

## RESEARCH NOTE



# Noninvasive DNA-based species and sex identification of Asiatic wild dog (*Cuon alpinus*)

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**Abstract.** Asiatic wild dog (*Cuon alpinus*) or dhole is an endangered canid with fragmented distribution in South, East and Southeast Asia. The remaining populations of this species face severe conservation challenges from anthropogenic interventions, but only limited information is available at population and demography levels. Here, we describe the novel molecular approaches for unambiguous species and sex identification from noninvasively collected dhole samples. We successfully tested these assays on 130 field-collected dhole faecal samples from the Vidarbha part of central Indian tiger landscape that resulted in 97 and 77% success rates in species and sex identification, respectively. These accurate, fast and cheap molecular approaches prove the efficacy of such methods in gathering ecological data from this elusive, endangered canid and show their application in generating population level information from noninvasive samples.

**Keywords.** dhole-specific primers; molecular sexing; canid conservation; dhole distribution; demography; large carnivore; faecal DNA.

## Introduction

Asiatic wild dog (*Cuon alpinus*), commonly also known as ‘dhole’, is the only species in the genus *Cuon*. Once, distributed across North America, Europe and Asia during Pleistocene, dholes are currently extinct from most of their historic range, and are currently found in small, fragmented populations restricted to Bhutan, Cambodia, China (including Tibet), India, Indonesia (Sumatra and Java), Laos, the Malaysian Peninsula, Myanmar, Thailand and Vietnam (Durbin *et al.* 2004). Their population decline is majorly driven by habitat loss, prey depletion, disease and human persecution globally (Hayward *et al.* 2014). They are categorized as ‘endangered’ according to IUCN classification (Durbin *et al.* 2004; Kamler *et al.* 2015) and ‘Schedule 2’ of Wildlife Protection Act of India (1972, i.e. higher level of protection) and their future survival is depended on detail ecological, demographic and population level information across their range (Ghaskadbi *et al.* 2016).

However, gathering information on this highly social, elusive canid in their natural habitat within dense forests (Johnsingh *et al.* 1985), where they co-occur with other canid species (fox, jackal etc.) is challenging. In this context, noninvasive methods can be very useful in generating information on dhole distribution and other population parameters. To date, the only study on Asiatic wild dog by Iyengar *et al.* (2005) has assessed phylogeography and genetic structure of dholes with faecal samples collected from Mudumalai Wildlife Sanctuary, India and Baluran National Park, Indonesia. No species-specific molecular assay was developed and species identification was conducted through sequencing. Also, molecular sexing was performed using a single Y chromosome marker to detect males. As these approaches are expensive, time consuming and potentially can produce false negative results during sexing, therefore, it is critical to develop an accurate species and sex identification assay for poor quality field-collected dhole samples. In this paper, we describe a simple and fast species identification approach from degraded biological samples and design a multiplex molecular sexing assay involving both X and Y chromosome markers for dholes.

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We successfully tested these assays on field-collected dhole faecal samples from the western part of central Indian tiger landscape and show their application in generating population level information from noninvasive samples.

## Materials and methods

### Study area

This study was conducted in the Eastern Vidarbha landscape of Maharashtra, India bordering with Madhya Pradesh in the north, Chhattisgarh in the east and Telangana in the south. This landscape comprises four major protected areas (covering both tiger reserve as well as wildlife sanctuary) of the state of Maharashtra. This entire landscape is semiarid and characterized by dry deciduous forest with vegetation mainly consisting of *Tectona grandis*, *Anogeissus latifolia*, *Lagerstroemia parviflora*, *Terminalia* spp., *Heteropogon contortus*, *Themeda quadrivalvis*, *Cynodon dactylon* etc. We conducted an intensive faecal sampling in tiger reserves, i.e. Pench Tiger Reserve (257.3 km<sup>2</sup>), Navegaon-Nagzira Tiger Reserve (152.8 km<sup>2</sup>), Tadoba-Andhari Tiger Reserve (627.5 km<sup>2</sup>) and Umred- Karhandla Wildlife Sanctuary (189 km<sup>2</sup>) (figure 1). All these four protected areas represent different diverse habitat types where dholes are found in this landscape. The entire study was conducted using field-collected faecal samples. Appropriate permissions for faecal sampling were provided by Maharashtra Forest Department (permit no. 09/2016).

### Field sampling

For faecal sampling, the entire study area was intensively surveyed using a vehicle as well as on foot to look for dhole latrine sites. Dholes have a communal latrine system where they defecate in groups generally at the junction of roads (Johnsingh 1982). All samples were collected fresh from latrine sites during our surveys. As dholes defecate in groups, it is challenging to ascertain samples to individual levels in latrine sites. During sampling we collected each bolus as different sample.

The sampling was conducted between November 2015 and February 2016. All the samples were collected with GPS coordinates along with supplementary information, i.e. track marks, substrata etc. The entire scat sample was collected in sterile zip-lock bags, dried in shade inside the field station and subsequently shipped to the laboratory, where they were stored at  $-20^{\circ}\text{C}$  until further processing. We collected a total of 130 scats from all four areas during our sampling period.

### Primer selection

As no species-specific molecular assay is available for dholes, we developed a polymerase chain reaction (PCR)-based approach by designing species-specific mtDNA

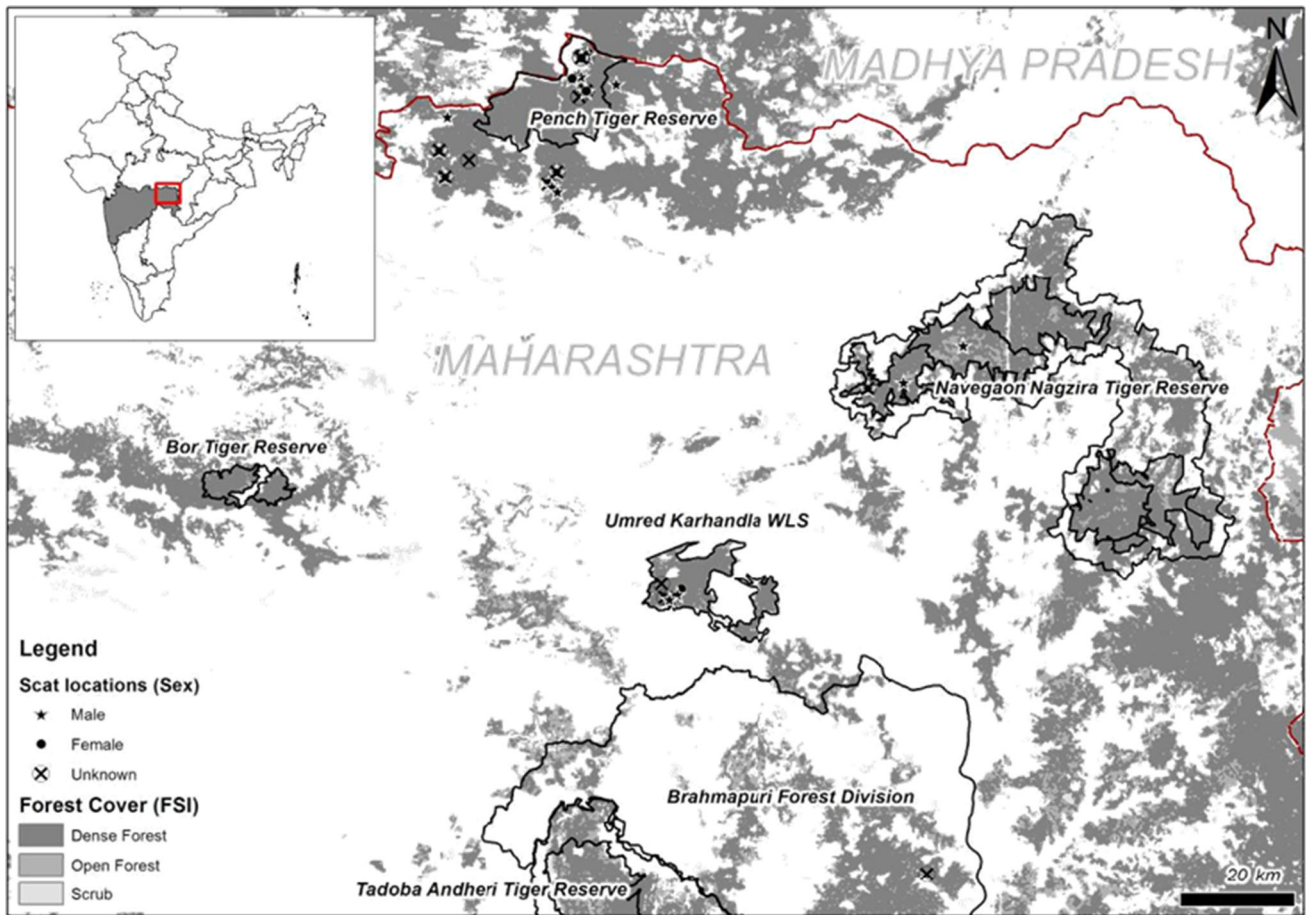
primers to unambiguously identify dholes from degraded, low quality biological samples. Complete mtDNA sequences of dhole and other canids including domestic dog, golden jackal, fox and Indian grey wolf were downloaded from the GenBank (accession numbers: dhole, 261840635; domestic dog, 17737322; golden jackal, 926459877; fox, 115345109 and Indian grey wolf, 294774473). The downloaded sequences were aligned using MEGA6 (Tamura et al. 2013) and screened visually to find regions with species-specific variations. We manually designed four sets of primers that amplify  $<250$  bp amplicon size to ensure higher amplification success from degraded DNA.

For molecular sexing, we combined two earlier described sexing markers, one used for wolves (DBY and AHT-X40) by Sastre et al. (2009) and another used for dholes (SRY) by Iyengar et al. (2005) to develop a multiplex PCR system.

### DNA extraction and primer standardization

All field-collected scat samples ( $n = 130$ ) were swabbed twice using sterile swabs (HiMedia, Pennsylvania, USA) in the laboratory (Ball et al. 2007). The swabs were placed in an Eppendorf tube and stored at  $-20^{\circ}\text{C}$  until further processing. DNA extraction was performed with a DNeasy Tissue kit (Qiagen, Hilden, Germany) using a modified approach. Lysis was performed overnight with 300  $\mu\text{L}$  of lysis buffer, followed by the manufacturer's protocol provided in the kit. DNA was eluted twice with 100  $\mu\text{L}$  of  $1 \times \text{TE}$  and stored at  $-20^{\circ}\text{C}$  for long-term storage. Each set of 22 extractions were accompanied with two negative controls to monitor possible contamination.

All the primers were standardized using three confirmed dhole faecal DNA collected from the state of Maharashtra, India, and tissue and hair DNA from other co-occurring canids. Following post-temperature standardizations, PCR reactions were performed with 4  $\mu\text{L}$  of hot start Taq mix (Qiagen), 4  $\mu\text{M}$  BSA, 0.5  $\mu\text{M}$  of primer mix and 3  $\mu\text{L}$  of DNA extract under conditions including initial denaturation ( $95^{\circ}\text{C}$  for 15 min); 50 cycles of denaturation ( $94^{\circ}\text{C}$  for 30 s), annealing ( $50^{\circ}\text{C}$  for 30 s) and extension ( $72^{\circ}\text{C}$  for 35 s); followed by a final extension ( $72^{\circ}\text{C}$  for 10 min). To check any cross-species amplification all primers were tested against domestic dog, Indian grey wolf, golden jackal, fox, tiger and leopard DNA samples. During all amplifications, both extraction controls and PCR negative controls (one PCR negative for every set of amplifications) were included to monitor any possible contamination. Finally, selected sets of dhole-specific primers were tested on all field-collected scats ( $n = 130$ ) to ascertain the species status. PCR products were visualized in 2% agarose gel, and 37 representative samples were randomly selected, cleaned using Exo-SAP and sequenced using forward primers for validation. All sequences were visually



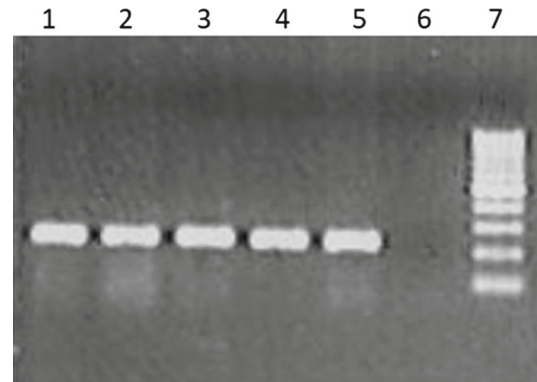
**Figure 1.** Field sampling locations for scats (male, female and unknown) in our study area within the state of Maharashtra.

examined and matched against the GenBank database for species confirmation.

For molecular sexing, PCR reactions were performed for dhole samples with multiplex buffer mix (Qiagen), 4  $\mu$ M BSA, 0.5  $\mu$ M of primer mix and 1–3  $\mu$ L of DNA extract under conditions including initial denaturation (95°C for 15 min); 50 cycles of denaturation (94°C for 30 s), annealing (55°C for 30 s) and extension (72°C for 30 s); followed by a final extension (72°C for 10 min). PCR products were electrophoresed in 5% intermediate melting temperature agarose (MetaPhor Agarose, Lonza) for 60 min at 120 V and sex determination was performed by analyses of sex-specific band patterns. We used reference tissue samples of three male dogs, two female dogs and one male wolf along with dhole positive samples (two male and one female) to standardize the multiplex sexing system. For all samples, three independent PCR reactions were conducted to confirm sex identification.

## Results and discussion

We developed four sets of potential dhole-specific mitochondrial markers for species identification. Of the four



**Figure 2.** Representative gel picture of PCR amplified products of dhole-specific mitochondrial primer designed in this study. Lane 1–4 shows amplification from field-collected dhole faecal samples, Lane 5 shows amplification from dhole positive sample, lane 6 is PCR negative and lane 7 contains 100-bp DNA ladder.

primer sets, only one pair of markers produced desired results with the reference samples. This set produced 236-bp dhole specific amplicons (figure 2) from the reference dhole samples (table 1) and showed no cross-species amplification with other canid species used in this

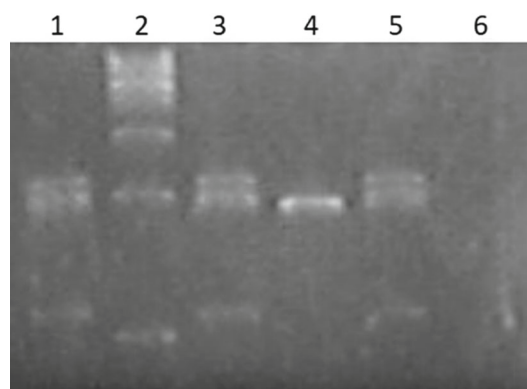


**Table 1.** mtDNA and sexing markers used in this study.

Primer name	Sequence	$T_a$	Amplicon size (bp)	Reference
DholespIDF	CAGCCTGTCCTATCTGAG	50	236	This study
DholespIDR	ATTAGTTTCAGAGGCGGTA			
DBY-F	TTGGGGGTGGTTTTATTGTC	55	Male: 112	Sastre <i>et al.</i> (2009)
DBY-R	CCATCTCAACATCGCTGAAC			
AHTx40-F	GTAGCCCCATTTGTTTATATTTGC	55	Female: 190	Sastre <i>et al.</i> (2009)
AHTx40-R	GATCACTGTCTTACACCACAGGC			
Canis SRY-F	ATGGCTCTAGAGAATCCCCA	55	Male: 199	Iyengar <i>et al.</i> (2005)
Canis SRY-R	GCAATTTGTGACTTTTCTGTGC			

study. The other three marker sets produced nonspecific amplifications with other species and were discarded subsequently. Our confirmatory sequencing results with randomly selected faecal samples ( $n = 37$ ) showed accurate dhole identification from all the samples when matched against the GenBank database (accession number: KY860107–KY860143). Therefore, any positive amplification observed subsequently from faecal samples with this band pattern was hence considered as originated from dholes. From all the field-collected faecal samples ( $n = 130$ ), we successfully ascertained 126 faecal samples to the species level as dholes (success rate of 97%), much higher than the earlier study by Iyengar *et al.* (2005) where species identification success rate was about 65%. Apart from higher success rate, this approach also helps in rapid screening of large number of samples due to less number of steps during sample processing (DNA extraction, PCR and electrophoresis) and is cheaper than sequencing-based approaches.

The sexing multiplex PCR resulted in a three-band pattern (112, 190 and 199 bp from DBY, AHTX-40 and SRY genes, respectively) for males and a single band (190 bp from AHTX-40 gene) for females (figure 3). We did not find any cross-gender amplification with any of the reference or field-collected samples. Success rate for sex discrimination for all field-collected dhole samples ( $n = 126$ ) was 77%. We identified 71 male and 26 female dhole scats from the field (sex ratio 2.7 : 1). Earlier ecological work on dholes in southern India (Venkataraman 1998) suggests male biased packs, supporting the pattern found in this study. This molecular sexing method with multiple markers is advantageous over earlier approaches used for canid sexing (for example, restriction digestion – Ortega *et al.* (2004); high-resolution melting analysis – Gonzalez *et al.* 2015; tagged fluorescent markers – Sastre *et al.* (2009)). This new approach is cheap, provides quick results and shows reduced sex misidentifications or ‘false negatives’ of males due to allelic dropout from the Y chromosome, specifically in poor quality samples. Positive amplifications from reference canid species (dog and wolf) suggest that this multiplex sexing approach would be useful in other canid species also. However, it is noteworthy to point out that during molecular sexing standardization



**Figure 3.** Representative gel picture of PCR amplified products of the multiplex molecular sexing assay developed for canids in this study. Lanes 1 and 5 show amplification from field-collected male faecal DNA samples, lanes 3 and 4 show amplification from a known male and female dhole sample respectively, lane 6 is PCR negative.

we used expensive intermediate melting agarose to differentiate two sex chromosome marker bands separated by 9 bp (Canis SRY, 199 bp and AHTX-40, 190 bp). It is possible to confirm male and female dholes by running two separate PCRs (first AHTX-40 and DBY and then with the SRY marker) if such expensive products are logistically difficult to arrange.

This PCR-based technique for species identification and molecular sexing is a fast, accurate and cheap molecular approach and it is advantageous over standard field-based observations as it allows the large sample coverage and availability for assessing dhole occurrence and demography at landscape levels. This PCR- and electrophoresis-based assay is simple, highly specific to dholes and showed high success rate (97% in species identification) from field-collected, poor quality degraded dhole samples. The multiplex sexing assay showed unambiguous results with high success rate (77%) in dholes and potentially useful in studying other canid species.

In conclusion, these molecular approaches will be crucial in gathering baseline ecological and genetic information of dholes, and therefore help in conservation and management across their current range.

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