each species and each primer set were used in separate reactions. The sensitivity test was performed using the DNA template at nine serial 10-fold concentrations from 0.01 fg to 1 ng. The thickness of the PCR products from each primer set was detected based on gel electrophoresis on 2% agarose gel.

Results and Discussion

Target selection for primer designing region

Many regions in mtDNA have been used to identify species and to examine the genetic diversity of rhinos. However, the D-loop has been intensively selected for several genetic studies. In the present study, the primers were designed by targeting the D-loop region. The sizes of PCR products in other rhino genetic studies were in the range 392–970 bp (Fernando et al., 2006; Zschokke et al., 2011; Margaryan et al., 2020; Das and Choudhury, 2021; Ghosh et al., 2022). Since aDNA samples, especially in tropical regions, have been severely damaged and fragmented, mostly 50–200 bp remains in the ancient specimens (Wannajuk et al., 2013; Cai et al., 2014; Siripan et al., 2019; Chittavichai et al., 2021; Zhang et al., 2022). Primer pairs that could be used to amplify aDNA should produce PCR products shorter than 200 bp. In the present study, in total, 40 complete D-loop sequences of Asian rhinos—18 Sumatran (*D. sumatrensis*), 5 Javan (*R. sondaicus*) and 17 Greater one-horned (*R. unicornis*)—from the GenBank database were aligned and scanned for the proper sites to use for species identification and nucleotide diversity analysis of the ancient or modern specimens that were extremely damaged.

The complete D-loop sequences of each rhino species were separately analyzed for nucleotide diversity at each nucleotide position (Fig. 1). The Sumatran rhinos had the highest diversity (33 positions) followed by the Greater one-horned (14 positions) and Javan (4 positions) rhinos, respectively (Figs. 1A, 1B and 1C, respectively). The positions of the intra-species variation in the Sumatran, Javan and Greater one-horned rhinos were mainly located at nucleotide positions in the ranges 100–400 bp, 200–400 bp and 100–400 bp, respectively. When combining all three analyses together, the most variable sites of all three species (60.7% of polymorphic sites) were clearly located in the range 100–400 bp of the D-loop region (Fig. 1D). The positions of the hypervariable region (HV-I) in mammals are located in the first half of the D-loop, with HV-I

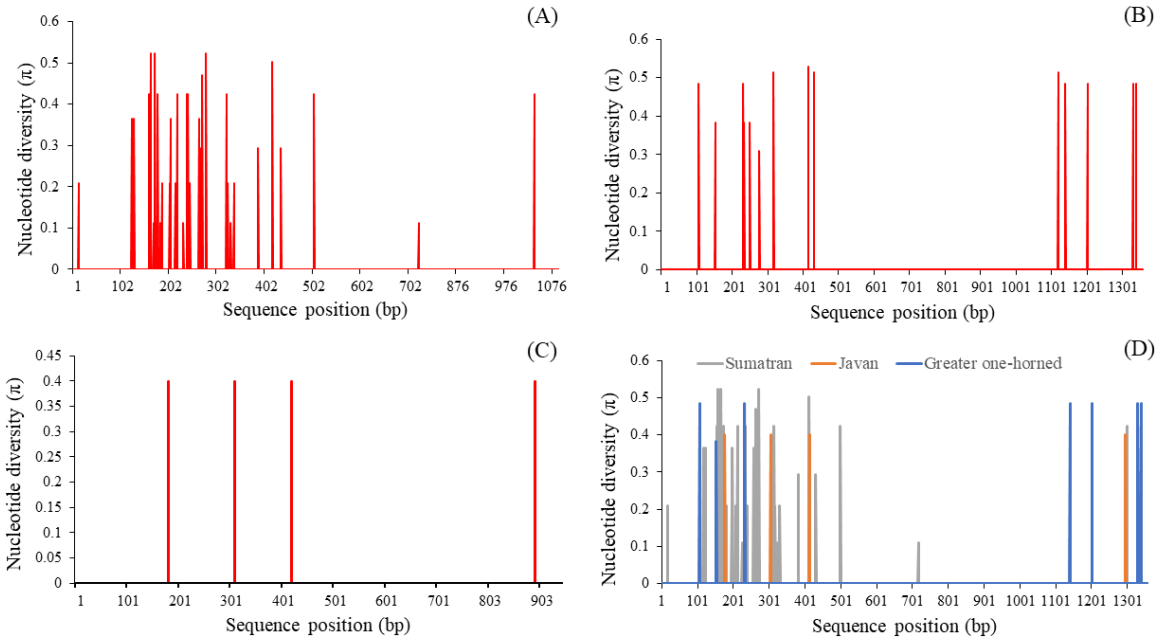

Fig. 1 Distribution of nucleotide diversity (π) of complete mtDNA D-loop in Asian rhinoceros: (A) Sumatran (n = 18); (B) Greater one-horned (n = 17); (C) Javan rhinos (n = 5); (D) combination of nucleotide diversity distribution of all three rhino species.

being used as a molecular marker for species identification and population genetics in mammals (Nakamura et al., 2009; Siripan et al., 2019). The results of such studies suggested that this is the main target for primer design. Consequently, in the present study, the region (positions 100–400 bp) was compared to other partial D-loop sequences (392–970 bp) from other genetic studies of rhinos to be comparable with further study (Fernando et al., 2006; Zschokke et al., 2011; Margaryan et al., 2020; Das and Choudhury, 2021; Ghosh et al., 2022). The 257 bp D-loop fragment was selected because it covers the majority of polymorphic sites of the D-loop and is located within all of the partial D-loop sequences obtained from the other studies.

Phylogenetic analysis of complete D-loop and selected region

Phylogenetic trees based on the complete D-loop and the 257 bp sequences of the rhino species were constructed to confirm the suitability of the 257 bp region as a good representative of the complete D-loop of all three Asian rhino species (Figs. 2A and 2B). The complete D-loop sequences of the 40 Asian rhino samples were aligned and trimmed to 936 bp fragments (from 1,089 bp of the Sumatran rhinos, 946 bp of the Javan rhinos and 1,358 bp of the Greater one-horned rhinos) before constructing the complete D-loop tree. The result showed that the 936 bp and 257 bp trees had similar topologies and that all three species could be separated with high bootstrap values (96–100). The phylogenetic tree based on the 936 bp sequences clearly separated these Asian rhinos with bootstrap values of 100 (Fig. 2A). Similarly, the phylogenetic relationship based on the 257 bp region had the same pattern with very high bootstrap values of 100, 99 and 96 for the Javan, Greater one-horned and Sumatran rhinos, respectively (Fig. 2B). Both trees showed that the Javan and the Greater one-horned rhinos were sister groups. The results indicated that the 257 bp region was a good representative for genetic analyses of Asian rhinos.

Primer design, phylogenetic analysis and polymorphism detection

Referring to the other aDNA works in tropical regions, especially in Thailand, successful D-loop amplification could produce 131–179 bp of PCR products (Wannajuk et al., 2013; Siripan et al., 2019; Chittavichai et al., 2021). The 257 bp length was too long and had a very high risk of failure in amplification with a single primer pair. Therefore, primer pairs were designed that produced PCR products shorter than 179 bp and covered the 257 bp fragment. The details of three primer sets (Primer I, Primer II and Primer III) are shown in Table 1.

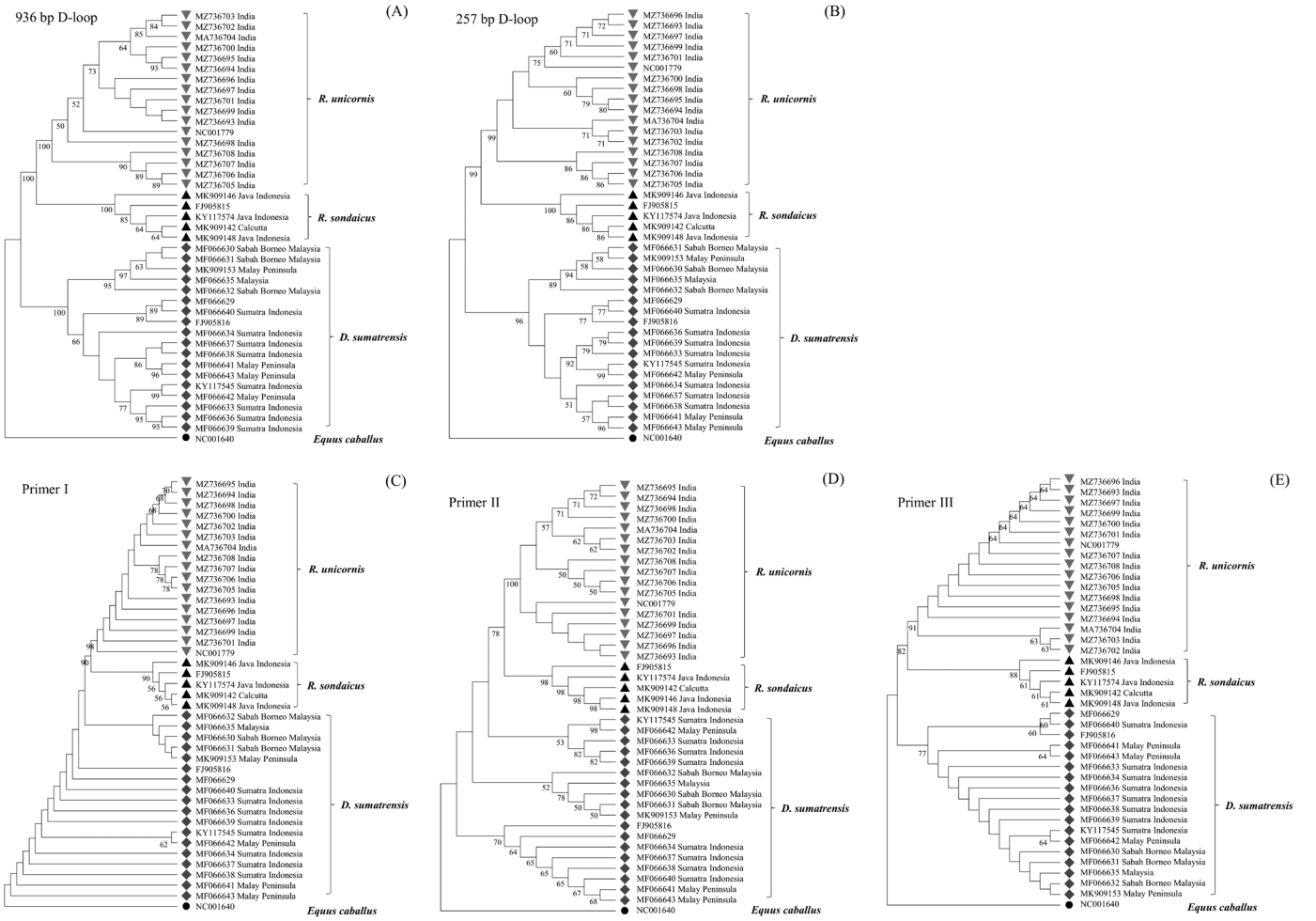

Fig. 2 Neighbor-joining trees reconstructed from: (A) 936 bp D-loop; (B) 257 bp D-loop; (C) sequences from Primer I; (D) sequences from Primer II; (E) sequences from Primer III, where ▼, ▲ and ♦ represent Greater one-horned (*Rhinoceros unicornis*), Javan (*R. sondaicus*) and Sumatran (*D. sumatrensis*), respectively, *Equus caballus* (NC001640) is included as outgroup represented by ● and numbers at nodes indicate frequency values above 50% based on 1,000 replications.

Both Primer I and Primer III had only one forward and one reverse primer, while Primer II had two forward primers specific to the different rhino genera—*Rhinoceros* (Javan and Greater one-horned rhinos) and *Dicerorhinus* (Sumatran rhinos)—due to the high nucleotide diversity in this region. For Primer I, the forward (RDL-F1) and the reverse (RDL-R1) primers were located at the positions 1–24 and 142–166, respectively, of the 257 bp fragment. For Primer II, the first forward (RDL-F2-R), the second forward (RDL-F2-D) and the reverse primers (RDL-R2) were located at the positions 114–134, 111–130 and 244–267, respectively. For Primer III, the forward (RDL-F3) and the reverse primers (RDL-R3) were located at the positions 142–166 and 282–301, respectively (Table 1). In addition, some nucleotide positions in all primers (except RDL-R3) had high levels of variation; in such cases, ambiguous bases (W, H, Y and R) were used in the primers. In addition, because the GC content was too low in certain primers, the sequences *HaeIII* (5'-GGCC-3') or *BseRI* (5'-GAGGA-3') for restriction sites were added at the 5' end of the four primers: RDL-R1, RDL-F2-R, RDL-F2-D and RDL-R3. The expected sizes of the PCR products were in the range 158–170 bp (Table 1).

The sequences within each primer set were examined based on phylogenetic analyses to determine whether the three divided regions from the three sets of primers would be suitable for intra- and inter-genetic studies of the Asian rhino species (Fig. 2C–2E). The result from Primer I showed that the Greater one-horn (*R. unicornis*) and Javan (*R. sondaicus*) species were separated with very high bootstrap values

of 98 and 90, respectively. However, the Sumatran rhino (*D. sumatrensis*) was split into 13 branches with bootstrap values lower than 50 that were not clustered into a single clade. For Primer II, the Greater one-horn, Javan and Sumatran rhinos were separated with very high bootstrap values of 100, 98, and 78, respectively, and the Sumatran rhino was split into three subclades with bootstrap values higher than 50 (52, 53 and 70). Lastly, based on the sequences within Primer III, the tree had three species clades with bootstrap values of 91, 88 and 77, respectively.

Even though the number of polymorphic sites within the sequences from Primer I was higher than for the other two primers (Tables 2S–4S), most of the Sumatran and Greater one-horned sequences were not clustered well in the phylogenetic analyses. In contrast, the phylogenetic trees based on sequences from Primers II and III presented a clearer separation of those rhino species, indicating that sequences from Primer II and III would be better candidates for species identification of all three Asian rhinos. However, the genetic relationship based on sequences from Primer II produced a more related pattern to the 936 bp tree. Therefore, Primer II might be the best for Asian rhino species identification. In addition, the phylogenetic tree based on the sequences from Primer II had high bootstrap values, with all branches within the Sumatran rhinos having bootstrap values higher than 50, indicating that this primer set might also be suitable for studying intra-species diversity, especially for Sumatran rhinos. However, all of these overlapping primer sets could be used together to produce 257 bp D-loop sequences for better results in species identification and genetic diversity analysis of Asian rhinos.

Primer assay validation

Specificity test

To test whether the three primer sets could produce the expected sizes of PCR products, PCR tests were performed using each primer set and the synthesized DNA templates of *R. sondaicus* and *D. sumatrensis*. Lanes J and S in Fig. 3 show the PCR products from the primer assay using the DNA templates of Javan and Sumatran rhinos, respectively. For the Primer I assay, approximately 170 bp were produced (Fig. 3A). In Fig. 3B, two different forward primers of Primer II (RDL-F2-R and RDL-F2-D), specific to different rhino genera (*Rhinoceros* and *Dicerorhinus*) were used and the 160 bp PCR products were amplified. In Fig. 3C, the PCR products approximately 160 bp in size were detected based on the Primer III assay (164 bp for Javan rhinos and 162 bp for Sumatran rhinos). The results showed that the expected targets were generated as predicted, indicating that all primers could specifically bind to the DNA templates and could be used in the study of Asian rhino genetics.

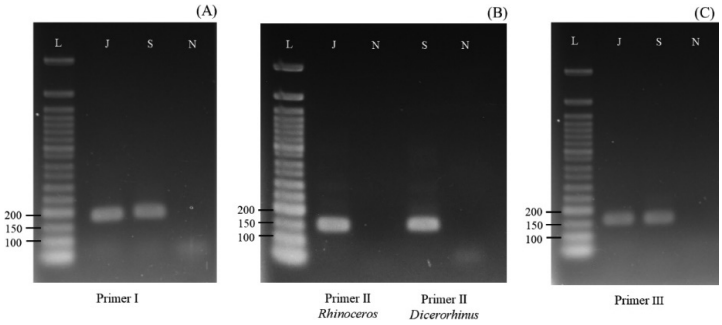

Fig. 3 Agarose gel electrophoresis for specificity testing of three primer assays: (A) Primer I; (B) Primer II; (C) Primer III, where lane L is 50 bp DNA ladder, lane N is negative control and lanes J and S represent PCR products amplified using DNA templates of Javan and Sumatran rhinos, respectively.

Sensitivity test

The detection limits of the primer assays were tested at 10-fold dilution concentration levels (0.01 fg to 1 ng), as shown in Fig. 4. Overall, the results showed a gradient of amplicon concentrations from high to low, according to the concentrations of the DNA templates; however, the detection limit of each primer set was different. The minimum concentrations of the DNA templates from both the Javan and Sumatran rhinos in the Primer I and II assays were 10 fg and 1 fg, respectively (Figs. 4A and 4B). However, for the Primer III assay, the detection limit was lower at 0.01 fg (Fig. 4C). These results indicated that Primer III had the highest sensitivity, followed by Primer II and Primer I, respectively. However, neither smear bands nor multiple bands were detected from any concentration of the DNA template in the Primer II assay, indicating the very high specificity of the primer sets. This might have been due to Primer II having two different forward primers, while the other primer sets contained only one forward primer. Even though Primers I and III produced smears or multiple bands, this occurred only when using high concentrations of the DNA templates. These results have been due to the primer attaching to the non-target region or the fragment matching

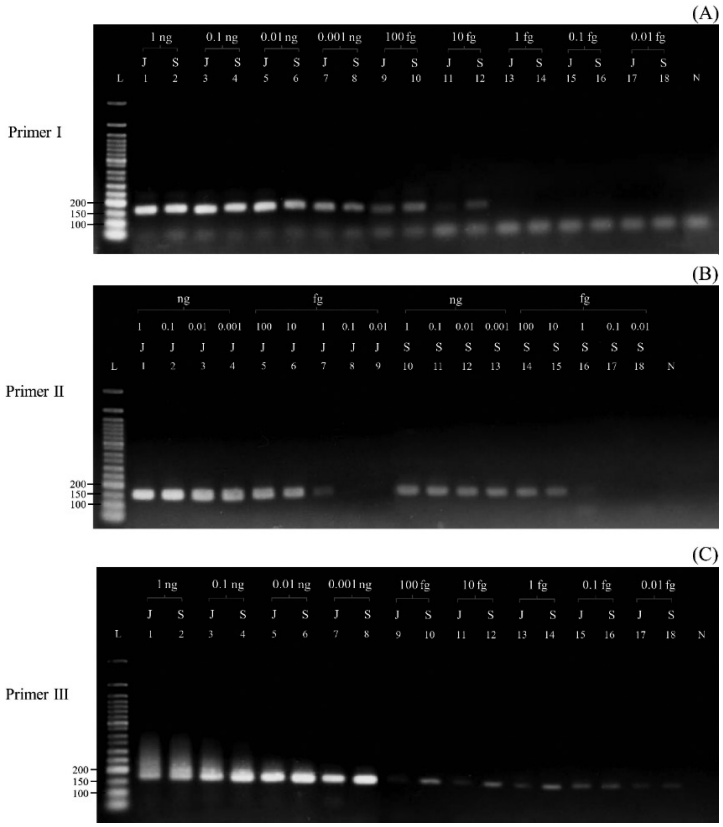

Fig. 4 Sensitivity testing of assays in 10-fold serial dilution concentrations (1 ng–0.01 fg) of: (A) Primer I; (B) Primer II; (C) Primer III (C), where lane L is 50 bp ladder, lane N is negative control and lanes J and S represent PCR products amplified using DNA templates of Javan and Sumatran rhinos, respectively.

to another fragment and forming the larger amplicon (Zrimec et al., 2013). In the present study, synthesized DNA was used, with the concentration of this type of DNA template being much higher than for DNA templates extracted from real samples. For the application of these primers in future studies, if either a smear or multiple bands were observed, dilution of the DNA templates or decreasing the number of PCR cycles might resolve the problem.

Notably, these detection limits of all the primer sets in the present study were lower than in other studies by around 2–5 orders of magnitude. The limitation sensitivity tests in the other studies were in the range 0.02 ng–0.1 pg (Ali et al., 2015; Suwannarat et al., 2017; Koehler et al., 2020), while the range in the present study was 1–0.01 fg. However, the sensitivity results were tested using high-quality synthesized templates, which would certainly differ from using real DNA samples. Further experimentation should include modern or aDNA samples from rhinos to confirm the specificity and sensitivity of the primers.

In summary, the present study discovered a 257 bp region, as a good representative of the complete D-loop, for species identification and genetic diversity examination of Asian rhino species. Even though the length is shorter than any of the D-loop fragments in other reported rhino genetic studies, the 257 bp fragment is still too long for extremely damaged DNA specimens. Hence, three overlapping primer sets (Primers I, II and III) covering the 257 bp region were designed, with all three having high specificity and high sensitivity. In the phylogenetic analyses, the tree based on the sequences within Primer II presented the most similar topology to that of the complete D-loop and could clearly separate all three Asian rhino species, based on high bootstrap values. Furthermore, all branches within the Sumatran sequences in this tree presented bootstrap values higher than 50, indicating that Primer II would be suitable for studying intraspecies diversity in Sumatran rhinos, a critically endangered species. Since the primer sets amplified very short amplicons (158–162 bp) with high specificity and sensitivity, they can be used for species identification and genetic diversity study of extremely damaged DNA specimens of Asian rhinos.

Conflict of Interest

The authors declare that there are no conflicts of interest.

Acknowledgements

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