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ORIGINAL ARTICLE

Two novel species-specific mitochondrial markers for non-invasive identification of rhesus macaques (*Macaca mulatta*)

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Abstract

Background The rhesus macaque (*Macaca mulatta*) is a widely studied model organism in biological research. However, accurate species identification from non-invasive genetic samples remains essential for robust field studies, especially in habitats shared with other primates. Morphological similarities in fecal or hair samples of sympatric species and cross-amplification of standard genetic markers across closely related primates complicate species assignment. This study targets to develop species-specific primers to enable accurate, low-cost species identification of *M. mulatta* from non-invasive samples.

Methods and results We designed two novel primer pairs targeting the mitochondrial *cytochrome b* gene, based on fixed nucleotide differences between *M. mulatta*, sympatric primates and other species with which rhesus feaces can possibly be confused. In silico screening ensured specificity and PCR suitability. The primers were tested in vitro using DNA from blood, hair, and fecal samples of rhesus macaques. Cross-amplification was assessed using DNA from tissue samples of eight non-target species, including five *Macaca* spp., *Semnopithecus entellus*, *Hoolock hoolock*, and humans. We performed endpoint PCRs using these two primer pairs and amplification success was verified via gel electrophoresis and sequencing. Both primer pairs amplified only rhesus macaque DNA and showed no cross-amplification in non-target species. They performed consistently across all sample types, including naturally degraded fecal samples. Sequencing and BLAST analysis confirmed 100% identity with the *M. mulatta* cytochrome b region.

Conclusions These primers offer a reliable, cost-effective, and efficient tool for confirming species identity in non- invasive samples. Their application will improve the accuracy of large-scale ecological, behavioral, and genetic studies on rhesus macaques, and can support broader monitoring of wild populations in mixed-species habitats.

Keywords Species detection · Species-specific primers · Non-invasive · Cost-effective tools · Mitochondrial markers · Primer design

Abbreviations

DNA	Deoxyribonucleic acid
SSP	Species specific primers
PCR	Polymerase chain reaction
mtDNA	Mitochondrial DNA
Ta	Annealing temperature
NCBI	National centre for biotechnology information
BLAST	Basic local alignment search tool

Introduction

Rhesus macaques (*Macaca mulatta*) are among the most extensively studied non-human primates, accounting for 65% of all non-human primate research in the United States [1]. Their widespread use in scientific studies is attributed to their ease of management in captivity [2], broad geographic distribution and abundance [3], and close anatomical, physiological, and genetic similarities to humans [4]. These characteristics make them invaluable in medical and biological research related to both human and animal health [2–4].

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Furthermore, their complex social structures—such as multimale-multifemale social groups [5] and despotic societies [6] provide opportunities to investigate social dynamics, including dominance hierarchies [7], cooperation [8] and kinship patterns [9]. Their adaptability in the Anthropocene era and remarkable niche flexibility further enhances their significance as model organisms for studying evolutionary ecology, particularly the relationship between sociality and fitness [10].

Despite their prominence as a model species, research on free-ranging rhesus macaques in Asia has primarily focused on demography, population biology, and geographic distribution, with relatively little emphasis on their ecology and behavior [11]. Studies have largely relied on direct observations, leaving several fundamental questions unanswered, in their natural range. These questions include how ecological and dietary differences shape life history, reproduction, social behaviour, and lifespan; the relative roles of behavioural plasticity versus evolutionary adaptations in responding to human-modified environments; and the evolutionary drivers and ecological implications of individual personality traits in urban success [10]. To explore these questions more deeply, genetic data can play a critical role. However, genetic data collection has traditionally relied on invasive sampling methods, which involve capturing and anesthetizing individuals to obtain biological samples such as blood, tissue biopsies, or invasive swabs. Although valuable for genetic studies, invasive sampling poses significant challenges including stress or harm to animals, logistical difficulties in the field, limited sample sizes, and difficulty targeting specific individuals [12]. In contrast, non-invasive sampling—such as collecting feaces, urine, hair, saliva, or shed skin— offers a practical, low-impact alternative that facilitates the collection of samples from a larger number of individuals with minimal disturbance [13–15].

While non-invasive methods are widely adopted in primate research, they present their own challenges, particularly in reliably identifying the species origin of samples collected in the field. This is especially problematic in habitats where multiple *Macaca* species coexist (due to morphological similarities among fecal or hair samples of sympatric species [16–18]. Some macaque species can also hybridize in the wild [19–21], and standard genetic markers can amplify across closely related primate species [22, 23], adding another layer of difficulty. Additionally, juveniles of some macaque species are morphologically indistinguishable in the field [24, 25], leading to potential misidentification when linking non-invasive samples to observed individuals. Misidentification at the species level can lead to misleading conclusions in ecological or behavioral studies.

One widely used and reliable approach for species identification is sequencing a portion of a mitochondrial gene, which is conserved within a species [26]. However, for large-scale studies, sequencing is not cost-effective. Instead, researchers often rely on species-specific primer (SSP)-based Polymerase Chain Reaction (PCR) and gel electrophoresis to confirm species identity [27]. The use of species-specific primers, which amplify only a defined region of highly conserved mitochondrial genes unique to a particular species, is a practical and efficient method for screening large numbers of samples in population studies.

Currently, no validated and publicly available species-specific primers exist that are designed exclusively for identifying rhesus macaques from non-invasive samples. Recognizing this gap, we developed and tested two novel primer pairs targeting the mitochondrial *cytochrome b* gene. These primers are intended to serve as a crucial component of a broader molecular identification workflow—enabling accurate species assignment when used with endpoint PCR and gel electrophoresis. Our objective was to develop a reliable, specific, and cost-effective genetic tool to support large-scale, non-invasive studies of rhesus macaque, particularly in regions where they coexist with other primate species.

Materials and methods

Primer designing and screening

We targeted the 'mitochondrial *cytochrome b*' region to design species-specific primers for rhesus macaques. *Cytochrome b* was selected because it has limited intraspecific variation, and contains regions with sufficient divergence between closely related species—making it a suitable target for species identification [28]. The *cytochrome b* sequences of rhesus macaque, sympatric primates and other species (with which rhesus feaces can possibly be confused), were retrieved from the NCBI database (species name and their reference sequences accession number provided in Table 1). A multiple sequence alignment was performed using the software MEGA v11.0.13 with the ClustalW algorithm, to identify fixed nucleotide differences between species (Supplementary file 1).

Potential primer pairs were designed using NCBI's Primer-BLAST tool and manual identification of fixed nucleotide differences in the *Cytochrome b* region unique to the rhesus macaque genome. Primer-BLAST was run under stringent specificity criteria, requiring at least 2 total mismatches to unintended targets, including at least 2 mismatches within the last 5 bp at the 3′ end, while ignoring potential off-targets with ≥ 5 mismatches. We targeted amplicon size less than 500 bp to facilitate amplification from degraded DNA. At this step, we got 12 primers pairs

Table 1 Name of species and their reference sequences NCBI accession number

S. No.	Species name	Accession No.
1	*Macaca mulatta*	AY612638.1
2	*Homo sapiens*	MF588857.1
3	*Semnopithecus entellus*	DQ355297.1
4	*Macaca assamensis*	NC 023795.1
5	*Macaca arctoides*	KM360179.1
6	*Macaca leonina*	NC 027604.1
7	*Semnopithecus schistaceus*	MN163131.1
8	*Macaca thibetana*	EU294187.1
9	*Nycticebus bengalensis*	NC 021958.1
10	*Trachypithecus phayrei*	NC 056326.1
11	*Trachypithecus pileatus*	NC 024529.1
12	*Hoolock hoolock*	NC 033885.1
13	*Trachypithecus geei*	NC 056324.1
14	*Macaca munzala*	DQ859976.1
15	*Macaca radiata*	LC225392.1
16	*Macaca fascicularis*	NC 012670.1
17	*Macaca silenus*	KM679363.1
18	*Loris lydekkerianus*	NC 021955.1
19	*Trachypithecus johnii*	NC 019583.1
20	*Canis lupus familiaris*	KU290632.1
21	*Vulpes vulpes*	GQ374180.1
22	*Vulpes ferrilata*	NC 027935.1

(Supplementary file 2). The suitability of the designed primers was evaluated using the 'Sequence Manipulation Suite' [29], which assessed parameters such as melting temperature (Tm ~ 58–64 °C), GC content (45–60%), PCR suitability, and potential for dimer or hairpin formation (self-complementarity scores of 0–6, and 3′ complementarity = 0–2) (Supplementary File 3). All 12 potential primer pairs were tested in-silico for cross-amplification using NCBI's Primer-BLAST tool, and only 10 primer pairs which were species specific to rhesus macaque were retained for further in vitro testing (Supplementary file 4).

Sample collection

For in vitro testing, we used 5 blood and 5 hair samples (for which positive samples were available in-house), and fecal samples of free-ranging rhesus macaques were opportunistically collected from identified group locations in Dehradun, India. Fecal samples were visually categorized into four freshness classes based on physical characteristics: **very fresh** (moist, glossy surface, strong odor), **fresh** (slightly moist, intact shape, mild odor), **old** (partially dry, surface cracks appearing, no odor), and **very old** (completely dry, fragmented, discolored). All fecal samples were collected using sterilized forceps, stored in sterile containers with silica gel, and transported to the lab, where they were stored at − 20 °C until DNA extraction [30]. We used only those fecal samples whose species identity was confirmed by sequencing the mitochondrial cytochrome b region using the universal primers MCB [31] (for reference, MCB sequences of three samples are provided in supplementary file 5). A total of 60 fecal samples of rhesus macaques were used for in-vitro testing, 15 in each degradation category (very fresh, fresh, old, and very old). To check potential cross-amplification in other related species, we included eight non-target species - bonnet macaque (*Macaca radiata*), lion-tailed macaque (*Macaca silenus*), crab-eating macaque (*Macaca fascicularis*), stump-tailed macaque (*Macaca arctoides*), Northern pig-tailed macaque (*Macaca leonina*), hanuman langur (*Semnopithecus entellus*), western hoolock gibbon (*Hoolock hoolock*), human (*Homo sapiens*). For each non-target species, 3–4 positive tissue samples were available from existing in-house collections.

DNA extraction

DNA extraction was performed using the Qiagen DNeasy Blood & Tissue Kit for tissue, blood and hair samples, while fecal DNA was extracted using a modified GuSCN-Silica-based manual method [32]. All extractions were performed with negative controls to monitor for contamination.

PCR (Polymerase chain Reaction) optimization and In-vitro testing of primers

We performed gradient PCRs (temperature range 48–70 °C) to optimize primer annealing conditions for each primer pair using standard endpoint PCR. PCRs were conducted in Eppendorf Mastercycler Nexus gradient machine, consisting a PCR reaction of 12 µL volume containing 4 µL of 2X MasterMix with HotStar Taq polymerase (Qiagen), 1.2 µL of 2 mg/mL Bovine Serum Albumin (BSA), 0.3 µM unlabelled forward and 0.3 µM unlabelled reverse primer, 1 µL of Coral Dye (Qiagen), and 1 µL of extracted DNA. PCR conditions were: initial denaturation (95 °C for 15 min), 35 cycles of denaturation (94 °C for 45 s), annealing (Ta for 45 s), and extension (72 °C for 45 s) and a final extension (72 °C for 10–30 min). Amplified PCR products were visualized on 2% agarose gel electrophoresis. Negative controls (extraction and PCR) were included in all reactions to ensure contamination-free results.

Primer pairs producing clear, single-band amplicons of expected size were further validated for specificity using DNA from additional eight non-target species. We performed gradient PCRs with temperature range of 48–70 °C to check for cross- species amplification in additional eight non-target species. For each species, a minimum of three replicates per primer pair were tested. The non-target species selected for this assay comprised phylogenetically closely related sympatric primate species within the genus

Macaca (bonnet macaque, lion-tailed macaque, crab-eating macaque, stump-tailed macaque, pig-tailed macaque) and two sympatric species from genus other than *Macaca* (hanuman langur, western hoolock gibbon) whose faeces are most likely to be confused with rhesus macaque's faeces in field identification and human (to screen out the contamination during handling and processing of the samples by researchers). PCR products were visualized on a 2% agarose gel to assess amplification specificity. Only two primer pairs that amplified DNA exclusively from rhesus macaques without cross-amplification in non-target species were considered specific. Summary of *in- vitro* testing results for 10 primer pairs is provided in Supplementary file 4.

Sequencing validation

To confirm that the amplified PCR products corresponded to the intended target sequence, we sequenced positive amplicons from six reference samples following gel electrophoresis. Amplicons were column-purified using the QIAquick Gel Extraction Kit and sequenced on an ABI 3730 automated DNA Sequencer. The resulting sequences were subjected to NCBI BLAST analysis to verify species identity.

Results

After a rigorous process of primer design and screening, two rhesus macaque-specific primer pairs (Mmu1 and Mmu2) targeting the *cytochrome b* region of mitochondrial DNA were finalized (Table 2). Both primers demonstrated high specificity during in silico analysis, with no predicted cross-amplification against non-target species, including sympatric macaques, other non-human primates, and humans. Figure 1 illustrates the sequence similarity of the primer-binding regions across closely related primate species. Although the Primer-BLAST stringency criterion (≥2 total mismatches to unintended targets, including≥2 mismatches within the last 5 bp at the 3′ end, while ignoring potential off-targets with ≥5 mismatches) was not strictly met for a few species (*Macaca radiata* and *M. fascicularis* for Mmu1; *M. arctoides* and *M. leonina* for Mmu2), none of these species showed amplification in vitro under optimized PCR conditions. This demonstrates that the primers are functionally species-specific despite minor deviations from the mismatch rule.

Table 2 Sequence details of the macaque-specific primers finalised through experimentation

	Primer name	Primer sequence (5'−3')	Product size (bp)	Ta (°C)
Mmu1	Mmu1_H15897	TTCATCGATTTACCCGCCCC	411	66 °C
	Mmu1_L16288	CGGGATTGCTGATAGCAGGT		
Mmu2	Mmu2_H16419	CTCACAACCGTGCACCTACT	294	64 °C
	Mmu2_L16693	TCCCAGTTTGTTGGGGATGG		

PCR amplification using Mmu1 and Mmu2 produced clear, distinct bands at the expected size range (411 bp and 294 bp respectively) exclusively for rhesus macaque DNA samples. Amplification success was 100% in both blood ($n=5$) and hair ($n=5$) samples of rhesus macaques. Among the 60 field-collected fecal samples, 56 (93.3%) amplified successfully with at least one of the two primer pairs. Since amplification by a single primer pair was sufficient for species identification, these samples were considered positive detections. Amplification success was 100% in both very fresh and fresh samples ($n=15$ each), while old samples showed ~ 93% success and very old samples ~ 80%. These results demonstrate that the primers perform reliably across various sample types and levels of DNA degradation, highlighting their robustness and utility for non-invasive genetic studies in field conditions.

No amplification was observed for DNA from non-target species, including bonnet macaque, lion-tailed macaque, crab-eating macaque, stump-tailed macaque, pig-tailed macaque, hanuman langur, western hoolock gibbon, or human (Fig. 2). Negative controls included in all reactions confirmed the absence of contamination. The sequencing results confirmed 100% identity with the rhesus macaque *cytochrome b* region when subjected to NCBI BLAST analysis. This validated the specificity of both Mmu1 and Mmu2 primers for identifying rhesus macaques from non-invasive genetic samples.

Discussion

We successfully designed and validated two species-specific primer pairs for rhesus macaques, addressing a critical gap in species identification from non-invasive genetic samples. Although empirical validation was conducted using fecal, hair, and blood DNA, these primers are potentially applicable to other non-invasive sample types such as urine, skin, and saliva. While rhesus macaques are generally easy to observe and sample due to their terrestrial habits and proximity to human settlements, misidentification can still occur, especially in mixed-species habitats (e.g., mixed social groups of rhesus macaques and bonnet macaques in India [33], or when dealing with unhabituated groups in dense forests where direct observation is challenging. Unlike universal mitochondrial markers, these primers demonstrate

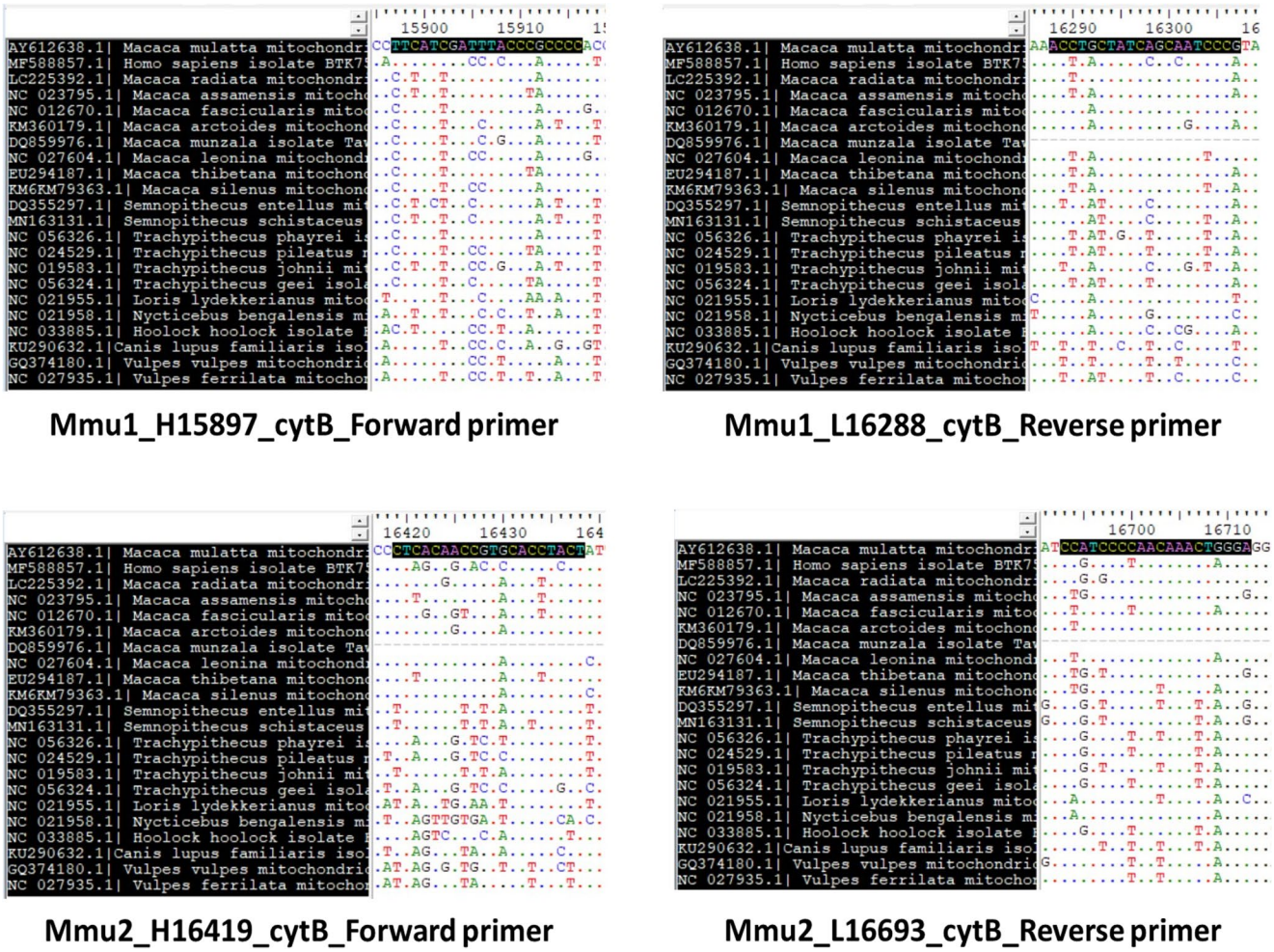

Fig. 1 Sequence similarity of the primer region in macaca mulatta when compared with other relevant species

high specificity, amplifying only rhesus macaque DNA and not that of closely related macaque species. This specificity is crucial for ensuring the reliability of non- invasive genetic studies, particularly in regions where multiple macaque species coexist However, potential hybridization among sympatric macaque species may still pose a limitation, as mitochondrial introgression can lead to shared haplotypes and obscure species boundaries.

Primer utility and specificity

The validated primer pairs consistently produced specific amplification in rhesus macaque samples while showing no amplification in any of the eight tested non-target species across a wide annealing temperature gradient (48–70 °C), confirming their specificity under both stringent and relaxed PCR conditions. Amplification success was 100% in blood and hair samples ($n = 5$ each), and in all fresh and very fresh fecal samples. Among fecal samples collected under field conditions, 56 of 60 samples (93.3%) amplified successfully using at least one of the primer pairs. Although each primer pair independently yielded species-specific amplification, using both primer pairs in parallel is recommended to increase detection reliability, particularly in degraded or low-quality DNA samples. Notably, amplification success was ~93% in old samples and ~80% in very old samples, with 15 samples per category. These findings confirm that the primers are effective even on naturally degraded DNA, which is common in field-collected non-invasive samples. The short amplicon sizes (294 and 411 bp), fixed species-specific polymorphisms, and Tm optimization through gradient PCR all contributed to the primers' robustness. This methodological approach—combined with rigorous in silico and in vitro testing—strengthens confidence in the primers' utility for distinguishing *M. mulatta* from sympatric primates, which has been a recognized challenge in field-based genetic studies involving non-invasive sampling methods.

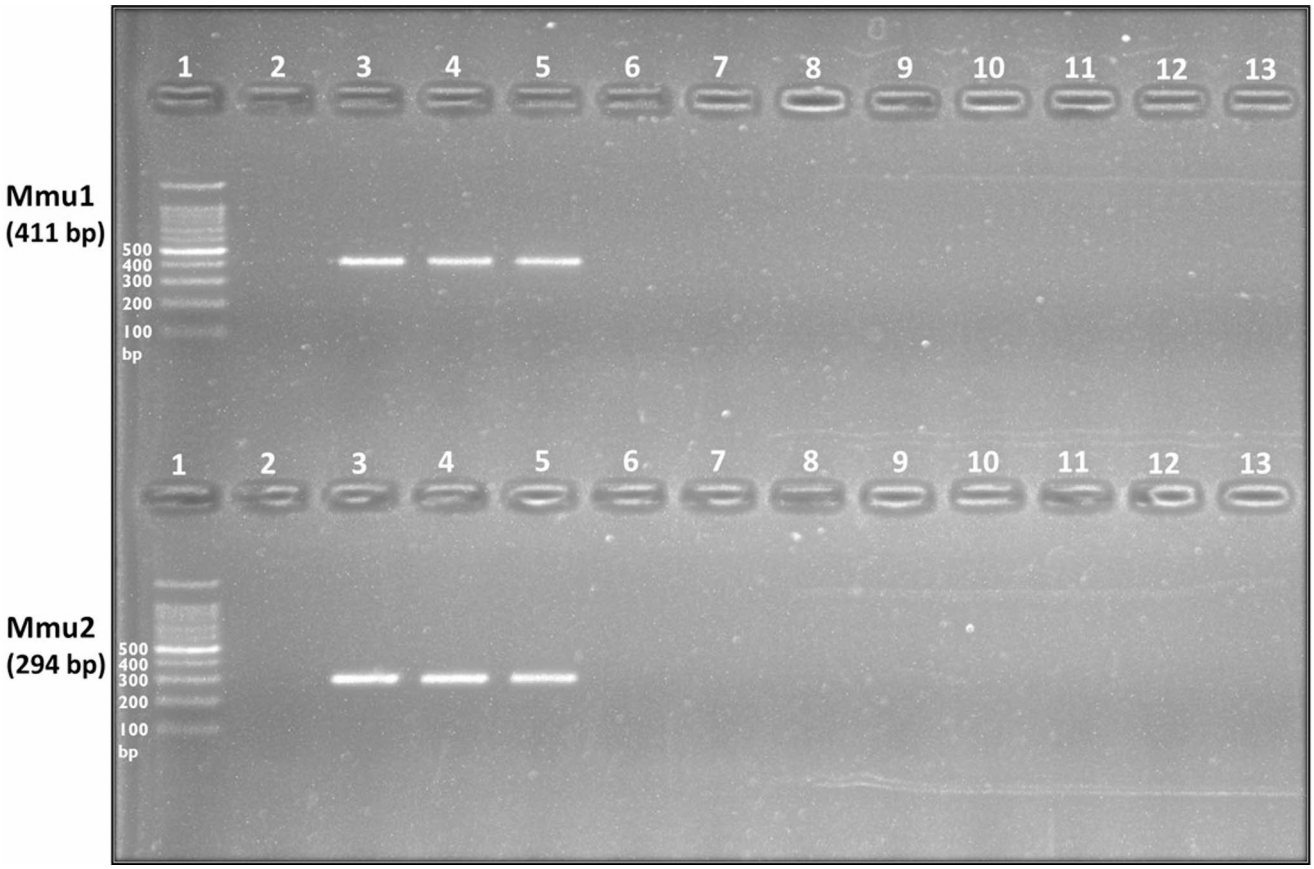

Fig. 2 Electrophoresis picture (2% agarose gel) showing amplication of both primer pairs Mmu1 and Mmu2 only in target species rhesus macaque's DNA sample at expected range, while no amplification was observed in other non-target species. (1: 100 bp Ladder, 2: PCR Negative Control, 3–5: Rhesus Macaque (3- Blood DNA; 4- Fecal DNA; 5-Hair DNA), 6: Bonnet Macaque, 7: Lion-tailed Macaque, 8: Crab-eating Macaque, 9: Stump-tailed Macaque, 10: Pig-tailed Macaque, 11: Hanuman Langur, 12: Western Hoolock Gibbon, 13: Human)

Comparison with previous work

Bhaskar et al. 2025 [34] recently developed COI-based primers targeting a 440 bp mitochondrial segment for identifying Indian rhesus macaques using degraded fecal samples. While their work focused on broader phylogenetic resolution and lineage differentiation within *M. mulatta*, our study emphasizes species-level discrimination among sympatric primates in field ecology contexts. Importantly, our primers were tested against a wide panel of potential confounding species and optimized for degraded DNA, making them better suited for ecological, behavioral, or population studies where rapid, low-cost species confirmation is required without full sequencing.

Applications in behavioural and ecological research

These species-specific primers offer a foundational tool for large-scale non-invasive sampling projects, particularly for initial species confirmation before applying more detailed nuclear genotyping methods. By eliminating the need for full sequencing to confirm species identity, they provide a time- and cost-effective solution that is well suited for large-scale or resource-limited field studies. This is especially valuable in multi-species habitats or when working with unhabituated groups where direct observation is challenging. Although the current primers are species-specific and not designed to reveal intraspecific polymorphism, they help reduce ambiguity in sample assignment and thus support ecological and behavioural studies. Once species identity is confirmed, additional genotyping methods — such as microsatellite or SNP-based analyses — can then be used to explore individual identity, kinship, reproductive skew, or mating patterns [35]. This layered approach is particularly relevant in behavioural ecology, where linking genetic data to observed social or reproductive patterns requires nuclear markers.

Limitations and future directions

While our primers demonstrated high specificity and performance in the tested samples, future studies could extend this

validation by evaluating amplification success in samples stored over longer durations and exposed to various environmental conditions (e.g., temperature, humidity, UV). Additionally, sequencing-based confirmation of amplified products from larger sample across broader geographic populations of *M. mulatta* will strengthen confidence in primer reliability. It is also worth exploring their performance on environmental DNA (eDNA) samples collected from substrates like soil or water. A further refinement could involve developing multiplex PCR assays that include a suite of macaque-specific primers or developing a barcoding pipeline that detects and differentiates multiple species from complex environmental samples, enabling community-level monitoring.

Conclusion

The development of species-specific primers for rhesus macaques offers a valuable addition to the molecular toolkit for non-invasive research on wild populations. By eliminating the risk of cross-species amplification, these primers will enhance the accuracy of ecological, behavioral, and genetic studies relying on non-invasive sampling methods. Their cost-effectiveness and scalability make them especially useful for large-scale non-invasive studies. As non-invasive genetic techniques continue to evolve, tools like these will play a key role in advancing ecological and conservation research on wild primate populations.

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Author contributions Deepika Boora: fieldwork, lab work, data curation, data analysis and visualization, writing-original draft; Shweta Singh: conceptualization, methodology; Sanath Krishna Muliya: funding acquisition; Lallianpuii Kawlni: supervision, funding acquisition, project administration and coordination, writing-reviews and edits; Kafil Hussain: project administration and coordination; Virendra Prasad Uniyal: supervision, coordination, writing-reviews and edits; Qamar Qureshi: conceptualization, methodology, supervision, funding acquisition, writing-reviews and edits; Vishnupriya Kolipakam: conceptualization, methodology, supervision, funding acquisition, project administration and coordination, writing-reviews and edits.

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Data availability All supporting data are included in the manuscript as well as additional files in the supplementary section.

Declarations

Competing interests The authors declare no competing interests.

Ethics approval Sample collection of Rhesus macaques was undertaken at the study site as per permissions granted by the competent authority i.e. Uttarakhand Forest Department, Govt. of Uttarakhand vide letter no. 1541/5–6, dated 12th November 2021, in accordance with the Wildlife Protection Act (1972), Govt. of India. Competent authority and the concerned institutional committee of the Wildlife Institute of India permitted the study with all due diligence for animal ethics.

Consent to participate Not applicable.

Consent to publish Not applicable.

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