UNDERSTANDING THE IMPACT OF HABITAT FRAGMENTATION ON SYMPATRIC LARGE CARNIVORE CONNECTIVITY ACROSS TIGER RESERVES IN MAHARASHTRA, INDIA

A THESIS
Submitted by

SHRUSHTI MODI

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DOCTOR OF PHILOSOPHY
IN
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Under the guidance of
Dr BILAL HABIB

Saurashtra University
Rajkot – 360 005

भारतीय वन्यजीव संस्थान
Wildlife Institute of India

Dehradun, Uttarakhand, India

FEBRUARY- 2022

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I, **Shrushti Modi**, declare that the thesis entitled **"Understanding the Impact of Habitat Fragmentation on Sympatric Large Carnivore Connectivity across Tiger Reserves in Maharashtra, India"** submitted by me for the degree of Doctor of Philosophy is the record of research work carried out by me during the period from January 2017 to October 2021 under the guidance of **Dr. Bilal Habib**, Scientist-E, Wildlife Institute of India and co-supervision of **Dr. Samrat Mondol**, Scientist-E, Wildlife Institute of India and has not formed the basis for the award of any degree, diploma, associateship, fellowship, titles in this or any other University or other institution of higher learning. I further declare that the material obtained from other sources has been duly acknowledged in the thesis. I shall be solely responsible for any plagiarism or other irregularities if noticed in the thesis

Shrushti Modi
Wildlife Institute of India, Dehradun, Uttarakhand (India)-248001 &
Saurashtra University, Rajkot, Gujarat (India)-360005

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Dr. Bilal Habib
Scientist E
Department of Animal Ecology & Conservation Biology
Supervisor

Dr. Samrat Mondol
Co-Supervisor

Forwarded:
Dr. Ruchi Badola,
Dean, Faculty of Wildlife Sciences

Place: Dehradun
Date: 2nd February 2022

SAURASHTRA UNIVERSITY P.G.T.R. Section
Main office, First Floor, University Road,
Rajkot - 360 005(Gujarat) Phone No.: 2578501
www.saurashtrauniversitv.edu
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2	**Modi, S.**, Habib, B., Ghaskadbi, P., Nigam, P., & Mondol, S. (2019). Standardization and validation of a panel of cross-species microsatellites to individually identify the Asiatic wild dog (*Cuon alpinus*). *PeerJ*, *7*, e7453.
3	**Modi, S.**, Mondol, S., Nigam, P., & Habib, B. (2021). Genetic analyses reveal demographic decline and population differentiation in an endangered social carnivore, Asiatic wild dog. *Scientific reports*, *11*(1), 1-1.
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1	**Modi, S.**, Mondol, S., & Habib, B. (2019). Social canid in a metapopulation network: Dhole genetics in Eastern Vidarbha landscape, Maharashtra, India. Oral talk presented at: *56th Annual Meeting of the Association for Tropical Biology & Conservation*, 2019 July 30 – Aug 3, Antananarivo, Madagascar.
2	**Modi, S**., Mondol, S., & Habib, B. (2021). The 3D view: Genetic evidences of differentiation, dispersal and demography of Asiatic wild dog in Maharashtra, India. Oral talk presented at: the 2021 International Congress for Conservation Biology, 2021 December 13 – 17, Kigali, Rwanda.

Executive Summary

In the present era of the Anthropocene, habitat loss and fragmentation are major concerns for the survival of any species in the wild. The adverse effects caused by fragmentation lead to the destruction of natural habitats, resulting in biodiversity loss. The conversion of large contiguous forest land into small patches surrounded by human habitation with no connectivity leads to an increased probability of extinction in the long run. The increased risk of extinction mainly arises due to loss of genetic diversity and increased stochastic events. Therefore, maintaining connectivity among the populations is the only possible way to conserve the populations in the wild. Protecting both functional and structural connectivity is essential for the conservation of biodiversity. The effects of fragmentation on large carnivores' ecological and genetic structure are alarming because of their relatively low numbers, interaction with humans, and large home ranges. Owing to their low fecundity and long generation time, they are more prone to reduction in genetic variation. Co-occurrence of these large carnivores is another challenge in the present era of human-dominated landscapes due to competition for prey, habitat and resources. It is essential to understand the effect of habitat fragmentation on their genetic structure and its long-term consequences on the existing population of these species to maintain the persistence and co-existence of large carnivores.

This thesis is an effort to understand the impact of habitat fragmentation on the connectivity of sympatric large carnivores across the tiger reserves of Maharashtra. The top predator assemblage in Maharashtra's major tiger reserves and surrounding areas consist of three species: tiger, leopard, and dhole. Most connectivity studies focus on the single species, which does not incorporate other species' requirements and conservation interests. As a result, given the current context of haphazard development, there is a need to develop a multispecies conservation plan that focuses on multidimensional aspects for several species of interest. Given this, the current study was conducted in major tiger reserves and surrounding protected areas, i.e., Melghat Tiger

Reserve (MTR), Tadoba-Andhari Tiger Reserve (TATR), Nawegaon-Nagzira Tiger Reserve (NNTR), Pench Tiger Reserve (PTR), Sahyadri Tiger Reserve (STR), Umred Karandhla Wildlife Sanctuary (UKWLS) in the state of Maharashtra. The objectives of the study were to (i) Develop and standardise molecular markers for species and individual identification, (ii) Meta-population dynamics of tiger, leopard and Asiatic wild dog across the tiger reserves of Maharashtra, (iii) Impact of habitat fragmentation on the gene flow and genetic connectivity across the habitat connectivity gradient of the tiger reserves.

Faecal sampling was opted for a landscape-level connectivity study of these three large carnivores due to its extensive coverage and non-invasive nature. The first challenge was to identify the species from the scat. There were already species-specific markers for tiger and leopard, but no such species-specific method available for dholes. Therefore, I first standardised the already available markers of tiger and leopard with the positive sample of tiger and leopard from Maharashtra. I developed a novel PCR-based method for species identification and molecular sexing for dholes. The markers were developed by aligning the mitochondrial genome of wolf, dog, dhole and jackal and then looked for site-specific differences exclusive for dhole. Using this approach, I selected three sets of primers, out of which one was finally selected based on amplification success and species specificity. The developed primers provided a 97% amplification success rate with faecal samples. I collected a total of 1156 scats, of which 250 were identified as tiger, 151 as leopard and 590 as dhole using the mitochondrial species-specific markers.

After confirming the species, I used nuclear microsatellite markers for population-level study to identify individuals. The cross-species markers panel for tiger and leopard were used from previous studies and standardised with the positive samples for this study. I standardised a new set of cross-species markers from dogs with a higher PID value than the prior dhole study. I generated a dataset of 200 tigers, 95 leopards and 305 dhole unique genotypes, attaining a success rate of 85%, 62% and 59.1%, respectively.

To determine the effect of fragmentation, I first looked at the genetic aspect, where I used multiple analyses to determine the genetic differentiation of the three species across the sampled areas. The Bayesian clustering and multivariate analyses showed the highest genetic differentiation in dholes while the lowest in the leopard population. I further used spatial multivariate analyses, which confirmed the same differentiation pattern. The gene flow pattern for the three species was conducted using the Bayesian approach again showed the same pattern as the differentiation analyses with the highest mean gene flow for leopard and lowest mean gene flow for dhole. I further looked at the demographic pattern of dholes as it was not explored earlier as opposed to tiger and leopard. I used a coalescent framework approach and found a population decline around 300 years back in both the major dhole populations of Maharashtra.

To confirm the dispersal pattern in dholes, I conducted STRUCTURE and spatial autocorrelation analyses on TATR and NNTR dhole populations. I found that males are more differentiated than females in TATR, while in NNTR, there is no such difference in the structure of males and females. The spatial autocorrelation test also reveals that NNTR males are positively autocorrelated at shorter distances and negatively autocorrelated at longer distances. In contrast, NNTR females are positively autocorrelated only at one distance class at 40-45 km. In the case of TATR, we found a similar pattern. According to the spatial autocorrelation results, males are often more similar to females at shorter distances, implying that males are more philopatric than females.

I used the Resistance GA package in the R programme to overlay the genetic difference among the three carnivores with geographical connectivity. I employed a linear mixed-effects model fit using genetic distance as the dependent variable and scaled and centred resistance distance as the predictor variable. Mixed models were fitted using the maximum-likelihood population effects parameterisation to account for the nonindependence of the pairwise genetic and ecological distances. The resistance surface of the optimised variables obtained from Resistance GA was used as an input for current map generation in Circuitscape using Julia. The results of univariate optimisation and model selection show that the most supported model for tiger is evapotranspiration and distance, whereas for leopard, it is ndvi and distance, and for dholes, it is evapotranspiration and population. For multi-surface optimisation, the bootstrap analysis for 1000 iterations predicts distance as the best model fit for tiger and leopard, and evapotranspiration as the best-supported model for dholes. Out of the three species, only dhole shows a difference in model fit for multivariate and univariate optimisation

The current map derived from the optimised resistance surface shows that there are extremes of connectivity current either very low or very high for tigers, indicating significant barriers in tiger movement. Still, telemetry and genetic data suggest otherwise, showing that tigers are exploring areas of the landscape that are not proper habitat. In the case of leopards, I get a network mesh with low to high current running over the entire landscape, which is quite similar to the genetic admixture pattern derived from genetic data. This also demonstrates that distance can be a crucial determining factor for leopards. I found higher current around the TATR complex and medium to low current around the PTR and MTR complex for dholes. However, this contradicts genetic differentiation results, which show that dholes can traverse far but cannot move outside the protected areas due to restricted habitat. Continuous small forest patches are essential for dhole movement.

Pinch points may be conservation priorities due to the lack of alternate movement routes. The loss of these areas may affect migration routes or other critical needs. The tiger pinch-point map found only one viable path for movement with pinch points areas. The leopard map indicates that preserving one or more pinch spots can conserve the travel path on the same scale as the tiger. Again, prioritising single moving pathways as corridors is critical for the dhole map. Due to habitat requirements and adaptations, the current map reveals similarities to genetic structure results for leopards but not for tigers and dholes.

The three species from the major carnivore guild suggests that landscape genetic patterns respond to the ecological differences in habitat characteristics, and disturbance can differentially affect landscape genetic structure. The study develops a framework for the landscape genetic study of any species starting from the sampling to understanding the impact of habitat fragmentation on the functional as well as structural connectivity of these species. The empirical findings of this study provide an outlook to delineate the corridor planning as per species need and do not follow the one for all approach. This study also developed the first PCR based protocol for dhole species identification and a better individual identification panel. The study will help in introducing species-specific modifications in connectivity corridors planning. It has paved the way to consider the requirements of each species while establishing priority-based conservation. As obtained in this study, landscape genetics outputs, coupled with multispecies connectivity models, could help prioritise the conservation of areas where maintaining connectivity is most critical.

Dedicated to

VatKir

CHAPTER 1

Habitat fragmentation and geographical connectivity across the tiger reserves of Maharashtra

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Habitat fragmentation and geographical connectivity across the tiger reserves of Maharashtra

1.1 Introduction

1.1.1 Fragmentation

Habitat fragmentation is a very bandied-out term casually used for any type of disturbance in a habitat. However, it means disrupting a large continuous habitat patch into small isolated patches with no connectivity (Fahrig, 2003). Fragmentation can arise from four scenarios (Collinge et al., 1998), i.e. perforation, shrinkage, fragmentation, bisection. Fragmentation alters the structure and pattern of habitats across the landscape, resulting in lower habitat quality than areal losses would suggest. Roads, railways, and other man-made barriers divide ecosystems, reducing landscape connection (Forman & Alexander, 1998). Habitat fragmentation can have a variety of adverse effects, including decreased abundance (Flather et al., 2002), altered movement patterns (Brooker & Brooker, 2002), disruption of social structure (Cale, 2003), and population isolation (Frankham, 1996), ultimately leading to decrease in genetic diversity, reduced survival and reproduction (Frankham, 1995) on species ranging from minute ants to large mammals like tigers and can lead to a decline in wildlife populations (Hansen & DeFries, 2007). Habitat fragmentation reduces the amount of structural core area available, resulting in smaller patches, higher edge-to-interior ratios, increased patch isolation, and variability in patch connectedness. (Saunders et al., 1991). It has the potential to disrupt food chains by altering trophic linkages, resulting in secondary extinctions in food webs. (Bolger et al., 2000; Woodward et al., 2012).

Fragmentation has a greater impact on higher-trophic-level species, which rely on vast stretches of undisturbed habitat and are extremely vulnerable to human activities (Crooks, 2002). Reduced connectivity can have an impact on foraging on a daily basis, annual migrations, genetic mixing rates, and population persistence (Crooks, 2002). Therefore, large carnivores are most vulnerable to the change in habitat (Liao et al., 2017). However, the species can further display various responses due to the differences in their dispersal range and the preference for their habitat quality: habitat generalists are affected lesser than habitat specialists (Fahrig, 2017). Also, the species' evolutionary predilection for crossing "gaps" or barriers in the landscape determines the extent of the effect caused by fragmentation (Fischer & Lindenmeyer, 2007). Mammalian predators, as area-dependent species that require movement corridors for survival, can be suitable focal organisms for assessing the degree of functional landscape-level connectedness

1.1.2 Connectivity & Corridors

Merriam (1984) originated the term "landscape connectivity," which was later defined by Taylor and others (1993) as "the degree to which the landscape facilitates or impedes movement among resource patches." It is further classified as functional and structural, where structural connectivity defines the contiguity of forest patches in the landscape and does not account for the actual land use by wildlife (Theobald et al., 2012). Functional connectivity, on the other hand, is dependent on species and their genes being able to move across areas of suitable habitat in a fragmented landscape (Fahrig, 2003; Hilty et al., 2006). To model the functional connectivity following criteria must be taken into consideration (i) the phenotypical traits of the mover, (ii) the behaviour of the moving object, and (iii) the characteristics of the medium that affect movement (Calabrese & Fagan, 2004).

It is essential to protect both the structural and functional connectivity for the conservation of biodiversity (Bennet, 1999). Structural isolation of species can be because of habitat loss which leads to facing dispersal barriers and reduced access to food and mates (Moilanen & Hanski, 1998). But the functional isolation is more critical in the form of decreased genetic diversity, which may further lead to inbreeding depression (Hedrick & Kalinowski, 2000) and higher disease susceptibility presenting the species at higher risk of extinction (Spielman et al., 2004;

Charlesworth, 2009). Extended functional isolation can lead to structural isolation of species present in small disconnected habitat patches. Combined functional and structural connectivity evaluation can be possible using the multidisciplinary field of landscape genetics.

The term "meta-population" was coined by Levins (1969) and elaborated by Hanski (1994) to describe a set of local populations occupying distinct habitat patches and connected through individual movement (Ricklefs & Miller, 2000). The proportion of patches inhabited is calculated using metapopulation theory as a function of the ratio of patch-level extinction and recolonization (Levins 1970). Even in ecosystems with limitless habitats, Lande (1987) theorised that substantial fragmentation will lead to extinction for territorial species. Metapopulation models are critical tools for analysing the dynamics of fragmented populations and assessing the impact of (de)fragmentation (Carmichael et al., 2007). Landscape ecology and meta-population ecology go hand in hand to develop measures determining the viability of local populations in the heterogeneous structured landscape (Hanski, 1999). This implies that the major determining factor for the survival of any species in the present scenario of discontinuous and fragmented habitats are dispersal and geographical as well as genetic connectivity.

The corridors assisting movement through these patches provide connectivity in a fragmented landscape. A corridor is defined as a "naturally existing or restored native linear landscape feature that connects two or more larger tracts of essentially similar habitat and functions as either a movement route for individuals or an avenue for gene-flow among native fauna and flora" (Saunders et al., 1991). Wildlife corridors are an effective tool to combat the ill effects of habitat fragmentation and to help in maintaining the balance. It is the most reasonable and possible solution for conserving biodiversity and the co-existence of humans and wildlife in the present world. The corridors can vary from species to species as each species has its degree of sensitivity to fragmentation. Species that are highly sensitive to fragmentation, such as north olive thrushes, do not prefer the edges; corridors for them should be at the minimum distance from the end of the habitat (Newmark, 1993). However, in a country like India, with the second-highest population globally, it is not possible to design artificial corridors for every single species. Therefore, it is of utmost importance to restore the natural patches acting as corridors among the protected areas, which can be possible by developing a better understanding of the contribution provided by these corridors at a multi-species level.

In the Anthropocene era, most of these corridors form a matrix of cleared agricultural land surrounding the habitat patches. The matrix is the determining factor for any species' movement and mainly consists of various landscape features and resources. Different species perceive this matrix differently depending on their habitat and resource requirement, which further affects their dispersal behaviour through these fragments (Desrochers & Hannon, 1997). With the heterogeneous nature of the matrix and the different species' abilities to penetrate it, isolation of habitat patches suggests two types of connectivity: structural connectivity (the availability of interconnected habitat elements such as habitat corridors) and functional connectivity (whether animals move successfully through the landscape) (Tischendorf & Fahrig, 2000; Kupfer et al., 2006). This means that to understand the impacts of fragmentation on different species, it is important to develop a species- and landscape-specific measurement of isolation for landscape connectivity throughout the meta-population network (Tischendorf & Fahrig, 2000).

1.1.3 Connectivity Modelling

To understand and model the connectivity of species, two models are generally used considering various biotic and abiotic factors:

Least-Cost Model (LCM)

It identifies the single best pathway between the locations to measure the extent of connectivity. LCM evaluates potential animal movements across a landscape based on the cumulative cost of the movement (Sawyer et al., 2011; Howey, 2011) and selects the single best path as the model output. The model requires two inputs for the evaluation, the two areas which are being connected and the 'cost' layer for the landscape. It allows to compare several cost landscapes with varying details (Sawyer et al., 2011) but comes at the expense of accuracy to model the movement of the concerned species.

Circuit theory

This model considers movement to be completely random and independent of previous knowledge of the route with no preferred directions (Codling et al., 2008). It allows multiple pathways to be evaluated rather than the best fit line (McRae et al., 2008). It utilizes selection bias to understand the movement behaviour of a species based on existing barriers (McRae et al., 2008). For example, when an animal moves in a fragmented landscape, its movement can be decided by factors such as (i) avoiding crossing the boundary into an urban area, (ii) immediately leaving the urban area if it has been entered, (iii) decreasing the time in the area by moving more often or at a greater rate, or (iv) some combination of the above (McRae, 2006). This theory is more accurate in predicting animal and gene dispersal rates due to its use of multiple pathways and incorporation of animals' selection bias (McRae & Beier, 2007).

In this model, the movement behaviour of any organism is represented as an electrical current influenced by the levels of resistance it encounters. The key elements of circuit theory include

a) Node: It represents the specific location of an organism or the centre of a habitat patch.
b) Voltage: The probability of an individual starting from a point to reach the destination.
c) Current: The total movement of the ecological processes from one node to another, where a higher current implies more unrestricted movement.
d) Resistance: It represents the barriers to animal movement in the landscape. Different landscape features have different resistance values depending on the animal ecology and behaviour.

Circuit theory can proficiently model the random movement of a species through the heterogeneous landscape. It can incorporate multiple resistance layers such as water availability, forest cover, roads, night light which represents the heterogeneity of a landscape to model the species-specific dispersal pattern (McRae and Shah, 2009). The output reflects the dispersal patterns in random directions with varying strength and identifies areas with high current and low resistance (McRae and Beier, 2007; Howey, 2011; Dutta et al., 2016). These areas with high current and low resistance are called pinch points and play a crucial role in the dispersal dynamics of species.

1.1.4 Present Scenario of Central Indian Highlands

The "Central Indian highlands" is a critical biogeographic zone (Rodger et al., 2002). The region harbours a diverse array of ecosystems from arid to semi-evergreen and correspondingly rich biodiversity. The Central Indian Landscape is a tiger conservation hotspot, home to 30% of India's total tiger population (Jhala et al., 2011), and has been designated as a Global Priority Landscape for tiger conservation (Sanderson et al., 2010). Owing to the haphazard development and urbanization, this landscape has lost 78% of the forest cover over the last 300 years.

Quite a few genetic studies have been done on the movement pattern of large carnivores in this landscape, showing the evidence of movement and genetic connectivity through the patched forests. The primary focus of the studies done on tiger, leopard and sloth bear by (Dutta et al., 2015; Dutta et al., 2013a; Sharma et al., 2013a; Sharma et al., 2013b) were on connectivity across Kanha Tiger Reserve, Satpura Tiger Reserve, Pench Tiger Reserve and Melghat Tiger Reserve and interconnecting corridors. These studies found that all three species have different extents of genetic differentiation considering their adaptability, dispersal range and habitat preference. Two other studies on tigers by Yumnam et al., 2014 & Joshi et al., 2013 covered a larger area comparatively and, with a difference of one or two sampling sites, found that still there is contemporary gene flow among the sub-populations of these protected areas. Other studies have used the GIS tools for the connectivity analysis using habitat variables, resistance surfaces, anthropogenic variables and other related data (Dutta et al., 2016; Dutta et al., 2018). A recent multi-species study on jungle cats, tigers, leopards and sloth bears by Thatte et al. (2020) showed the differential impact of the Anthropocene on these species. All these studies prove that the restoration of connectivity corridors in this landscape will be a stepping stone in the conservation scenario of this landscape.

Many studies suggest distance is not the only limiting factor for the movement of any species, but it is also affected by landscape features as perceived by the species. The environmental, as well as landscape barriers, can help understand the movement and genetic differentiation of species in a better way. The role of these variables can be visible in a wide range of taxa and can affect them differently. For example, the pattern of forest distribution has a significant effect on the genetic differentiation of alpine butterflies (Keyghobadi et al., 1999), while for roe deer, specifically woody plants and trees, provided the landscape connectivity (Coulon et al., 2004). The natterjack toadlets prefer a more complex habitat that provides them with a hiding place as well as food (Stevens et al., 2004). The landscape between rivers created a total barrier to recent gene flow in the Mallorcan midwife toad (Alytes muletensis), according to a study (Kraaijeveld-Smit et al., 2005). This proves that there is no thumb rule to generalize the habitat connectivity across the multiple species.

1.2 Study area

The state of Maharashtra, located in the western part of India, forms a major part of the Central Indian Landscape. It has a tropical monsoon climate, which includes hot, rainy, and chilly seasons, as well as dry summers. The annual rainfall ranges from 400 to 6,000 millimetres. It harbours 16.91% forest out of the state's total geographical area. Out of which 2.83% comes under Very Dense Forest (VDF), 6.69% under Moderately Dense Forest (MDF), 6.98% under Open Forest (OF) and 1.38% under scrub forests type. Six Tiger Reserves, 48 Wildlife Sanctuaries, and six Conservation Reserves make up the state's Protected Area network, which covers 3.03 percent of the state's land area. The main forest types are southern-moist mixed deciduous forest, moist teak forest, dry teak forest and west-coast semi-evergreen forest. Following are the major protected areas in Maharashtra where the study has been conducted (Figure 1).

1.2.1 Melghat Tiger Reserve (MTR)

It is situated in the southern offshoot of Satpura hill ranges of Central India and lies in the Vidarbha region bordering Madhya Pradesh in the north and east. It is geographically located from 21°26′45″N to 77°11′50″E, an altitude of 312M to 1178 M above MSL. It was declared as a tiger reserve in 1974, being the first tiger reserve of Maharashtra and one of the biggest tiger reserves in the world in terms of area with a total of 2768.52 sq. km. (core – 1500.49 sq. km.; buffer – 1268 sq. km.) (NTCA 2020). The name 'Melghat' means the confluence of various 'ghats' or valleys, and it is characterized by a tropical dry-deciduous forest type dominated by Tectona grandis. The tiger reserve is an abode to tiger, sloth bear, dhole, Indian gaur, sambar, leopard, forest owlet, flying squirrel etc.

1.2.2 Tadoba-Andhari Tiger Reserve (TATR)

TATR is situated in the Chandrapur district of Maharashtra in central India and is the second declared tiger reserve established in 1995. It is a mega-bio-diversity site in the Central Plateau Province of the Deccan Peninsula bio-geographic zone. The geographical location of the park is 79° 11' 50" E and 20° 29' 44" N at an altitude of 188 M. It is characterized by southern tropical dry-deciduous forest inhabiting the largest tiger population of Maharashtra. It is rich in flora and fauna, including flora like teak, ain, bija, arjun, bamboo etc. and fauna like tiger, leopard, sloth bear, gaur, dhole, nilgai, small Indian civet, sambar, chital etc. The total area of the reserve is 1727.59 sq. km. (core - 625.82 sq. km.; buffer - 1101.77 sq. km.) (NTCA 2020).

1.2.3 Nawegaon-Nagzira Tiger Reserve (NNTR)

Nawegaon-Nagzira Tiger Reserve is situated in the Gondia and Bhandara districts of Maharashtra with an area of 1894.94 sq. km. (core - 653.67 sq. km.; 1241.27 sq. km.) (NTCA 2020). The reserve is characterized by moist deciduous forest and is therefore considered "Green Oasis" in the Vidarbha region, the easternmost part of Maharashtra. This was recently declared as a tiger reserve in 2012. The reserve is rich in bio-diversity with a very high number of dholes and a comparatively lesser number of tigers. The topography is undulating, with the highest point at around 702 MSL.

1.2.4 Pench Tiger Reserve (PTR)

Pench Tiger Reserve is located in the Satpuda- Maikal hills of Nagpur district and shares its boundary as well as another half part with Madhya Pradesh. It covers a total area of 741.22 sq. km. (core - 257.26 sq. km.; buffer - 483.96 sq. km.) (NTCA 2020). The reserve gets its name from the Pench river flowing north to south, 74 km through the reserve. It was declared as a tiger reserve in 1999 by the state of Maharashtra. The major forest type in reserve is southern tropical dry-deciduous forest supporting a wide range of flora and fauna. The major fauna includes sambar, chital, barking deer, leopard, tiger, dhole, sloth bear etc. In contrast, the major flora includes teak, ain, haldu, dhauda, amaltas, tendu etc., with a mixture of understorey.

1.2.5 Sahyadri Tiger Reserve (STR)

The Sahyadri Tiger Reserve is located in the Sahyadri Ranges of the Western Ghats in Maharashtra. This tiger reserve covers 1166.57 square kilometres (core - 600.12 sq. km.; buffer - 565.45 sq. km.) (NTCA 2020) at an elevation of 1500 metres above sea level, constituting a shared border between Maharashtra, Karnataka, and Goa, and consisting of dense evergreen, semi-evergreen, and moist deciduous woods. It is Western Maharashtra's first Tiger Reserve and the state's fourth tiger reserve, having been established in 2008. The wildlife of Sahyadri is typical of the western ghats bio-diversity with several endemic species from different taxa.

1.2.6 Umred Karandhla Wildlife Sanctuary (UKWLS)

UKWLS is situated across the Bhandara and Nagpur district and has connections with Tadoba-Andhari tiger reserve through the forests. It was declared a sanctuary in 2012 and acted as one of the most important links for the major tiger reserves of Maharashtra. This sanctuary is home to tigers, leopards, wild dogs, gaurs, pangolins, sambars etc., in an area of 189.30 sq. km.

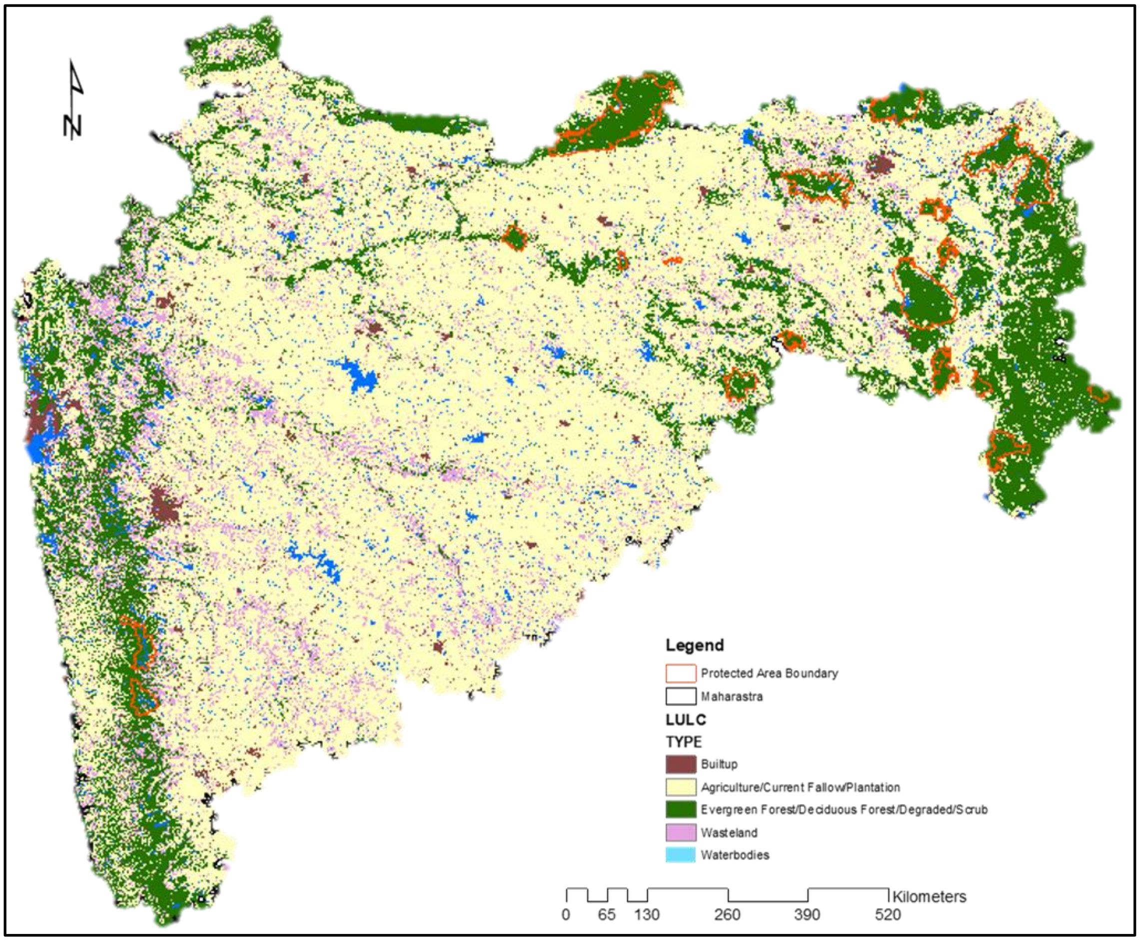


Figure 1.1: Land use land cover map of Maharashtra with the major protected areas

1.3 Geographical Connectivity

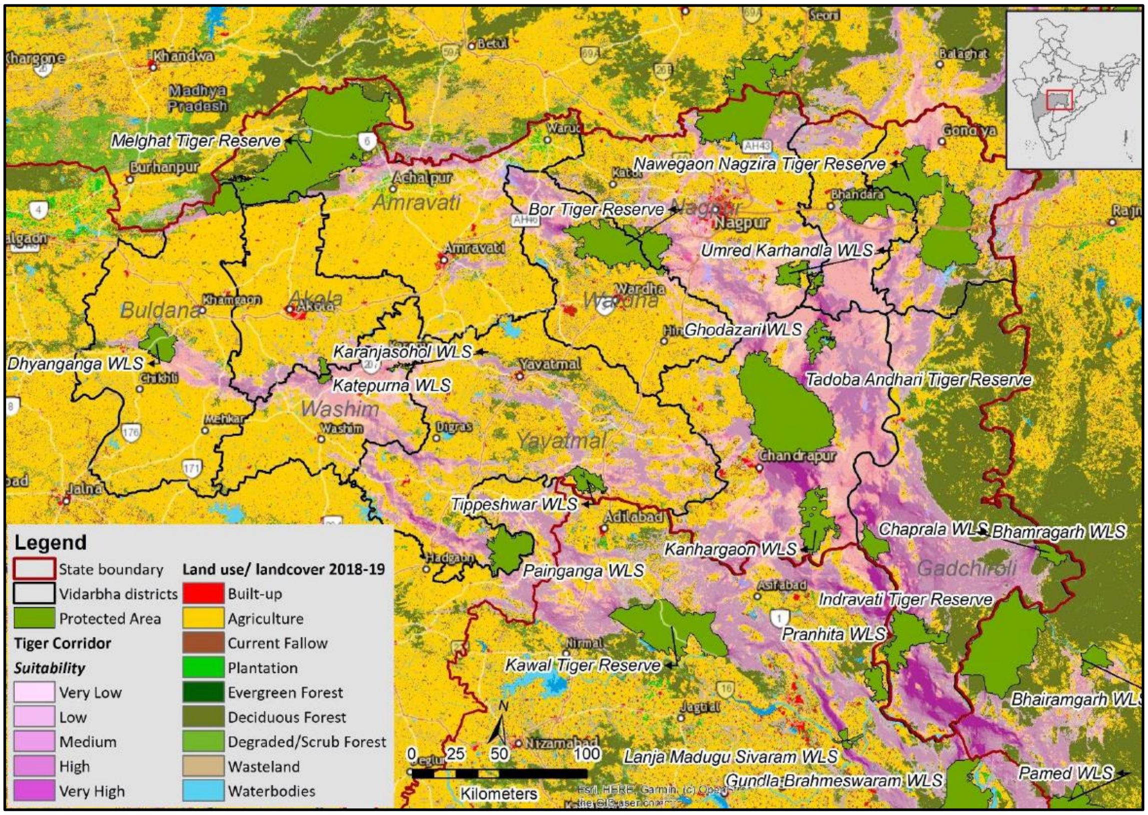
Maharashtra harbours three major corridor complexes of Central India, which includes

Complex A (Melghat – Bor – Pench)

Complex B (NNTR – TATR – Umred Karandhla)

Complex C (Painganga – Tipeshwar –- Chaprala – Pranhita)

These major corridor complexes combine to form the Vidarbha landscape in Maharashtra supports a decent portion of the bio-diversity of the state and is a major habitat for tigers (Figure 2). It is home to approximately 396 tigers and serves as a link between the central and southern Indian tiger populations. Therefore, studying the effect of habitat fragmentation at the Maharashtra level considering these corridors and their roles is of utmost importance from the conservation viewpoint.



(Habib et al. 2021a)

Figure 1.2: Major corridors map obtained from the telemetry data of tiger

The doctoral work for my thesis was conducted in the five major tiger reserves and their surrounding protected areas. Historically, the major protected areas of Maharashtra comprising Melghat Tiger Reserve, Tadoba-Andhari Tiger Reserve, Pench Tiger Reserve, Umred-Karandhala Wildlife Sanctuary, Bor Tiger Reserve, Tippeshwar Wildlife Sanctuary, Navegaon-Nagzira Tiger Reserve and Brahmapuri Division have more or less a connected mesh of forests. Over time, due to various anthropogenic pressures, the entire landscape got fragmented with varying extents of connectivity. For example, still, there is high connectivity between Umred Karandhla Wildlife Sanctuary (UKWLS) and Brahmapuri forest division (BFD), while there are a few small patches of forest between Pench Tiger Reserve (PTR) and Navegaon Nagzira Tiger Reserve (NNTR). Radio telemetry and camera trapping data showed the movement of tiger from UKWLS to BFD, NNTR to UKWLS, TATR to UKWLS, respectively (Habib et al., 2021a).

CHAPTER 2

Ecological traits of species and its influence on their gene flow

Chapter 2

Ecological traits of species and its influence on their gene flow

2.1 Introduction

Dispersal is one of the fundamental processes which is majorly affected by habitat fragmentation. Impediment in dispersal reduces the genetic connectivity among different populations resulting in decreased gene flow, as found in the mountain goat population (Parks et al., 2015). Dispersal is one of the least understood phenomena in the wild, and at the same time, it is the most crucial part of the life history of any species (Macdonald et al., 2001). In mammals, it can be dependent on various factors such as social structure, age and time of dispersal, dispersal distance, and availability of resources. Dispersal of individuals from a population is a well-planned, non-random phenomenon governed by several factors (Clobert et al., 2009). For continuous propagation and persistence of any species, reproduction is the essential requirement which is assisted by dispersal for a healthy population. In the case of large carnivores, dispersal can be fueled by a variety of factors, including reduced competition for resources and mates, avoidance of inbreeding, and so on (Pusey and Wolf, 1996).

The cost-benefit of movements across the surrounding patches that can be traversed, as well as the presence of corridors that ease transit between foraging patches, might influence behavioural responses to the fragmented landscape. (Laurance, 1995; Nupp et al., 2000). Being one of the costliest decisions in an animal's life history, it also differs from species to individual level due to differences in their choices. These choices refer to preferable corridors, kin competition, inbreeding, resource selection, and environmental stochasticity (Clobert et al., 2004). Dispersal is important as it helps maintain genetic diversity by spatial exchange of genetic material. Dispersal has a rescue effect for small isolated populations on the verge of extinction by introducing the immigrants and their set of new gene pool (Kodric-Brown, 1977).

The effect of habitat fragmentation on large carnivores' ecological and genetic structure is alarming because of their relatively low numbers, interaction with humans, and large home ranges (Crooks, 2002). Also, low fecundity and long generation times make them more prone to suffer from decreasing genetic variation, e.g. giant panda (*Ailuropoda melanoleuca*) (Lu et al., 2001). Therefore, it is crucial to understand the effect of habitat fragmentation on the ecology and population structure of these species. There are reports about sex-biased dispersal in large carnivores, both social and solitary. In solitary animals like tigers and leopards, most studies showed that females are generally philopatric, and males disperse to establish their territory (Smith, 1993). While in the case of social carnivores such as Ethiopian wolves, kit foxes, Asiatic wild dogs, etc., this prompts the question of which sex should disperse given that subordinates are typically the offspring of the dominant pair (Moehlman, 1989). Studies on Ethiopian wolves, bat-eared foxes, San Joaquin kit foxes, swift-foxes, and African wild dogs showed a distinct array of dispersal patterns (Randall et al., 2007; Kamler et al., 2013; Koopman et al., 2000; Kamler et al., 2004; Girman et al., 1997). However, the most studied reason behind dispersal for most species is inbreeding avoidance (Pusey and Wolf, 1996). Studies also suggest that dispersal's decision depends on the balance between the advantages of philopatry and the costs of intra-sexual competition (Moore et al., 1984). Therefore, a question arises why the pattern of dispersal is species-specific if the sole reason for dispersal is the same. It is crucial to understand the factors causing the difference in dispersal patterns of these species.

Ecological Interactions of the carnivores

It is of utmost importance to understand the co-occurrence of sympatric species in the present era of human-dominant landscapes. Tiger, leopard, and dhole form three species top predator assemblage in the major tiger reserves of Maharashtra and the surrounding habitats. The food habits of all three species include the killing of larger prey than themselves. The three predators killed different prey based on species, body size, and age-sex groups, allowing them to coexist.

(Karanth & Sunquist, 1995). According to Steinmetz et al. (2013), tigers' distribution is exclusively determined by prey availability. Leopard and dhole, on the other hand, appeared to be influenced by prey availability and tigers' avoidance, as mediated by habitat structure. Even if the dense habitats provide less food, subordinate carnivore species generally avoid open areas in favour of sites with a denser cover that is safer (Creel et al., 2001; Mukherjee et al., 2009).

The evolution of various anatomical adaptations for prey selection has also aided the coexistence between felids and canids (Biknevicius et al., 1996). Dholes surpass their victims by pursuit, lethal bites, and death by blood loss and shock, whereas the two felids have morphologically specialised for leaping and grasping prey with their sharp and retractile claws (Johnsingh, 1983). Tigers and leopards are solitary hunters, while dholes hunt in packs. Hunting in groups can aid to reduce morphological disadvantages when it comes to killing larger prey (Rosenzweig, 1966; Gittleman, 2019), while in other predators hunting success rate may determine prey selection patterns through the selection of disadvantaged individuals only where prey species are difficult to capture (Temple, 1987). Tigers and leopards are more active during night, whereas dholes show a diurnal activity pattern (Steinmetz et al., 2013).

Although there are few instances of intra-guild predation, which can be a sign of intense competition owing to prey insufficiency and habitat requirements (Polis et al.,1989). Tigers have been known to kill dholes and leopards (Karanth & Sunquist, 1995), as well as dhole groups deterring tigers or other niche competitors displaying aggressive behaviour (Schaller, 1984; Venkataraman, 1995). The multi-predator system represented by tigers, leopards, and dholes has been intensively researched to understand the dynamics of interspecific competition, primarily for prey availability (Karanth and Sunquist, 2000; Wang and Macdonald, 2009; Steinmetz et al., 2013). With the increasing pace of urbanisation, habitat availability would also be the determining factor for this predator assemblage survival in Central Indian forests and plays a vital role in shaping the inter-specific dynamics of this sympatric guild.

The Maharashtra part of the central Indian tiger landscape serves as a major abode for large carnivores in the country. Tiger (*Panthera tigris*), leopard (*Panthera leo*), and Asiatic wild dog (*Cuon alpinus*) are the three major carnivore species in this landscape. *Panthera tigris* is a solitary carnivore with specialised habitat requirements. Tigers define their territory and have a comparatively large range of size. Leopard (*Panthera pardus*), another solitary felid, co-exists with the tiger in this landscape. Leopards are generalists and less affected by the disturbance as compared to the tiger. Co-existing together in this landscape surrounded by human habitation is a great challenge for these species. Dhole (*Cuon alpinus*), a canid, is another carnivore found in this landscape that is very much prone to human disturbance. They live in packs and prefer dense forests.

These three large carnivores are distributed in the protected areas which are interspersed with patches of forest and human habitation forming a meta-population network.There are few studies on the genetic structure of tiger and leopard in the country and the meta-population network of these two felids in the Central Indian landscape. But for Asiatic wild dogs, only one study has been done till now, which mainly talks about phylogeography.

In terms of ecology, a considerable amount of conservation efforts and ecological data is available for these species, which makes Maharashtra an ideal location to conduct genetic studies for a deeper understanding of population dynamics in this landscape. Over the years, the change in landscape structure due to human disturbance has affected these three species differently. The difference in ecological and behavioural attributes results in different response types from these species.

To maintain the persistence and co-existence of the three large carnivores, it is essential to understand the effect of habitat fragmentation on their genetic structure and its long-term consequences on the existing population of these species. This study will highlight the importance of examining the temporal scale and species-specific responses to habitat fragmentation and its effect on the genetic diversity of these species. It will also determine the extent of connectivity among the populations of these species across the entire landscape.

2.2 Ecological and Behavioral traits

2.2.1 Tiger

Tigers (*Panthera tigris*) are the solitary top predators and one of the most charismatic species. There are eight subspecies described based on size, skull patterns, and pelage colouration worldwide, out of which *Panthera tigris tigris* is found in India. Out of eight subspecies, three are extinct (Seidensticker et al., 2010).

Tigers can be identified based on their unique stripe patterns. The body length and weight of a male are 270-310 cm and 175-200 kg and of a female are 240-265 cm and 240-265 kg. The generation time for males is 4.8 years, while for females, it is 3.8 years. The dispersal in tigers is

Figure 2.1: Tiger (*Panthera tigris*)

Tigers were distributed over a wide geographical range extending from Russia through southern China, Southeast Asia, the Indian subcontinent, and into the Indus River valley in Pakistan (Schnitzler et al., 2019). Tigers are mainly adaptable and tolerate temperatures ranging from $–50^{o}$ C in the snow-bound Russian Far East to 50^{o} C in Western India, but they cannot bear the arid climate. They live in a wide range of environments, from the dry forests of India and Indochina to the tropical rainforests of Sumatra and Malaysia, as well as the mangroves of Bangladesh and India's Sundarbans and China's taiga forests (Goodrich et al., 2015). With increasing urbanisation and the more fragmented landscapes, the tigers also utilise agricultural fields for dispersal. In India, currently, tigers occur mainly in the forest areas of 17 states within 42 tiger reserves (Jhala et al., 2011).

Tiger has been classified as 'Endangered' by The IUCN 2015 Red List of Threatened Species, and it is listed in Appendix I of CITES. Indian Wildlife (Protection) Act, 1972 has placed it under Schedule I. The primary causes for its depletion are habitat loss, poaching, prey depletion ((Nowell & Jackson, 1996; Karanth, 1999), and illegal trade of their body parts.

2.2.2 Leopard

Leopards are solitary, reclusive carnivores and are considered one of the most adaptable species. Seven subspecies of leopard have been identified based on variations in coat colour and spot size (Hes, 1991), while based on DNA, eight subspecies have been described (Miththapala et al., 1996). The sub-species found in the Indian subcontinent is *Panthera pardus fusca*.

Leopards are uniquely identifiable with the help of a rosette pattern. The home range is estimated to be 8 -15 sq. km. in the human-dominated landscape in India (Odden et al., 2014, Snider et al., 2021), with males using a larger area than females. Like other felids, dispersal in leopards is also male-biased. Leopards are most active between sunset and sunrise, and this is when they kill the most prey (Chaudhary et al., 2020; Bailey, 1993).

Leopards have the largest geographic range due to their adaptability (Nowell & Jackson, 1996). They can be found in Africa, Central Asia, Southeast Asia, and Russia's North Amur Valley. Except for deserts, the Sundarbans mangroves, and densely populated areas, leopards can be found throughout the Indian subcontinent (Khan, 2013; Odden et al., 2014). They can even persist near major towns like in India there is a population that resides in the heart of one of the busiest metro cities (Odden et al., 2014). The Indian subspecies of leopard, *Panthera pardus fusca* is found in all the forested habitats across the country except the arid deserts and above the timberline (Prater, 2005).

The common leopard is classified as a vulnerable species in the IUCN Red list data book and Appendix-1 of CITES. It had been given the highest level of protection (listed as a Schedule I species) under the Indian Wildlife (Protection) Act, 1972. The major threats to the persisting population in India are poaching, habitat loss, and man-animal conflict. The present population trend for leopards is declining (Bhatt et al., 2020).

Figure 2.2: Leopard (*Panthera pardus*)

2.2.3 Asiatic Wild Dog (Dhole)

Asiatic wild dog is one of the major predators of the wild and one of the least studied social canids in the world (Fox, 1984). Unlike its distant cousin, only a few studies have been done on the ecological and behavioural aspects of dholes. It is a monophyletic genus that mainly thrives in deep forests. Dholes are endemic to Asia, with a total of 6 subspecies, out of which the species found in central India is *Cuon alpinus dukhunensis.* The name dhole means recklessness and daring, which explains their persecution and bounty hunting in the past, due to which there was a drastic decline in the dhole population. Dholes are monomorphic and cannot be distinguished easily. However, the size and colouration of wild dogs vary regionally (Sheldon, 2013; Menon, 2003). Dholes are mostly diurnal in nature, course after their prey, and then hunt together (Fox, 1984). The average pack size of five to twelve dholes is considered standard for hunting success (Davidar, 1975). However, the numbers can increase to 25 (Venkatraman et al., 1995) or decrease up to 3-5 (TATR) as per the resource availability (Bhandari et al., 2021). Males dominate the pack composition in dholes in a 2:1 ratio (Venkatraman, 1998; Johnsingh, 1982). This biased sex ratio also indicates sex-biased dispersal in dholes with a higher probability of female dispersal and more related males in the pack (Venkatraman, 1998). The average home range size for a dhole pack is 58.67 sq. km. (Habib et al., 2021b). The average weight of the males is 15-20 kg and females 10-13 kg (Cohen, 1978).

The distribution of Asiatic wild dogs extends throughout eastern and central Asia (Sheldon, 2013) and south, east, and Southeast Asia (Durbin et al., 2004, 2008). In India *Cuon alpinus dukhunensis* still common (Johnsingh, 1983), especially in central Indian highland and southern states of India. Dholes mainly prefer deep forests and scrub jungles, making them difficult to find and observe (Krishnan, 1972; Davidar, 1975).

The population of Asiatic wild dogs is particularly vulnerable to extinction due to anthropogenic pressure, particularly habitat loss and hunting. Poisoning, den digging, and killing of pups caused the Asiatic wild dogs' major decline in the past (Fox, 1984; Johnsingh, 1985; Durbin, 2004). After which, they were brought under the Indian Wildlife Protection Act of 1972 in the 'Endangered' category (Ginsberg & Macdonald, 1990; Kamler et al., 2015).

Figure 2.3: Dhole aka Asiatic wild dog (*Cuon alpinus*)

2.3 Landscape Genetics

Genetic factors are considered crucial for the long-term perpetuation of biodiversity at three different levels for conservation purposes: gene, species, and ecosystem (McNeely et al., 1990; Jamieson et al., 2012). The haphazard urbanisation and environment modification development of effective conservation strategies require prior biological and genetic knowledge of the concerned species. The most important is the knowledge of functional connectivity in the fragmented landscape. This information can help in future landscape planning and management, the extent of population persistence, and predicting species' response to the changing landscape (Sunnucks et al., 2008). To understand the connectivity pattern of carnivores at the landscape scale, standard observations and long-term demographic changes are logistically difficult to employ in the wild, and data generated are infrequent at the temporal scale.

Landscape genetics is a novel concept that incorporates data and analysis from landscape ecology, spatial statistics, geography, and population genetics to characterise genetic variation's spatial aspect (Manel et al., 2003; Storfer et al., 2007). It provides powerful tools for quantifying processes such as metapopulation dynamics with respect to landscape barriers (Manni et al., 2004), gene flow, genetic differentiation in real landscapes. Landscape genetics is currently focusing on analysing the degree to which landscapes allow the migration of species (landscape connectivity) by linking gene-flow patterns to landscape structure in the perspective of habitat fragmentation (Holderegger & Wagner, 2008). We need to understand both the structural and functional aspects of landscape connectivity for these species (Brooks, 2003), and landscape genetics is ideally suited for testing the effect of structural landscape connectivity (e.g., the distance between habitat patches and the nature of the intervening habitat types) on functional landscape connectivity (i.e., dispersal and gene flow between habitat patches) (Holderegger & Wagner, 2008).

Landscape genetics can also help in answering the temporal effect of fragmentation on any species by enabling the comparison between the contemporary and historical gene flow (Orsini et al., 2008; Kettle, 2014), estimating source-sink dynamics (Lowe et al., 2006) and quantifying diversity (Segelbacher et al., 2008) and in tracking the genetic signatures for assessing the mating system and social organisation of lesser studied species. Landscape genetics can deal with the problem of direct observations and can reveal the specific habitat variables, which can facilitate or act as a barrier to the geneflow of respective species (Epps et al., 2007). It has also been useful in determining the counterintuitive features that can facilitate gene flow, such as for the populations of the blotched tiger salamander *(Ambystoma tigrinum melanostictum)* (Spear et al., 2005). Landscape genetics research has offered some of the greatest case studies of how recent habitat change is affecting population dynamics like connectivity in terrestrial species, ranging from insects (e.g., Keyghobadi et al., 2005), amphibians (e.g., Funk et al., 2005; Murphy et al., 2010a, 2010b; Spear & Storfer, 2012), reptiles (e.g., Stow et al., 2001; Clark et al., 2010), birds (e.g., Pavlacky et al., 2009), and mammals (e.g., Epps et al., 2005; Vignieri et al., 2005; Wasserman et al., 2012). But most of the landscape level connectivity studies have focused on single species, but there are a few comparative multispecies studies (Cleary et al., 2017; Thatte et al., 2020; Zancolli et al., 2014). There is a need for such multispecies to identify connectivity networks common to multiple species.

Landscape genetics studies increasingly focus on the spatial distribution of adaptive and neutral loci (Manel et al., 2010). Many recent studies have proved the importance of high-resolution molecular markers based on microsatellite or simple sequence repeats in determining the levels of gene flow among wild populations (Lindsay et al., 2008). Another advantage of this tool is utilising its non-invasive nature allowing a large sample size, wide coverage, and feasibility. The low-cost polymorphic markers provide potent approaches to determining the gene flow dispersal among populations due to the difficulty of directly tracking endangered species in the wild (Ugelvig et al., 2012).

2.4 Objectives

This doctoral work aims to understand the impact of habitat fragmentation on the genetic connectivity of large carnivores, considering the difference in connectivity matrix, landscape variables, and ecology of species using genetics as a tool. I delineated and compared the genetic structure, gene flow, dispersal, and functional connectivity of three large carnivores, i.e. tiger (*Panthera tigris*), leopard (*Panthera leo*) and Asiatic wild dog aka dhole (*Cuon alpinus*), incorporating the effect of different environmental, landscape and anthropogenic variables.

The specific objectives of the study were:

i) Development and standardisation of molecular markers for species and individual identification.
ii) Meta-population dynamics of tiger, leopard and Asiatic wild dog across the tiger reserves of Maharashtra.
iii) Impact of habitat fragmentation on the gene flow and genetic connectivity across the habitat connectivity gradient of the tiger reserves

The following three chapters of this thesis address the objectives mentioned above using a combined approach of sampling, multivariate analyses, GIS, and ecological traits of species to delineate genetic differentiation and the effect of habitat fragmentation, as well as the need to determine species-focused connectivity in the landscape on their connectivity.

Chapter 3 is the technical chapter which describes the markers used for the data generation of the entire study. It talks about the importance of species-specific markers for a population-level study. The mitochondrial and nuclear markers for tiger and leopard have been procured and standardised from previous studies. In contrast, the species-specific markers for species, individual, and sex identification of Asiatic wild dogs have been developed and standardised for the first time.

Chapter 4 describes the second objective. It examines the metapopulation dynamics for the three species using the genotypic data generated from the microsatellite markers. Based on the ecology of the three carnivores, it was hypothesised that the leopard population would be the least differentiated while the dhole population would be the most genetically differentiated. This compares the genetic differentiation and dispersal pattern of the three carnivores. The chapter also talks about the effect of competition on the dispersal pattern of a subordinate carnivore. It also looked at the demographic pattern for the carnivores.

Chapter 5 describes the third objective. It determines the impact of habitat fragmentation on the three ecologically different carnivore species. It describes the differential landscape and environmental requirements of the three carnivores for traversing through the fragmented landscape. It starts with optimisation of different variables and uses it to develop a current map for depicting the movement path of the species. It also features the pinch point maps, which show the specific crucial location facilitating the species movement.

CHAPTER 3

Development and standardisation of markers for species and individual identification

Chapter 3

Development and standardisation of markers for species and individual identification

3.1 Species identification

Dhole

3.1.1 Introduction

Dhole is the only species in the genus *Cuon*. Once distributed across North America, Europe and Asia during the 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 driven by habitat loss, prey depletion, disease and human persecution globally (Hayward et al., 2014). Their future survival depends on detailed 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, 1985), where they co-occur with other canid species (fox, jackal etc.), is challenging. On one hand where a lot of studies have been conducted on the genetic aspect of tigers and leopards (Mondol et al., 2015; Mondol et al., 2009a; Mondol et al., 2009b; Dutta et al., 2013a; Cao et al., 2011); 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.

In this context, non-invasive methods can be very useful in generating information on dhole distribution and other population parameters. 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. This chapter describes a simple and fast species identification approach from degraded biological samples and designs a multiplex molecular sexing assay involving both X and Y chromosome markers for dholes. These assays were successfully evaluated on field-collected dhole faecal samples from the western part of the central Indian tiger habitat, and their use in obtaining population-level information from non-invasive samples was proved.

3.1.2 Methods

I conducted an intensive faecal sampling in tiger reserves, i.e. Pench Tiger Reserve, Navegaon-Nagzira Tiger Reserve, Tadoba-Andhari Tiger Reserve and Umred- Karandhla Wildlife Sanctuary. The entire study area was intensively surveyed using a vehicle as well as on foot to look for tiger leopard and dhole scats. 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 the surveys. As dholes defecate in groups, it is challenging to ascertain samples to individual levels in latrine sites. Therefore, in the case of dholes, I collected each bolus as a 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 the shade inside the field station and subsequently shipped to the laboratory, and stored at -20°C until further processing.

Primer selection

As no species-specific molecular assay is available for dholes, I 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. I manually designed four sets of primers that amplify<250 bp amplicon size to ensure higher amplification success from degraded DNA. For molecular sexing, I have 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.

All field-collected scat samples 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°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 μL of lysis buffer, followed by the manufacturer's protocol provided in the kit. DNA was eluted twice with 100 μL of TE and stored at -20°C for long-term storage. Each set of 22 extractions was accompanied by two negative controls to monitor possible contamination.

All the primers were standardised 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 standardisations, PCR reactions were performed with 4μL of hot-start Taq mix (Qiagen), 4 μM BSA, 0.5 μM of primer mix and 3 μL of DNA extract under conditions including initial denaturation (95°C for 15 min); 50 cycles of denaturation (94°C for 30 s), annealing (50°C for 30 s) and extension (72°C for 35 s); followed by a final extension (72°C for 10 min). All primers were tested against domestic dogs, Indian grey wolves, golden jackals, foxes, tigers, and leopards DNA samples to check any cross-species amplification. During all amplifications, both extraction controls and PCR negative controls (one PCR negative for every set of amplification) 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 visualised 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 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 μM BSA, 0.5 μM of primer mix and 1–3 μL 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. I 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 standardise the multiplex sexing system. Three independent PCR reactions were conducted to confirm sex identification for all samples.

3.1.3 Results

I developed four sets of potential dhole-specific mitochondrial markers for species identification. Of the four primer sets, only one pair of markers produced desired results with the reference samples. This set produced 236-bp dhole specific amplicons (Figure 3.1 (a)) from the reference dhole samples and showed no cross-species amplification with other canid species used in this study. The other three marker sets produced nonspecific amplification with other species and were discarded subsequently. The 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), I 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 a higher success rate, this approach also helps in the rapid screening of many samples due to fewer steps during sample processing (DNA extraction, PCR and electrophoresis) and is cheaper than sequencing-based approaches.

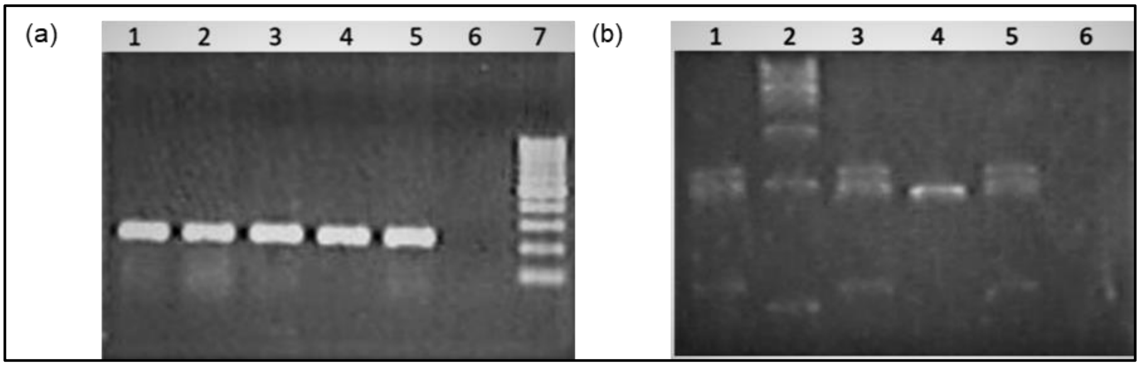


Figure 3.1 (a): Gel picture of PCR amplified products of dhole-specific mitochondrial primer designed in this study. Lane 1–4 shows amplification from field-collected dhole faecal samples; Lane 5 shows amplification from dhole positive sample, lane 6 is PCR negative, and lane 7 contains a 100-bp DNA ladder. (**b) 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.

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 3.1(b)). I did not find any cross-gender amplification with a reference or field-collected samples. The success rate for sex discrimination for all field-collected dhole samples (*n* 126) was 77%. I 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 also be useful in other canid species. However, it is noteworthy that during molecular sexing standardisation, I 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). If such expensive products are logistically difficult to arrange, 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).

Primer name	***Sequence***	***T_a***	***Amplicon size (bp)***	***Reference***
DholespIDF	CAGCCTGTCCTATCTGAG	50	236	Modi et al., 2018
DholespIDR	ATTAGTTCAGAGGCGGTA			
DBY-F	TTGGGGGTGGTTTTATTGTC	55	Male-112	Sastre et al., 2009
DBY-R	CCATCTCAACATCGCTGAAC			
AHTx40-F	GTAGCCCCATTTGTTTATATTTGC	55	Female-190	Sastre et al., 2009
AHTx40-R	GATCACTGTCTTACACCACAGGC			
Canis SRY-F	ATGGCTCTAGAGAATCCCCA	55	Male-199	Iyengar et al., 2005
Canis SRY-R	GCAATTTGTGACTTTTCTGTGC			

Table 3.1: Mitochondrial and molecular sexing markers for dhole

3.1.4 Discussion

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 the electrophoresis-based assay is simple, highly specific to dholes and showed a high success rate (97% in species identification) from field-collected, poor quality degraded dhole samples. The multiplex sexing assay showed unambiguous results with a high success rate (77%) in dholes and was 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.

3.1.5 Markers for tiger and leopard species identification

Tiger and leopard are the sympatric felids inhabiting similar areas as dholes. Though tiger and leopard can be identifiable through stripes and spots, DNA-based approaches have become a preferred alternative for population-level study compared to camera trapping. These methods can overcome the challenges during species assignment for landscape-level study, which requires a large dataset (Prugh & Ritland, 2005) and do not merely rely on the sign-track surveys or faecal morphology (size, shape, scent and dietary contents), which can be error-prone in co-occurring species (Fernandes et al., 2008).

I collected the suspected tiger and leopard faecal samples during the field sampling in tiger reserves, i.e. Pench Tiger Reserve, Navegaon-Nagzira Tiger Reserve, Tadoba-Andhari Tiger Reserve and Umred- Karandhla Wildlife Sanctuary. The entire study area was intensively surveyed using a vehicle and on foot to look for tiger leopard scats. The sampling was conducted between November 2015 and February 2016. All the samples were collected fresh with GPS coordinates and supplementary information, i.e. track marks, substrata etc. The entire scat sample was collected in sterile zip-lock bags, dried in the shade inside the field station and subsequently shipped to the laboratory, where they were stored at -20˚C until further processing.

I have used the already available species-specific mitochondrial markers for species identification of tiger and leopard from the collected faecal samples. These markers were first standardised with the tiger and leopard positive blood samples from this landscape and then used for identifying the scats. The mitochondrial primers used for the two felids were from the previous studies by Mondol et al., 2014 and Mukherjee et al., 2007**.** For species identification of tigers, I have used two different methods described in Mondol et al. (2014) and Mukherjee et al. (2007). PCR reactions were carried out in 10 µl reaction volumes containing 4 µl of Qiagen master mix (QIAGEN Inc.), 3µl of BSA, 0.5 µl of 10µM primer mix and 2 µl of template DNA. The PCR conditions included an initial denaturation (95 °C for 15 min); 50 cycles of denaturation (95 °C for 30 sec); annealing (57°C for 30 sec); extension (72 °C for 30 sec); followed by a final extension (72 °C for 15 min) in an ABI thermocycler (Table 3.2).

While for leopard, the species identification was done with the primers described in Mondol et al., 2014. PCR reactions were carried out in 10 µl reaction volumes containing 4 µl of Qiagen master mix (QIAGEN Inc.), 3µl of BSA, 0.5µl of 10µM primer mix and 2 µl of template DNA. The PCR conditions included an initial denaturation (95 °C for 15 min); 50 cycles of denaturation (95 °C for 30 sec); annealing (50°C for 30 sec); extension (72 °C for 30 sec); followed by a final extension (72 °C for 15 min) in an ABI thermocycler (Table 3.2).

Primer name	***Sequence***	***T_a***	***Amplicon size (bp)***	***Reference***
Tig490F	TTACTAGGACTCCTCCTAGCC	57	225	Mukherjee et al., 2007
Tig490R	GAATAGGGTTGTGATGGCCCC			
Tig509F	TATTCTTTGCACTCCTGGGG	57	164	Mukherjee et al., 2007
Tig509R	GGTCATTTGTGGGATGGTC			
TigParND2-F1 Common forward	TGTAGGYTGAATARCAGC			Mondol et al., 2014
TigND2-R1 Tiger	GGGCTGTATATGAGTAAC	50	110	
ParND2-R1 Leopard	GGGGACATTATTAGAACC	50	190	
TigParND4-F1 Common forward	TRATAGCTGCYTGATGAC			Mondol et al., 2014
TigND4-R1 Tiger	GCTACAAATAGCTCTGCA	50	85	
ParND4-R1 Leopard	GTTTGTGCCTATAAGGAC	50	130	

Table 3.2: List of mitochondrial markers used for tiger and leopard.

This combination of approaches has given an unambiguous species identification from the field-collected samples. The tiger-specific fragments of 85 and 110 bp in the multiplex and 164 and 225 bp while leopard-specific fragments of 190 and 130 bp, respectively, were produced. Amplifications with reference samples and test samples always produced species-specific bands for only tigers and leopards. Sequencing results of these fragments always gave accurate species identification when checked against the Genbank database.

3.2 Individual identification:

Dhole

3.2.1 Introduction

Given the current anthropogenic disturbance scenario across its range, the future survival of the carnivore guild depends on integrated conservation measures involving detailed, accurate information on ecology, demography and genetics. However, generating reliable information for the elusive, forest-dwelling, sympatric carnivores like tiger, leopard and dhole at the landscape scale is challenging. Also, in the case of dholes, traditional ecological techniques such as the regular photographic capture approach are ineffective due to the absence of unique coat patterns and their monomorphic forms. Physical tagging methods are impractical at landscape scales due to logistical constraints, difficulties, high costs and small numbers of captures possible. In this context, genetic tools have tremendous potential to generate critical information such as population size estimation (Mondol et al., 2009a), phylogeography (Luo et al., 2014; Waits et al., 1998), pack dynamics and reproductive fitness (Sillero-Zubiri et al., 1996; Girman et al., 1997), dispersal patterns (Epps et al., 2007; Gour et al., 2013) for elusive species conservation across large landscapes (Mondol et al., 2013). The ability to identify individuals from non-invasive samples collected over an ample space provides a feasible option to generate detailed information on elusive, forest-dwelling dholes as they cannot be identified using other approaches. Among the three carnivores, the dhole is least studied in terms of genetic details, and the panel used for individual identification (Iyengar et al., 2005) did not have adequate PID values and included and considered different parameters.

In this study, I addressed key methodological issues related to selecting and standardising a set of molecular markers for the individual identification of dholes. Subsequently, I used field-collected dhole samples from five protected regions of the central Indian landscape in Maharashtra, India, to evaluate these markers for individual identification. In addition to utilising these markers in dhole population estimation at the landscape level, I believe that this approach has broader relevance in non-invasive, faecal DNA based population assessments of many other low density, elusive, wide-ranging species.

3.2.2 Methods

Field sampling

A total of 249 samples were tested (PTR-92, MTR-76, NNTR-37, UKWLS-34, TATR-10, respectively) for this study. For blood sampling, four animals (three males and one female) were remotely administered with a reconstituted lyophilised mixture of Telatamine-Zolezepam (Zoletil 100; Virbac, Carros, France) at the dose rate of 8.6 mg/kg body weight using Dan-inject syringe projector (Model-IM) at a distance of 15–25 m. After ensuring sedation was safe for handling, the animal was approached, blindfolded, and one ml of blood was collected through femoral-saphenous vein puncture. Blood was collected in EDTA vacutainers and preserved at −20°C in the laboratory for genetic work.

DNA extraction and primer standardisation

DNA from blood samples was extracted using the standard protocol given in the QIAamp DNA Tissue Kit (QIAGEN Inc., Hilden, Germany). Negative control was incorporated to monitor any possible contaminations.

Selection of microsatellite markers

There are no dhole specific microsatellites developed so far, and the only study focusing on dhole population genetics had used 13 cross-species markers from domestic dogs to study genetic variation (Iyengar et al., 2005). These markers showed low levels of polymorphism and low

PIDsibs value (3.3×10^{-4}), providing a misidentification rate of 1 in 3,000 siblings. Given that India is considered to retain a high (about 1,500–3,000) number of dholes (Kamler et al., 2015), this panel will not provide sufficient statistical power for unambiguous individual identification at landscape levels with large population sizes. I developed a panel following a stringent cross-species marker selection and testing process for this study. The entire process was conducted in two steps: marker selection and rigorous testing before developing a final microsatellite panel for dhole individual identification.

As most of the cross-species markers were found to be from dogs and earlier used markers were less polymorphic for individual identification, I decided first to examine if both species (domestic dog and wild dog) share genetic similarities. Earlier karyotype and chromosomal banding studies (Graphodatsky et al., 2008) showed almost identical G-banding patterns, indicating high chromosomal level similarity between both species. Subsequently, I identified 37 dog microsatellite loci from earlier published literature (Holmes et al., 1995; Ostrander et al., 1993; Fredholm et al., 1995; Ostrander et al., 1995; Francisco et al., 1996; Neff et al., 1999). These markers were selected based on their polymorphism (number of alleles (Na), PIC, observed heterozygosity (Ho) etc.) and amplicon sizes in published literature. Further, I mapped all the markers on available dog genome canFam 3.1 in UCSC Genome Browser (http://genome.ucsc.edu/; Accession ID: GCA_000002285.2) to assess the chromosome number associated with each marker. Finally, a total of 18 microsatellites were selected based on their amplicon size, chromosome number and polymorphism (based on published data) for further testing. The details of the markers are given in Table 3.3.

Locus	***Primer sequence 5'-3'***	***Motif***	***Dye***	***Ta***	***Chromosome number (in dog)***	***Na***	***Ho***	***He***	***Allelic Range***	***PID (unbiased) (cumulative)***	***PID (sibs) (cumulative)***	***Success rate (%)***	***Genotyping error (%)***			***Reference***
													ADO	***FA***	***NA***	
WD2201 (Panel 4)	ATCAACAATGCATGCCACAT GAGAACAAATAAATGCAAGCCC	Tetra	FAM	59ºC	7	9	0.63	0.78	170-202	7.40E-02	3.81E-01	73.97	10.4	6.9	3.5	Francisco et al. (1996)
PEZ6 (Panel 3)	ATGAGCACTGGGTGTTATAC ACACAATTGCATTGTCAAAC	Tetra	NED	53ºC	27	7	0.38	0.78	206-230	5.88E-03	1.46E-01	72.05	9.1	8.2	19.2	Neff et al. (1998)
WD2140$^{\#}$ (Panel 2)	GGGGAAGCCATTTTTAAAGC TGACCCTCTGGCATCTAGGA	Tetra	HEX	56ºC	5	9	0.4	0.76	122-178	5.00E-04	5.69E-02	69.65	10.2	7.9	4.8	Francisco et al. (1996)
AHT130$^{\#}$ (Panel 2)	CCTCTCCTGGTAAGTGCTGC TGGAACACTGGTCCCCAG	Di	FAM	56ºC	18	7	0.44	0.74	98-112	4.69E-05	2.29E-02	71.47	13.9	2.4	0	Holmes et al. (1994)
PEZ3 (Panel 3)	CACTTCTCATACCCAGACTC CAATATGTCAACTATACTTC	Tetra	PET	53ºC	19	8	0.52	0.73	110-146	5.26E-06	9.53E-03	88.45	11	16.5	16.4	Neff et al. (1998)
WD2137 (Panel 4)	GCAGTCCCTTATTCCAACATG CCCCAAGTTTTGCATCTGTT	Tetra	FAM	56ºC	3	7	0.46	0.66	156-180	7.29E-07	4.33E-03	68.94	9	1.6	22.1	Francisco et al. (1996)
PEZ5 (Panel 3)	GCTATCTTGTTTCCCACAGC TCACTGTATACAACATTGTC	Tetra	FAM	53ºC	12	8	0.15	0.61	150-254	1.39E-07	2.14E-03	57.37	12.9	6.9	16	Neff et al. (1998)
CXX251 (Panel 2)	TACCACTGTCATTTTTCCATGC AAGAGGATACCGGTGGCAG	Di	NED	56ºC	1	3	0.23	0.58	128-136	3.32E-08	1.11E-03	78.99	8.8	5.6	11	Ostrander (1995)
WD2096$^{\#}$ (Panel 1)	CCGTCTAAGAGCCTCCCAG GACAAGGTTTCCTGGTTCCA	Tetra	FAM	59ºC	11	3	0.66	0.51	93-101	1.05E-08	6.38E-04	81.98	8.6	8.5	24	Francisco et al. (1996)
CXX279$^{\#}$ (Panel 1)	TGCTCAATGAAATAAGCCAGG GGCGACCTTCATTCTCTGAC	Di	PET	59ºC	22	5	0.2	0.45	125-135	3.29E-09	2.33E-04	84.05	7.0	6.9	13	Ostrander (1995)

WD2001[#] (Panel 1)	TCCTCCTCTTCTTTCCATTG TGAACAGAGTTAAGGATAGACAC	Tetra	HEX	59ºC	23	3	0.41	0.48	134-142	1.24E-09	3.86E-04	78.92	10.4	8.2	6.2	Francisco et al. (1996)
CXX30 (Panel 1)	GCCTTTTAGGGAGCTTTCTTT GAGTCTGCTTTTCTCCTTCCC	Di	NED	59ºC	2	3	0.38	0.41	122-142	4.68E-10	1.50E-04	77.28	9.4	9.9	3.3	Ostrander (1995)
Mean						6	0.40	0.62				75.26	10.1	9.2	11.2	
AHT136*	GAGAGGGCTGGTGGTAGGGG CGTGGCTATCTTTGGAGGGA	Di	HEX	NA	11	-	-	-	-	-	-	-	-	-	-	Neff et al.1998, Holmes et al.1994
WD2159*	GAATCCCACATCGGGCTC ATTAAGTTTTGAAAGCCAGGTAAG	Tetra	HEX	NA	24	-	-	-	-	-	-	-	-	-	-	Francisco et al. (1996)
CPH6*	CATTGGCTGTTTGACTCTAGG ACTGATGTGGGTGTCTCTGC	Di	FAM	56ºC	23	4	0.189	0.577	107-136	-	-	-	-	-	-	Fredholm et al. (1995)
CPH16*	CTACACCAGTTAGGGAATCTAGC CAGATTCAAATCCACTCTCAGAC	Di	HEX	NA	20	-	-	-	-	-	-	-	-	-	-	Fredholm et al. (1995)
CXX140*	CAGAGGTGGCATAGGGTGAT TCGAAGCCCAGAGAATGACT	Di	PET	56ºC	4	2	0.012	0.059	149-151	7.42E-08	9.87E-04	-	-	-	-	Ostrander (1995)
CXX608*[#]	TATTGTAAGTCTTCCTTGAC TCTACCGTCTACAACAAAAGGG	Di	HEX	53ºC	15	2	0	0.04	134	7.42E-08	9.87E-04	-	-	-	-	Ostrander (1995)

* Loci omitted from final analyses

[#] Loci used in the study by Iyengar et al.

Table 3.3: Details of microsatellite loci used for Asiatic wild dog standardised and validated in this study. Na=number of alleles, H_o= Observed heterozygosity, H_e= Expected heterozygosity, PID= Probability of identity.

PCR standardisation and data validation

All initial standardisation of the markers was conducted using dhole blood samples (n = 4). PCR reactions were performed for selected 18 microsatellites in 10 µl reactions containing 3.5 µl Qiagen multiplex PCR buffer mix (QIAGEN Inc., Hilden, Germany), 0.2 µM labelled forward primer (Applied Biosystems, Foster City, CA, USA), 0.2 µM unlabelled reverse primer, 4 µM BSA and 2 µl of 1:50 dilution of blood DNA extract. The PCR conditions included an initial denaturation (95°C for 15 min); 50 cycles of denaturation (94°C for 30 s), annealing (50–60°C gradient for 30 s) and extension (72°C for 35 s); followed by a final extension (72°C for 30 min). Following post-temperature standardisations, markers with the same annealing temperatures but with different labels or allele sizes were standardised as multiplex assays (see Table 3.3 for details). Both extraction and PCR negative controls (one PCR negative every set of 11 reactions) were included during all amplifications to monitor any possible contamination. Post amplification, 2 µl of PCR product was mixed with HiDi formamide (Applied Biosystems, Foster City, CA, USA) and LIZ 500 size standard (Applied Biosystems, Foster City, CA, USA) and genotyped in an ABI genetic analyser (Applied Biosystems, Foster City, CA, USA). The fragment lengths were scored manually using the program GENEMARKER (Softgenetics Inc., State College, PA, USA). Each reaction was repeated three times to ensure good data quality.

Once the initial temperature and multiplexing standardisations were performed using reference blood DNA samples, final standardisation was conducted with dhole faecal DNA. Species identification was performed for all field-collected faeces using specific mtDNA primers described in Modi et al. (2018). For faecal samples, data validation was performed through a modified multiple-tube approach as described in Mondol et al. (2009b). All faeces that had amplified in 50% of the loci in the panel during the first PCR were repeated two more times for all loci. Following allele calling, a consensus genotype was prepared using the ‘Quality index’ protocol (Miquel et al., 2006), during which alleles were called manually and scored as ‘1’ if the repeat is identical with the first call, or ‘0’ if calls do not match due to no amplification, allelic dropout (one allele in the heterozygote is erroneously not amplified), false allele (FA; slippage artefact during PCR) etc. The scores assigned to each repetition are added together and divided by the total number of repeats to determine the quality index for each locus/sample, and only quality indexes of 0.75 or higher (at least three out of four repeats) were considered for downstream analyses. As indicated by Broquet et al., (2004), I estimated average amplification success as the per cent positive PCR for each locus (2004). In addition to using MICROCHECKER v 2.2.3 (Van Oosterhout et al., 2004), I manually calculated allelic dropout and FA rates as the number of dropouts or FAs over the total number of amplification (Broquet et al., 2004). For both homozygous and heterozygous genotypes, the FA frequency was calculated as the ratio of the number of amplification with one or more FAs at a particular locus to the total number of amplification, whereas the allele dropout rate was calculated as the ratio of the observed number of amplification with loss of one allele to the number of positive amplification in heterozygous individuals. Programme FreeNA (Chapuis et al., 2007) was used to determine the frequency of null alleles (NAs), which estimates the NA frequency using EM algorithm (Dempster et al., 1977).

Data analyses

By comparing data from all samples for all amplified loci, the identity analysis module in programme CERVUS (Kalinowski et al., 2007) was used to find identical genotypes (or recaptures). The PID(sibs) for all of the individuals was calculated using GIMLET (Valière 2002). After that, any allele with a frequency of less than 10% across all amplified loci was rechecked for allele verification. Hardy Weinberg equilibrium and linkage disequilibrium for all loci were determined using ARLEQUIN (Excoffier et al., 2005).

3.2.3 Results

During initial standardisations, I tested all 18 selected markers (see Table 3.3) with four wild-caught dhole blood DNA samples. Three of these tested markers (WD2159, CPH16, AHT136) did not show any amplification in the blood DNA samples and were removed from subsequent analyses. The remaining 15 markers were amplified with 225 genetically confirmed dhole faecal samples. Following data validation through multiple repeats, amplification success rates and polymorphism for these loci were calculated. The results show that loci CXX608 and CXX140 were monomorphic in all amplified samples, and locus CPH6 has a low amplification success rate (~35%) from faecal DNA and thus were removed from the panel. The remaining 12 markers were finally standardised as four multiplex panels (see Table 3.3) for dhole individual identification.

None of these final 12 loci showed any signatures of large-scale allelic dropouts. The average allelic dropout rate was 0.1, while the average FA frequency across all 12 loci was 0.092. The overall frequency of NAs was calculated to be 0.11, showing that the genotyping error rates for this 12 loci panel are minimal. From dhole faecal DNA, amplification success ranged from 57% to 88 per cent. Polymorphism levels ranged from high (WD2201-9 alleles, Ho = 0.63) to low (CXX251-3 alleles, Ho = 0.23) at the loci (Table 3.3). Except for locus WD2001, none of the other loci deviated from the Hardy-Weinberg equilibrium, and no evidence of substantial linkage disequilibrium between any two loci was detected. Summary statistics for various measures of polymorphism (H_o and expected heterozygosity, N_a and allelic size range) for all loci in the final panel are presented in Table 3.3.

I only considered samples that produced good quality data for at least seven of the 12-panel loci for individual identification. This cut-off value of an average of seven loci was decided based on the statistical support (PID_{sibs} value of 1 in 500 siblings) produced by these loci. Given that any single largest dhole population is about 250–300 individuals (Kamler et al., 2015), this value is sufficient for individual identification at local scales in India. Out of the 225 field-collected dhole faecal samples amplified with the panel of 12 markers, 98 produced seven or more loci data. Overall, I generated genetic data from a total of 102 samples (four blood and 98 faecal samples). About 70% of these samples (n = 71) have successfully amplified for 10-12 loci. Cumulative PID $_{\text{sibs}}$ and PID $_{\text{unbiased}}$ values were found to be 1.5×10^{-4} and 4.7×10^{-10}, respectively, suggesting good statistical support for unambiguous individual identification.

3.2.4 Discussion

In this study, I standardised protocols for individual identification of Asiatic wild dogs from poor-quality DNA samples, and the final marker panel could unambiguously identify individual dholes in this field-based pilot study from five protected areas of Maharashtra, India. The systematic protocols followed here offer some advantages over earlier efforts on dhole individual identification from faecal samples by Iyengar et al. (2005). Firstly, the use of a large panel of 37 microsatellite loci for preliminary assessment of marker suitability along with a genomic mapping-based selection of final markers (n = 18) allowed the study to ascertain a combination of loci for unambiguous individual identification with high statistical power. The rigorous testing of the loci with a large number of DNA samples from different sources also allowed me to exclude loci that might be problematic due to low amplification success from non-invasive samples. The final panel consisting of 12 markers were further standardised into four multiplex reactions to provide time and cost-effective options during data generation. I was very careful to initially select a large number of tetranucleotide markers as they are known to have low stutter peak problems and better allele characteristics from poor quality samples (Walsh et al., 1996), while dinucleotide markers generally have higher amplification success (Broquet et al., 2007). Thus, the final panel with a ratio of 2:1 tetra vs dinucleotide microsatellites would provide the ideal combination in terms of high success rate and fewer technical issues in allele calling during dhole individual identification. The amplification success rate for all loci was >70% except locus PEZ5 (~60%), but it was found to be polymorphic and was included in the panel. The overall genotyping error frequency was found to be <0.2 from dhole faeces, which is within the recommended limits for non-invasive population genetic research (Smith et al., 2014).

My motivation in this study was to develop effective protocols that could be applied for individual identification of Asiatic wild dogs as they are difficult to identify from physical characteristics (spots, marks, stripes etc.). Their elusive nature also makes it challenging to estimate population size using traditional techniques (photographic capture, field-based observations etc.) at landscape levels. Waits et al. (2001) recommended a threshold PIDsibs value that is at least double the approximate number of animals in any given area for genetic estimation of population size. The cumulative PIDsibs value of 1.5×10^{-4} achieved in this study is better than Iyengar et al. (2005) (PIDsibs of 3.3×10^{-4}) and should be sufficient to study dhole genetics and specifically population estimation across its range. Among all the dhole range countries, India is considered to retain the highest (about 1,500–3,000) number of individuals (Kamler et al., 2015), and the misidentification rate achieved in this study (1 in 6,700 siblings) would provide strong statistical power in individual identification. The most recent assessment suggests that the largest dhole population in the Western Ghats, India holds about 207–304 individuals (Kamler et al., 2015), thereby assuring that the seven loci cut-off (misidentification rate 1 in 500 siblings) to select samples and 12 loci panel would be useful in population estimation at the local scale. However, it is important to point out that I generated individual-level information from about 43.5% (98 out of 225 faeces) of the field-collected samples in this study. Similar patterns of low amplification success rate from field-collected faecal samples have been observed in the earlier genetic study of dhole (Iyengar et al., 2005), leopard (Mondol et al., 2009b) and other species (Smith et al., 2014). Considering dhole cryptic nature, social behaviour and ecology in corroboration with low amplification success rate, I suggest an intensive faecal sampling effort to estimate population size for this species.

In the broader context of understanding dhole population dynamics at local or landscape scales, genetic sampling is possibly the only way to generate information with spatial and temporal coverage for this elusive, social carnivore as photographic sampling or conventional tagging cannot be employed due to lack of distinguishing natural marks and logistical difficulties of physical captures of a large number of animals. Results from this study provide a robust tool to generate individual-level information from field-collected faecal samples. In combination with a good sampling strategy, these methods can be used cost-effectively to investigate species biology (including patterns of genetic diversity, relatedness and population connectivity) and estimate the population abundance of dholes in the wild.

3.2.5 Markers for tiger and leopard individual identification

Tiger and leopard possess the distinguishable stripes and spots pattern and can be identified individually through it but generating reliable information at the landscape level through this for the co-occurring felids is challenging. Non-invasive marker-based individual identification has tremendous potential in developing data at the landscape scale, which can be further used for different population genetic studies (Epps et al., 2007).

For identifying individual genotypes from tiger and leopard confirmed samples, I, standardised the microsatellite markers from a previous study by Mondol et al. (Mondol et al., 2009a; Mondol et al., 2009b) with the positive blood samples from Maharashtra. I have standardised two panels of 12 microsatellite markers for tiger and leopard, respectively. These loci were selected in earlier studies based on their polymorphism, observed heterozygosity (HO) and amplification success rate from low-quality samples (Joshi et al., 2013). The summary statistics of these loci for tiger and leopard are presented in Tables 3.4 and 3.5, respectively. All the standardisation and genotyping procedures were similar for the three species.

S.no.	***Locus***	***Success rate (%)***	***No. of alleles (NA)***	***Expected heterozygosity (HE)***	***Observed heterozygosity (HO)***
1.	FCA090	92.11	10	0.86765	0.30435
2.	msFCA506	33.33	08	0.81908	0.14583
3.	FCA672	24.12	11	0.84444	0.38235
4.	FCA304	49.56	11	0.76432	0.24561
5.	FCA232	92.98	04	0.50761	0.01429
6.	FCA230	94.74	03	0.69967	0.10294
7.	FCA279	79.39	06	0.67921	0.39286
8.	FCA441	54.39	12	0.58221	0.29630
9.	FCA069	73.68	13	0.48979	0.39130
10.	msFCA453	99.12	09	0.50267	0.95714
11.	msF115	68.86	01	0.0	0.0
12.	msHDZ170	99.12	07	0.77937	0.52239
Mean			**7.3**	**0.62**	**0.30**

Table 3.4: List of microsatellite loci and the summary statistics used for tiger individual identification.

S. no.	***Locus***	***Success rate (%)***	***No. of alleles (NA)***	***Expected heterozygosity (HE)***	***Observed heterozygosity (HO)***
1.	FCA090	47.06	7	0.77796	0.44000
2.	FCA279	31.93	13	0.79933	0.47273
3.	FCA230	81.51	8	0.75158	0.07143
4.	FCA672	67.23	9	0.77812	0.46296
5.	msHDZ170	51.26	9	0.68615	0.25000
6.	msFCA453	52.10	5	0.59508	0.33929
7.	FCA232	78.99	8	0.74749	0.51852
8.	FCA391	81.51	8	0.83333	0.31250
9.	msFCA441	34.45	9	0.82236	0.29545
10.	FCA309	50.42	8	0.82032	0.26531
11.	msFCA506	67.22	10	0.81456	0.27500
12.	msFCA052	84.03	7	0.81010	0.42857
Mean			**8.41**	**0.77**	**0.34**

Table 3.5: List of microsatellite loci and the summary statistics used for leopard individual identification.

PCR reactions were performed for selected 12 microsatellites in 10 µl reactions containing 3.5 µl Qiagen multiplex PCR buffer mix (QIAGEN Inc., Hilden, Germany), 0.2 µM labelled forward primer (Applied Biosystems, Foster City, CA, USA), 0.2 µM unlabelled reverse primer, 4 µM BSA and 2 µl of 1:50 dilution of blood DNA extract. The PCR conditions included an initial denaturation (95°C for 15 min); 40 cycles of denaturation (94°C for 30 s), annealing (51°C for 30 s) and extension (72°C for 40 s); followed by a final extension (72°C for 10 min). During all amplifications, both extraction and PCR negative controls (one PCR negative every set of 11 reactions) were included to monitor any possible contamination. Post amplification, 2 µl of PCR product was mixed with HiDi formamide (Applied Biosystems, Foster City, CA, USA) and LIZ 500 size standard (Applied Biosystems, Foster City, CA, USA) and genotyped in an ABI genetic analyser (Applied Biosystems, Foster City, CA, USA). The fragment lengths were scored manually using the program GENEMARKER (Softgenetics Inc., State College, PA, USA). Each reaction was repeated three times to ensure good data quality.

Once the initial temperature and multiplexing standardisations were performed using reference blood DNA samples, final standardisation was conducted with tiger and leopard faecal DNA. For faecal samples, data validation was accomplished through a modified multiple-tube approach as described in Mondol et al. (2009a). All faeces that had amplified in 50% of the loci in the panel during the first PCR were repeated two more times for all loci. Following allele calling, a consensus genotype was prepared using the ‘Quality index’ protocol (Miquel et al., 2006), during which alleles were called manually and scored as ‘1’ if the repeat is identical to the first call, or ‘0’ if calls do not match due to no amplification, allelic dropout (one allele in the heterozygote is erroneously not amplified), false allele (FA; slippage artefact during PCR) etc. To calculate the quality index for each locus/sample, the scores assigned to each repeat are summed and divided by the total number of repetitions and only a quality index of 0.75 or more (at least three out of four repeats) for each locus was considered for downstream analyses. I calculated average amplification success as the per cent positive PCR for each locus, as described by Broquet et al. (2004). The detailed results for population-level analyses have been discussed in Chapter 4 for both tiger and leopard.

The 12 markers microsatellite panel was standardised and used for the population scale study across the major tiger reserves and surrounding areas of Maharashtra. This panel provided a cost-effective way to investigate species biology, i.e. genetic diversity pattern, connectivity, differentiation etc., in the wild.

CHAPTER 4

Meta-population dynamics of tiger, leopard and Asiatic wild dog across the tiger reserves of Maharashtra

Chapter 4

Meta-population dynamics of tiger, leopard and Asiatic wild dog across the tiger reserves of Maharashtra

4.1 Genetic differentiation and Gene flow

4.1.1 Introduction

On the backdrop of rampant global industrialisation, urbanisation, and agricultural intensification, the long-term survival of most wild animals and their habitats are severely challenged by the drastic reduction in available habitats through fragmentation (Wilcove et al., 1986). Fragmentation events, in their most extreme form, cause negative edge effects along the borders of habitat patches, limiting animal migration and gene flow, severing landscape connectivity, and causing major demographic shifts in the long run (Crooks et al., 2017). Although species adapt differently to such pressures (Fahrig, 2011), generally, animals having large body sizes with apex positions in the food chain (Okie et al., 2009), low growth rates (deCastro et al., 2004), wide home ranges (Feeley et al., 2008), and habitat specialists (Prugh et al., 2008) are at higher risk of facing the detrimental effects of fragmentation. In this regard, the mammalian carnivore guild is one of the most fragmentation-effected groups of species, making them the ecological indicators of landscape connectivity (Crooks et al., 2011). Apex predators are most affected by the landscape change due to their requirements for large areas, and higher energy requirements. A large number of studies demonstrated the impacts of habitat fragmentation on carnivores (for example, ocelot (Janecka et al., 2016); African wild dog (Creel, 2001); mountain lion and coyote (Crooks, 2002); grey wolf (Ripple et al., 2014) and established that habitat-specialist pack-living carnivores are more prone to degraded habitats owing to their smaller niche breadth, smaller range of dispersal and Allee effect (Sanderson et al., 2014).

Several studies have been conducted in Central India to understand the dynamics of the tiger and leopard population. The pioneer works done by Dutta et al., 2013a; Dutta et al., 2013b; Joshi et al., 2013; Yumnam et al., 2014; Sharma et al., 2013a; Sharma et al., 2013b used the non-invasive approach to delineate the connectivity pattern across the tiger reserves of Central India. The common notion which came out from all the studies is that all the tiger reserves share a fair amount of connectivity with each other for tiger and leopard movement. In all the studies, there were some missing links from the complete frame, such as low sample size (Joshi et al., 2013), insufficient sampling in Pench (Maharashtra), and absence of sampling in the NNTR which has connectivity with both KTR and PTR (Dutta et al., 2012; Dutta et al., 2013a; Sharma et al., 2013a; Sharma et al., 2013b) and insufficient sampling in TATR which is one of the major tiger habitats in the whole mosaic of connectivity (Yumnam et al., 2014).

While in the case of Asiatic wild dogs, no single study has been done to understand the impact of fragmentation on this habitat specialist apex carnivore. To date, most of the studies have focused on their behaviour (Ghaskadbi et al., 2016; Karanth et al., 2000; Johnsingh, 1982), occupancy (Ngoprasert et al., 2019), population pattern (Selvan et al., 2014; Jenks et al., 2015), genetics (Modi et al., 2018; Modi et al., 2019; Iyengar et al., 2005) at local/regional scales; still, an in-depth understanding of population/demographic patterns are lacking.

I hypothesised that dholes have the highest genetic differentiation due to their smaller dispersal range, strict dependence on forested habitat, and group living nature (Habib et al., 2021b; Johnsingh, 1985). While tiger and leopard show lesser genetic differentiation and higher gene flow due to their larger home ranges (Simcharoen et al., 2014) and comparatively more adaptable habitat requirements as reported from the Vidarbha landscape (Habib et al., 2021a; Jacobson et al., 2016; Athreya et al., 2016). For this, I investigated the patterns of genetic differentiation and diversity of the top carnivore guild (tiger, leopard, and dhole) and demography of dhole population across Maharashtra, a part of a larger landscape of Central India. Using non-invasive genetic tools, I evaluated (1) the extent of genetic diversity of three carnivores across all tiger reserves in the state; (2) compared the population structure of tiger, leopard, and dhole in this area; and (3) estimated the effective population size and demographic history of the major populations of dhole within Maharashtra. I addressed these questions using 12 microsatellite loci (Mondol et al., 2009a, Mondol et al., 2009b, Modi et al., 2019) surveyed in 200 tigers, 95 leopards, and 305 dholes individual genotypes from six protected areas. Finally, I interpreted the results in the light of ecology and historical changes in the habitat of these carnivores in the Central Indian Landscape.

4.1.2 Methods

Study Area and Sampling

I sampled major known habitats of tiger, leopard, and dholes across the state, covering five tiger reserves: Pench Tiger Reserve (PTR), Melghat Tiger Reserve (MTR), Sahyadri Tiger Reserve (STR), Tadoba-Andhari Tiger Reserve (TATR) and Navegaon-Nagzira Tiger Reserve (NNTR) (Fig. 1.1). Apart from these areas, I also sampled the surrounding regions of Umred-Karandhla Wildlife Sanctuary (UKWLS). This mosaic of the tiger reserves and surrounding regions is already established as tiger corridors (Habib et al., 2021a), making it important to see if obligate forest-dwelling dholes are also using the same corridors. STR is completely disconnected from all other sites (Fig. 1.1). The remaining areas form a complex network of habitat patches, where the remaining sites are known to have varying degrees of habitat connectivity (Habib et al., 2021a). MTR is an exception to this complex as it does not share direct connectivity with the tiger reserves in this complex. All of these areas are characterised by dry deciduous to moist deciduous forests (Champion et al., 1968).

I sampled the entire region between January 2016 to April 2019, covering PTR (741.22 sq. km.), MTR (2768.52 sq. km.), NNTR (1894.94 sq. km.), TATR (1727.59 sq. km.), STR (1166.57 sq. km.) and UKWLS (189.30 sq. km.), Maharashtra. Each site was sampled intensively once for scats.

Extensive scat sampling resulted in 1156 scats across all seven study areas through foot and vehicle surveys. I only collected fresh samples during field surveys, where one bolus/scat was stored in butter paper following approaches described in Biswas et al. (2019). I also recorded each sample's GPS coordinates and other associated field information (substrate, track marks). The samples were temporarily stored in a large box containing silica gel in the field. In some cases, I sprayed a small amount of absolute ethanol to minimise fungal growth (Hallsworth et al., 1998). The samples were kept in the field for a maximum period of 10 days. Once transferred to the laboratory, all the scat samples were stored in a − 20 °C freezer till further processing.

DNA extraction and species identification

I performed DNA extraction from all field-collected scats using already established approaches described in Modi et al. (2018). In brief, I either swabbed twice (samples with no dust) or scraped (samples covered with dust) the top layer of the samples with sterile swabs or blade, respectively. They were lysed overnight in a lysis buffer at 56 °C, and extraction was performed following QIAamp DNA Tissue Kit (Qiagen Inc, Hilden, Germany) protocol. Final elution was performed twice in 100 μl of 1X TE buffer, and the DNA was stored at − 20 °C for long-term use. I conducted molecular species identification using species-specific mitochondrial primers described in Mondol et al., 2014; Mukherjee et al., 2007; Modi et al., 2018.

Individual identification

For individual identification from the respectively confirmed scats, I used the earlier validated 12 microsatellite loci panel described in Mondol et al., 2009a; Mondol et al., 2009b; Modi et al., 2019. All samples were genotyped three independent times to ensure good data quality for subsequent analyses.

Data Analyses

To generate the best quality data for analyses, I prepared consensus genotypes of each locus following the multiple tube approach combined with the quality index protocol described in Modi et al. (2019). I only considered the genotypes, which produced data for at least seven out of 12 loci in the consensus (Modi et al., 2019). The quality index threshold of 0.66 per loci, while the mean quality index of 0.75 across loci was set for the samples to be considered for downstream analyses. I used MICROCHECKER v 2.2.3 (van Oosterhout et al., 2004) to determine large allele dropouts as well as genotyping error estimation module of GIMLET (Modi et al., 2019; Broquet et al., 2004) to calculate the overall genotyping error rates (allelic dropout and false alleles). I used FreeNa (Chapuis et al., 2007) to determine the frequency of null alleles (NAs), which estimates the NA frequency using the EM algorithm (Dempster et al., 1977). I removed all genetic recaptures using the identity analyses module of CERVUS (Kalinowski et al., 2007), allowing up to two mismatches, and calculated the cumulative $PID_{(unbiased)}$ (probability of identity) and $PID_{(sibs)}$ value (Waits et al., 2001) using GIMLET (Valière, 2002) to assess the differentiation power of the panel. I estimated the allelic richness using the rarefaction approach in HP-RARE, considering the varying sample size of populations. I used GENPOP and ARLEQUIN (Excoffier et al., 2005) to check deviations from Hardy–Weinberg equilibrium (HWE) and linkage disequilibrium (LD).

Inferring population structure

To infer any possible genetic structure among the carnivores across the sampled areas, I used a combination of Bayesian clustering and multivariate analyses. Multivariate clustering analyses will help in determining patterns and clusters incorporating several variables in the population at a time. These analyses were conducted for only those populations with data from at least ten different individuals. I implemented the Bayesian clustering approach through program STRUCTURE v.2.3.4 (Pritchard et al., 2000), where 10 independent runs were performed for a range of population values (K = 1 to 10) with 100,000 burnin and 500,000 iterations. The models were run with admixture models considering correlated allele frequency and ancestry from one or more of K genetically distinct sources. The optimal number of clusters was determined by the deltaK approach (Evanno et al., 2005) implemented in STRUCTURE HARVESTER (Earl et al., 2011). The admixture proportion of individuals over ten replicates was averaged using CLUMPAK (Kopelman et al., 2015).

Further, I used the program Discriminant Analysis of Principal Component (DAPC) (Jombart et al., 2010) to identify genetic clusters in the present data. This is a multivariate analytical approach where no spatial information is required, and the population does not require to be under Hardy–Weinberg Equilibrium (Jombart, 2008a; Jombart et al., 2010). The genetic data is first transformed into principal components, then clustered using the discriminant function to define a group of individuals with the least within-group variation and the most between-group variation. The analyses were carried out using the adegenet package 2.1.1 in R studio 1.1.453 (R Development Core Team 2018), with the Bayesian Information Criterion (Jombart et al., 2010) determining the optimal number of clusters and the number of clusters being assessed using the find. clusters dapc function in R.

Finally, I used another multivariate clustering method implemented in the program spatial Principal Component Analysis (sPCA) that investigates cryptic spatial patterns of genetic variability using georeferenced multilocus genotypes (Jombart et al., 2008b). sPCA incorporates the spatial information along with the genotype data to ascertain local and global patterns of variations (Thioulouse et al., 1995). The global pattern (positive autocorrelation) would differentiate between two spatial groups, whereas the local pattern (negative autocorrelation) would determine the genetic differences among neighbours. The analysis was carried out using the nearest neighbour as the connection network which determines the extent to which the set of individual genotypes are clustered or uniform spatially. The variance was plotted against spatial autocorrelation (Moran's I) (Moran, 1948) to visually estimate any spatial structure in the genetic data. I used the Monte Carlo test with 10,000 iterations to statistically test global and local spatial structure.

Genetic differentiation among populations

I estimated genetic differentiation through different indices (G'st and Jost D) (Hedrick, 2005; Jost, 2008) using the R package DiveRsity 1.9 (Keenan et al., 2013) in R studio 3.1. I used both the differentiation indices to elucidate the asymmetric migration (Sundqvist et al., 2016) and differentiation among the sub-populations (Ryman et al., 2009; Meirmans et al., 2011).

Assessment of gene flow among different subpopulations

I used a Bayesian approach implemented in BAYESASS ver. 3.0.3 (Wilson et al., 2003) to infer the contemporary migration rate (m) among the detected subpopulations. This approach detects recent, low immigration rates in a population based on the genotype disequilibrium relative to the sampled populations without assuming HW equilibrium within the populations. The run parameters included 3×10^6 iterations and 10^6 burn-in with sampling every 2000 iterations. Delta values were adjusted to maintain an MCMC state change acceptance ratio of 20–40%. I averaged the results of multiple runs for the best model fit, as indicated by the Bayesian deviance measure (Faubet et al., 2007). Migration rates were depicted as circos plot using circlize ver. 0.3.4 in R ver. 1.2.5 (Zhang et al., 2013).

Effective population size (Ne)

I used the program Ne estimator v.2.01 (Do et al., 2014) to estimate the Ne for dhole from genotype data. I used the random mating model which refers to mating in a population in proportion to their genotypic frequencies with the following critical values (Pcrit:0.05, 0.02, & 0.01), and a jackknife 95% confidence interval for the analyses. I calculated the Ne for each subpopulation separately based on the number of putative clusters determined with a critical value of 0.02. I further used LDNe (Waples et al., 2008) to estimate the effective population size using the linkage disequilibrium approach with bias correction.

Demography analyses

I used qualitative and quantitative approaches to determine past demographic patterns of dhole subpopulations based on population substructure analysis results. For qualitative analysis, I used two different summary statistics-based approaches to detect any signal of population decline in dholes. These approaches are the Ewens, Watterson, Cornet, and Luikart method implemented in program BOTTLENECK ver 1.2.02 (Piry et al., 1999) and the Garza-Williamson index/ M-ratio approach implemented in program ARLEQUIN (Excoffier et al., 2005). Simulations were run on BOTTLENECK using three different mutation models: infinite allele model (IAM), single stepwise model (SMM), and two-phase model (TPM). During the simulations for the TPM model, 30% of multi-step mutation events were allowed. This approach identifies deviations from mutation-drift equilibrium and neutrality that can be explained by any departure from the null model, such as selection, population expansion, or decline. To analyse population decrease, the Garza-Williamson index uses data on the frequency and the total number of alleles, as well as the allele size difference.

Further, I used the R package VarEff 1.2 (Nikolic et al., 2014) in the R software version 3.1 to quantify dhole demographic patterns. This approach uses a coalescent framework to estimate the variation in effective population size (Ne) from present to ancestral time and determines the time of population decline from genetic data. I performed the analysis assuming the stepwise mutation model (SMM) (Kimura et al., 1978) with a generation time of 5 years for Asiatic wild dogs (Kamler et al., 2015). I used the SMM model to describe the mutation process for microsatellites in a more wholesome way (Ruiz-Garcia et al., 2013) which assumes that while the product of the effective population size and the mutation rate remains constant, the mean number of distinct alleles contained in the population rapidly hits a plateau that is not significantly bigger than the effective number of alleles. I considered a constant mutation rate of 3.5×10^{-3} per generation as described for canid microsatellites (Xu et al., 2000) over the past 1000 generations. The models were set with parameter DMAXPLUS values of 4 and 6 from the allele frequency histograms (maximum distance observed with a frequency ≥ 0.005 at 4 and 6) (Nikolic et al., 2014), along with prior values for Ne (parameter NBAR, range provided by theta), and the variances of the prior log-distributions for Ne (parameter VARP1, value of 3) and time intervals with constant population size (parameter VARP2, value of 3). The prior correlation coefficient between successive population sizes (parameter RHOCORN) was set to zero, and Jmax value was set at 2. The run parameters included the number batch to 10,000 lengths and space batch to 10, acceptance rate of 0.25 with a diagonal of 0.5. The demographic analyses were performed for only NNTR and TATR dhole populations, with adequate sample sizes.

4.1.3 Results

Genetic diversity

A total of 1156 samples were collected from six protected areas during the study period (2016-2019). I identified 250 tiger, 151 leopard, and 590 dhole faeces through species-specific molecular assay (Figure 4.1, 4.2, 4.3). Using a panel of 12 microsatellite markers (Modi et al., 2019), I generated a dataset of 200 tiger, 95 leopard and 305 dhole unique genotypes, attaining a success rate of 85%, 62%, and 59.1%, respectively, after removing 36 tiger genotypes, 44 dhole genotypes and 25 leopard genotypes as recaptures (Table 4.1). The PIDsibs and PIDunbiased values were low for the three species indicating the power of microsatellites to identify related genotypes as individual genotypes, indicating a statistically robust value for individual identification. The genotyping error rate is within the threshold of 20% suggested for non-invasive population-level studies (Smith et al., 2014; Godinho et al., 2015). The summary statistics of each protected area and species are provided in Table 4.2. The panel showed no evidence for strong linkage disequilibrium between any pair of loci. However, two to four loci from the panel were out of HW equilibrium in the individual population, but not a single loci were found to be out of HW equilibrium in all populations (Modi et al., 2019).

Parameter	***Scats confirmed***	***Individual genotypes***	***No. of recaptures***
Species			
Tiger	250	200	36
Leopard	151	95	25
Dhole	590	305	44

Table 4.1: Details of no. scats confirmed as species and identified.

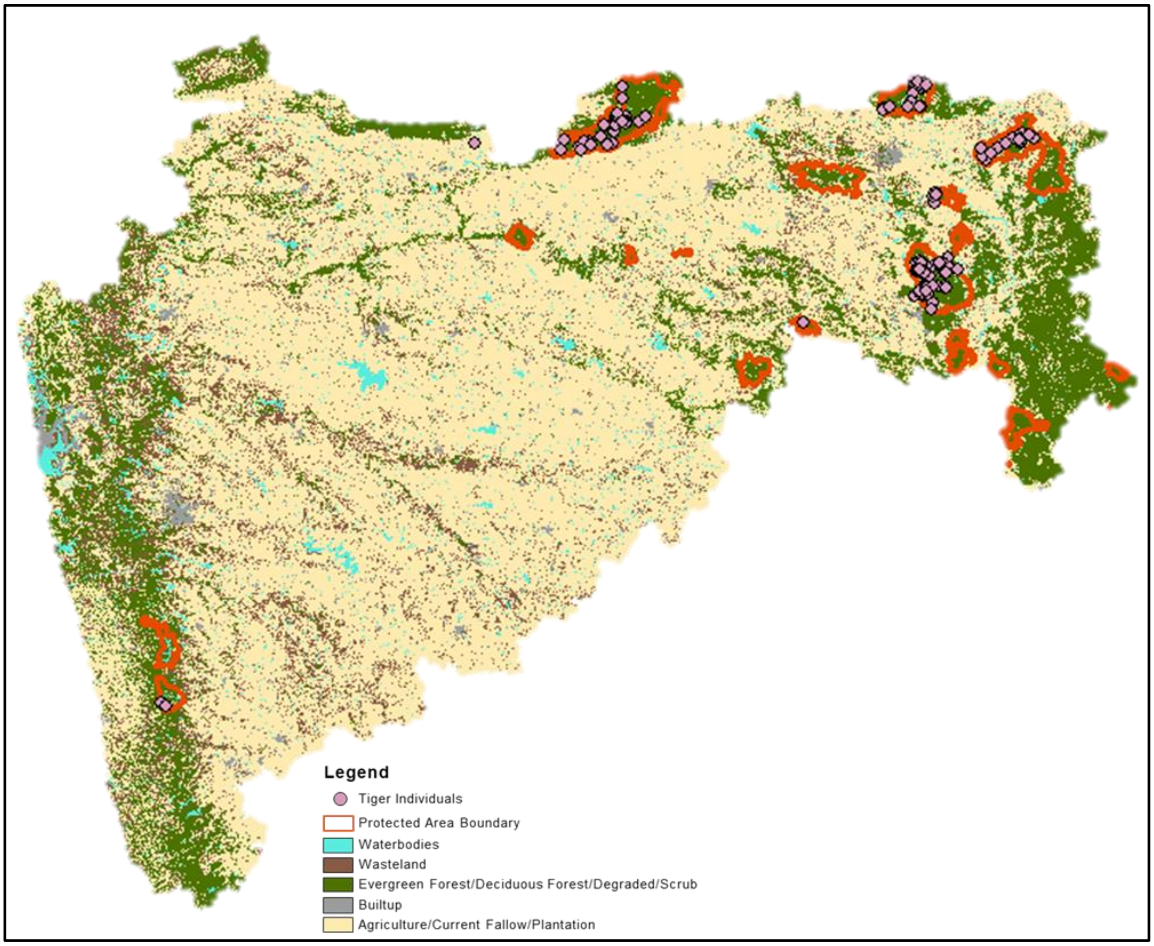


Figure 4.1: Map representing the total number of tiger individuals identified from the scats collected.

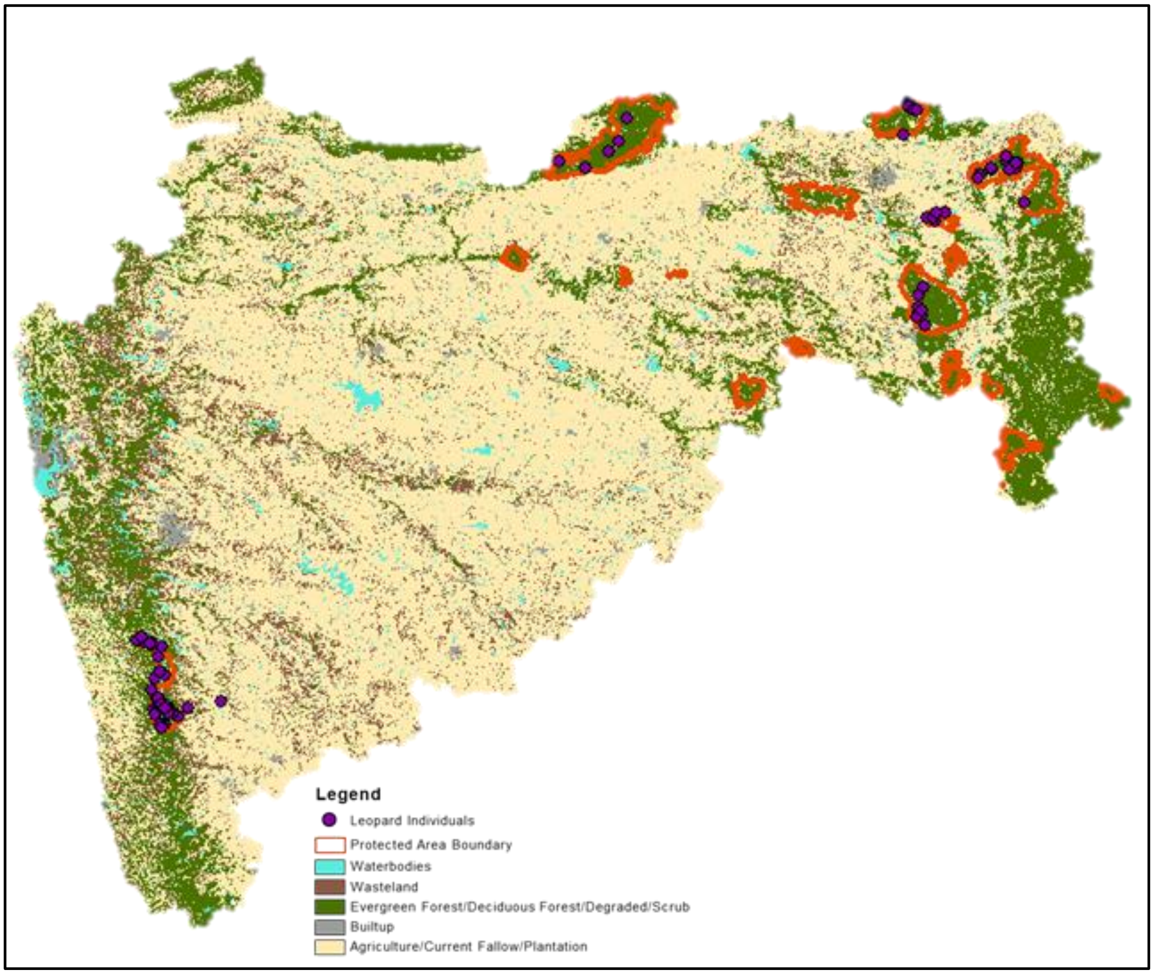


Figure 4.2: Map representing the total number of leopard individuals identified from the scats collected.

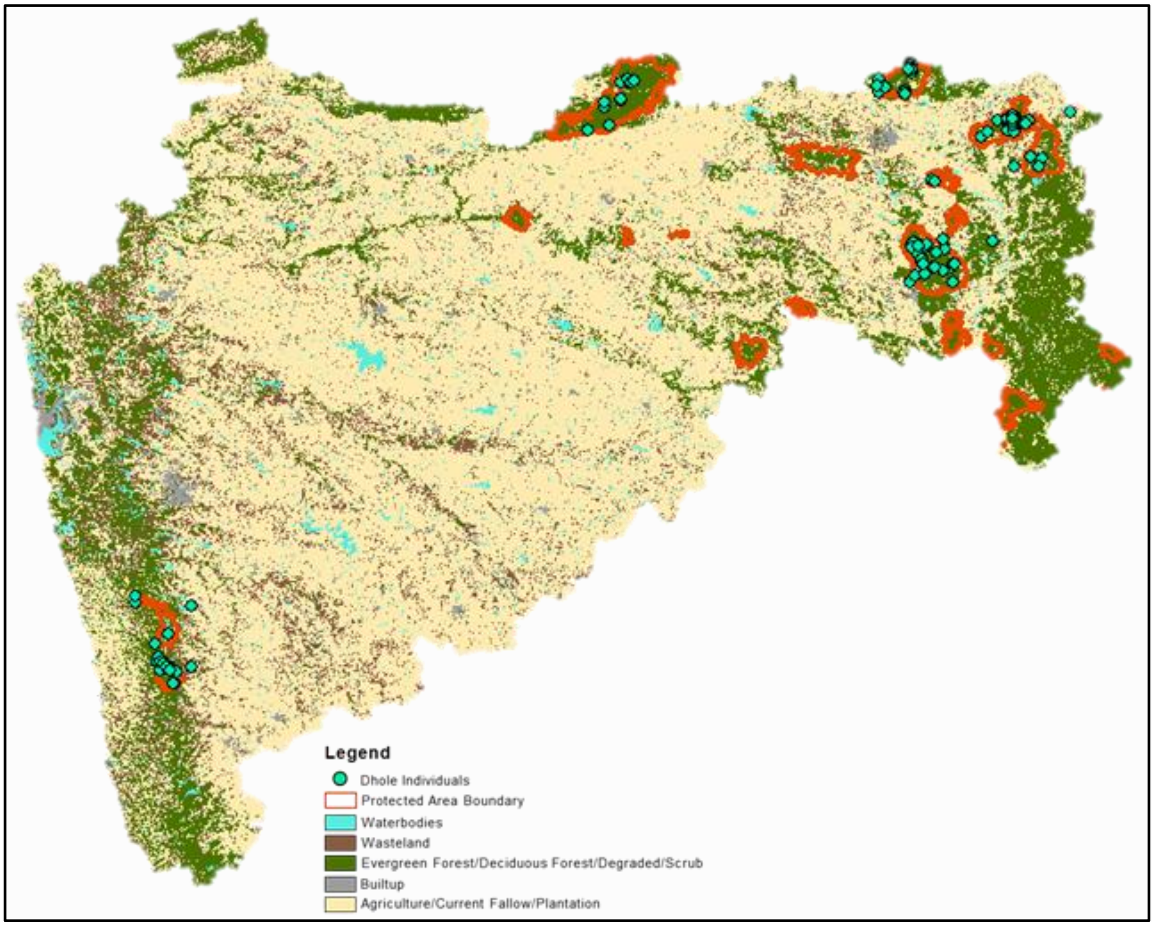


Figure 4.3: Map representing the total number of dhole individuals identified from the scats collected.

Parameters		***Tiger***	***Leopard***	***Dhole***
Error Rate		0.04	0.08	0.07
PID(sibs) value		9.6x10-7	6.9x10-6	1.06x10-4
Ho/He	PTR	0.43(0.17)/0.80(0.03)	0.48(0.27)/0.62(0.22)	0.45(0.22)/0.60(0.11)
	MTR	0.46(0.16)/0.81(0.04)	0.40(0.24)/0.71(0.12)	0.50(0.26)/0.50(0.20)
	NNTR	0.43(0.22)/0.78(0.07)	0.44(0.15)/0.74(0.13)	0.39(0.17)/0.50(0.20)
	TATR	0.39(0.17)/0.80(0.05)	0.48(0.18)/0.76(0.06)	0.49(0.20)/0.54(0.17)
	STR	NA	0.56(0.15)/0.75(0.04)	0.55(0.16)/0.59(0.06)
	UKWLS	0.33(0.16)/0.76(0.05)	0.5(0.35)/0.63(0.12)	NA

Table 4.2: Population-wise summary statistics of the three carnivores.

Population structure

My sampling strategy focused on maximum coverage of unique individuals across a relatively small region of species distribution to assess any possible population structure. Bayesian clustering analysis with 12 microsatellite loci revealed K=4 for tiger with no clear separation among clusters, K=5 for dhole (cluster 1- TATR, cluster 2- PTR+UKWLS, cluster 3- NNTR, cluster 4- MTR, cluster 5- STR) and K=2 with a similar pattern as tiger, without proper segregation between cluster for leopard (Figure 4.4 & 4.5)

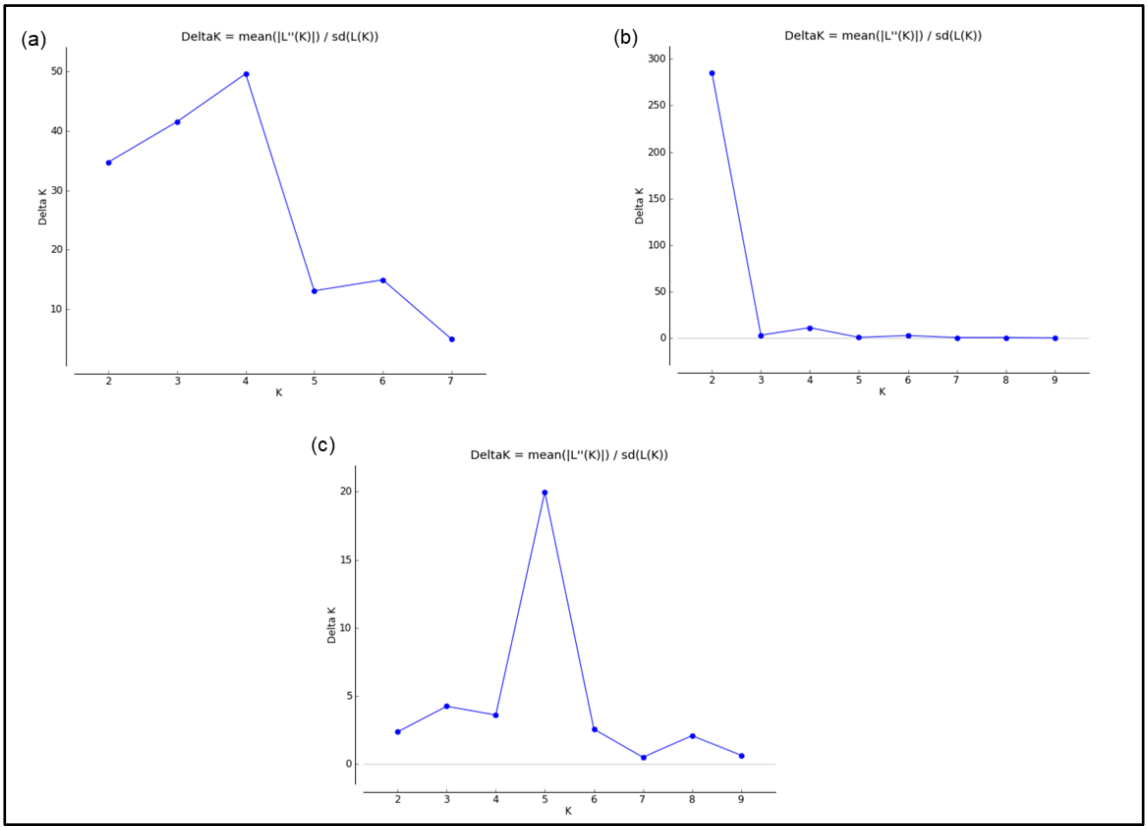


Figure 4.4: delta K graph using Evanno's coefficient to determine the value of K for (a) Tiger, (b) Leopard, and (c) Dhole.

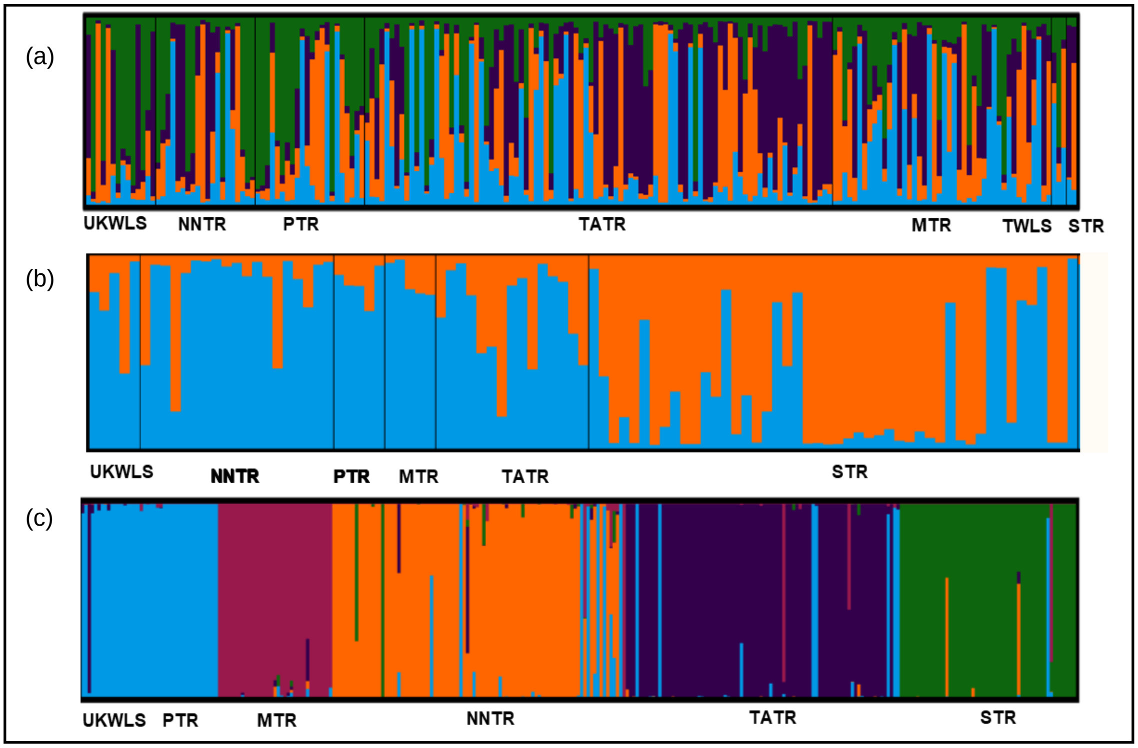


Figure 4.5: shows the results of Bayesian clustering approach implemented in STRUCTURE. Each colour represents a cluster, and a single bar plot represents the individual. The-X axis represents the population while the Y-axis corresponds to the probability of assignment of an individual to each cluster (a)shows the population genetic structure of 200 tiger individual genotypes with (k=4) (b) shows the population genetic structure of 95 leopard individual genotypes with (k=2) (c) shows population genetic structure of 305 dhole individual genotypes with (k=5).

Admixture level was higher among the tigers for the four clusters showing no clear signature of its own. BIC showed four genetic clusters for tiger, two for leopard, and seven for dhole using DAPC, almost similar to the STRUCTURE results (Figure 4.6).

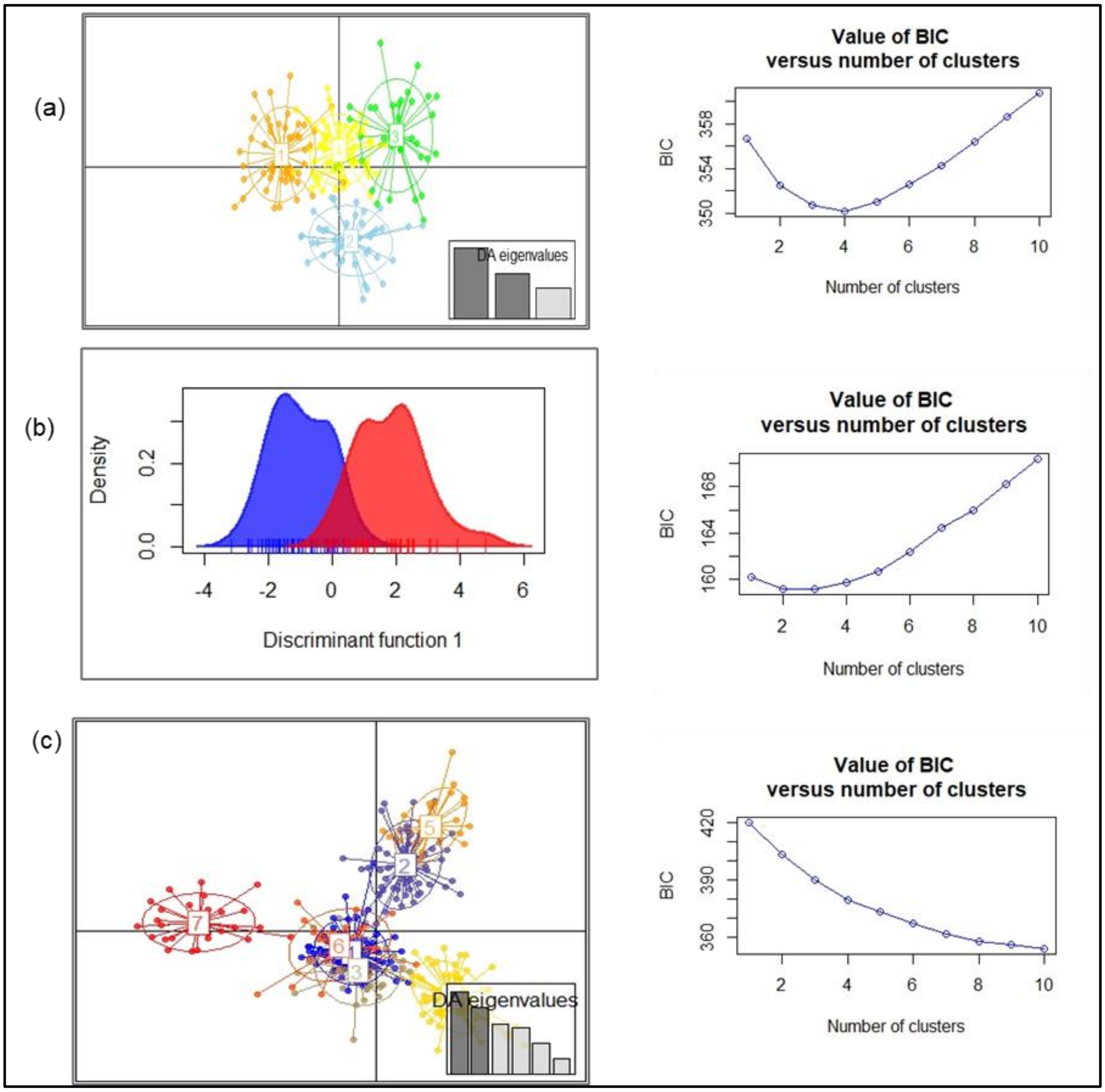


Figure 4.6: Represents the genetic clusters and BIC value from DAPC analysis for (a) For tiger (k=4) (b) For leopard (k=2) (c) For dhole (k=7) where TATR (cluster 2 and 3) and NNTR (cluster 5 and 6) populations show two overlapping clusters, making a total of five genetic subpopulations.

The global spatial pattern was found to be significant for dhole (p = 0.0001), indicating strong signatures between population separations while non-significant for tiger and leopard (Figure 4.7).

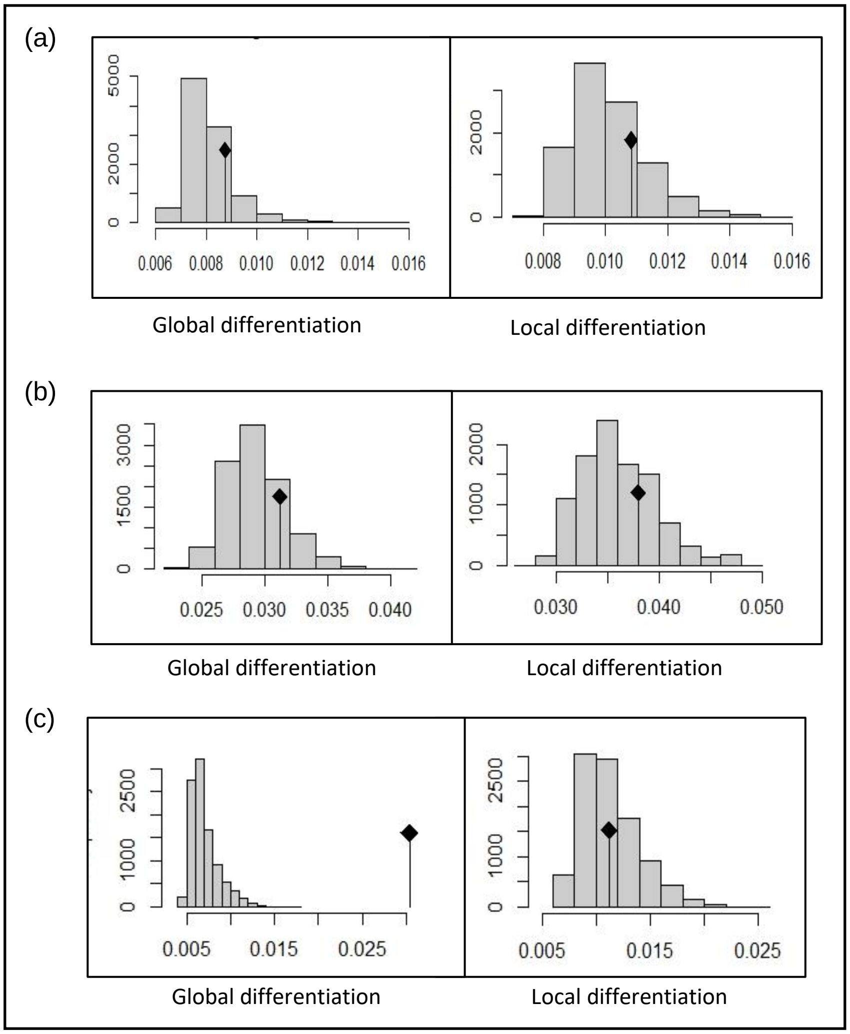


Figure 4.7: Results of eigenvalue test of global and local differentiation by sPCA analysis for (a) tiger, (b) leopard, and (c) dhole.

For tiger, the G'st value was similar for most of the combinations with a range from 0.065-0.154 (Table 4.3). The range of differentiation was found to be comparatively higher as opposed to the expectation from 0.107 – 0.406 (Table 4.4). This vast difference could be because of the small sample size from some populations of leopard. In the case of dholes, the G'st values ranged between 0.22–0.40, with the highest differentiation found between MTR-STR (0.40) and MTR-

PTR (0.40), respectively, and the lowest value between PTR-UKWLS (0.20) as expected (Table 4.5).

G'st / D Jost	***UKWLS***	***NNTR***	***PTR***	***TATR***	***MTR***
UKWLS		0.036 (-0.05-0.16)	0.047 (-0.06- 0.17)	0.065 (-0.00-0.17)	0.056 (-0.02-0.16)
NNTR	0.093 (-0.01-0.24)		0.111 (0.01-0.23)	0.026 (-0.02-0.09)	0.034 (-0.02-0.12)
PTR	0.121 (0-0.26)	0.154 (0.03-0.27)		0.099 (0.03-0.16)	0.044 (-0.01-0.11)
TATR	0.102 (0.00-0.23)	0.065 (-0.00-0.16)	0.150 (0.08-0.22)		0.024 (-0.01-0.06)
MTR	0.105 (0.00-0.22)	0.107 (0.03-0.20)	0.117 (0.41-0.20)	0.073 (0.02-0.13)	

Table 4.3: Pairwise value for G'st (lower diagonal) and Jost's D (upper diagonal) for tiger.

,G'st / D Jost	***UKWLS***	***NNTR***	***PTR***	***MTR***	***TATR***	***STR***
UKWLS		0.084 (-0.04-0.29)	0.111 (-0.03-0.30)	0.038 (-0.14-0.29)	0.095 (-0.45-0.27)	0.094 (-0.01-0.27)
NNTR	0.203 (0.03-0.44)		0.184 (0.01-0.36)	0.006 (-0.17-0.26)	0.028 (-0.08-0.16)	0.174 (0.09-0.27)
PTR	0.332 (0.16-0.51)	0.286 (0.12-0.48)		0.117 (-0.14-0.40)	0.062 (-0.10-0.29)	0.162 (0.04-0.31)
MTR	0.175 (-0.03-0.41)	0.165 (-0.01-0.41)	0.406 (0.24-0.58)		0.0146 (-0.17-0.25)	0.140 (-0.008-0.34)
TATR	0.229 (0.05-0.44)	0.207 (0.08-0.34)	0.330 (0.13-0.52)	0.172 (-0.05-0.40)		0.039 (-0.04-0.14)
STR	0.234 (0.08-0.42)	0.258 (0.17-0.36)	0.299 (0.17-0.45)	0.275 (0.11-0.46)	0.107 (0.0-0.23)	

Table 4.4: Pairwise value for G'st (lower diagonal) and Jost's D (upper diagonal) for leopard.

G'st / D Jost	***UKWLS***	***PTR***	***MTR***	***NNTR***	***TATR***	***STR***
UKWLS		0.095 (0.04-0.16)	0.229 (0.16-0.32)	0.146 (0.09-0.22)	0.097 (0.02-0.20)	0.189 (0.12-0.30)
PTR	0.203 (0.11-0.33)		0.126 (0.09-0.17)	0.186 (0.14-0.24)	0.115 (0.08-0.15))	0.183 (0.13-0.23)
MTR	0.402 (0.32-0.50)	0.297 (0.24-0.35)		0.190 (0.14-0.23)	0.170 (0.13-0.20)	0.207 (0.16-0.25)
NNTR	0.351 (0.28-0.44)	0.337 (0.29-0.40)	0.354 (0.29-0.41)		0.139 (0.11-0.16)	0.187 (0.15-0.22)
TATR	0.227 (0.12-0.38)	0.258 (0.21-0.30)	0.333 (0.28-0.39)	0.295 (0.26-0.34)		0.219 (0.18-0.26)
STR	0.394 (0.31-0.51)	0.332 (0.28-0.39)	0.404 (0.35-0.45)	0.340 (0.29-0.39)	0.370 (0.32-0.42)	

Table 4.5: Pairwise value for G'st (lower diagonal) and Jost's D (upper diagonal) for dholes.

Gene flow

Contemporary migration rates estimation suggested an average migration rate of 0.05 (range 0.003–0.15) for tigers, 0.06 (range 0.01-0.14) for leopards, and 0.02 (range 0.003-0.015) for dholes across the eastern Vidarbha landscape (Figure 4.8). The rate of migration, representing the proportion of individual movement, was found to be highest from TATR to UKWLS and NNTR for tigers (Table 4.6). For leopards, the gene flow was found to be highest from NNTR to UKWLS and TATR (Table 4.7), while for dholes, it showed very low and non-significant gene flow among the genetic subpopulations corroborating the distinct population structure patterns. The highest value for gene flow was from PTR to UKWLS, and the lowest was between UKWLS and NNTR (Table 4.8).

A (horizontal row) / B (vertical row)	***UKWLS***	***NNTR***	***PTR***	***TATR***	***MTR***
UKWLS	**0.7865 (0.0387)**	0.0176 (0.0169)	0.0544 (0.0281)	0.1236 (0.0389)	0.0180 (0.0170)
NNTR	0.0593 (0.0378)	**0.6805 (0.0133)**	0.0692 (0.0286)	0.1560 (0.0444)	0.0350 (0.0228)
PTR	0.0861 (0.0418)	0.0211 (0.0185)	**0.7933 (0.0448)**	0.0288 (0.0222)	0.0708 (0.0301)
TATR	0.0064 (0.0058)	0.0039 (0.0038)	0.0780 (0.0161)	**0.8873 (0.0179)**	0.0245 (0.0089)
MTR	0.0090 (0.0084)	0.0075 (0.0074)	0.0918 (0.0220)	0.1133 (0.0272)	**0.7785 (0.0269)**

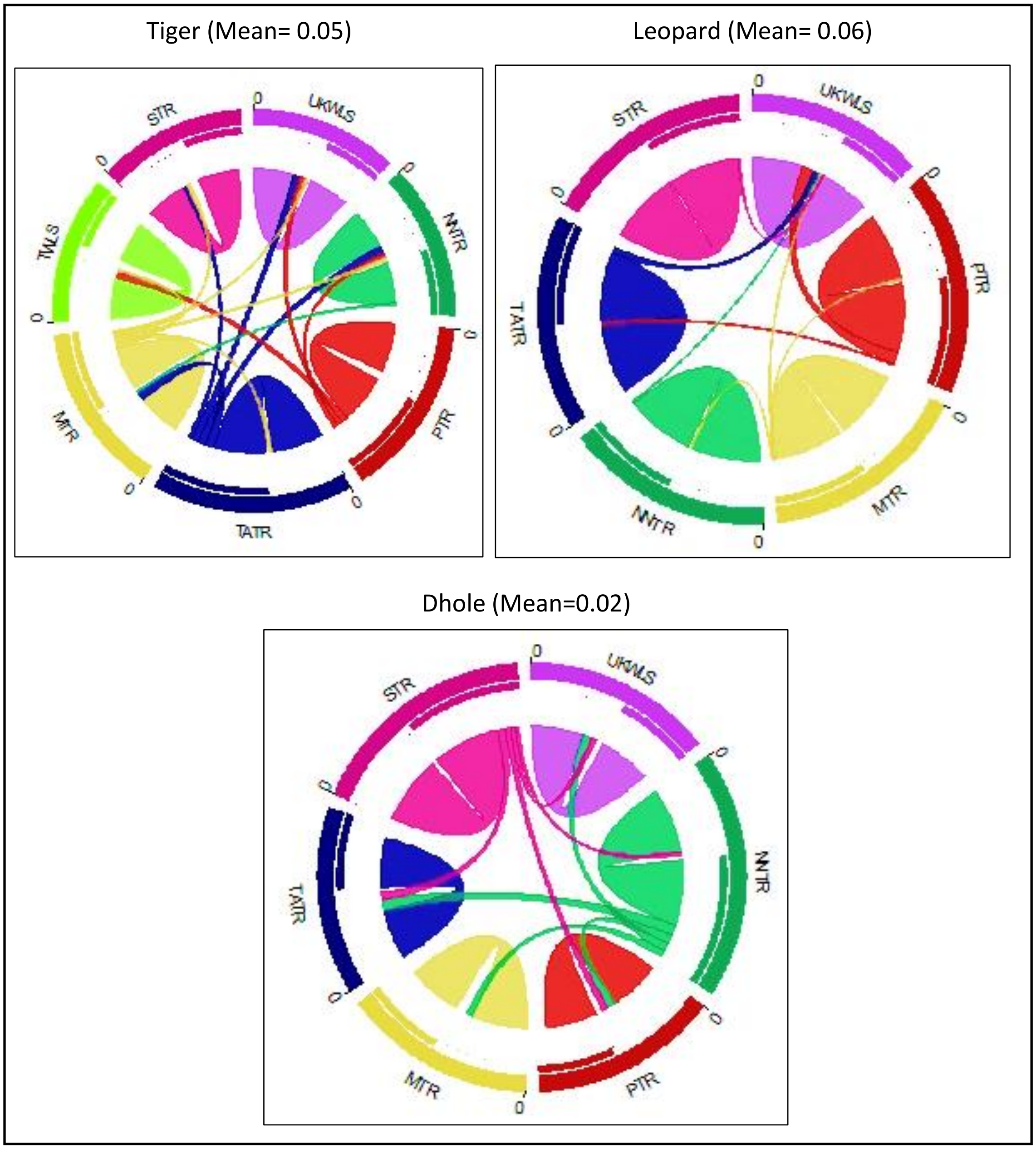
Table 4.6: Results of gene flow analysis of tiger using BAYESASS from Eastern Vidarbha region. The posterior distribution values of migration rates (m) with 95% CI are presented. The direction of gene flow is from A to B.

A (horizontal row) / B (vertical row)	***UKWLS***	***NNTR***	***PTR***	***MTR***	***TATR***
UKWLS	0.7099 (0.0398)	0.1438 (0.0562)	0.0344 (0.0314)	0.0714 (0.0504)	0.0406 (0.0359)
NNTR	0.0150 (0.0143)	0.8858 (0.0350)	0.0166 (0.0160)	0.0652 (0.0289)	0.0173 (0.0169)
PTR	0.0339 (0.0305)	0.1289 (0.0510)	0.7091 (0.0381)	0.0904 (0.0484)	0.0377 (0.0337)
MTR	0.0702 (0.0460)	0.0953 (0.0475)	0.0376 (0.0333)	0.7519 (0.0480)	0.0451 (0.0382)
TATR	0.0212 (0.0197)	0.1432 (0.0457)	0.0243 (0.0216)	0.1107 (0.0376)	0.7007 (0.0317)

Table 4.7: Results of gene flow analysis of leopard using BAYESASS from Eastern Vidarbha region. The posterior distribution values of migration rates (m) with 95% CI are presented. The direction of gene flow is from A to B.

A (horizontal row) / B (vertical row)	UKWLS	NNTR	PTR	MTR	TATR
UKWLS	0.7099 (0.0370)	0.0248 (0.0230)	0.1598 (0.0555)	0.0243 (0.0228)	0.0811 (0.0441)
PTR	0.0090 (0.0090)	0.0095 (0.0093)	0.9489 (0.0208)	0.0204 (0.0145)	0.0122 (0.0112)
MTR	0.0085 (0.0082)	0.0097 (0.0095)	0.0166 (0.0167)	0.9527 (0.0229)	0.0124 (0.0118)
NNTR	0.0040 (0.0039)	0.9528 (0.0137)	0.0241 (0.0119)	0.0112 (0.0083)	0.0079 (0.0064)
TATR	0.0038 (0.0037)	0.0046 (0.0045)	0.0244 (0.0115)	0.0059 (0.0056)	0.9614 (0.0135)

Table 4.8: Results of gene flow analysis of dhole using BAYESASS from Eastern Vidarbha region. The posterior distribution values of migration rates (m) with 95% CI are presented. The direction of gene flow is from A to B.


Tiger (Mean= 0.05)

Leopard (Mean= 0.06)

Dhole (Mean=0.02)

Figure 4.8 BayesAss output in the form of circos plot represents the gene flow among the different populations for tiger, leopard and dhole (left to right).

Effective population size and demography

Two independent, effective population size estimation approaches showed low values, ranging from 6–16.3 across the dhole subpopulations, suggesting potential inbreeding (Table 4.9). Based on the data of unique individuals from each subpopulation and calculated effective population sizes, I found a very skewed ratio of Ne/N in TATR (0.16), NNTR (0.18), and MTR (0.17) but a balanced value in STR (0.29) and PTR (0.35). The estimates should be taken with caution due to the use of surrogate census population size. The inbreeding coefficient (Fis) value ranged between 0.005–0.296 (Table 4.9) among the subpopulations. Careful investigation revealed a pattern where populations with lower Fis showed higher effective population sizes.

	PTR (n=33)	**MTR (n=35)**	**NNTR (n=90)**	**TATR (n=84)**	**STR (n=54)**
Ne estimator	11.8 (7.8-18.6)	6.0 (2.9-10.4)	16.3 (12.7-21.0)	13.5 (8.9-20.0)	16.0 (10.6-25.1)
LDNE	12.2 (8.3-18.6)	6.0 (3.1-9.7)	16.6 (13.4-20.7)	11.8 (9.7-14.4)	18.4 (13.3-26.3)
Fis value	0.223	0.29	0.074	0.071	-0.0005

Table 4.9: Estimates of effective population sizes (Ne) (C.I.-95%) and inbreeding coefficient (Fis) values at five sampled areas from the LD approach.

Both qualitative analyses revealed signatures of population decline in all the five dhole subpopulations. BOTTLENECK results showed significant heterozygote excess in seven out of twelve loci using both SMM and TPM models across all subpopulations, suggesting a loss of rare alleles during a possible population decline. Similarly, the Garza-Williamson index showed low values (compared to M_{cric} 0.68 for stable populations) in all populations (M-ratio$_{PTR}$- 0.27611 (SD 0.12); M-ratio$_{MTR}$- 0.35809 (SD 0.24); M-ratio$_{NNTR}$- 0.33504 (SD 0.15); M-ratio$_{TATR}$-0.28588 (SD 0.12) and M-ratio$_{STR}$-0.34592 (SD 0.09), indicating signals of population decline (Table 4.10).

Population	***Sign Test: Number of loci with heterozygosity excess (probability)***		
	IAM	***TPM***	***SMM***
PTR	**6.59 (0.007)**	6.96 (0.383)	7.02 (0.374)
MTR	**5.89 (0.010)**	6.43 (0.260)	6.44 (0.506)
NNTR	6.68 (0.546)	**7.05 (0.019)**	**7.14 (0.016)**
TATR	6.81 (0.162)	**7.12 (0.003)**	**7.09 (0.003)**
STR	6.85 (0.056)	**7.07 (0.003)**	**7.18 (0.002)**

Table 4.10: Sign test results from BOTTLENECK analysis for dhole under IAM, TPM, and SMM mutation model. P-values are in the bracket, and significant results are in bold.

The quantitative VarEff approach showed a steep decline in the effective population size in both NNTR and TATR subpopulations. Results indicate 77-85% decline in dhole effective population size for NNTR and TATR subpopulations. The timing of this decline was quantified at ~60-90 generations ago, making it about 300-450 years before the present (with five years of generation time for dholes) (Figure 4.9). The current effective size ranged between 21-114 (median 58) for TATR and 14-110 (median 45) for NNTR at a 95% confidence interval.

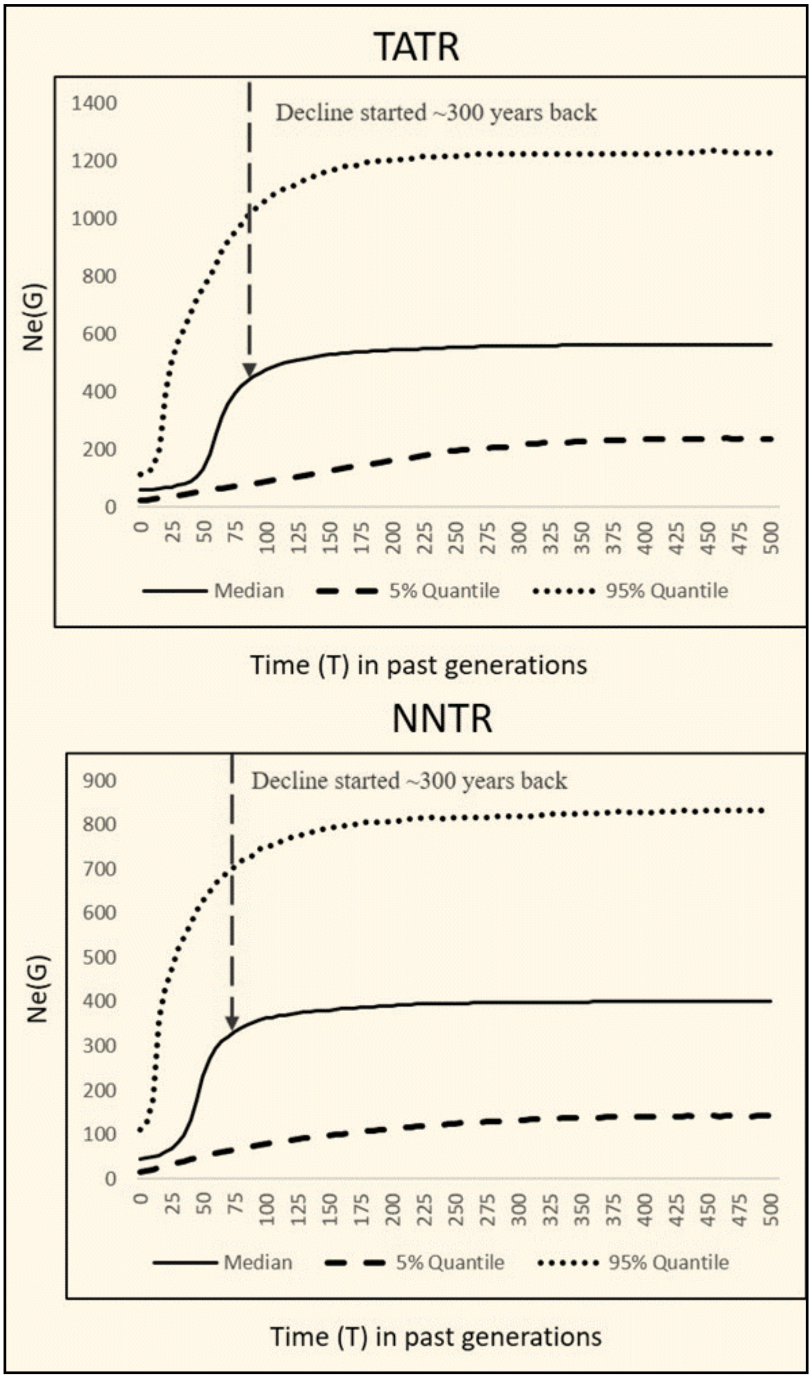


Figure 4.9: Demographic history of dholes in TATR and NNTR, Maharashtra through quantitative VarEff approach. Demographic changes have been presented as posterior distribution (median estimates) of the effective population sizes (0-500 generations ago) based on simulations with 12 microsatellite loci data from TATR (n=84 individuals) and NNTR (n=90 individuals). The decline timing has a median distribution value of ~300 years from the present.

A.4.4. Discussion

Generating detailed information on the population parameters of large carnivores in present anthropogenically altered and fragmented landscapes to preserve & restore their genetic diversity and connectivity is crucial for their conservation. Comparative genetic studies to determine the response of sympatric species to habitat fragmentation are required to develop a wholesome framework for conservation and management planning for the conservation of multiple species. The main aim of this study was to understand the population dynamics in terms of genetic diversity, genetic structure, and gene flow of the three large carnivores using non-invasive genetic sampling. This study provides the first comparative genetic inference of three large carnivores with similar niches in the state of Maharashtra.

I adopted a multi-disciplinary approach involving field sampling, genetic information, and multivariate & Bayesian analytical frameworks to address spatial genetic patterns and demography of the three species in the tiger reserves of Maharashtra. This region faces significant changes in land-use patterns from rapid urbanisation, expanding agriculture, infrastructure development, acquisition of minerals, and economic growth (Reddy et al., 2013; Dutta et al., 2018). Thus, this study has important conservation/management implications for these sympatric felid and canid species and their habitat.

Firstly, multiple genetic analyses with landscape-scale microsatellite data revealed low to moderate levels of genetic differentiation, with leopard showing the lowest genetic differentiation and dhole showing the highest. Out of six sampled areas, the five major tiger reserves (MTR, PTR, TATR, NNTR, and STR) represent the five clusters for dholes. While the UKWLS, a known connecting habitat between PTR and TATR (Habib et al., 2021a), showed a mixture of genetic signals from the four major clusters (TATR, NNTR, MTR, and PTR). The genetic clusters were clearly separated with very few mixed genetic signals in UKWLS. Despite any assignment cluster for UKWLS, it was included further in connectivity analysis due to its strategic geographical location in the landscape acting as a connecting link (Habib et al., 2021a). For tiger, I found no clear differentiation in clusters representing the protected areas, while in the case of leopard, a generalist, I found two major clusters, one representing the eastern Vidarbha landscape (MTR, PTR, TATR, NNTR, UKWLS) while the second was representing the western ghats population of STR. The distinct genetic structure for leopard of STR shows that even the most adaptable species out of the three, which have the least differentiation and highest gene flow, is not connected with the rest of the Vidarbha population resulting in a distinct signature of its own. It also suggests a complete loss of connectivity between STR and the rest of the eastern Vidarbha landscape.

The patterns of genetic differentiation are not surprising and support my hypothesis of a differential effect of habitat fragmentation on these carnivores. Since dholes are highly forest-dependent and short-range dispersers (Srivathsa, 2019), this situation highlights their vulnerability in the current scenario of continuing land-use change and urbanisation. Similar group-living species have been reported to show genetic differentiation due to their adaptations to a specific habitat, group cohesion, and local philopatry (Lowther et al., 2012). For example, social canids such as grey wolves and African wild dogs have also shown higher genetic differentiation in fragmented landscapes (Marsden et al., 2012). Contrary to this, other co-occurring large carnivores (tiger-Yumnam et al., 2014; leopard-Dutta et al., 2013a) and omnivore (sloth bear-Thatte et al., 2020) showed much less genetic differentiation, possibly due to longer dispersal capabilities resulting in higher gene flow.

Given the small population size of dholes across their range (Kamler et al., 2015) and possible genetic differentiation (based on the results of this study), the species face potential demographic impact (Lourenço et al., 2017; Slatkin, 1994). Kamler et al. (2015) suggested that dholes require area five times larger than tigers for long-term viability, which seems to be improbable in the current scenario. Thus, it will be critical to maintain population connectivity through corridor restoration and facilitate gene flow at the landscape scale. The NNTR and TATR clusters show a comparatively higher number of first-generation migrants with respect to other sub-populations. One of the major reasons behind such a pattern is the larger pack size due to low tiger density in NNTR (Bhandari et al., 2021) and higher turnover in TATR due to higher tiger density. In low tiger density areas, breeding opportunities reduce because of the larger pack size required to suppress the recovery of top-predator, while in high tiger density areas, immigrants often fail to establish themselves hence, contributing less to the gene pool. These dynamics of pack size and top-predator density influence the genetic structure of a population.

The demography analyses with two qualitative approaches indicate a strong decline in dhole population size for all five genetic subpopulations, as expected from fragmented and small, isolated populations (Davies et al., 2000). The quantitative approach with VarEff revealed a 77–85% decline in NNTR and TATR dhole populations, respectively. The magnitude of decline for dholes corroborates with other co-occurring large carnivores in this landscape. For example, earlier studies have shown a 90% and 98% decline in the leopard and tiger population in the central Indian region, respectively (Bhatt et al., 2020; Mondol et al., 2009c). The relatively less decline for dholes compared to other larger carnivores is possibly due to fewer demands of dholes as trophies/illegal wildlife trade. Tiger and leopard body parts (pelt, bones, claw, meat, fat, whisker) are highly sought products in the trans-national illegal wildlife trade (Nijman et al., 2019), whereas dhole populations have faced historical pressures from bounty hunting and human persecutions as vermin during British colonial period (Kamler et al., 2015). However, it is difficult to validate these decline patterns with other information as no robust quantitative data on actual population size (both historical and current) are available (Srivathsa et al., 2020). Another important finding is the relatively old timing of decline for dholes. Demographic results suggest a median dhole decline timing of ~ 300 years in both NNTR and TATR, much older than tiger/leopard decline timing in central India (tiger- decline ~ 200 years ago (Mondol et al., 2009c), leopard- decline ~ 125 years ago (Bhatt et al., 2020)). This could be explained by a combination of habitat loss driven population decline in historical times followed by hunting during the British era (Durbin et al., 2004). The study on global land-use change over the last 300 years has also estimated a forest loss of 40% for the Indian sub-continent during the last century (Richards et al., 1994; Goldewijk et al., 2009). Sharma et al. (2013b) showed that this landscape had experienced a major change in land-use patterns during the last 300 years, leading to ~ 77% loss of forested habitats to agricultural areas and urbanisation. Rangarajan (1999) also reported severe fragmentation of historically contiguous habitats of the Central Indian Highland during the last few centuries. Subsequently, over-exploitation of teak started during the early British period (1750–1990) for navy and railway lines which further resulted in the conversion of forests into commercial plantations by large scale clearing (Gadgil, 2011; Bebarta, 1999), thereby further reducing the available habitats for dholes. Such drastic changes possibly had severe impacts on the population size of obligate forest-dwelling dholes. Although comprehensive data on the exact effects of such large-scale habitat loss on dhole population size is lacking, this available information suggests that continuing habitat fragmentation starting since last 300 years coupled with massive hunting pressure during colonial bounty-hunting rules resulted in dhole population decline over a longer time.

One of the most important aspects of this study is the assessment of inbreeding status (Fis value) and effective population size (Ne) of dholes which are critical population parameters, and summarise the history of any population (Waples et al., 2011). For both NNTR and TATR populations (relatively higher population size compared to the other areas), the Ne is approximately 20% of the total population (Ne/N ratio of 0.16 and 0.18 for TATR and NNTR, respectively) which is similar to 0.11 across different taxa (Frankham et al., 2014). Such low values for Ne are not unusual and have been earlier described in social animals with dominance hierarchy (for example, lions (deManuel et al., 2020), African wild dogs (Creel, 1998), dwarf mongooses (Creel, 1998)) as well as in endangered species with small population sizes (Lande et al., 1987). However, the Ne values obtained from VarEff were comparatively higher and probably more realistic than the linkage dis-equilibrium based approach as this approach can substantially underestimate the Ne in inbred populations (Bhandari et al., 2021; deManuel et al., 2020; Creel, 1998; Neel et al., 2013). The number of individuals observed in each population could be related to the sampling size, which is a limitation in this study due to the unavailability of population estimates. I have only used the population size from this study as a surrogate to calculate the Ne/N ratio which is independent of Ne calculation. In addition, I also found out that inbreeding coefficient values (Fis) were different for each population and indicated an inverse relationship with Ne. For example, dhole data from MTR showed the highest Fis value and lowest Ne, whereas STR showed the lowest Fis and high Ne value. This pattern makes sense for a species with social dominance hierarchy where only the dominant member of the pack has the highest mating opportunities (thus low Ne) and will have more inbred individuals in a small group or population (high Fis).

Finally, the results from this study also showed that relatively large dhole populations such as NNTR and TATR still retain reasonably high genetic variation despite the severe decline and strong population structure. The genetic variations of NNTR and TATR are comparable with other social canid species such as African wild dogs from Kruger National Park (Girman et al., 2001). However, the rest of the populations (need urgent management interventions (possibly in the form of translocations as well as better habitat connectivity) to increase the genetic variation and ensure the future survival of the populations in this landscape as a whole.

I acknowledge the chances of bias resulting from individuals identified with low recaptures, which can be correlated with complex interactions between this specific sampling strategy and relatively low amplification success rates from field-collected faeces. Since my field sampling strategy focused on maximum coverage within six protected areas of Maharashtra and was conducted only once, I surveyed the entire area and collected only fresh samples for DNA analyses. Because of this, I might have missed recapturing the same individuals, thus giving low recapture rates.

This can be dealt with with a genetic capture-recapture method in future with a more intensive sampling strategy. The high difference in the expected and observed heterozygotes could be a complex combination of species-population marker scenarios found in other canids (grey wolf, coyote, golden jackal) studies where cross-species dog primers were used (Sacks et al., 2005; Stronen et al., 2014).

With the ongoing habitat fragmentation scenario, the threats of habitat loss, prey depletion, disease transmission, and human persecution of these carnivores must be addressed to ensure their long-term persistence. Deciphering the patterns of genetic structure, gene flow, and demography of multiple co-occurring carnivores is crucial for developing informed conservation strategies. This study provides baseline information of the genetic database and comparative differences in the genetic differentiation and diversity of the three sympatric carnivores. I hope that the results and suggestions from this study will lead to the generation of critical information on the genetics of these species from Maharashtra, which possess three major tiger corridors of the Central Indian region, and will aid in understanding the effects in a similar landscape across the distribution range.

4.2 Dispersal

4.2.1 Introduction

Dispersal of individuals from the natal group in order to pass on their genes is one of the most important aspects of an organism's life history (Handley et al., 2007). Effective dispersal is the key to population persistence and individual fitness (Bowler et al., 2005; Lowe et al., 2010) and affects the genetic structure across populations or social groups (Ross, 2001). It is one of the most expensive but inevitable decisions in an individual's life history, including an array of risks, which is further determined by many factors such as dispersal distance, habitat quality (patched or connected), mating system, and individual's personality as well as sex (Burgess et al., 2016; van Overveld et al., 2014). Depending on selective factors such as the availability of resources and mates, as well as the risk of inbreeding and competition with kin, dispersal tactics might differ from species to species (Pusey, 1987; Dieckmann et al., 1999; Gandon, 1999; Perrin & Mazalov, 2000; Szulkin & Sheldon, 2008). Species, populations, and individuals do not necessarily react in the same way to the triggers that cause dispersal, resulting in divergent dispersal strategies (Baguette et al., 2013).

In the present Anthropocene era, with limited resources and habitat patches, a few other factors can play a decisive role in an individual's dispersal, such as landscape connectivity, interspecific, and intraguild competition. Interspecific competition impacts nearly all species and has long been recognised as a key factor in the organisation of large carnivore guilds (Courchamp & Macdonald, 2001; Palomeres & Caro, 1999). Tiger, leopard, and the Asiatic wild dog represent the major carnivore guild in the Indian sub-continent, with the tiger as the dominant predator. The dispersal pattern of tiger and leopard has been widely studied with the prevalence of long-range male-biased dispersal (Gour et al., 2013; Singh et al., 2021; Fattebert et al., 2015a; Fattebert et al., 2015b). While the Asiatic wild dog is the least studied group-living species with unique social characters and is among the least monogamous canid (Johnsingh, 1985). Evolutionary dholes are cooperative breeders and possess a highly organised social structure. This monophyletic species mainly thrives in deep forests and comes under the Endangered category by IUCN (Kamler et al., 2015). Preference for a deeply forested habitat and small home range of 58.67 sq. km (± 4.08) (Habib et al., 2021b) makes this species vulnerable to habitat fragmentation and disrupted gene flow posing major concerns for the long-term survival of this species.

Previous ecological studies on dholes have found a bias towards female dispersal (Johnsingh, 1982; Venkataraman, 1998; Iyengar et al., 2005), but no extensive study has been done to comprehend the dispersal pattern of dholes. Though the male-biased packs point toward the biased nature of dispersal in dholes (Venkataraman, 1998; Modi et al., 2018), for a pack living animal, the dispersal pattern is further complexed by other variables. A study on red colobus monkeys found that female dispersal is more frequent in larger groups (Miyamoto et al., 2013). In the case of dholes, there is a significant variation in the pack size in the nearby protected areas within the landscape. The difference in pack size is a response to the tiger density (Bhandari et al., 2021) since asymmetric intraguild interactions are known to shape group sizes in social carnivores.

To understand the evolutionary pressures giving rise to asymmetric dispersal owing to the difference in sex and pack size and its genetic and ecological consequences, we need to delineate the pattern of dispersal of this group-living canid. This information on dispersal pattern will also provide a baseline for developing conservation strategies for one of the least studied carnivores (Fox, 1984). However, determining dispersal bias in the wild can be difficult for elusive species like dholes. The direct methods based on field observations using mark-recapture and telemetry can be impractical, considering the need for a large sample size for such experiments. Most importantly, the direct methods only tell us about the present migration but do not reveal the effective dispersal followed by passing on the genes. The recent developments in the field of population genetics have provided tools for inferring dispersal patterns via indirect estimates coupled with faster, more accessible, and wide sampling coverage with the use of bi-parental markers (Andrew et al., 2013; Goudet, 2002).

In this study, I hypothesised a dispersal bias in dholes due to their complex social structure, cooperative breeding, and male-biased sex ratio of packs. I predicted that the dispersal of this sub-ordinate predator would be further governed by top predator density and pack size as well as the habitat contiguity. To test my hypothesis, I used the microsatellite dataset of two different populations (NNTR & TATR) having different average pack sizes, which is an outcome of different top predator and prey densities between the two populations (Bhandari et al., 2021), to determine the dispersal pattern and its relationship with pack size. Pertaining to the present findings of strong genetic structure among the sub-populations, I presumed the nature of dispersal to be short and within the sub-population.

B.4.2. Methods

Study area

Sampling

The study was conducted from the scat samples collected from the two tiger reserves of Maharashtra, possessing similar population sizes and differences in average pack size (Bhandari et al., 2021), i.e. Tadoba-Andhari Tiger Reserve (smaller packs) and Nawegaon-Nagzira Tiger Reserve (larger packs). A total of 180 scats from TATR and 194 scats from NNTR were considered for this study.

Molecular sexing

The sex of the confirmed individual genotypes was determined using the multiplex sexing approach (Modi et al., 2018). I conducted three independent PCR repeats for the sex identification of all the individuals identified.

Data analyses

Genetic diversity

Observed (Ho) and expected (He) heterozygosity per locus H-W equilibrium and linkage disequilibrium were calculated using Arlequin 3.1 for both populations (Excoffier et al., 2005). I also calculated the genetic diversity in terms of allelic richness per locus, expected heterozygosity (Ho), and expected heterozygosity (He) of males and females separately for both populations.

Fine-scale genetic structure

To develop a broad understanding of the population structure at the reserve level I used the Bayesian clustering method implemented in STRUCTURE (Pritchard et al., 2000). The analysis was conducted separately on males and females to understand their assignment within the population. Ten independent runs for k=1 to 10 were performed using an admixture model with 100,000 burn-in steps and 1000000 Markov Chain Monte Carlo (MCMC) repetitions to ensure chain stabilisation. I also used the Locprior model to improve the genetic assignment in case of weak structure within the population. The optimum k (the number of clusters) was determined with the help of the highest estimated log-likelihood, the ad hoc ΔK (Evanno's method) using STRUCTURE HARVESTER v6.8. ((Evanno et al., 2005; Earl and vonHoldt, 2012)

Sex-biased dispersal

To understand the dispersal pattern of dholes I conducted and compared different genetic indices between males and females for both the reserves based on bi-parentally inherited markers (Goudet et al., 2002).

Fis value

I calculated the inbreeding coefficient (Fis) to determine how well the genotype frequencies within the population fit in the H-W equilibrium. The evaluation was done using the demerelate function for Fis calculation incorporated in the r package 'Demerelate' (Kraemer et al., 2017) with R version 1.1.453 (R Development Core Team 2018). Fis value for the dispersing sex should be higher than the philopatric sex. I used the most common and unbiased method, Weir and Cockerham's estimator (1984), to determine Fis value.

Relatedness

I estimated the relatedness within male and female groups of NNTR and TATR with all the twelve microsatellites using the Queller & Goodnight index (Queller et al., 1989) incorporated in Genalex 6.502 (Paekall et al., 2012). This index calculates the relatedness between any two individuals by comparing the alleles shared by these individuals with the allele frequency of the group and relatedness coefficients value R ranging from -1 to 1 (Queller et al., 1989) with jackknifing over the loci to estimate the standard error. I estimated the relatedness in three categories males(M-M), females (F-F), and female-male (F-M) for the two populations. In the presence of sex-biased dispersal, the dispersing sex is expected to have a lower mean relatedness than the individuals of more philopatric sex and also between opposite-sex pairs as compared to the members of more philopatric sex. There will be no significant difference in the mean relatedness value between the sexes if there is bisexual philopatry.

Spatial autocorrelation analyses

Genalex 6.502 was used to compare the spatial genetic structure between the sexes, referred to as spatial genetic autocorrelation (Paekall et al., 2012). It uses the matrices of pairwise genetic and geographic distance to determine the spatial autocorrelation coefficient r. I first calculated the pairwise codominant genetic distance and linear genetic distance for male and female individuals separately. The GPS coordinates of the samples were registered in the UTM (Universal Transversal Mercator) coordinate system, and the geographical distances were calculated using GenAlEx.

The autocorrelation coefficient r (-1 to 1) indicates whether two individuals are genetically similar ($r > 0$) or dissimilar ($r < 0$). When r lies outside a 95 percent upper and lower confidence interval calculated by permutation with 9,999 replicates, significant spatial structure (P 0.05) is observed. Samples were distributed in fourteen distance classes of 5 km intervals based on the maximum geographic distance between two samples in a particular area. I used the squared paired sample tests and omega tests to compare the heterogeneity between correlograms. Male and female correlograms should be distinct, and the philopatric sex should have significant positive genetic structure at shorter distances and considerable negative genetic structure at longer distances if there is sex-biased dispersal (Smouse et al., 1999). Spatial autocorrelation has proved to be a powerful and flexible tool to detect fine-scale genetic structure in animals, and this method does not make any assumption about the relationship between geographic and genetic distance.

Assignment index

Program FSTAT 2.9.4 was used to compare the assignment index of males and females in a population. This statistic was first introduced by Paetkau et al. (1995) and modified later by Favre et al. (1997) to differentiate the dispersers from residents in a population. I calculated the corrected assignment index, mAIC, and vAIC to determine the biases in the dispersal of dholes. The AIc has a zero-centered distribution, with a positive value indicating a genotype that is more likely than average to occur in the subpopulation in which the individual was sampled (presumably resident), and a negative value indicating a prospective immigrant (Goudet et al., 2002). In comparison to the more philopatric sex, the dispersing sex would have a lower mean of AIc (mAIc) and a higher variance of AIc (vAIc).

B.4.3. Results

Genotyping and sex determination

Out of the total samples collected, 84 were individually identified from the TATR, and 90 were identified from the NNTR population. The probability of identity for the NNTR and TATR population using the set of 12 markers was 5.98 $\times 10^{-8}$ & 4.52 $\times 10^{-8}$, while the probability of identity sibs was 8.52 $\times 10^{-4}$ and 6.16 $\times 10^{-4}$ respectively. Molecular sexing confirmed 54 males and 35 females in the NNTR population, while 44 males and 32 females in the TATR population (Figure 4.10). Rest of the individuals which did not confirm the sexing were discarded for further analysis. Males are proportionately more than females in both the population.

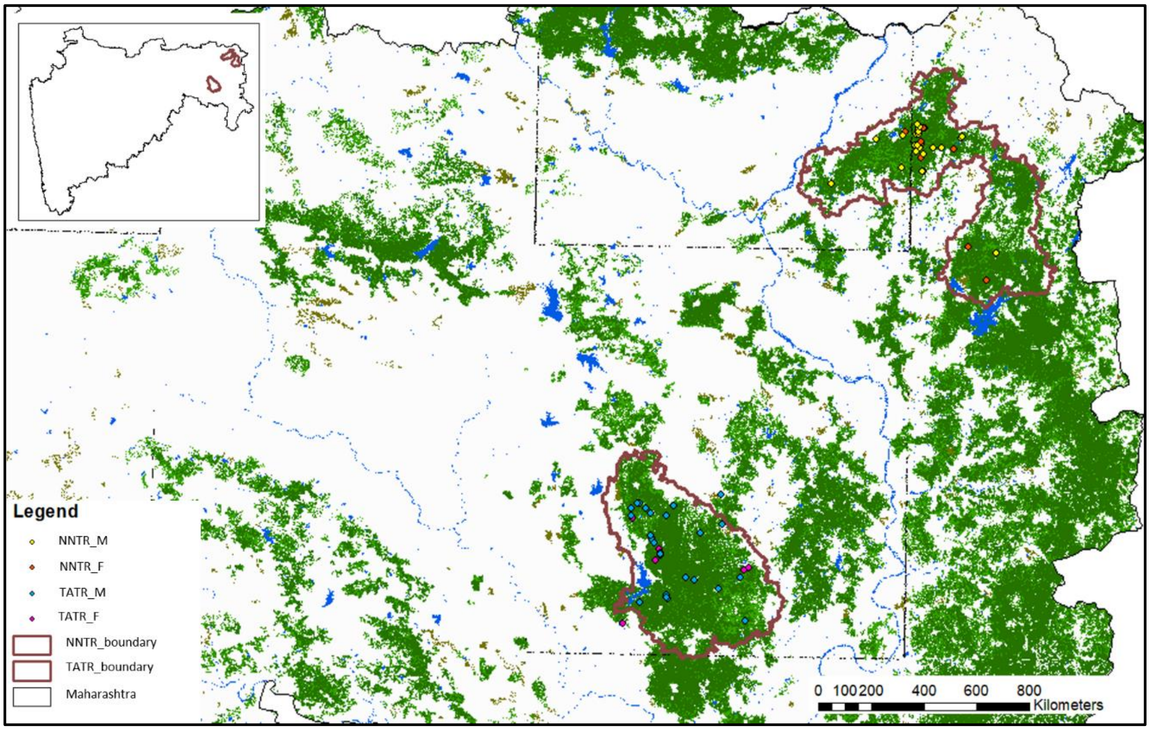


Figure 4.10: Locations of male and female dholes identified from NNTR and TATR populations.

Genetic diversity

The genetic diversity of NNTR was found to be 3 to 12 alleles per locus with expected heterozygosity He=0.51 and observed heterozygosity Ho=0.32 while the genetic diversity of TATR was 3 to 8 alleles per locus with expected and observed heterozygosity He=0.54 and Ho=0.42 respectively. The details of genetic diversity for males and females are provided in Table 4.11.

Parameters	***NNTR_F***	***NNTR_M***	***TATR_F***	***TATR_M***
Na	4.5	5.2	4.2	4.8
He	0.52	0.50	0.51	0.53
Ho	0.29	0.33	0.38	0.46

Table 4.11: Detailed estimates of genetic diversity (number of alleles per locus, expected (He), and observed (Ho) heterozygosity for males and females in dhole.

Fine-scale genetic structure

The k value obtained by running the STRUCTURE for NNTR and TATR individuals suggests k=3 and k=2, respectively, as per the largest log-likelihood and highest posterior probability. The plot for ranked average partial membership q for each cluster shows q>0.75 for a majority of individuals with only 10 and 12 first-generation migrants or admixed individuals for NNTR and TATR, respectively. Males and females did not show any distinct genetic clustering for both the protected areas.

When STRUCTURE was run for males and females separately, the log-likelihood values for TATR_M suggest k=4 while for TATR_F, it was found to be k=2. In the case of NNTR, both males and females showed the same value of log-likelihood (k=2) (Figure 4.11). This gives an idea that males are more structured in TATR than females, suggesting males are more philopatric sex than females in TATR. While in the case of NNTR, I did not find any difference in the structure pattern of males and females.

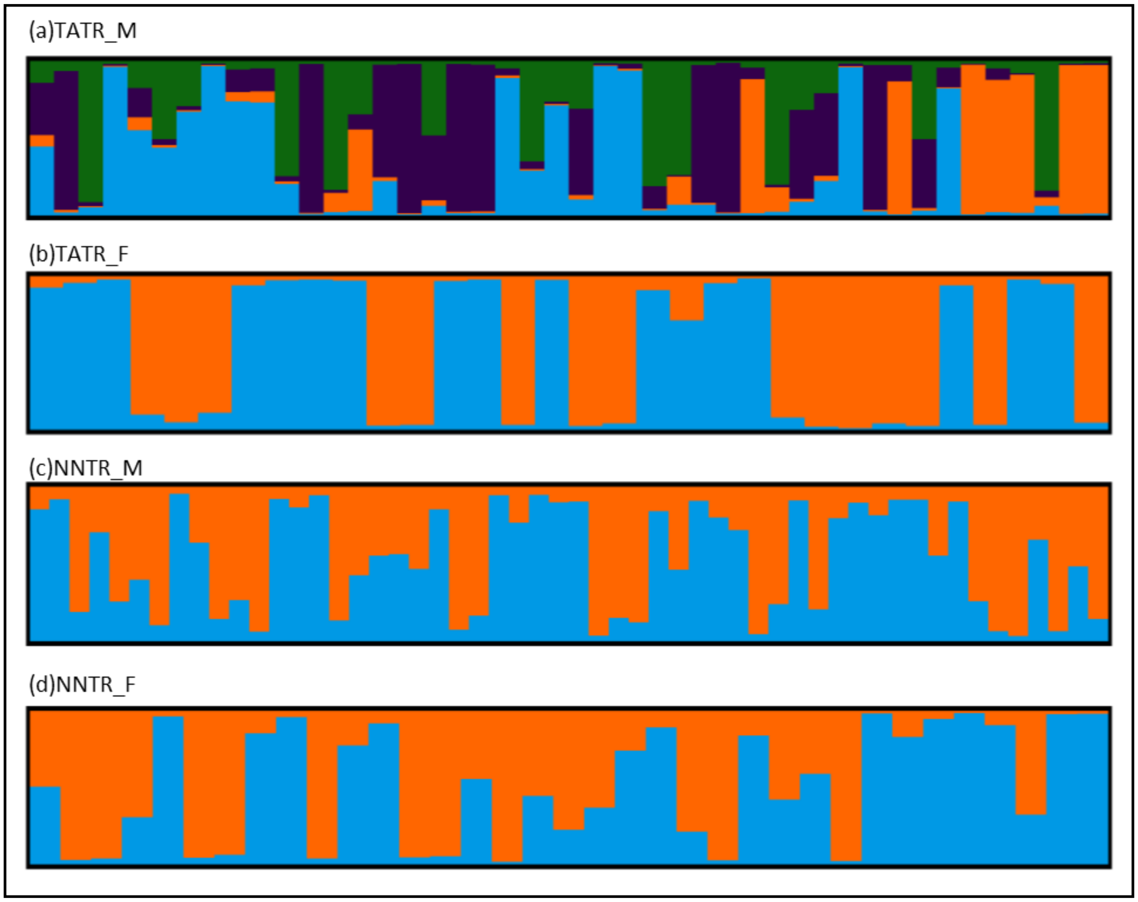


Figure 4.11: Bar-plot graph of the estimated membership coefficient of dhole male and female genotypes separately using STRUCTURE. The log-likelihood value confirms number of cluster (a) TATR_M (K=4), (b) TATR_F (K=2), (c) NNTR_M (K=2) and (d) NNTR_F (K=2)

Sex-biased dispersal

The Fis value for NNTR males is significantly lower than the NNTR females. Similarly, the Fis value for TATR males is lower than the TATR females, but there is no significant difference (Table 4.12). This suggests that the males are more philopatric sex than females due to the Wahlund effect resulting in higher homozygosity in males (Figure 4.12). Relatedness coefficient r as Queller Goodnight mean showed no difference in the relatedness value of NNTR males and females, while in the case of TATR, males showed a higher R-value compared to females but with an insignificant p-value (Table 4.13) (Figure 4.13). This can be indicative that males are more related than females suggesting that females have a higher likelihood of being immigrants to the population sampled from TATR. But I cannot conclude anything significantly.

Fis value (p-value)	
NNTR_F	0.438 (0.053)
NNTR_M	0.353 (0.042)
TATR_F	0.28 (0.072)
TATR_M	0.149 (0.067)

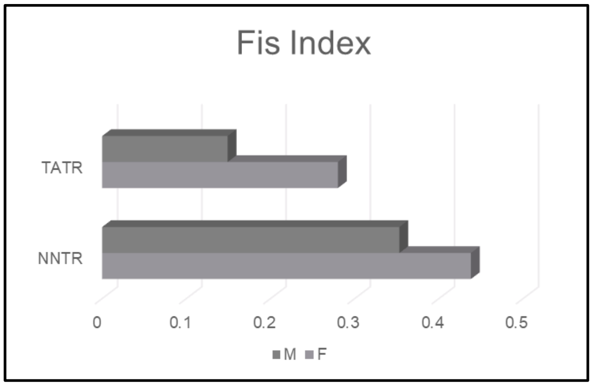


Table 4.12: Mean relatedness between same-sex and opposite-sex pairs of individual dhole genotypes of NNTR and TATR using Queller-Goodnight index.

Figure 4.12: Bar graph showing the difference in Fis value of males and females.

NNTR males for neighbouring pairwise comparisons were found to be positively autocorrelated at the distance of 0-10 km, P=0.001, while they were found to have negative spatial autocorrelation at a distance of 10-15 km & 35-40 km, P=0.001 & 0.014, respectively. While for NNTR females, significant positive autocorrelation was found only at one distance class at 40-45 km, P=0.009.

The rest of the distance classes did not show any significant results for NNTR males and females (Figure 4.14 a & b).

Protected area	***F-F (SE)***	***M-M (SE)***	***F-M (SE)***
NNTR (QGM)	-0.025 (0.014)	-0.020 (0.010)	-0.004 (0.006)
TATR (QGM)	-0.050 (0.018)	-0.020 (0.006)	-0.024 (0.007)

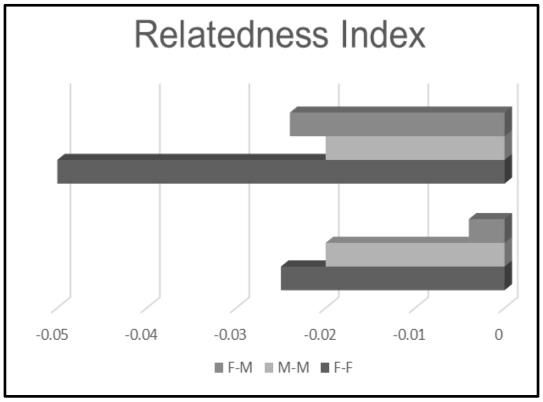


Table 4.13: Mean relatedness between same-sex and opposite-sex pairs of individual dhole genotypes of NNTR and TATR using Queller-Goodnight index.

Figure 4.13: Bar graph of relatedness index between male and female individuals of dholes determined using Queller-Goodnight index.

For TATR males, the spatial correlogram was found to be significant with a positive autocorrelation at 0-10 & 20-25 km, P=0.001 & 0.003 and negative autocorrelation at 10-15; 15-20 & 30-35 km, P= 0.33; 0.006 & 0.005 respectively. While for TATR females, I found no significant auto-correlation across the distance classes. The spatial autocorrelation results show that males are generally more similar than females at shorter distances suggesting that males are more philopatric than females (Figure 4.14(c), 4.14(d)).

The assignment test conducted for NNTR showed a lower mean AIc and higher variance for females compared to males, while in the case of TATR the females showed a higher mAIC and lower variance than males, but both the comparisons showed a non-significant p-value. The t-test conducted to compare the mean and variance of males and females was also insignificant (Table 4.14). Therefore, the assignment test could not be used to make any further conclusion regarding the dispersal bias in dholes.

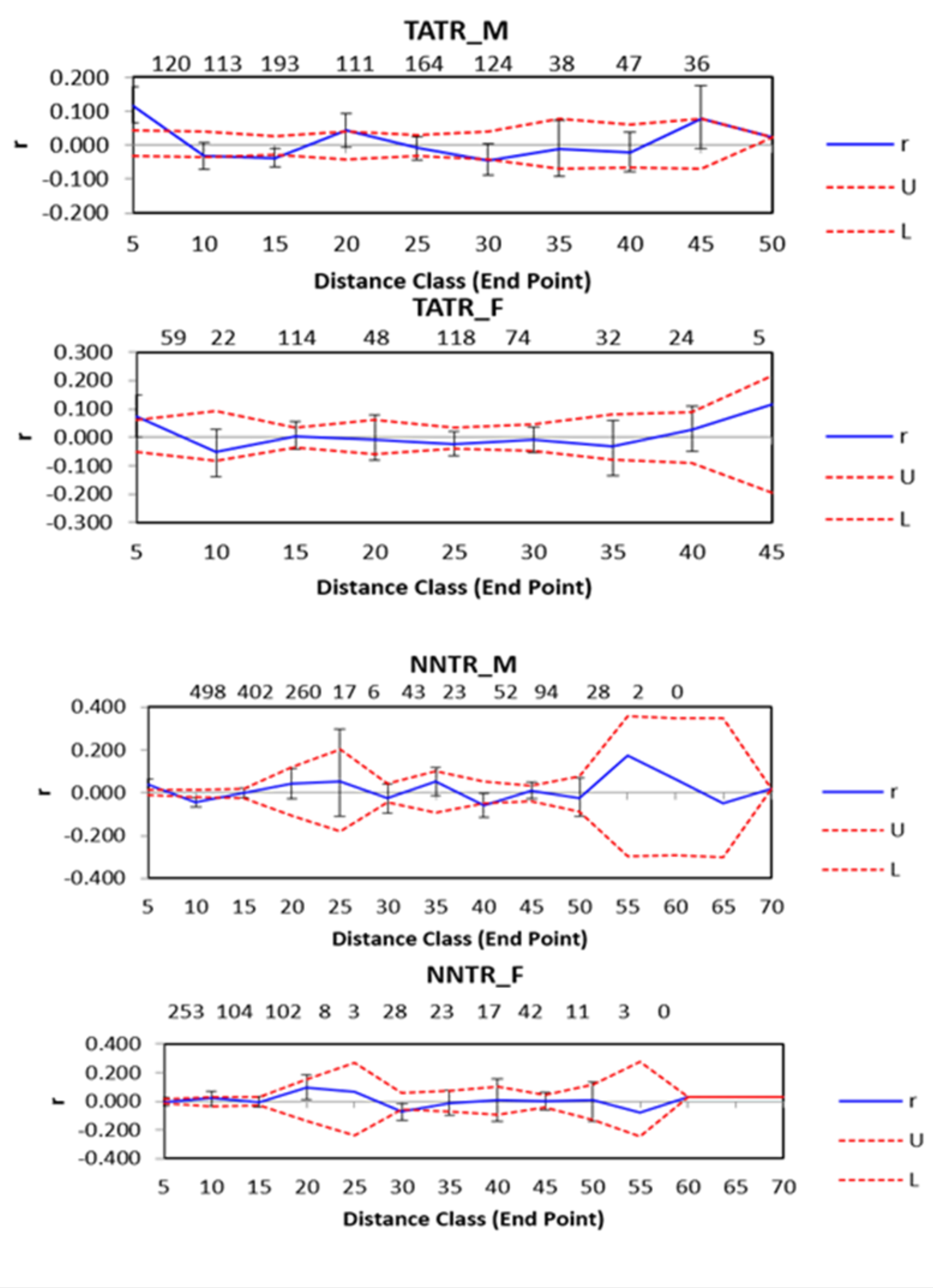


Figure 4.14: Spatial genetic structure correlograms for the two dhole populations NNTR and TATR. The sample size at each distance class is provided above each correlogram. Spatial genetic structure correlograms for (a) TATR males; (b) TATR females; (c) NNTR males; (d) NNTR females

t-test	***TATR_M vs. TATR_F***	***NNTR_M vs. NNTR_F***
mAIC	0.814	-0.970
p-value	0.42	0.33
vAIC	0.717	1.240
p-value	0.80	0.378

Table 4.14: Test for the differences between sexes in the mean (mAIC) and variance (vAIc) of the corrected Assignment Index with the significant values ($P<0.05$).

B.4.4. Discussion

The selective pressures which determine the decision of a particular sex in a species to disperse always remained controversial. In a multi-predator system, the density of a larger co-predator proves to be a detrimental factor in controlling the density of subordinate predators which can further reflect in the dispersal pattern of the latter. I evaluated the dispersal pattern of dholes from two genetically differentiated populations of NNTR and TATR at different tiger densities to understand the role of varying dhole pack size in the dispersal pattern since the average dhole pack size in TATR is comparatively lower than NNTR (Bhandari et al., 2021). Results showed that males are more structured in TATR than females, indicating the female dispersal (da Silva et al., 2018), while in NNTR, both the males and females showed a similar level of differentiation with the same number of clusters (k=2). Other corresponding analyses for sex-biased dispersal also revealed a significant difference between males and females of TATR, while for NNTR, the differences were not very profound.

The different tests conducted to determine the dispersal pattern of dholes indicate two possibilities, either it is female-biased or there is no bias in dispersal. The relatedness index and the mean & variance of the assignment test did not show any significant difference between the dispersal patterns. However, the other two sex-biased dispersal tests for dholes showed a significant difference in Fis and spatial autocorrelation, indicating the biased nature of dispersal. I found a lower Fis value and a positive spatial autocorrelation at a shorter distance for males of both populations. Both results indicate that males are more philopatric than females. I did not use the Fst index as indicated by Goudet et al. because dholes are considered to have a short dispersal distance with a home range of 58.67 sq. km (± 4.08) (Habib et al., 2021b). Contrary to their African counterparts, I did not find any significant inter-dispersal events between these two populations. This study, therefore, focuses primarily on the dispersal bias within the population at the inter-pack level.

Dhole is among the least monogamous canid and exhibits cooperative breeding, often with multiple breeding females in a pack (Macdonald et al., 2019; Fox, 1984). The pack also possesses an alpha breeding pair and dominance hierarchy (Johnsingh, 1985). Therefore, one primary reason for highly structured male-biased packs in TATR can be the intra-specific competition due to which females disperse more. At the same time, males remain in the packs as there are higher chances of females getting accepted in a new pack. Another major reason for such a pattern can be the higher tiger density in TATR, giving rise to a high turnover rate.

Contrary to this in NNTR, there is no interspecific competition pressure due to lower tiger density and the larger pack size provide similar opportunities to both male and female. The difference in the turnover rate of any species can influence the genetic structure of the population (Serfass et al., 1998) such as higher competition in TATR leads to a larger turnover of male members of the pack giving rise to a higher structured male population (Williams et al., 2003). The tiger population modulates the pack size of dholes in both the tiger reserves with a larger pack size in NNTR due to a very low number of tigers and a smaller pack size in TATR for better avoidance strategy (Groom et al., 2016; Bhandari et al., 2021) in high-density tiger area. This could be because tiger presence is primarily connected with prey-rich areas, whereas dhole presence was a trade-off between prey availability and tigers' active spatial avoidance (Steinmetz et al., 2013).

TATR has sufficiently good connectivity with the adjacent Brahmapuri forest division, which can act as a dispersal escape, while in the case of NNTR, the connectivity to the nearest protected area is very patchy. A tentative reason behind the high genetic structure in TATR males can also be that due to better geographical connectivity, the males are dispersing to farther distances, while the females are dispersing to shorter distances within the park. Such dispersal pattern has been observed in African wild dogs where males disperse less frequently but traverse longer distances while females travel shorter distances but disperse more frequently (McNutt, 1996), and a similar pattern was also observed in a previous study on dholes (Iyengar et al., 2005). However, I did not find any significant results supporting this hypothesis.

Although, the male-biased sex ratio in both populations indicates the possibility of female-biased dispersal, which is further supported by previous ecological studies (Johnsingh, 1982; Venkataraman, 1998). The studies on the species having hierarchical cooperative breeding also indicate that pack bias towards the more helping sex according to the local resource enhancement model (LRE) (McNutt et al., 2008). The present genetic study revealed a strong bias in dispersal pattern in the case of TATR, but due to larger pack size and less competition in NNTR, dispersal bias is not evident. However, the sex bias dispersal test, despite being non-significant, points toward the female-biased nature of dispersal in the case of NNTR also.

Combining the knowledge of ecology and the findings obtained during this study, I found that dispersal is a complex phenomenon governed by the local dynamics, competition, and landscape connectivity of a population. Though African wild dogs are the social canid with a rigid structure of dominant hierarchies and share a sister lineage with dholes often found to have female-biased dispersal, it cannot be generalised in the case of dholes due to various ecological, environmental, and geographical local factors. I have used the bi-parental markers to delineate the dispersal pattern by comparing the genetic diversity of males and females after dispersal and before reproduction. This approach is consistent with the sampling strategy for just one generation and also provides the advantage of using the genetic diversity of both sexes (Prugnolle, 2002).

Most solitary carnivores are reported to have a male-biased dispersal pattern (Gour et al., 2013; Biek et al., 2006; Janecka et al., 2007) and are generally characterised by uniparental care. As found through the analysis, the biased dispersal can be because of the biparental care and pack living nature of socially structured dholes (Stockley et al., 2016). The present study has paved the way to develop a fundamental understanding of future research on dholes' dispersal. This is the first and foremost effort to define the dispersal of a lesser-studied social pack-living endangered canid that thrives in Indian forests incorporating the pack size and competition. This has helped to develop a notion of the possibility that while most dispersal decisions are male-biased, mainly governed by the search for mate and territory, for pack-living social species such as dhole, it can be either female-biased or unbiased at all. Future investigations in this field are needed to delineate a detailed dispersal pattern in dholes with broader coverage at the landscape level and a multi-disciplinary approach.

Understanding the dispersal pattern of this endangered monophyletic species is critical in developing a better framework for the long-term persistence of populations in this anthropogenic era. This study showed that dispersal is not based on a set of rules; it is further an outcome of several ecological, geographical variables and an individual's life history decisions. The results validate the Asiatic wild dogs are one of the genera where dispersal decision is further determined by pack size, top-predator density, prey density, and geographical connectivity. This will further help in developing focused conservation strategies for short-ranging species with lesser-known ecology. I support that a comprehensive knowledge of dispersal patterns is required to develop any conservation management plan.

CHAPTER 5

Impact of habitat fragmentation on the gene flow and genetic connectivity across the landscape connectivity gradient of the tiger reserves

Chapter 5

Impact of habitat fragmentation on the gene flow and genetic connectivity across the landscape connectivity gradient of the tiger reserves

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5.1 Introduction

The increasing pace of human footprints on earth is alarming for large carnivores because of their large ranges, relatively low numbers, sensitivity to fragmentation, and human persecution (Weber & Rabinowitz, 1996; Ripple et al., 2014). This charismatic guild has lost 62% of its historical range globally, with the minimum range of 5% left in remaining Asia (excluding southeast Asia) (Wolf & Ripple, 2017). Anthropogenic disturbances have increased the isolated patches for the surviving population in the wild (Fahrig, 2003; Ezcurra, 2016). Top predators are pivotal to the integrity of the entire system, and their removal or decline can lead to trophic cascades (Ripple et al., 2014). Therefore, mammalian carnivores can be a valuable tool to determine the extent of disturbance and conservation planning. Habitat fragmentation reduces the contiguous habitat into pieces that may be too small to maintain a viable population of large carnivores considering the extensive home range (Crooks & Soule, 1999; Tigas et al., 2002), leading to decreased gene flow (Schlaepfer et al., 2018), genetic variation (Lu et al., 2001) and increased probability of extinction (Fahrig, 2002). It can reduce the functional connectivity or the movement of species among the populations (Tischendorf & Fahrig, 2000). Maintaining connectivity among the remnant populations is the key to preventing the detrimental effects of fragmentation (Ritchie et al., 2019) by identifying the movement corridors in the present development pressure (Fattebert et al., 2015b).

Landscape connectivity is considered species-specific derived as a response to landscape structure and species behaviour (Crooks et al., 2011; Marrotte et al., 2017). However, large carnivores have been considered umbrella species for conservation and planning due to their wide movement range (Beier et al., 2008). Connectivity planning across the guild, taking one species' requirements into account, does not benefit each concerned species. Multi-species connectivity can be a solution to this problem, accounting for the needs of different species with contrasting life history, body size, home range, interspecific competition, and movement ecologies (Nicholson et al., 2006; Albert et al., 2017; Creel et al., 2019; Habib et al., 2021b). Persistent gene flow across populations is critical for the genetic connectedness of landscapes. The concept of multi-species genetic connectivity is recent and has been explored in a few terrestrial systems (Mech & Hallett, 2001; Wultsch et al., 2016; Thatte et al., 2020; Creel et al., 2019).

India is a bio-diverse nation harbouring 23% of the world's large terrestrial carnivores and the second-highest human population in the world (Saini & Budhwar, 2004). The predators are interspersed in the patches of protected areas separated by vast stretches of agriculture, industries, roads, railways, and human habitation. In such a scenario, maintaining the functional connectivity of large carnivores through corridors is a challenge. Understanding the landscape level connectivity at the gene flow level among protected sites and across the landscape matrix is essential. Previous works on the genetic landscape connectivity in India mainly focused on single species (Reddy et al., 2017; Yumnam et al., 2014; Joshi et al., 2013). The only multi-species genetic connectivity study done in India focuses on diverse taxa with different habitat requirements and food preferences (Thatte et al., 2020) which do not deal with the factors such as prey requirement, niche separation and interspecific competition of the carnivore guild (Creel et al., 2019).

This study focused on understanding the comparative genetic connectivity of three sympatric carnivores tiger (*Panthera tigris*), leopard (*Panthera leo*), and dhole (*Cuon alpinus*). The two felids, tiger and leopard, are solitary, while dhole is a pack living canid. The species under study are sympatric and show interspecific competition but have different body sizes, dispersal ability and sensitivity to fragmentation (Hand et al., 2014). Interspecific competition and dispersal ability are major factors to be considered while determining genetic connectivity.

I hypothesised that dhole being habitat specialists are most affected by habitat fragmentation, and the leopard will be least affected. This study aims to understand the factors defining the movement of species with different sensitivity to habitat fragmentation, dispersal ability, and interspecific competition across the human-modified matrix. I tried to determine the movement patterns using resistance surface maps considering the ecology and habitat specificity of species. I also investigated the pinch points which can be a part of prospective corridors for multi-species movement. My primary objective is to determine whether the multi-species connectivity map can predict the genetic connectivity of all three species despite the differences in their ecology, home range and movement behaviour.

5.2 Methods

I used the population genetic dataset of the three species for the study explained in chapter 4 across the Eastern Vidarbha Landscape. The dataset included microsatellite profiles of tiger (198 genotypes), leopard (47 genotypes), and dhole (251 genotypes) at 12 loci from the protected area of Eastern Vidarbha Landscape (i.e. TATR, MTR, PTR, NNTR, UKWLS). I did not include the samples from the Sahyadri Tiger reserve since the study from the previous chapter proves that the leopard and dhole populations of STR are entirely different, and it also does not share any connectivity with the Eastern Vidarbha complex.

Landscape resistance surfaces

I developed a landscape resistance surface to estimate the influence of landscape features among the genetically defined populations of each species. I used nine variables to construct the resistance surface. 1) LULC layer for Maharashtra, a categorical variable with eight classes including built-up, agriculture, fallow land, forest, plantation, wasteland, and waterbodies. 2) Distance from roads and railways, I combined the linear structures of roads and railways and derived the distance from roads and railways using Euclidean distance in ArcMap. Road data downloaded as vector layer from Open Street Map. 3) NDVI data were obtained from the USGS database, and the average was done for the three months (December to February) of sampling from the year 2016 to 2019. 4) Livestock data was derived from the Food and Agriculture Organisation of United Nations (FAO) database, and layers for cattle, sheep, goat, and buffalo were summed for the final livestock layer. 5) Climatic variables of annual mean temperature, mean precipitation, and evapotranspiration were downloaded as bioclimatic variables from the WorldClim database (Fick & Hijmans, 2017). 6) Population density layer was derived from WorldPop Project (Stevens et al., 2015). All these variables were selected based on the available knowledge from telemetry, and habitat mapping studies were done on the species and its association with the habitat (Habib et al., 2021b; Schaller, 2009; Areendran et al., 2012, Thinley et al., 2021; Aryal et al., 2015). The variables were further delineated into two groups: anthropogenic variables (land use land cover, NDVI, population density, distance to roads, and livestock density) and environmental variables (temperature, precipitation, evapotranspiration, elevation). Spatial scale can also have varied responses to species depending on their ecology; therefore, I analysed each variable at different resolutions of 1 km, 2 km, 5 km, and 10 km. I used a buffer of 20 km to prevent the overestimation of resistance due to map boundaries (Koen et al., 2010).

Landscape connectivity modelling

The raster data was converted into ASCII format using export to Circuitscape tool in Arc GIS and provided the same projection to each variable. To assess the relationship between genetic distance and landscape resistance distance and select Rmax for each landscape variable model, I used the optimisation methods implemented in ResistanceGA (Peterman, 2018) in Rstudio ver

1.2.5. ResistanceGA determines the linear mixed-effects model fit with genetic distance measured as ar (Rousset, 2000), calculated using Genepop (Rousset, 2008), as the dependent variable and the scaled and centred resistance distance as the predictor variable. I used GenAlEx to conduct a mantel test to determine whether there is any correlation between pairwise codominant genotypic and geographical distance. Further, I conducted the spatial autocorrelation function for each species to examine the spatial extent of genetic structure. Mixed models were fitted using the maximum-likelihood population effects (MPLE) parameterisation (Clarke et al., 2002) implemented in the R package LME4 (Bates et al., 2011) for accounting for the non-independence among the pairwise genetic and ecological distances. I measured the cost distance using CIRCUITSCAPE v. 5.0.0 written with the JULIA programming language (Hall et al., 2021) (v. 0.6.4, accessed July 2021 at https://julialang.org/).

For all the continuous variables except LULC, I tested nine possible transformations to assess the best fit relationship between the two variables. The pairwise geographic distance was used as a null variable, and I conducted the optimisation three times independently for robust results. Model fits were determined by AIC values calculated from linear mixed effect models. The covariate structure in maximum-likelihood population effects model (MLPE) was explicitly designed to account for the non-independence of values within pairwise distance matrices (Clarke et al., 2002; Van Strien et al., 2012) and the random effect in these models indicates the dependencies between each of the pairwise distances. I resampled 75% of the samples without replacement; each surface was fit to the subset of samples using its optimised resistance surface to control for potential bias in the analysis. The procedure was repeated 10,000 times to determine the AICc-best model (π) and each model's marginal R^2 value. Multi-surface optimisation evaluated the importance of each landscape feature by calculating the percent contribution of each surface by dividing each transformed resistance surface by the sum of composite multi-surface resistance surfaces.

Connectivity maps

I visualised the current flow and corridors across the focal nodes with the optimised resistance surface using two programs for each focal species. Circuitscape provided the current density map while linkage mapper 1.0.2 ArcGIS tool determines the least-cost corridor and identifies the routes that facilitate or impede species movement among the focal areas (McRae et al., 2012). Both analyses used a habitat raster map that specified the conductivity or resistance of each cell (raster pixel) in the landscape (optimised resistance surface) and a map of focal regions (Strictly Protected Areas or Mosaics of Protected Areas) to identify the connections between the focal regions (Mcrae and Shah, 2011; McRae et al., 2012). I further ran Centrality Mapper (McRae, 2012(a)) and Pinchpoint Mapper (McRae, 2012(b)), which use circuit theory to identify the importance of individual nodes in keeping the overall connectivity intact.

For the multi-species connectivity map, I overlaid resistance maps for best-supported models to identify locations important to multiple species. I used the function Cell statistics – Mean in ArcGIS v.10.8 for the parameters ranked as first, second, and third by the AIC to build a cumulative map for the three carnivores. Through this map, I attempted to identify areas with high priority for connectivity conservation by considering multi-species movements and the pressures and threats in the landscape.

5.3 Results

Spatial autocorrelation

The mantel test results for leopard and dhole showed significant correlation (leopard r = 0.227, p= 0.10 & dhole r = 0.137, p = 0.010) while for tiger it showed non-significant autocorrelation (r = -0.036, p = 0.120) between genetic and geographical distance. The spatial autocorrelation test conducted to determine the spatial extent of differentiation showed non-significant autocorrelation at all distances for tiger, significant positive autocorrelation at first 100 km in leopard and significant negative autocorrelation at different interval of distance classes.

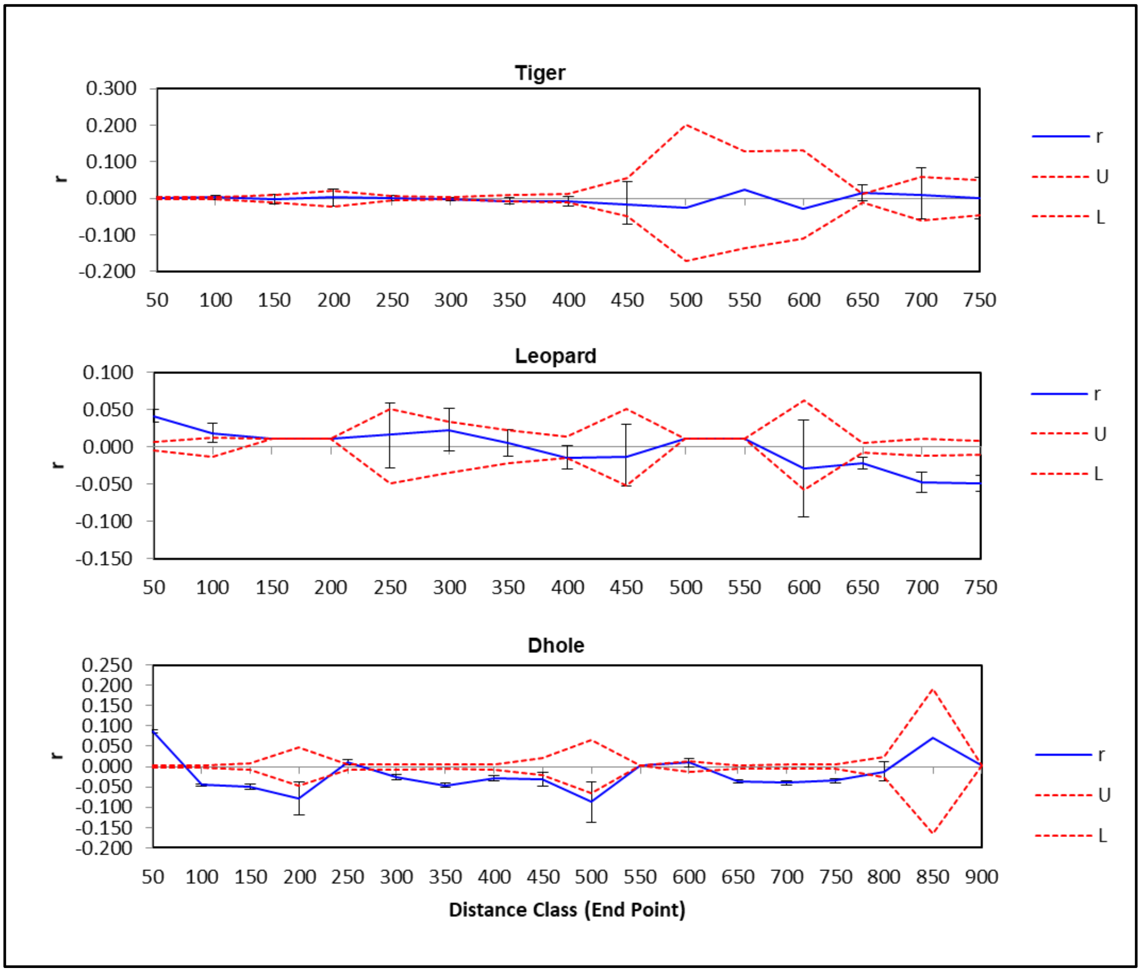


Figure 5.1: Spatial genetic structure correlograms for the three carnivores. X-axis represents distance class in km and Y-axis is the coefficient between genetic and geographic distance with 95% confidence interval.

Univariate Optimisation

For optimisation of the resistance surface, I removed the samples with nearby or overlapping locations to avoid the Circuitscape error due to the presence of more than one sample in a pixel. Out of the total confirmed genotypes of the individual species, I have used 85 tiger genotypes, 89 dhole genotypes, and 46 leopard genotypes. Single surface optimisation aims to identify the best parameter value that describes the relationship between individual landscape variables and geneflow. I considered models based on both AICc and marginal R^2 criteria. The univariate optimisation and model selection with AICc results show evapotranspiration and distance as the best-supported model for tigers; ndvi and distance as the best-supported model for leopards while evapotranspiration and population as the best fit model for dholes among the different variables used. The evapotranspiration optimised response curve with inverse reverse monomolecular and monomolecular shape shows an increase in resistance value with increasing evapotranspiration for both tiger and dhole, respectively. The optimised surface for ndvi, the second-best model with inverse monomolecular shape, shows a decrease in resistance with increasing vegetation cover. The function selection for the best-supported model of all the three species has been provided in Table 5.1, and the single surface optimisation curves for the best-supported model are given in Figure 5.2.

Surface	***K***	***Equation***	***AIC***	***Average weight***	***Average rank***
			Tiger		
Distance	2		-1234.61	0.318	1.42
Evapotranspiration	4	Inverse-Reverse Monomolecular	-1234.02	0.096	3.82
Temperature	4	Inverse-Reverse Monomolecular	-1233.54	0.086	6.06
NDVI	4	Inverse-Reverse Monomolecular	-1233.54	0.103	4.18
Road	4	Inverse Monomolecular	-1233.44	0.083	4.70
Elevation	4	Inverse Monomolecular	-1233.18	0.072	5.78
			Leopard		
Distance	2		-571.332	0.487	1.15
NDVI	4	Inverse Monomolecular	-570.623	0.142	2.30
Precipitation	4	Inverse Monomolecular	-568.826	0.073	4.21
Livestock	4	Inverse Monomolecular	-568.556	0.058	4.42
Road	4	Inverse Monomolecular	-567.791	0.043	6.06
Evapotranspiration	4	Inverse Monomolecular	-567.67	0.058	5.51
			Dhole		
Evapotranspiration	4	Monomolecular	-1270.84	0.324	2.19
Population	4	Monomolecular	-1268.43	0.243	5.43
Livestock	4	Inverse-Reverse Monomolecular	-1265.58	0.098	8.58
Elevation	4	Inverse Monomolecular	-1262.67	0.028	9.48
Road	4	Inverse Monomolecular	-1260.5	0.024	12.0
NDVI	4	Inverse-Reverse Monomolecular	-1259.64	0.052	12.8

Table 5.1: Model selection results for the generalised linear mixed-effects models optimised on genetic distance.

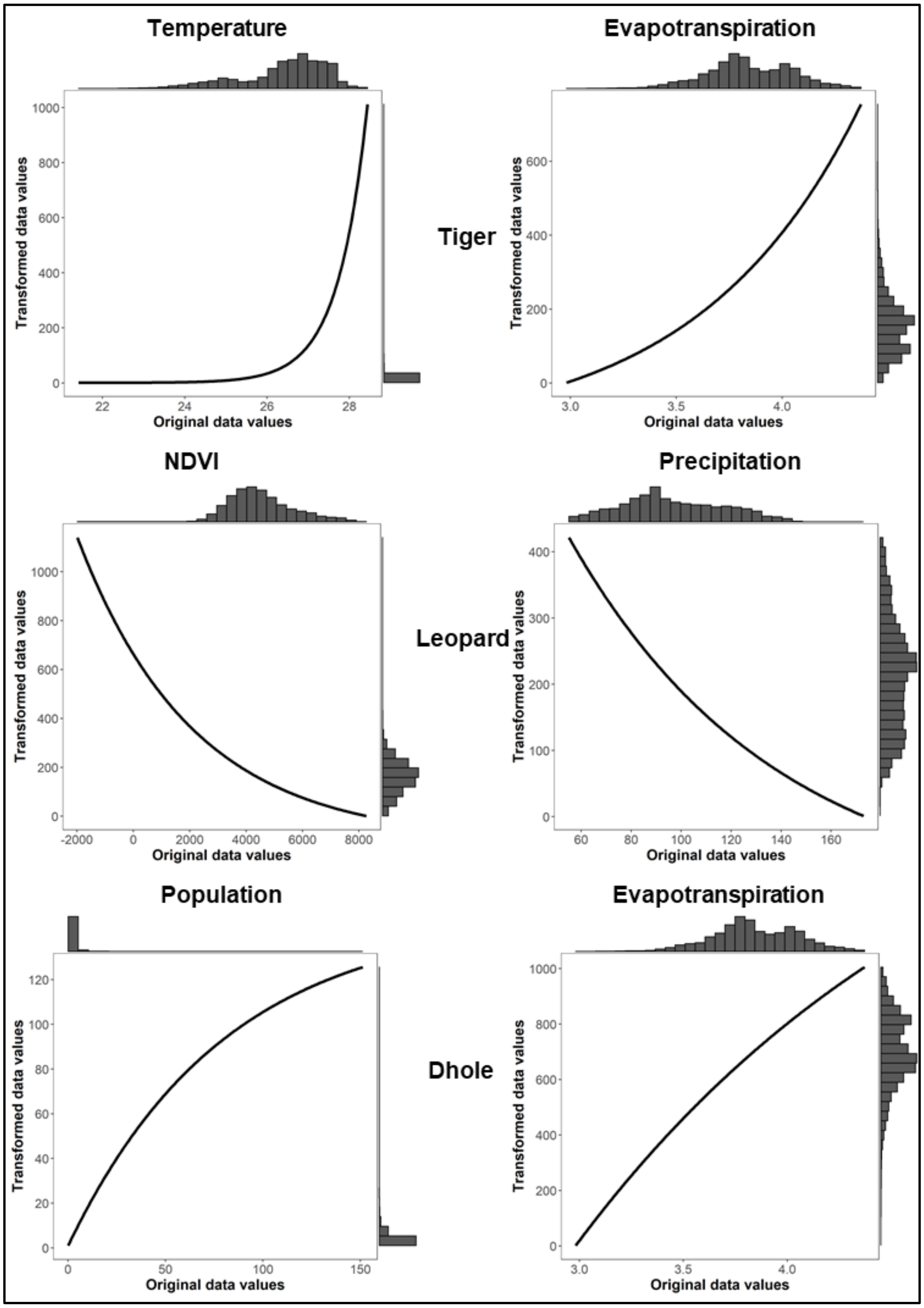


Figure 5.2: Single surface optimisation response curves of the best two models for tiger, leopard and dhole.

Multivariate Optimisation

Composite surfaces were constructed from the top four best models for optimisation of three species. Results of the boot-strap analysis with both single surface and composite surface show the distance as the best model (79%), followed by temp (7.9%), followed by evapotranspiration (4.7%) and ndvi (3.9%) for tiger. For leopard boot-strap analysis shows the distance as the best-supported model with a maximum per cent of 86.9%, followed by ndvi (12.7%). In contrast, the result for dholes shows evapotranspiration, population, livestock and population + evapotranspiration as best supported models with per cent contribution of 45%, 31.9%, 9% and 8.5%, respectively. The single surfaces outperform the multi-surface models in terms of AICc and top rank models. Out of the three species, only dhole shows a difference in model fit for multivariate and univariate optimisation. The multivariate optimisation result for the top five models for dhole is shown in table 5.2.

Dhole					
Surface	k	Per cent Contribution	AIC	Average weight	Average rank
Evapotranspiration	4		-671.608	0.324	2.19
Population	4		-669.528	0.243	5.43
Population + Evapotranspiration	7	0.07%+99.2%	-668.061	0.063	7.74
Livestock	4		-667.409	0.098	8.58
Livestock + Evapotranspiration	7	36%+63%	-666.641	0.011	9.00

Table 5.2: Model selection results for both individual and composite surfaces for dhole.

The Circuitscape heat map was generated from a combination of the top five best models fit optimised surface for all the three species showed their movement current map over the landscape. For tiger, the pattern was found to be either extreme high current or very low current. However, the high movement pattern was found near the TATR, which has better connectivity due to the presence of smaller patches of protected areas nearby. A mesh pattern of movement was detected for leopard, ranging from high to current over the entire landscape. The current map for dhole showed a high current around the TATR complex and a low current around the MTR -PTR complex (Figure 5.3). The pinch point maps with the same width of corridors showed very few pinch points in single available paths for both tiger and dhole. While for leopards, there were comparatively more pinch points with alternate ways available as expected from the species ecology.

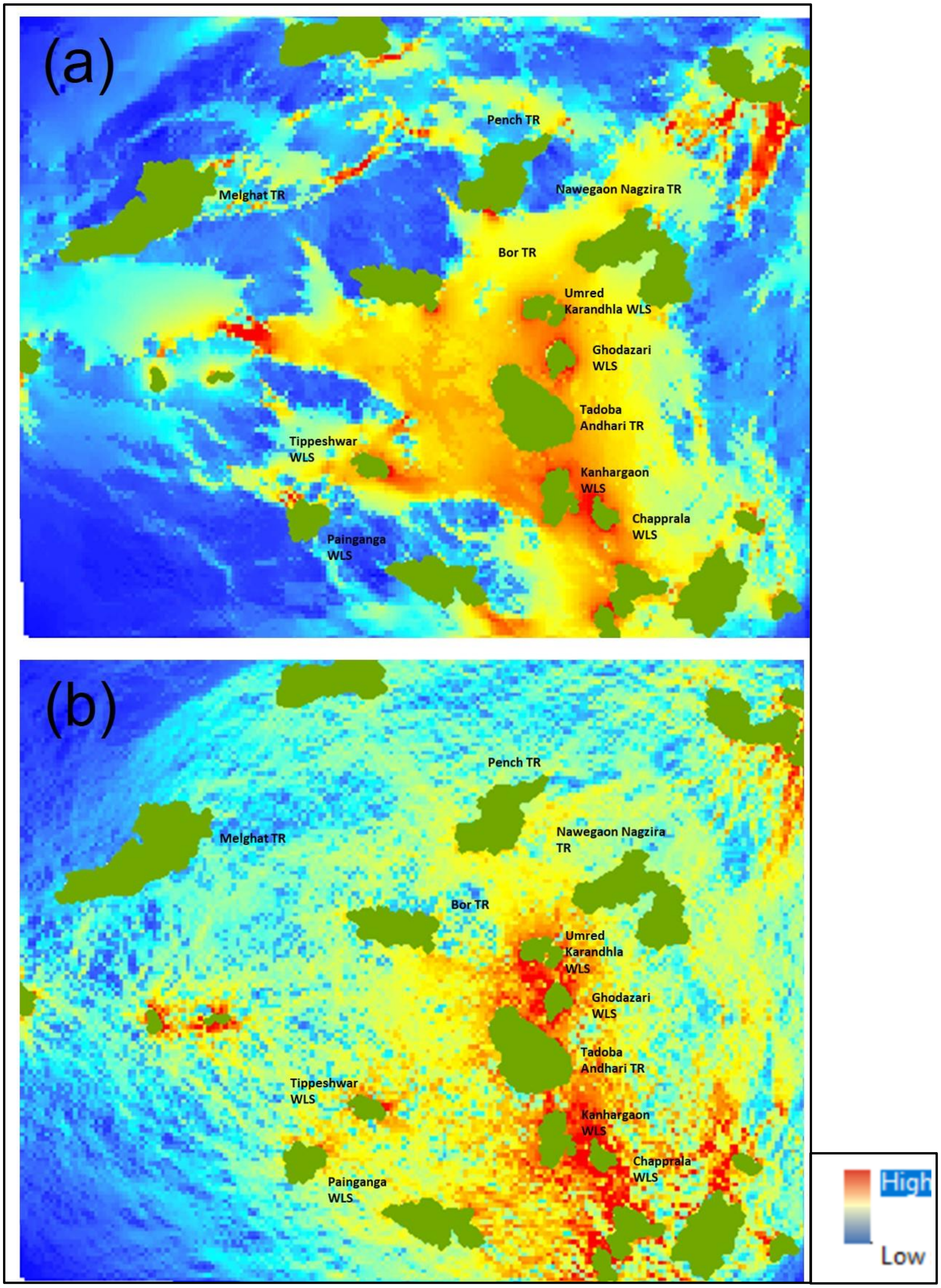


Figure 5.3 Current map of the carnivores based on the optimised resistance surfaces from ResistanceGA for (a) Tiger, and (b) Leopard.

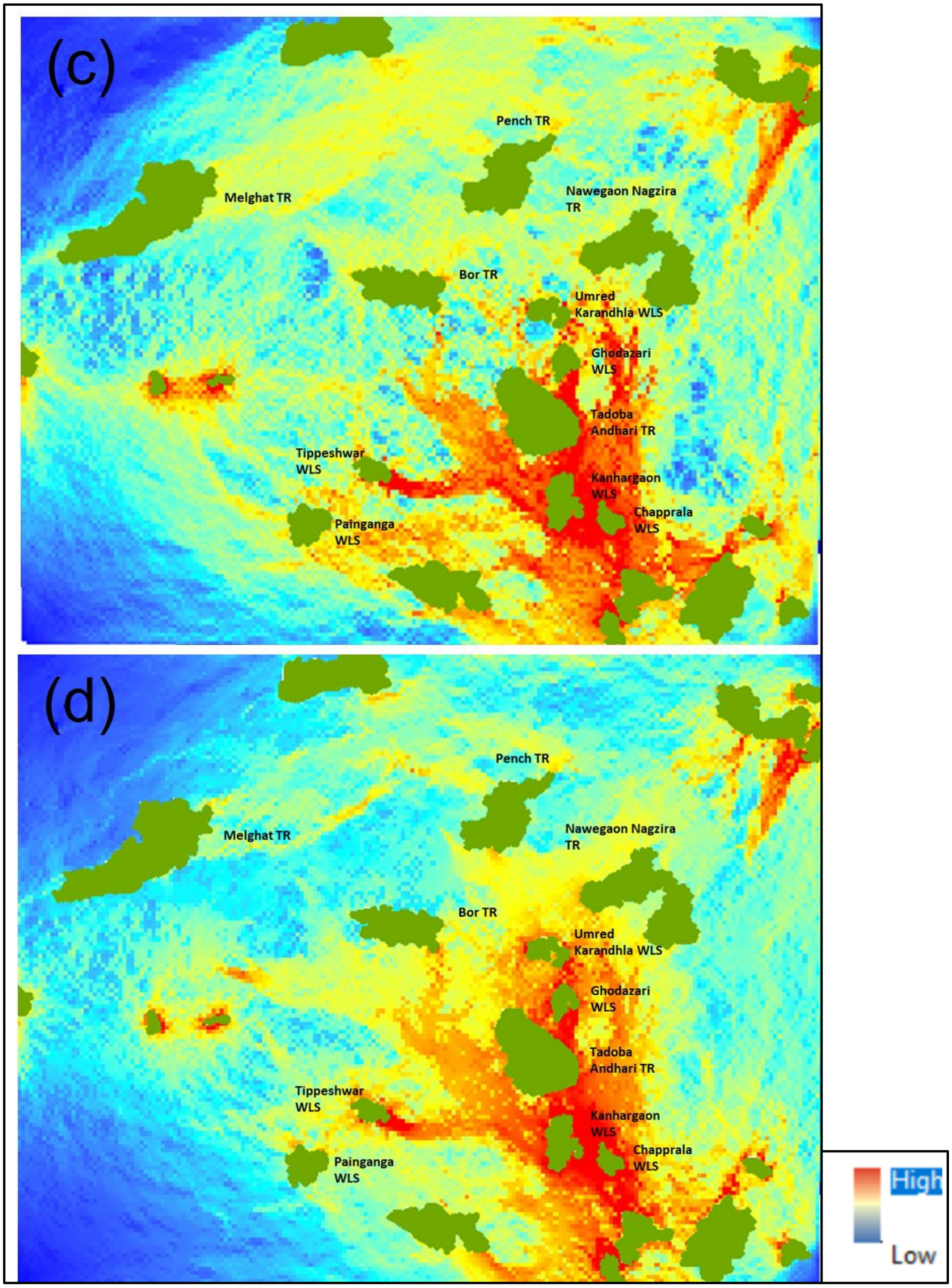
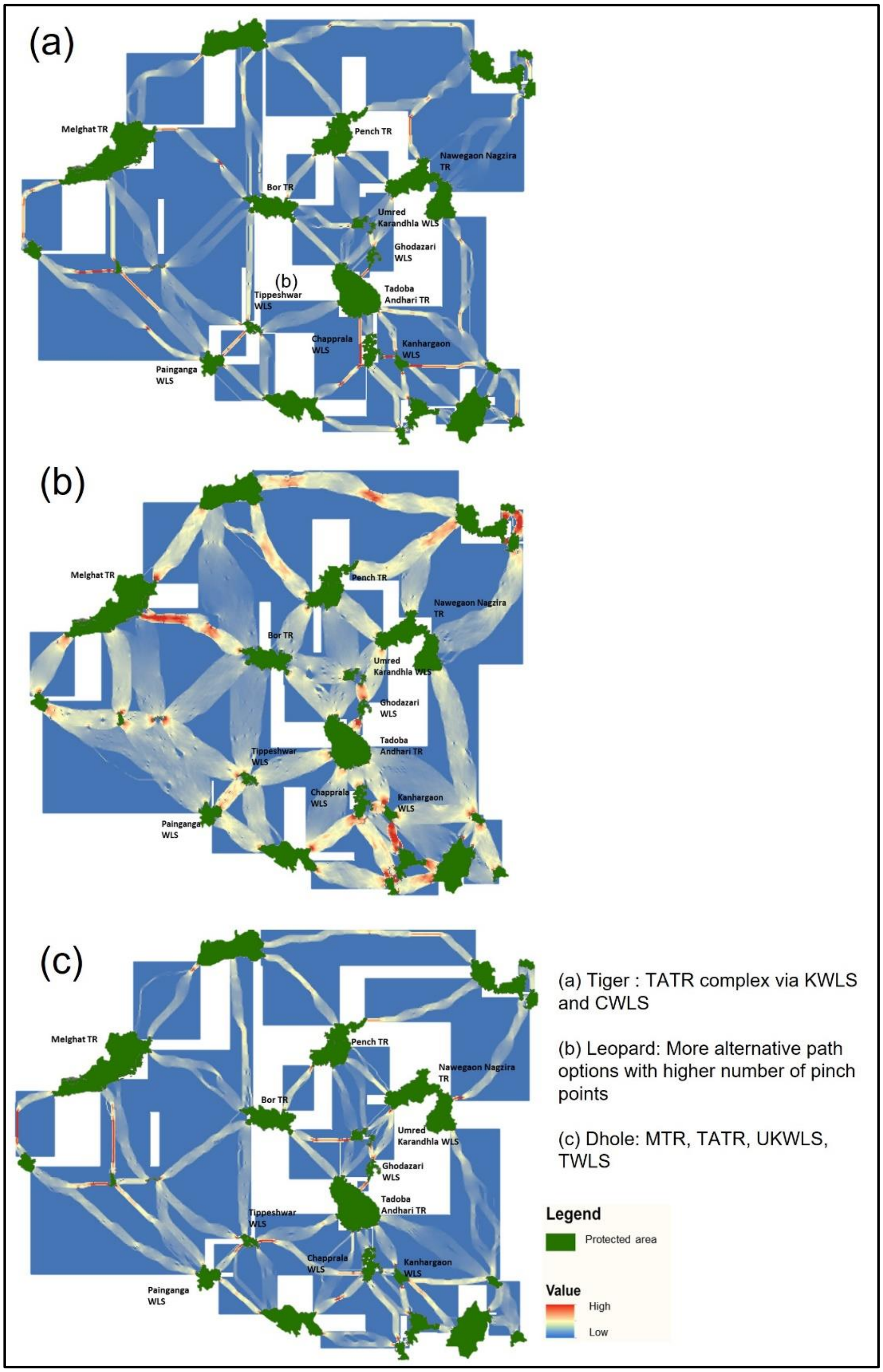


Figure 5.3 Current map of the carnivores based on the optimised resistance surfaces from ResistanceGA for (c) Dhole and (d) Multispecies.



(a) Tiger : TATR complex via KWLS and CWLS

(b) Leopard: More alternative path options with higher number of pinch points

(c) Dhole: MTR, TATR, UKWLS, TWLS

Figure 5.4: Pinch points for the three species across the major connectivity linkages generated using Pinch Point Mapper for (a) Tiger, (b) Leopard, and (c) Dhole.

5.4 Discussion

Improving our understanding of the connectivity and movement pattern of large carnivores is essential to effectively manage and conserve their population in the fragmented landscape due to present anthropogenic pressure. Developing conservation plans by considering the ecological as well as genetic traits of multiple species can help in devising better plans serving the needs of different species. The main objective of this study was to determine how the landscape and environmental variables can differentially shape the meta-population by differentially impacting movement of three top carnivores in the fragmented landscape of Maharashtra. I used the individual genotypes dataset of three carnivores, i.e. tiger, leopard and Asiatic wild dog, from six protected areas of Maharashtra to determine the effect of Anthropocene on these top carnivores. These protected areas in Maharashtra form a major part of the Central Indian Highlands, a conservation priority area for tigers. I used the linear mixed-effects model fit with genetic distance as the dependent variable and the scaled and centred resistance distance as the predictor variable. Mixed models were fitted using the maximum-likelihood population effects parameterisation to account for the non-independence among the pairwise genetic and ecological distances. I found geographical distance to be the most determining factor for the movement of tiger and leopard, while for dholes, it was evapotranspiration. The second-best model was found to be ndvi for leopard, evapotranspiration for tiger and population for dhole. The simultaneous optimisation of multiple raster surfaces allowed to explain the variation in genetic data for the three species suggesting their relevance for the three species.

The results support my hypothesis of maximum impact of habitat fragmentation on dhole followed by tiger and leopard. I found that population, livestock, and evapotranspiration are the key features, exhibiting that geneflow is strongly affected by the presence of high population density, and livestock presence implies the effect of nearby villages. For a forest-dependent species like dhole (Kamler et al., 2015), movement from one to another habitat node can only be facilitated by continuous small habitat patches. The results confirm the major role of anthropogenic factors in the genetic differentiation of the dhole population, as evident from the previous study. For tigers, geographical distance, evapotranspiration, vegetation cover and temperature play a crucial role in determining the gene flow, which suggests that tigers are comparatively less affected by anthropogenic factors. For leopards, geographical distance, vegetation cover, evapotranspiration and precipitation were found to be the key factors. The rate of evapotranspiration was found to have a major effect to different extents on the genetic differentiation of the three carnivores. Results showed higher evapotranspiration as an impeding factor for animal movement and generally, it is assumed that the evapotranspiration from forests is larger than for any crop compared (Ladekarl, 1998). But, it further depends on the type of forest and agriculture. This study is characterised by deciduous forest, and previous studies have found that coniferous and deciduous vegetation has a lower evapotranspiration rate than agricultural crops (Bastiaanssen et al., 2001). Evapotranspiration can also serve as a proxy for hot and dry conditions, limiting species through thermal stress and poor forage conditions (Tattersall et al., 2012) in the remnant patches of a fragmented landscape. Studies have shown that habitat fragmentation can change thermal conditions, resulting in unpredictable evapotranspiration rates and greater desiccation influencing the animal's distribution and activity patterns (Tuff et al., 2016). Another best model fit common for the three species is ndvi surface, where higher ndvi refers to vegetation cover and supports the animal movement.

The current map for leopards is supported by the genetic structure result from the analyses conducted for determining the extent of differentiation. Both current map and genetic structure show comparatively higher gene flow across the landscape for leopard than dhole and tiger. The current map for both tiger and dhole suggests that the presence of continuous forested patches is required for maintaining gene flow across the landscape. The use of the current flow model helps in mapping the pinch point, a relatively restricted area representing a landscape feature that can act as a dispersal passage. The haphazard development and industrialisation do not allow the possibility for wider corridors; therefore, pinch points are crucial for the movement of species across the landscape interspersed with hostile conditions for animal movement.

A comparison of the three species from the major carnivore guild suggests that landscape genetic patterns are a response to the ecological differences in habitat characteristics, and disturbance can differentially affect landscape genetic structure. The landscape genetics results in this study for tiger and leopard differ from the previous studies (Thatte et al., 2020; Dutta et al., 2015), which investigated lesser variables and smaller sample sizes. Previously, Thatte et al. found land use land cover to be the major factor affecting both tiger and leopard connectivity followed by linear features while in the present study in present study human population, human interference as agriculture, livestock and ndvi prove to be the major influencing factors. For dholes, the study was conducted for the first time; therefore, no reference is available, but the previous ecological studies consider dhole as a highly forest-dependent species, which is further supported by univariate optimisation results with the best model fit for population, livestock and evapotranspiration.

5.5 Conservation Implications

Species on the highest trophic level have on average small population size and, therefore, are more prone to adverse effects on genetic diversity caused by habitat fragmentation. It is evident that fragmentation affects biodiversity and ecosystem functioning (Haddad et al., 2015). The relative similarity or dissimilarity between habitat and non-habitat areas and matrix quality can affect both individual organisms and material fluxes (Prevedelo & Vieira, 2010). The impact of habitat fragmentation is faced by multiple species still; the mitigation plans are targeted mainly on an umbrella single species connectivity. The landscape connectivity concept is species-specific, and therefore incorporating the effect of fragmentation by considering a range of variables on multiple species provide a wholesome and more practical approach to mitigate the effects on a larger range of species.

Metapopulation ecology is a fundamental principle of conservation ecology. A species with a metapopulation structure lives in a habitat composed of patches suited for and accessible to the species' individuals. In this study, the three large carnivores show different requirements for maintaining a meta-population network. Asiatic wild dog, a habitat specialist, requires continuous patches to maintain the well-connected genetic populations due to their affinity for deep forests and shorter dispersal range than the other two carnivores in the study. Tigers, a long-ranging species, are traversing very large distances in Maharashtra, as evident from the telemetry data and which often leads to crossing non-protected human-dominated areas. In the case of tigers and Asiatic wild dogs, the current density was found to be high around the TATR complex with nearby wildlife sanctuaries. These wildlife sanctuaries, i.e. Ghodazari WLS, Umred-Karandhla WLS, and Kanhargaon WLS are critical in maintaining the long-term connectivity among the subpopulations for both species. These protected areas are crucial for tiger connectivity as they act as a small refuge for the dispersing individual. While for dholes, these protected areas may harbour small populations for maintaining continuous connectivity. For habitat generalists like leopards, these forested patches can act as a hideout and prevent the human-wildlife conflict, a major menace in the present Anthropocene era. Therefore, these protected areas are crucial links for a healthy meta-population network. The Melghat-Pench complex is also an important habitat for all three species, but it cannot support the metapopulation network because of the absence of interspersing small patches.

Studies have shown the importance of landscape patterns when habitat amount is low (Marrotte et al., 2017). In the present scenario of industrialisation and man-made fragmentation of habitat, with very less favourable habitat, the role of landscape variables should also be considered while planning mitigation measures and connectivity planning. All species exist in populations with varying degrees of spatial structure, ranging from many discrete populations that are completely independent of one another to a single large well-mixed population. A metapopulation exists between these two extremes and is composed of a collection of weakly interconnected individuals. Future multi-species connectivity studies should investigate approaches to prioritizing species based on vulnerability to connectivity loss and other factors because species respond to human disturbance and fragmentation differently (Pliscoff et al., 2020). Requirements of each species should be considered while establishing priority-based conservation, but multi-species maps could provide important insights to maintain as well as improve connectivity and also improvise the decisions for mitigation measures. As obtained in this study, landscape genetics outputs, coupled with multi-species connectivity models, could help to prioritise the conservation of areas where maintaining connectivity is most critical.

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Appendices

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RESEARCH NOTE

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

SHRUSHTI MODI, SAMRAT MONDOL, PALLAVI GHASKADBI, ZEHIDUL HUSSAIN, PARAG NIGAM and BILAL HABIB*

Wildlife Institute of India, Chandrabani, Dehradun 248 001, India
*For correspondence. E-mail: bh@wii.gov.in.

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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.

Shrushti Modi and Samrat Mondol contributed equally to this work.

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^2), Navegaon-Nagzira Tiger Reserve (152.8 km^2), Tadoba-Andhari Tiger Reserve (627.5 km^2) and Umred- Karhandla Wildlife Sanctuary (189 km^2) (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°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°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 μL of lysis buffer, followed by the manufacturer's protocol provided in the kit. DNA was eluted twice with 100 μL of 1 × TE and stored at −20°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 μL of hot start Taq mix (Qiagen), 4 μM BSA, 0.5 μM of primer mix and 3 μL of DNA extract under conditions including initial denaturation (95°C for 15 min); 50 cycles of denaturation (94°C for 30 s), annealing (50°C for 30 s) and extension (72°C for 35 s); followed by a final extension (72°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

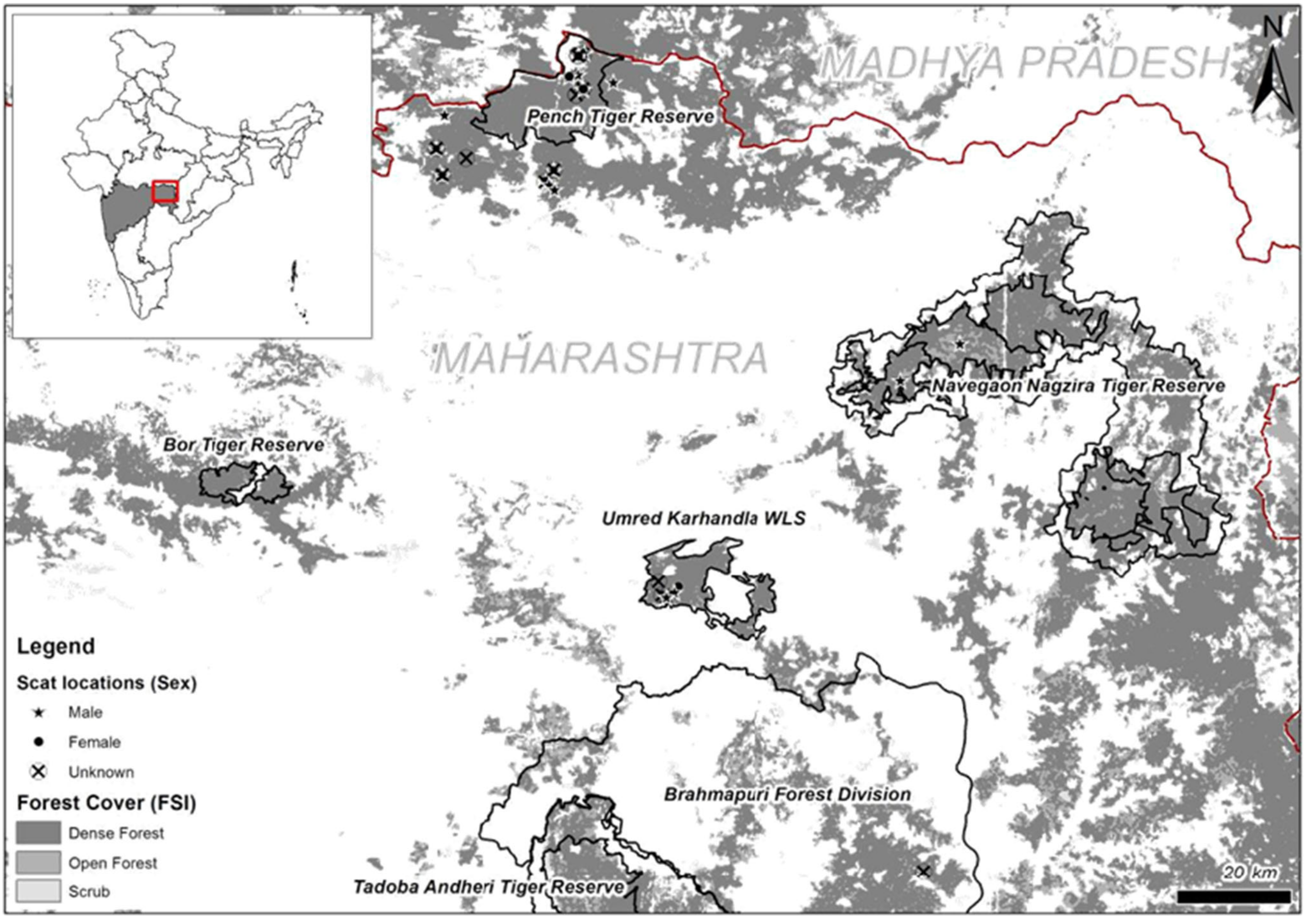

Figure 1. Field sampling locations for scats (male, female and unknown) in our study area within the state of Maharashtra.

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 μM BSA, 0.5 μM of primer mix and 1–3 μL 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.

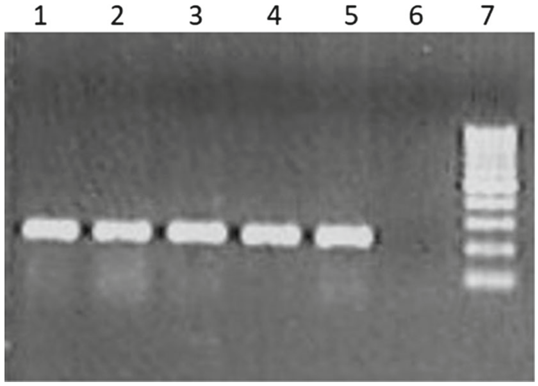

Figure 2. Representative gel picture of PCR amplified products of dhole-specific mitochondrial primer designed in this study. Lane 1–4 shows amplification from field-collected dhole faecal samples, Lane 5 shows amplification from dhole positive sample, lane 6 is PCR negative and lane 7 contains 100-bp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

Results and discussion

We developed four sets of potential dhole-specific mitochondrial markers for species identification. Of the four 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	ATTAGTTCAGAGGCGGTA			
DBY-F	TTGGGGGTGGTTTTATTGTC	55	Male: 112	Sastre *et al.* (2009)
DBY-R	CCATCTCAACATCGCTGAAC			
AHTx40-F	GTAGCCCCATTTGTTTATATTTGC	55	Female: 190	Sastre *et al.* (2009)
AHTx40-R	GATCACTGTCTTACACCACAGGC			
Canis SRY-F	ATGGCTCTAGAGAATCCCCA	55	Male: 199	Iyengar *et al.* (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 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.

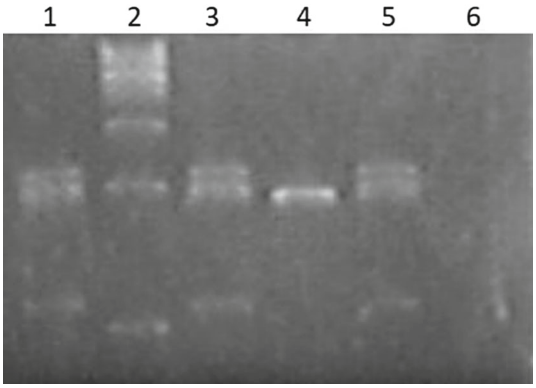

Figure 3. Representative gel picture of PCR amplified products of the multiplex molecular sexing assay developed for canids in this study. Lanes 1 and 5 show amplification from field-collected male faecal DNA samples, lanes 3 and 4 show amplification from a known male and female dhole sample respectively,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

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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Standardization and validation of a panel of cross-species microsatellites to individually identify the Asiatic wild dog (*Cuon alpinus*)

Shrushti Modi*, Bilal Habib*, Pallavi Ghaskadbi, Parag Nigam and
Samrat Mondol

Wildlife Institute of India, Chandrabani, Dehradun, Uttarakhand, India
* These authors contributed equally to this work.

ABSTRACT

Background: The Asiatic wild dog or dhole (*Cuon alpinus*) is a highly elusive, monophyletic, forest dwelling, social canid distributed across south and Southeast Asia. Severe pressures from habitat loss, prey depletion, disease, human persecution and interspecific competition resulted in global population decline in dholes. Despite a declining population trend, detailed information on population size, ecology, demography and genetics is lacking. Generating reliable information at landscape level for dholes is challenging due to their secretive behaviour and monomorphic physical features. Recent advances in non-invasive DNA-based tools can be used to monitor populations and individuals across large landscapes. In this paper, we describe standardization and validation of faecal DNA-based methods for individual identification of dholes. We tested this method on 249 field-collected dhole faeces from five protected areas of the central Indian landscape in the state of Maharashtra, India.

Results: We tested a total of 18 cross-species markers and developed a panel of 12 markers for unambiguous individual identification of dholes. This marker panel identified 101 unique individuals from faecal samples collected across our pilot field study area. These loci showed varied level of amplification success (57–88%), polymorphism (3–9 alleles), heterozygosity (0.23–0.63) and produced a cumulative misidentification rate or $\mathrm{PID}_{(\mathrm{unbiased})}$ and $\mathrm{PID}_{(\mathrm{sibs})}$ value of 4.7×10^{-10} and 1.5×10^{-4}, respectively, indicating a high statistical power in individual discrimination from poor quality samples.

Conclusion: Our results demonstrated that the selected panel of 12 microsatellite loci can conclusively identify dholes from poor quality, non-invasive biological samples and help in exploring various population parameters. This genetic approach would be useful in dhole population estimation across its range and will help in assessing population trends and other genetic parameters for this elusive, social carnivore.

Subjects Conservation Biology, Genetics

Keywords Dhole, Individual identification, Non-invasive sampling, Population parameters, Maharashtra tiger landscape

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Corresponding author
Samrat Mondol, samrat@wii.gov.in

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INTRODUCTION

The Asiatic wild dog or dhole (*Cuon alpinus*) is a highly elusive, endangered, social canid distributed in south and southeast Asia (*Johnsingh, 1982*; *Durbin et al., 2004*) occupying a range of habitat types including alpine, temperate, sub-tropical and tropical forests (*Durbin et al., 2004*). Driven by habitat loss, prey depletion, disease transmission from domestic dog, human persecution and interspecific competition (*Hayward, Lyngdoh & Habib, 2014*; *Kamler et al., 2015*), dholes are currently found in about 75% of their historical global range (*Durbin et al., 2004*; *Kamler et al., 2015*). Global dhole population is roughly estimated to be about 4,500–10,500 with only 949–2,215 mature individuals, but accurate estimates and population trends are not available from any part of its range (*Kamler et al., 2015*). They are considered as 'Endangered' by IUCN under criteria C2a(i) and included in Appendix II of the Convention on International Trade in Endangered Species. The Indian subcontinent currently retains majority of the remaining dhole populations (*Kamler et al., 2015*) in the Western Ghats and central Indian forests (*Karanth et al., 2009*), along with smaller populations in the Eastern Ghats (*Karanth et al., 2009*), northeast India (*Gopi, Lyngdoh & Selvan, 2010*; *Lyngdoh et al., 2014*) and Himalayan region (*Bashir et al., 2014*). The species has faced about 60% decline in their historical distribution in the subcontinent (*Karanth et al., 2010*).

Given the current anthropogenic disturbance scenario across its range, the future survival of this monotypic genus depends on integrated conservation measures involving detailed, accurate information on ecology, demography and genetics. However, generating reliable information for this elusive, forest-dwelling and pack-living canid at landscape scale is challenging. Traditional ecological techniques such as regular photographic capture approach are ineffective for dholes due to absence of unique coat patterns and their monomorphic forms, and physical tagging methods are impractical at landscape scales due to logistical difficulties, high costs and small numbers of captures possible. In this context, genetic tools have tremendous potential to generate critical information such as population size estimation (*Mondol et al., 2009b*), phylogeography (*Luo et al., 2014*; *Waits et al., 1998*), pack dynamics and reproductive fitness (*Sillero-Zubiri, Gottelli & Macdonald, 1996*; *Girman et al., 1997*), dispersal patterns (*Epps et al., 2007*; *Gour et al., 2013*) for elusive species conservation across large landscapes (*Mondol, Bruford & Ramakrishnan, 2013*). The ability to identify individuals from non-invasive samples collected over large space provides a feasible option to generate detailed information on elusive, forest-dwelling dholes as they cannot be identified using other approaches.

In this study, we addressed key methodological issues related to selection and standardisation of a set of molecular markers for individual identification of dholes. Subsequently, we tested these markers on field-collected dhole samples from five protected areas of the central Indian landscape in the state of Maharashtra, India for individual identification. In addition to the utilisation of these markers in dhole population estimation at landscape level, we believe that this approach has wider relevance in non-invasive, faecal DNA based population assessments of many other low density, elusive, wide-ranging species.

METHODS

Research permits and ethical considerations

All required permissions for fieldwork and sampling were provided by the Maharashtra Forest Department (Permit No. 09/2016). The entire study was non-invasive through field-collected faecal samples, and thus did not require any ethical clearance from the institute. Reference dhole blood samples ($n = 4$) were collected as part of another ongoing study in Tadoba-Andhari Tiger Reserve (TATR; Permit no. SPP-12/2016), where blood sampling was conducted during radio collaring of dholes.

Study area

The study was focused in five protected areas Melghat Tiger Reserve (MTR), Pench Tiger Reserve (PTR), Navegaon-Nagzira Tiger Reserve (NNTR), TATR and Umred-Karandhla Wildlife Sanctuary (UKWLS) of the central Indian landscape in the state of Maharashtra, India. The entire area is a complex of forested areas (core zone) with different levels of connectivity. NNTR and PTR are geographically closer as compared to MTR–NNTR and MTR–PTR. MTR and PTR are part of the Satpura-Maikal-Pench corridor in the Satpura-Maikal landscape. The forest type is of dry deciduous to moist deciduous nature with major vegetation consisting of *Tectona grandis, Anogeissus latifolia, Lagerstroemia parviflora, Terminalia* spp., *Heteropogon contortus, Themeda quadrivalvis, Cynodon dactylon* etc.

Field sampling

Dholes prefer dense forested habitats (*Johnsingh, 1985*) where the social groups defaecate in communal latrine sites mostly found on the junctions of roads/trails (*Johnsingh, 1982*). Their elusive nature and highly social behaviour present unique challenges in scat sampling for individual identification. In this study, sampling was conducted through intensive foot and vehicle surveys covering the entire study area. We sampled a total of 49 latrine sites covering five protected areas. Once a latrine site was found only fresh scats were targeted for collection. One bolus from each fresh scat was collected assuming it to derive from one individual. Separate gloves were used to collect each sample. All samples were collected directly in wax paper and stored in separate ziplock bags. Once brought to the field station, the sample containing ziplock bags were temporarily stored in a large box containing silica gel to minimise fungal growth in humid conditions. Samples were then shipped to the laboratory, where they were stored in a −20 °C freezer. GPS co-ordinates and other associated information (track marks, substrata etc.) were collected for each sample. Entire sampling was conducted once per site between January 2015 and June 2017 ensuring maximum coverage of the study area, covering PTR (257.3 km^2), MTR (1,500.49 km^2), NNTR (152.8 km^2), TATR (627.5 km^2) and UKWLS (189 km^2), Maharashtra. A total of 249 samples were collected (PTR—92, MTR—76, NNTR—37, UKWLS—34, TATR—10, respectively) for this study. Details of all sample locations are given in Fig. 1.

For blood sampling, four animals (three males and one female) were remotely administered with reconstituted lyophilised mixture of Telatamine-Zolezepam (Zoletil 100; Virbac, Carros, France) at the dose rate of 8.6 mg/kg body weight using Dan-inject

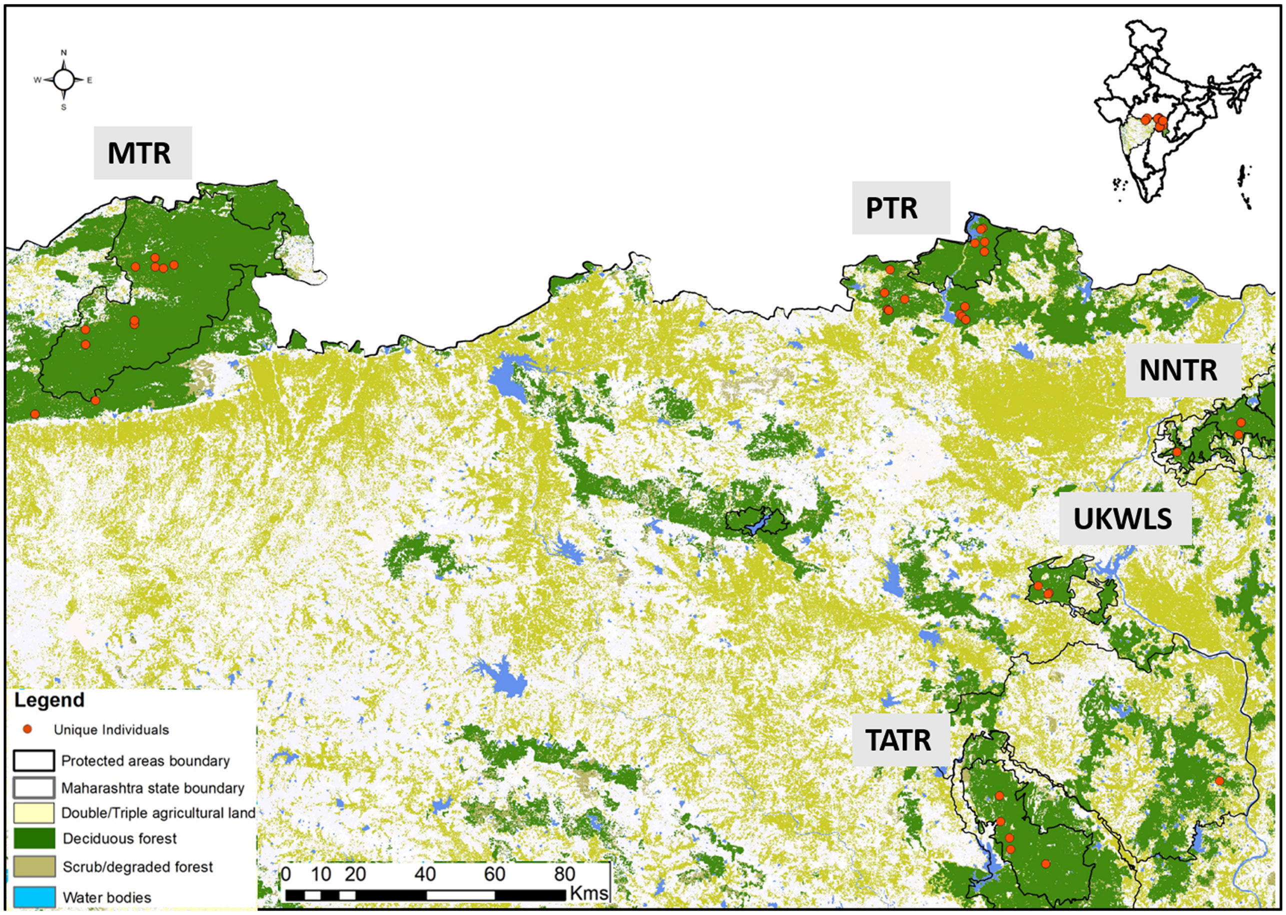

Figure 1 Protected area map with locations of unique genotypes identified in the study area of Maharashtra, India. The study area names are as following: MTR, Melghat Tiger Reserve; PTR, Pench Tiger Reserve; TATR, Tadoba-Andhari Tiger Reserve; NNTR, Navegaon-Nagzira Tiger Reserve; UKWLS, Umred-Karandhla Wildlife Sanctuary. Full-size DOI: 10.7717/peerj.7453/fig-1

syringe projector (Model-IM) at a distance of 15–25 m. After ensuring sedation safe for handling, animal was approached, blindfolded and one ml of blood was collected through femoral-saphenous vein puncture. Blood was collected in EDTA vacutainers and preserved at −20 °C in the laboratory for genetic work.

DNA extraction

DNA extraction was performed in the laboratory from the frozen faecal samples using QIAamp DNA Tissue Kit (QIAGEN Inc., Hilden, Germany) with a modified approach, depending on sample quality. If the sample had the entire top mucous layer available (i.e. not covered by dust, soil etc.) then it was swabbed with phosphate buffer saline soaked sterile cotton swab and was stored in sterile Eppendorf tube at −20 °C (*Biswas et al., in press*). However, if the mucous layer was covered then the top layer was scraped using sterile blades and stored in similar conditions (*Biswas et al., in press*). Subsequently, faecal samples collected by both methods were lysed overnight in 300/600 µl of lysis buffer for swabs and scraped samples, respectively and 20 µl proteinase K followed by extraction using the kit's protocol. DNA was eluted twice with 100 µl of 1X TE and stored in −20 °C for long-term use. Each set of 22 extractions was accompanied with two negative controls to monitor possible contamination.

DNA from blood samples was extracted using standard protocol given in the QIAamp DNA Tissue Kit (QIAGEN Inc., Hilden, Germany). Negative control was incorporated to monitor any possible contaminations.

Selection of microsatellite markers

There are no dhole specific microsatellites developed so far and the only study focusing on dhole population genetics had used 13 cross-species markers from domestic dogs to study genetic variation (*Iyengar et al., 2005*). These markers showed low levels of polymorphism and low $\mathrm{PID_{sibs}}$ value (3.3×10^{-4}), providing a misidentification rate of 1 in 3,000 siblings. Given that India is considered to retain high (about 1,500–3,000) number of dholes (*Kamler et al., 2015*), this panel will not provide sufficient statistical power for unambiguous individual identification at landscape levels with large population sizes. For this study, we developed a panel following stringent cross-species marker selection and testing process. The entire process was conducted in two steps: marker selection and rigorous testing before developing a final microsatellite panel for dhole individual identification.

As most of the cross-species markers were found to be from dogs and earlier used markers were less polymorphic for individual identification, we decided to first examine if both species (domestic dog and wild dog) share genetic similarity. Earlier karyotype and chromosomal banding studies (*Graphodatsky et al., 2008*) showed almost identical G-banding patterns, indicating high chromosomal level similarity between both species. Subsequently, we identified a total of 37 dog microsatellite loci from earlier published literature (*Holmes et al., 1995*; *Ostrander, Sprague & Rine, 1993*; *Fredholm & Winterø, 1995*; *Ostrander et al., 1995*; *Francisco et al., 1996*; *Neff et al., 1999*). These markers were selected based on their polymorphism (number of alleles (Na), PIC, observed heterozygosity ($\mathrm{H_o}$) etc.) and amplicon sizes in published literatures. Further, we mapped all the markers on available dog genome canFam 3.1 in UCSC Genome Browser (http://genome.ucsc.edu/; Accession ID: GCA_000002285.2) to assess the chromosome number to which each marker is associated with. Finally, a total of 18 microsatellites were selected based on their amplicon size, chromosome number and polymorphism (based on published data) for further testing. The details of the markers are given in Table 1.

PCR standardisation and data validation

All initial standardisation of the markers was conducted using dhole blood samples ($n = 4$). PCR reactions were performed for selected 18 microsatellites in 10 µl reactions containing 3.5 µl Qiagen multiplex PCR buffer mix (QIAGEN Inc., Hilden, Germany), 0.2 µM labelled forward primer (Applied Biosystems, Foster City, CA, USA), 0.2 µM unlabelled reverse primer, four µM BSA and two µl of 1:50 dilution of blood DNA extract. The PCR conditions included an initial denaturation (95 °C for 15 min); 50 cycles of denaturation (94 °C for 30 s), annealing (50–60 °C gradient for 30 s) and extension (72 °C for 35 s);

Table 1 Details of microsatellite loci used for Asiatic wild dog.

Locus	Primer sequence 5′–3′	Repeat motif	Dye	T_a (°C)	Chromosome number (in dog)	Na	H_o	H_e	Allelic range	$PID_{(unbiased)}$ (cumulative)	$PID_{(sibs)}$ (cumulative)	Success rate (%)	Genotyping error (%)			Reference
													ADO	FA	NA	
WD2201 (Panel 4)	ATCAACAATGCATGCCACAT GAGAACAAATAAATGCAAGCCC	Tetra	FAM	59	7	9	0.63	0.78	170–202	7.40*E*-02	3.81*E*-01	73.97	10.4	6.9	3.5	*Francisco et al. (1996)*
PEZ6 (Panel 3)	ATGAGCACTGGGTGTTATAC ACACAATTGCATTGTCAAAC	Tetra	NED	53	27	7	0.38	0.78	206–230	5.88*E*-03	1.46*E*-01	72.05	9.1	8.2	19.2	*Neff et al. (1999)*
WD2140[#] (Panel 2)	GGGGAAGCCATTTTTAAAGC TGACCCTCTGGCATCTAGGA	Tetra	HEX	56	5	9	0.4	0.76	122–178	5.00*E*-04	5.69*E*-02	69.65	10.2	7.9	4.8	*Francisco et al. (1996)*
AHT130[#] (Panel 2)	CCTCTCCTGGTAAGTGCTGC TGGAACACTGGTCCCCAG	Di	FAM	56	18	7	0.44	0.74	98–112	4.69*E*-05	2.29*E*-02	71.47	13.9	2.4	0	*Holmes et al. (1995)*
PEZ3 (Panel 3)	CACTTCTCATACCCAGACTC CAATATGTCAACTATACTTC	Tetra	PET	53	19	8	0.52	0.73	110–146	5.26*E*-06	9.53*E*-03	88.45	11	16.5	16.4	*Neff et al. (1999)*
WD2137 (Panel 4)	GCAGTCCCTTATTCCAACATG CCCCAAGTTTTGCATCTGTT	Tetra	FAM	56	3	7	0.46	0.66	156–180	7.29*E*-07	4.33*E*-03	68.94	9	1.6	22.1	*Francisco et al. (1996)*
PEZ5 (Panel 3)	GCTATCTTGTTTCCCACAGC TCACTGTATACAACATTGTC	Tetra	FAM	53	12	8	0.15	0.61	150–254	1.39*E*-07	2.14*E*-03	57.37	12.9	6.9	16	*Neff et al. (1999)*
CXX251 (Panel 2)	TACCACTGTCATTTTTCCATGC AAGAGGATACCGGTGGCAG	Di	NED	56	1	3	0.23	0.58	128–136	3.32*E*-08	1.11*E*-03	78.99	8.8	5.6	11	*Ostrander et al. (1995)*
WD2096[#] (Panel 1)	CCGTCTAAGAGCCTCCCAG GACAAGGTTTCCTGGTTCCA	Tetra	FAM	59	11	3	0.66	0.51	93–101	1.05*E*-08	6.38*E*-04	81.98	8.6	8.5	24	*Francisco et al. (1996)*
CXX279[#] (Panel 1)	TGCTCAATGAAATAAGCCAGG GGCGACCTTCATTCTCTGAC	Di	PET	59	22	5	0.2	0.45	125–135	3.29*E*-09	2.33*E*-04	84.05	7.0	6.9	13	*Ostrander et al. (1995)*
WD2001[#] (Panel 1)	TCCTCCTCTTCTTTCCATTG TGAACAGAGTTAAGGATAGACAC	Tetra	HEX	59	23	3	0.41	0.48	134–142	1.24*E*-09	3.86*E*-04	78.92	10.4	8.2	6.2	*Francisco et al. (1996)*
CXX30 (Panel 1)	GCCTTTTAGGGAGCTTTCTTT GAGTCTGCTTTTCTCCTTCCC	Di	NED	59	2	3	0.38	0.41	122–142	4.68*E*-10	1.50*E*-04	77.28	9.4	9.9	3.3	*Ostrander et al. (1995)*
Mean						6	0.40	0.62				75.26	10.1	9.2	11.2	
AHT136[*]	GAGAGGGCTGGTGGTAGGGG CGTGGCTATCTTTGGAGGGA	Di	HEX	NA	11	–	–	–	–	–	–	–	–			*Neff et al. (1999), Holmes et al. (1995)*

Table 1 **(continued).**

Locus	Primer sequence 5′–3′	Repeat motif	Dye	T_a (°C)	Chromosome number (in dog)	Na	H_o	H_e	Allelic range	$PID_{(unbiased)}$ (cumulative)	$PID_{(sibs)}$ (cumulative)	Success rate (%)	Genotyping error (%)			Reference
													ADO	FA	NA	
WD2159*	GAATCCCACATCGGGCTC ATTAAGTTTTGAAAGCCAGGTAAG	Tetra	HEX	NA	24	–	–	–	–	–	–	–	–			*Francisco et al. (1996)*
CPH6*	CATTGGCTGTTTGACTCTAGG ACTGATGTGGGTGTCTCTGC	Di	FAM	56	23	4	0.189	0.577	107–136	–	–	–	–			*Fredholm & Winterø (1995)*
CPH16*	CTACACCAGTTAGGGAATCTAGC CAGATTCAAATCCACTCTCAGAC	Di	HEX	NA	20	–	–	–	–	–	–	–	–			*Fredholm & Winterø (1995)*
CXX140*	CAGAGGTGGCATAGGGTGAT TCGAAGCCCAGAGAATGACT	Di	PET	56	4	2	0.012	0.059	149–151	7.42*E*-08	9.87*E*-04	–	–			*Ostrander et al. (1995)*
CXX608*#	TATTGTAAGTCTTCCTTGAC TCTACCGTCTACAACAAAAGGG	Di	HEX	53	15	2	0	0.04	134	7.42*E*-08	9.87*E*-04	–	–			*Ostrander et al. (1995)*

Notes:

Na, number of alleles; H_o, observed heterozygosity; H_e, expected heterozygosity; PID, probability of identity; T_a, annealing temperature; ADO, alleleic dropout; FA, false alleles; NA, null alleles.

* Loci omitted from final analyses.

\# Loci used in the study by *Iyengar et al. (2005)*.

followed by a final extension (72 °C for 30 min). Following post-temperature standardisations markers with same annealing temperatures but with different labels or allele sizes were standardised as multiplex assays (see Table 1 for details). During all amplifications, both extraction and PCR negative controls (one PCR negative every set of 11 reactions) were included to monitor any possible contamination. Post amplification, two µl of PCR product was mixed with HiDi formamide (Applied Biosystems, Foster City, CA, USA) and LIZ 500 size standard (Applied Biosystems, Foster City, CA, USA) and genotyped in an ABI genetic analyser (Applied Biosystems, Foster City, CA, USA). The fragment lengths were scored manually using the programme GENEMARKER (Softgenetics Inc., State College, PA, USA). Each reaction was repeated three times to ensure good data quality.

Once the initial temperature and multiplexing standardisations were performed using reference blood DNA samples, final standardisation was conducted with dhole faecal DNA. Species identification was performed for all field-collected faeces using specific mtDNA primers described in *Modi et al. (2018)*. PCR reactions were performed with four µl of hotstart taq mix (QIAGEN Inc., Hilden, Germany), four µM BSA, 0.5 µM of primer mix and three µl of DNA extract with conditions including initial denaturation (95 °C for 15 min); 50 cycles of denaturation (94 °C for 30 s), annealing (50 °C for 30 s) and extension (72 °C for 35 s); followed by a final extension (72 °C for 10 min). Negative controls were included to monitor contaminations. Samples that produced species-specific bands ($n = 225$) were further processed for microsatellite analyses.

For faecal samples, data validation was performed through a modified multiple-tube approach as described in *Mondol et al. (2009b)*. All faeces that had amplified in 50% of the loci in the panel during first PCR were repeated two more times for all loci. Following allele calling, a consensus genotype was prepared using the 'Quality index' protocol (*Miquel et al., 2006*), during which alleles were called manually and scored as '1' if the repeat is identical with the first call, or '0' if calls do not match due to no amplification, allelic dropout (one allele in heterozygote is erroneously not amplified), false allele (FA; slippage artefact during PCR) etc. To calculate the quality index for each locus/sample the scores assigned to each repeat are summed and divided by the total number of repeats, and only quality index of 0.75 or more (at least three out of four repeats) for each locus was considered for downstream analyses. We calculated average amplification success as the percent positive PCR for each locus, as described by *Broquet & Petit (2004)*. We quantified allelic dropout and FA rates manually as the number of dropouts or FAs over the total number of amplifications, respectively (*Broquet & Petit, 2004*), as well as using MICROCHECKER v 2.2.3. (*Van Oosterhout et al., 2004*). The FA frequency was calculated for both homozygous and heterozygous genotypes as the ratio of the number of amplifications having one or more FAs at a particular locus and the total number of amplifications while allele dropout rate was calculated as the ratio between the observed number of amplifications having loss of one allele and the number of positive amplifications of the heterozygous individuals. Programme FreeNA (*Chapuis & Estoup, 2007*) was used to determine the frequency of null alleles (NAs), which estimates the NA frequency using EM algorithm (*Dempster, Laird & Rubin, 1977*).

Molecular sexing of the identified individuals was conducted using already developed multiplex sexing approach (*Modi et al., 2018*), where three sex chromosome specific markers (DBY, AHT-X40 and SRY) were combined to generate a three-band pattern for males and a single band for females. This approach reduces identification of 'false negatives' of males due to allelic dropout from the Y chromosome from poor quality samples.

Data analyses

The identity analysis module implemented in programme CERVUS (*Kalinowski, Taper & Marshall, 2007*) was used to identify identical genotypes (or recaptures) by comparing data from all samples for all amplified loci. All genetic recaptures were removed from the data set. GIMLET (*Valière, 2002*) was used to calculate the $PID_{(sibs)}$ for all the individuals. Following this, any allele having less than 10% frequency across all amplified loci were rechecked for allele confirmation. ARLEQUIN (*Excoffier, Laval & Schneider, 2005*) was used to determine Hardy Weinberg equilibrium and linkage disequilibrium for all the loci.

RESULTS

During initial standardisations we tested all 18 selected markers (see Table 1) with four wild-caught dhole blood DNA samples. Three of these tested markers (WD2159, CPH16, AHT136) did not show any amplification in the blood DNA samples and were removed from subsequent analyses. The remaining 15 markers were then amplified with 225 genetically confirmed dhole faecal samples. Following data validation through multiple repeats, amplification success rates and polymorphism for these loci were calculated. The results show that loci CXX608 and CXX140 were monomorphic in all amplified samples, and locus CPH6 has low amplification success rate (~35%) from faecal DNA and thus were removed from the panel. The remaining 12 markers were finally standardised as four multiplex panels (see Table 1) for dhole individual identification.

None of these final 12 loci showed any signatures of large-scale allelic dropouts. The mean allelic dropout rate was found to be 0.1, whereas mean FA frequency for all the 12 loci was 0.092. Overall frequency of NAs was calculated as 0.11, indicating this 12 loci panel has low genotyping error rates. Amplification success ranged between 57% and 88% from dhole faecal DNA. The loci showed relatively higher (WD2201-9 alleles, $H_o = 0.63$) to medium (CXX251-3 alleles, $H_o = 0.23$) levels of polymorphism (Table 1). Except locus WD2001, none of the other loci were found to deviate from the Hardy–Weinberg equilibrium and there were no evidences for strong linkage disequilibrium between any pair of loci. Summary statistics for various measures of polymorphism (H_o and expected heterozygosity, Na and allelic size range) for all loci in the final panel are presented in Table 1.

For individual identification, we only considered samples that produced good quality data for at least seven of the 12 panel loci. This cut-off value of average of seven loci was decided based on the statistical support (PID_{sibs} value of 1 in 500 siblings) produced by these loci. Given that any single largest dhole population is about 250–300 individuals (*Kamler et al., 2015*), this value is sufficient for individual identification at local scales in

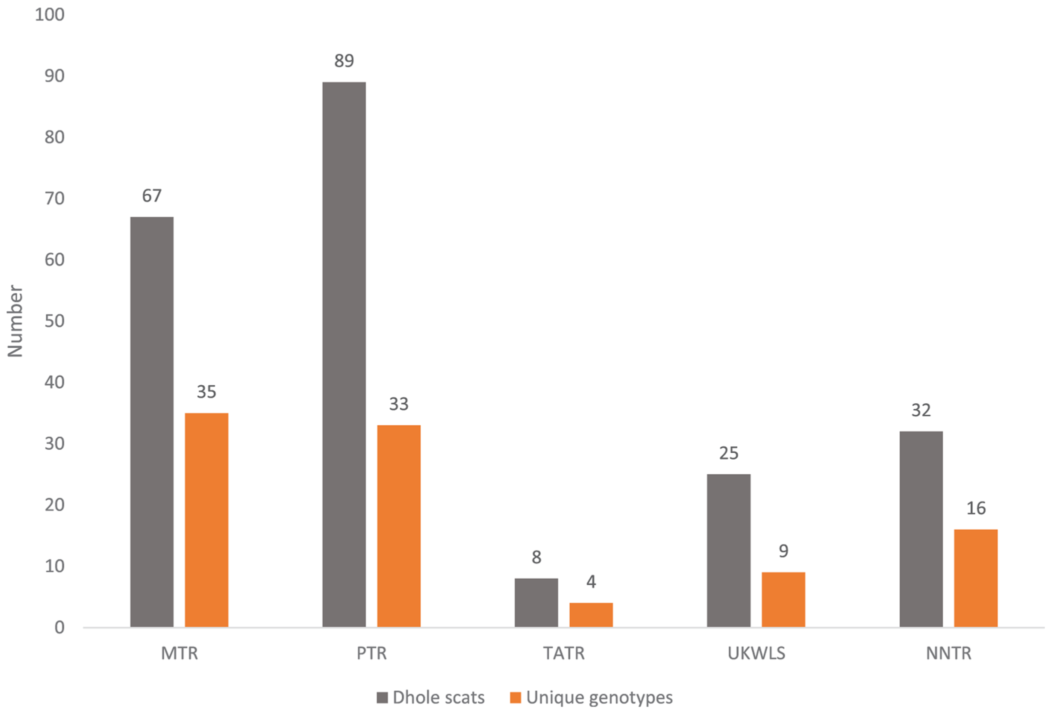

Figure 2 **Graph showing the number of confirmed dhole scats collected from the field and area-wise unique genotypes identified from them.** Full-size DOI: 10.7717/peerj.7453/fig-2

India. Out of the 225 field-collected dhole faecal samples amplified with the panel of 12 markers, 98 produced seven or more loci data. Overall, we generated genetic data from a total of 102 samples (four blood and 98 faecal samples) (Fig. 1). About 70% of these samples (n = 71) have successfully amplified for 10–12 loci. Following analyses with CERVUS, we identified 101 unique dhole individuals from the entire data, whereas one individual from NNTR was found to be a 'genetic recapture'. Cumulative PID_{sibs} and $PID_{unbiased}$ values were found to be 1.5×10^{-4} and 4.7×10^{-10}, respectively, indicating a strong statistical support for unambiguous individual identification. The number of unique individuals from each sampled area was found to be: PTR—33, MTR—35, NNTR—16, UKLWS—9 and TATR—4 (Fig. 2). Molecular sexing showed a success rate of 67%, with a male:female sex ratio of 4:1 in all identified dhole individuals (n = 101).

DISCUSSION

In this paper, we standardised protocols for individual identification of Asiatic wild dogs from poor quality DNA samples, and the final marker panel could unambiguously identify individual dholes in our field-based pilot study from five protected areas of Maharashtra, India. The systematic protocols followed here offer some advantages over earlier efforts on dhole individual identification from faecal samples by *Iyengar et al. (2005)*. Firstly, use of a large panel of 37 microsatellite loci for preliminary assessment of marker suitability along with genomic mapping-based selection of final markers (n = 18) allowed us to ascertain a combination of loci for unambiguous individual identification with high statistical power. The rigorous testing of the loci with large number of DNA samples from different sources also allowed us to exclude loci that might be problematic due to low amplification success from non-invasive samples. The final panel consisting 12 markers were further standardised into four multiplex reactions to provide time and cost-effective options during data generation. We were very careful to initially select a large number of tetranucleotide markers as they are known to have low stutter peak problems and better allele characteristics from poor quality samples (*Walsh, Fildes & Reynolds, 1996*), while dinucleotide markers generally have higher amplification success (*Broquet, Ménard & Petit, 2007*). Thus, our final panel with a ratio of 2:1 tetra vs. dinucleotide microsatellites would provide the ideal combination in terms of high success rate and less technical issues in allele calling during dhole individual identification. The amplification success rate for all loci was >70% except locus PEZ5 (~60%), but it was found to be polymorphic and was included in the panel. The overall genotyping error frequency was found to be <0.2 from dhole faeces, which is within the recommended limits for non-invasive population genetic research (*Smith & Wang, 2014*).

Our motivation in this study was to develop effective protocols that could be applied for individual identification of Asiatic wild dogs as they are difficult to identify from physical characteristics (spots, marks, stripes etc.). Their elusive nature also makes it challenging to estimate population size using traditional techniques (photographic capture, field-based observations etc.) at landscape levels. For genetic estimation of population size *Waits, Luikart & Taberlet (2001)* recommended a threshold PID_{sibs} value that is at least double than the approximate number of animals in any given area. The cumulative PID_{sibs} value of 1.5×10^{-4} achieved in this study is better than *Iyengar et al. (2005)* (PID_{sibs} of 3.3×10^{-4}) and should be sufficient to study dhole genetics and specifically population estimation across its range. Among all the dhole range countries India is considered to retain the highest (about 1,500–3,000) number of individuals (*Kamler et al., 2015*) and our misidentification rate achieved in this study (1 in 6,700 siblings) would provide strong statistical power in individual identification. The most recent assessment suggests that the largest dhole population in Western Ghats, India holds about 207–304 individuals (*Kamler et al., 2015*), thereby assuring that our seven loci cut-off (misidentification rate 1 in 500 siblings) to select samples and 12 loci panel would be useful in population estimation at local scale. However, it is important to point out that we generated individual level information from about 43.5% (98 out of 225 faeces) of the field-collected samples in this study. Similar patterns of low amplification success rate from field-collected faecal samples have been observed in earlier genetic study of dhole (*Iyengar et al., 2005*), leopard (*Mondol et al., 2009a*) and other species (*Smith & Wang, 2014*). Considering dhole cryptic nature, social behaviour and ecology in corroboration with low amplification success rate, we suggest an intensive faecal sampling effort for estimation of population size for this species. It is also noteworthy to point out that literature survey for dhole marker selection in this study was mostly based on available information on non-invasive canid population genetic research with specific information available on markers such as marker polymorphism (Na, H_o) and amplicon size etc. (*Holmes et al., 1995*; *Ostrander, Sprague & Rine, 1993*; *Fredholm & Winterø, 1995*; *Ostrander et al., 1995*; *Francisco et al., 1996*; *Neff et al., 1999*). However, future studies should also consider additional markers those are tested as part canid forensic studies (for example see *Van Asch et al., 2009*; *Berger et al., 2014*; *Hellmann et al., 2006*; *Eichmann, Berger & Parson, 2004*) on dholes. In addition, already available dhole genome information (*Campana et al., 2016*; *Habib et al., 2018*) also can be used to develop a suitable SNP panel for in depth analyses of dhole population and demographic parameters.

During individual identification we had identified only one genetic recapture from the field-collected faecal samples. This pattern of low dhole recapture could be attributed to our sampling strategy to cover large geographical area and maximise collection of faeces from potentially different individuals, as well as relatively low amplification success rates from faecal samples. We have sampled the entire study area only once and focused on collecting fresh samples, thereby probably missed recapturing the same individuals from same latrine sites. Further, low amplification success rate (101 genotypes from 225 fresh samples) might have resulted in getting lesser number of recaptures. We got a male biased sex-ratio (4:1) in this pilot study. While there is no conclusive information on dhole sex ratio across its range, our earlier study (*Modi et al., 2018*) in the same landscape has shown a male biased (M:F ratio of 3:1) sex ratio, and ecological study in southern India by *Venkataraman (1998)* suggested male biased packs. Future studies with extensive genetic sampling across this landscape would potentially provide more accurate sex ratio for dholes.

CONCLUSION

In the broader context of understanding dhole population dynamics at local or landscape scales, genetic sampling is possibly the only way to generate information with spatial and temporal coverage for this elusive, social carnivore as photographic sampling or conventional tagging cannot be employed due to lack of distinguishing natural marks and logistical difficulties of physical captures of large number of animals. Results from this study provide a robust tool to generate individual level information from field-collected faecal samples. In combination with a good sampling strategy, our methods can be used in a cost-effective way to investigate species biology (including patterns of genetic diversity, relatedness and population connectivity) as well as to estimate population abundance of dholes in the wild.

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ADDITIONAL INFORMATION AND DECLARATIONS

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Competing Interests

The authors declare that they have no competing interests.

Author Contributions

- Shrushti Modi conducted field sampling, performed the experiments, analysed the data, prepared figures and/or tables, authored or reviewed drafts of the paper, approved the final draft.
- Bilal Habib conceived and designed the experiments, contributed reagents/materials/analysis tools, approved the final draft.
- Pallavi Ghaskadbi approved the final draft, field sampling.
- Parag Nigam approved the final draft, field sampling for reference samples.
- Samrat Mondol conceived and designed the experiments, analysed the data, contributed reagents/materials/analysis tools, authored or reviewed drafts of the paper, approved the final draft.

Animal Ethics

The following information was supplied relating to ethical approvals (i.e. approving body and any reference numbers):

The study has been conducted using field-collected non-invasive samples (faeces) with appropriate permission from the Maharashtra Forest Department (Permit No. 09/2016). No ethical approval is required for non-invasive work.

Field Study Permissions

The following information was supplied relating to field study approvals (i.e. approving body and any reference numbers):

Reference dhole blood samples ($n = 4$) were collected as part of another ongoing study in Tadoba-Andhari Tiger Reserve under Permit no. SPP-12/2016, granted by the Office of the Principal Chief Conservator of Forests (HOFF), Maharashtra State.

Feacal sampling was done under Permit No. 09/2016, granted by the Principal Chief Conservator of Forests (Wildlife) & Chief Wildlife Warden, Maharashtra State.

Data Availability

The following information was supplied regarding data availability:

The raw data of microsatellite genotypes are available in the Supplemental File and Dryad: DOI 10.5061/dryad.17r4585.

Supplemental Information

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Genetic analyses reveal demographic decline and population differentiation in an endangered social carnivore, Asiatic wild dog

Shrushti Modi, Samrat Mondol, Parag Nigam & Bilal Habib✉

Deforestation and agricultural intensification have resulted in an alarming change in the global land cover over the past 300 years, posing a threat to species conservation. Dhole is a monophyletic, social canid and, being an endangered and highly forest-dependent species, is more prone to the loss of favorable habitat in the Anthropocene. We determined the genetic differentiation and demographic history of dhole across the tiger reserves of Maharashtra using the microsatellite data of 305 individuals. Simulation-based analyses revealed a 77–85% decline in the major dhole sub-populations. Protected areas have provided refuge to the historically declining dhole population resulting in clustering with strong genetic structure in the remnant dhole population. The historical population decline coincides with the extreme events in the landscape over the past 300 years. The study highlights the pattern of genetic differentiation and diversity of a highly forest-dependent species which can be associated with the loss of forest cover outside tiger reserves. It also warrants attention to develop conservation plans for the remnant surviving population of dholes in India.

On the backdrop of rampant global industrialisation, urbanisation, and agricultural intensification, long-term survival of most wild animals and their habitats are severely challenged by drastic reduction in available habitats through fragmentation[1]. In the most intensified form, fragmentation events create detrimental edge effects along the boundaries of habitat patches leading to restricted animal movement and gene flow, severed landscape connectivity and drastic retrogressive demographic changes in the long run[2]. Although species adapt differently to such pressures[3], generally animals having large body size with apex position in the food chain[4], low growth rates[5], wide home ranges[6], and habitat specialists[7] are at higher risk of facing the detrimental effects of fragmentation. In this regard, the mammalian carnivore guild is one of the most fragmentation-effected groups of species, making them the ecological indicators of landscape connectivity[8]. Large number of studies demonstrated the impacts of habitat fragmentation on carnivores (for example, ocelot[9]; African wild dog[10]; mountain lion and coyote[11]; gray wolf[12]) and established that habitat-specialist pack-living carnivores are more prone to degraded habitats owing to their smaller niche breadth, smaller range of dispersal[8] and allee effect[13].

Asiatic wild dog (*Cuon alpinus*) or dhole is a typical example of a pack living, habitat-specialist, apex carnivore. They are considered 'Endangered' by IUCN under criteria C2a(i) (small, declining and fragmented population with less than 2500 mature individuals) and are already at serious risk from habitat loss, prey depletion, disease transmission from domestic dog, human persecution and interspecific competition[14]. Their global population is approximately 4,500–10,500 with only 949–2,215 mature individuals with a decreasing population trend[14]. The Indian subcontinent is the major stronghold for the remnant dhole populations[14] distributed mostly within the forested areas of the Western Ghats and the central Indian landscape. Several smaller populations have been reported from north-eastern India, Eastern Ghats and the Himalayan region[15,16], but their long-term viability is under serious concern due to low population sizes. Throughout its distribution in India, this obligate forest-dwelling species is greatly affected by habitat loss (60% loss of its historical range)[17] mostly from agriculture intensification, urbanisation and developmental activities[14], leading to continuous insularisation of these populations along with restricted dispersal events. These fragmented populations may suffer from a reduction in genetic diversity[18] and genetic drift at a longer temporal scale, leading to possible strong population structure

Wildlife Institute of India, Chandrabani, Dehradun 248001, India. ✉email: bh@wii.gov.in

and inbreeding depression[19]. Despite the knowledge of these ongoing situations of dhole biology and conservation, appropriate studies to verify genetic diversity and differentiation patterns are limited[14]. To date, most of the studies have focused on their behaviour[20–22], occupancy[23], population pattern[24,25], genetics[26–28] at local/regional scales, still an in-depth understanding of population/demographic patterns are lacking.

In this paper, we investigated the patterns of genetic differentiation, diversity and demography in the dhole population across Maharashtra, a part of a larger landscape of Central India, having possibly the largest dhole population[29]. Using non-invasive genetic tools, we evaluated (1) the extent of dhole genetic diversity across all known dhole habitats in the state; (2) population structure of dhole in this area; and (3) demographic history of the major populations within Maharashtra. We addressed these questions using 12 microsatellite loci[26] surveyed in 305 individual dholes from six protected areas. Finally, we interpret the results in the lights of dhole ecology and historical changes on dhole habitat in the Central Indian Landscape.

Results

Genetic diversity. A total of 623 samples were collected from six protected areas during the study period (2016–2019). We identified 590 dhole faeces through species-specific molecular assay[27], attaining an amplification success of 94%. Using a panel of 12 microsatellite markers[26], we generated a dataset of 349 genotypes attaining a success rate of 59.1%. Out of them, 305 were identified as unique genotypes while 44 genotypes were removed as replicates from further analysis. Out of these, 101 individual genotypes were used in a previous study[26], while 204 individual genotypes from three protected areas (TATR, STR, NNTR) were identified in this study. These loci provided a cumulative misidentification rate or $PID_{(unbiased)}$ and $PID_{(sibs)}$ value of 1.09×10^{-10} and 1.06×10^{-4}, respectively, indicating a statistically robust value for dhole individual identification. Overall, the panel showed a low genotyping error rate where the mean allelic dropout rate was 0.040 per allele per locus, mean false allele frequency was 0.071 per allele per locus, and null allele frequency was 0.01, respectively. The genotyping error rate is within the threshold of 20% suggested for non-invasive population level studies[30,31]. The panel showed no evidence for strong linkage disequilibrium between any pair of loci. However, two to four loci from the panel were out of HW equilibrium in individual population, but not a single loci was found to be out of HW equilibrium in all populations (Supplementary Table 1)[26]. Mean pairwise relatedness was low for all the sub-populations in the four estimators (Supplementary Table 3).

Population structure. Our sampling strategy focused on maximum coverage of unique individuals across a relatively small region of dhole distribution to assess any possible dhole population structure. Bayesian clustering analysis with 12 microsatellite loci showed five distinct genetic groups (K = 5 clusters) (Supplementary Fig. 1a,b) (Fig. 1a). The ancestry coefficient (Q-matrix) indicated five different focal ancestry points, as presented in Fig. 1b. Majority of the individuals (n = 285, 93.5%) showed group-specific ancestries, while a few individuals from UKWLS (n = 9), NNTR (n = 9), MTR (n = 5), TATR (n = 5), STR (n = 4) and PTR (n = 2) showed mixed ancestry signals. Careful investigation revealed five genetic clusters i.e. PTR (n = 33), MTR (n = 35), NNTR (n = 90), TATR (n = 84) and STR (n = 54). Structure plot for k = 2 to k = 4 also followed the same pattern (Supplementary Fig. 1d).

The BIC value from DAPC analyses suggested k = 7 with the lowest value, after which there is a subtle difference in the BIC value (Supplementary Fig. 1c). The seven identified clusters represent four clusters overlapping between TATR and NNTR, respectively (Fig. 1c). Subsequent DAPC runs with a prior value of K = 5 showed the same pattern seen in the STRUCTURE analysis. sPCA analysis revealed highly significant global (p = 0.0001) but non-significant (p = 0.4327) local spatial structures (Supplementary Fig. 2), indicating strong signatures of between population separations. Assessment of three major global principal component axes (based on eigenvalue) (Supplementary Fig. 3) indicates strong structure among STR, NNTR and TATR-MTR-PTR with PC1, STR and TATR-NNTR-MTR with PC2 and MTR with other subpopulations with PC3 (Fig. 2), corroborating with the earlier results. Combinedly, we interpret that our sampling area has five genetic clusters. Two independent analyses of population differentiation indices (G'st and Jost D) reveal significant levels of genetic differences among these five clusters. The G'st values ranged between 0.22–0.40, with the highest differentiation found between MTR-STR (0.40) and MTR-PTR (0.40), respectively, and the lowest value between PTR-UKWLS(0.20). Table 1 shows the cluster-wise genetic differentiation values for both indices.

The summary statistics for the amplified markers (n = 12 loci) among the five clusters showed a higher mean number of alleles in NNTR and TATR (NNTRNa = 5.5 (SD 2.7) & TATRNa = 5.4 (SD 1.3), respectively) when compared with other three sub-populations (PTRNa = 4.3 (SD 1.5), STRNa = 4.9 (SD 1.1) and MTRNa = 3.4 (SD 1.1)). The highest observed heterozygosity was found in STR (Ho = 0.55 (SD 0.16)) followed by MTR (Ho = 0.50 (SD 0.26)), TATR (Ho = 0.49 (SD 0.20)), PTR (Ho = 0.45 (SD 0.22)) and NNTR (Ho = 0.39 (SD 0.17)), respectively. The mean allelic richness estimated from rarefaction method range from 3.35 (SD = 1.2) in MTR to 4.76 (SD = 2.2) in NNTR, with higher private alleles in STR and PTR (Supplementary Table 2).

Gene flow and effective population size. The BAYESASS results showed very low and non-significant gene flow among the genetic subpopulations corroborating the distinct population structure patterns for dholes. The highest value for gene flow was from PTR to UKWLS and the lowest between UKWLS and NNTR (see Table 2 for details). Two independent, effective population size estimation approaches showed low values, ranging from 6–16.3 across the dhole subpopulations, suggesting potential inbreeding (See Table 3). Based on our data on unique individuals from each subpopulation and calculated effective population sizes, we found a very skewed ratio of Ne/N in TATR (0.16), NNTR (0.18) and MTR (0.17) but a balanced value in STR (0.29) and PTR (0.35) (Supplementary Table 4). The estimates should be taken with caution due to the use of surrogate census population size. The inbreeding coefficient (Fis) value ranged between 0.005–0.296 (Table 3) among the

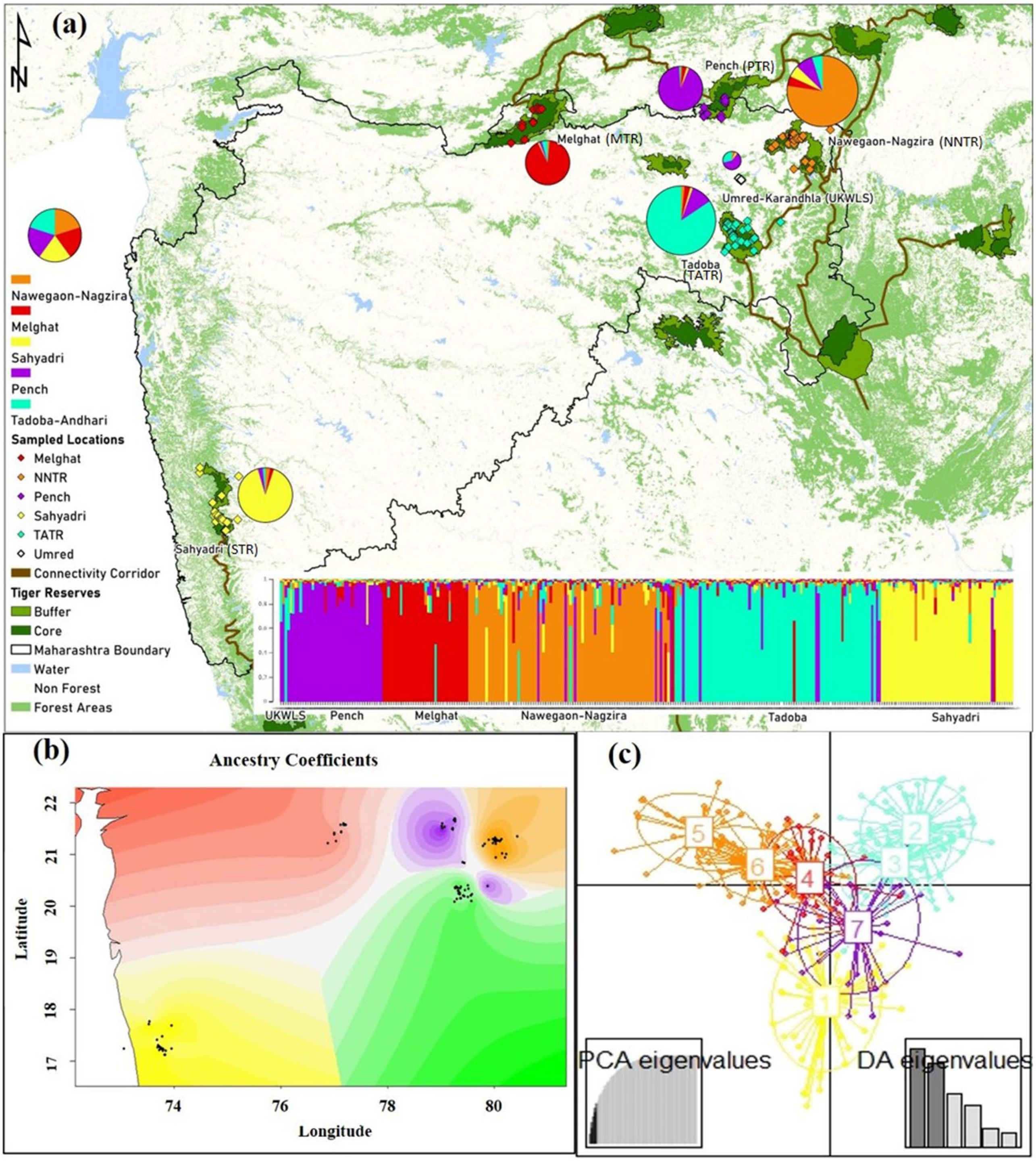

Figure 1. Dhole sampling and population structure across the study area. (**a**) shows the locations of the unique dhole individuals (n = 305) from the sampled protected areas (Nawegaon-Nagzira Tiger Reserve-NNTR, Melghat Tiger Reserve-MTR, Sahyadri Tiger Reserve-STR, Pench Tiger Reserve-PTR, Tadoba-Andhari Tiger Reserve-TATR, Umred Karandhla WLS-UKWLS) , along with population genetic structure of 305 dhole individual genotypes derived using a Bayesian clustering approach implemented in STRUCTURE. Each color represents a cluster and a single bar plot represents the individual. The-X axis represents the population while the Y-axis corresponds to the probability of assignment of an individual to each cluster. The pie chart presented for each protected area represents the respective proportion of genetic assignment in each population. The size of the pie-chart is proportional to the number of individual genotypes at each site. (**b**) represents the STRUCTURE ancestry coefficient (Q-matrix) through colour gradient for respective areas. (**c**) shows the genetic clusters (k = 7) from DAPC analysis. The TATR (cluster 2 and 3) and NNTR (cluster 5 and 6) populations show two overlapping clusters, making a total of five genetic subpopulations. Study area map was created using ArcGIS 10.3 (https://enterprise.arcgis.com/en/portal/10.3/use/get-started-with-maps.htm). (**c**) was generated using the package "adegenet" in R studio R Core Team (2019). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. (https://www.R-project.org/).

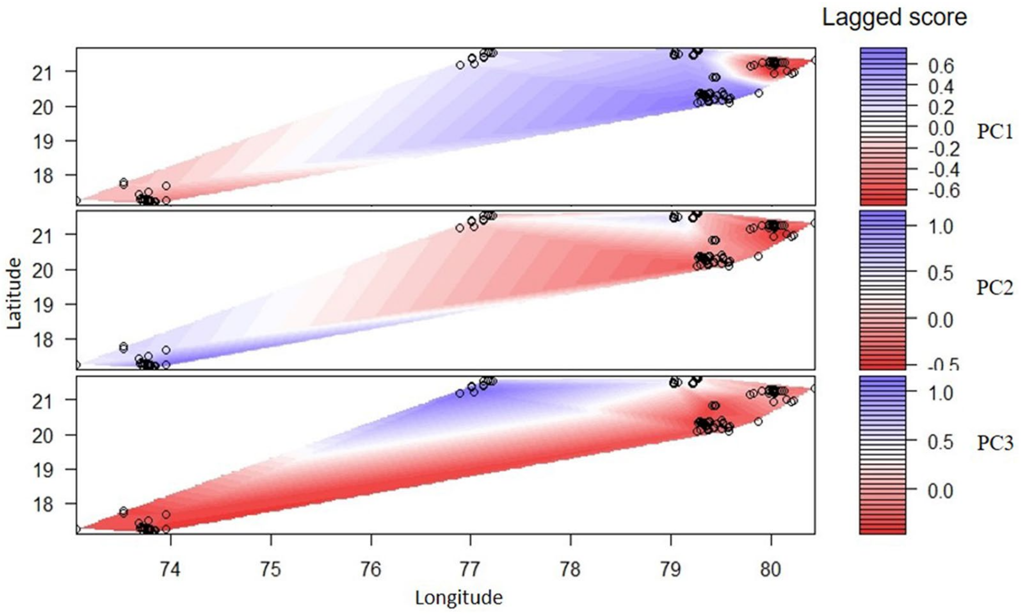

Figure 2. Results of genetic differentiation from sPCA analysis. The results are presented as heatmaps where higher differentiation corresponds to positive eigenvalue score of significant global genetic structure (presented as red colour), whereas lower values are shown as blue colour. The black circles represent the unique dhole individuals from different areas of this landscape. The figure was generated using the package "adegenet" in R studio R Core Team (2019). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. (https://www.R-project.org/).

G'st/D Jost	UKWLS	PTR	MTR	NNTR	TATR	STR
UKWLS		0.095(0.04–0.16)	0.229(0.16–0.32)	0.146(0.09–0.22)	0.097(0.02–0.20)	0.189(0.12–0.30)
PTR	0.203(0.11–0.33)		0.126(0.09–0.17)	0.186(0.14–0.24)	0.115(0.08–0.15))	0.183(0.13–0.23)
MTR	0.402(0.32–0.50)	0.297(0.24–0.35)		0.190(0.14–0.23)	0.170(0.13–0.20)	0.207(0.16–0.25)
NNTR	0.351(0.28–0.44)	0.337(0.29–0.40)	0.354(0.29–0.41)		0.139(0.11–0.16)	0.187(0.15–0.22)
TATR	0.227(0.12–0.38)	0.258(0.21–0.30)	0.333(0.28–0.39)	0.295(0.26–0.34)		0.219(0.18–0.26)
STR	0.394(0.31–0.51)	0.332(0.28–0.39)	0.404(0.35–0.45)	0.340(0.29–0.39)	0.370(0.32–0.42)	

Table 1. Pairwise value for G'st (lower diagonal) and Jost's D (upper diagonal).

A(horizontal row)/ B(vertical row)	UKWLS	PTR	MTR	NNTR	TATR	STR
UKWLS	**0.6891(0.0207)**	0.1647(0.0462)	0.0223(0.0207)	0.0228(0.0215)	0.0788(0.0402)	0.0223(0.0205)
PTR	0.0085(0.0082)	**0.9382(0.0234)**	0.0212(0.0160)	0.0093(0.0092)	0.0117(0.0110)	0.0110(0.0106)
MTR	0.0080(0.0079)	0.0133(0.0128)	**0.9490(0.0215)**	0.0095(0.0092)	0.0121(0.0114)	0.0081(0.0080)
NNTR	0.0038(0.0038)	0.0121(0.0097)	0.0144(0.0095)	**0.9525(0.0133)**	0.0061(0.0056)	0.0111(0.0064)
TATR	0.0039(0.0038)	0.0236(0.0114)	0.0062(0.0060)	0.0046(0.0045)	**0.9578(0.0140)**	0.0039(0.0038)
STR	0.0058(0.0057)	0.0086(0.0077)	0.0058(0.0057)	0.0062(0.0060)	0.0070(0.0065)	**0.9666(0.0136)**

Table 2. Results of gene flow analysis using BAYESASS. The posterior distribution values of migraion rates (m) with 95% CI is presented. Bold values represent the proportions of individuals derived from their source population. The direction of gene flow is from A to B in this table.

subpopulations. Careful investigation revealed a pattern where populations with lower Fis showed higher effective population sizes.

Detection of demographic changes. Both qualitative analyses revealed signatures of population decline in the dhole subpopulations. BOTTLENECK results showed significant heterozygosity excess for NNTR, TATR and STR populations under all the three mutation models (IAM, TPM, SMM), suggesting a loss of rare alleles

	PTR (n = 33)	MTR (n = 35)	NNTR (n = 90)	TATR (n = 84)	STR (n = 54)
Ne estimator	11.8 (7.8–18.6)	6.0 (2.9–10.4)	16.3 (12.7–21.0)	13.5 (8.9–20.0)	16.0 (10.6–25.1)
LDNE	12.2 (8.3–18.6)	6.0 (3.1–9.7)	16.6 (13.4–20.7)	11.8 (9.7–14.4)	18.4 (13.3–26.3)
Fis value	0.223	0.29	0.074	0.071	− 0.0005

Table 3. Estimates of effective population sizes (Ne) (C.I.-95%) and inbreeding coefficient (Fis) values at five sampled areas from LD approach.

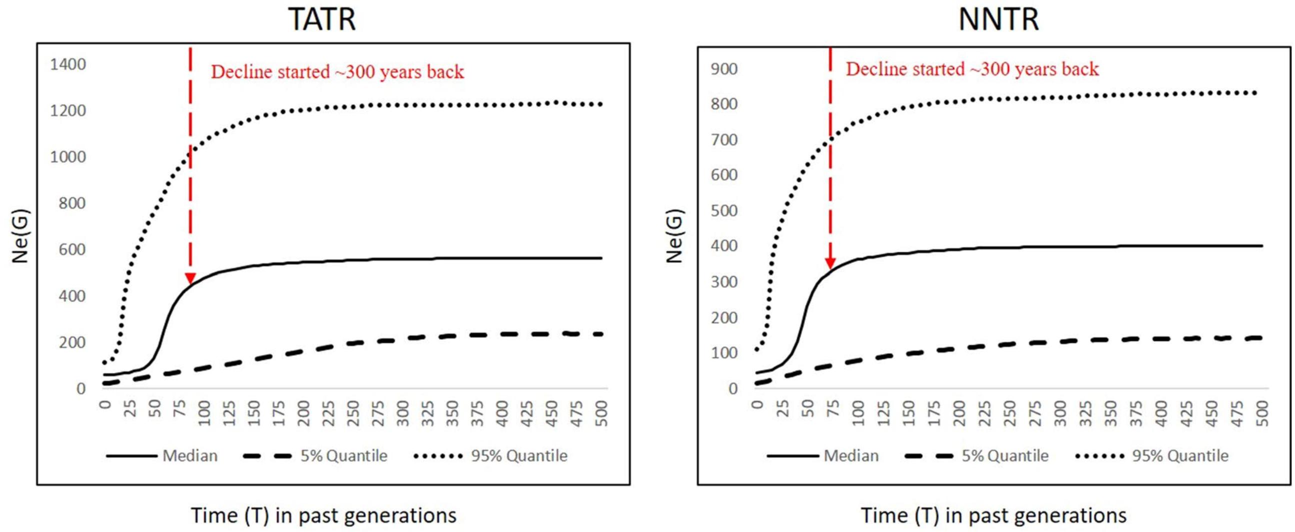

Figure 3. Demographic history of dholes in TATR and NNTR, Maharashtra through quantitative VarEff approach. Demographic changes have been presented as posterior distribution (median estimates) of the effective population sizes (0–500 generations ago) based on simulations with 12 microsatellite loci data from TATR (n = 84 individuals) and NNTR (n = 90 individuals). The decline timing has a median distribution value of ~ 300 years from present. The figure was generated using the package "VarEff" in R studio R Core Team (2019). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria.(https://www.R-project.org/).

during a possible population decline (Supplementary Table 5). Similarly, the Garza-Williamson index showed low values (compared to M_{cric} 0.68 for stable populations) in all populations (M-ratio$_{PTR}$- 0.27611 (SD 0.12); M-ratio$_{MTR}$- 0.35809 (SD 0.24); M-ratio$_{NNTR}$- 0.33504 (SD 0.15); M-ratio$_{TATR}$-0.28588 (SD 0.12) and M-ratio$_{STR}$-0.34592 (SD 0.09), indicating signals of population decline.

The quantitative VarEff approach showed a steep decline in the effective population size in both NNTR and TATR subpopulations. Results indicate a decline of 77–85% in dhole effective population size for NNTR and TATR subpopulations. The timing of this decline was quantified at ~ 60–90 generations ago, making it about 300–450 years before present (with five years of generation time for dholes) (Fig. 3). The posterior distribution of estimates of Log(Ne) in the past 500 generations are provided in Supplementary Fig. 4. The current effective population size ranged between 21–114 (median 58) for TATR and 14–110 (median 45) for NNTR at a 95% confidence interval.

Discussion

Generating detailed information on dhole population parameters at the landscape scale is highly challenging due to their obligate forest dependency, elusive nature and sensitivity towards anthropogenic activities. Therefore, dhole research has generally been focused on local habitat scales (for example, behavioural studies by[20,22,32], population patterns by[28]) or at their distribution and occupancy using standard approaches[33]. The present study is perhaps the most exhaustive primary information on dhole genetics and population patterns across the species distribution to the best of our knowledge. We adopted a multidisciplinary approach involving field sampling, genetic information, and multivariate & Bayesian analytical frameworks to address spatial genetic patterns and demography of the largest dhole population in the central Indian landscape. This region is currently facing significant changes in land use pattern from rapid urbanisation, expanding agriculture, infrastructure development, acquisition of minerals and economic growth[34,35]. Our results from this study thus have important conservation/management implications for dholes and their habitat.

Firstly, multiple genetic analyses with landscape-scale microsatellite data reveal five distinct dhole genetic clusters. Out of six sampled areas, the five major tiger reserves (MTR, PTR, TATR, NNTR and STR) represents the five clusters. While the UKWLS a known connecting habitat between PTR and TATR[36] showed a mixture of genetic signals from the four major clusters (TATR, NNTR, MTR and PTR). The genetic clusters were clearly separated with very few mixed genetic signals in UKWLS (Fig. 1a). Despite any assignment cluster for UKWLS, it was included further in connectivity analysis due to its strategic geographical location in the landscape acting as a connecting link[36]. However, such pattern is not surprising as dholes are highly forest-dependent and short-range dispersers[33]; this situation highlights their vulnerability in the current scenario of continuing land-use change and urbanization. Similar group-living species have been reported to show genetic differentiation due to their adaptations to specific habitat, group cohesion and local philopatry[37]. For example, social canids such as gray wolf, African wild dog have also shown higher genetic differentiation in fragmented landscapes[38]. Contrary to this, other co-occurring large carnivores (tiger[39]; leopard[40]) and omnivore (sloth bear[41]) showed much less genetic differentiation, possibly due to longer dispersal capabilities resulting in higher gene flow. Given the small population size of dholes across their range[14] , and possible genetic differentiation (based on the results of this study), the species face potential demographic impact[19,42] Kamler et al.[14] suggested that dholes require an area five times larger than tiger for long-term viability, which seems to be improbable in the current scenario. Thus, it will be critical to maintain population connectivity through corridor restoration and facilitate gene flow at the landscape scale. The NNTR and TATR clusters show a comparatively higher number of first-generation migrants with respect to other sub-populations. One of the major reasons behind such a pattern can be the larger pack size owing to low tiger density in NNTR[43] and higher turnover in TATR due to higher tiger density. In low tiger density areas, breeding opportunities reduce because of the larger pack size, required to suppress the recovery of top-predator, while in high tiger density areas, immigrants often fail to establish themselves hence, contribute less to the gene pool. These dynamics of pack size and top-predator density influence the genetic structure of a population.

Our demography analyses with two qualitative approaches indicate strong decline in dhole population size for all five genetic subpopulations, as expected from fragmented and small, isolated populations[44]. The quantitative approach with VarEff revealed a 77–85% decline in NNTR and TATR dhole populations, respectively. The magnitude of decline for dholes corroborates with other co-occurring large carnivores in this landscape. For example, earlier studies have shown a 90% and 98% decline in leopard and tiger population in the central Indian region, respectively[45,46]. The relatively less decline for dholes compared to other larger carnivores is possibly due to fewer demands of dholes as trophies/illegal wildlife trade. Tiger and leopard body parts (pelt, bones, claw, meat, fat, whisker) are highly sought products in trans-national illegal wildlife trade[47], whereas dhole populations have faced historical pressures from bounty hunting and human persecutions as vermins during British colonial period[14]. However, it is difficult to validate these decline patterns with other information as no robust quantitative data on actual population size (both historical and current) is available[48]. Another important finding is the relatively old timing of decline for dholes. Our results suggest a median dhole decline timing of ~ 300 years in both NNTR and TATR, much older than tiger/leopard decline timing in central India (tiger- decline ~ 200 years ago[46], leopard- decline ~ 125 years ago[45]). This could be explained by a combination of habitat loss driven population decline in historical times followed by hunting during the British era[29]. The study done on global land-use change over the last 300 years have also estimated a forest loss of 40% for the Indian sub-continent during the last century[49,50]. Sharma et al.[51] showed that this landscape had experienced a major change in land-use patterns during the last 300 years, leading to ~ 77% loss of forested habitats to agricultural area and urbanization. Rangarajan[52] also reported severe fragmentation of historically contiguous habitats of the Central Indian Highland during the last few centuries. Subsequently, over-exploitation of teak started during the early British period (1750–1990) for navy and railway lines which further resulted in the conversion of forests into commercial plantation by large scale clearing[53,54], thereby further reducing the available habitats for dholes. We feel that such drastic changes possibly had severe impacts on the population size of obligate forest-dwelling dholes. Although comprehensive data on the exact effects of such large-scale habitat loss on dhole population size is lacking, this available information suggests that continuing habitat fragmentation starting since last 300 years coupled with massive hunting pressure during colonial bounty-hunting rules resulted in dhole population decline over a longer time..

One of the most important aspect of this study is the assessment of inbreeding status (Fis value) and effective population size (Ne) of dholes which are critical population parameters, and summarise the history of any population[55]. For both NNTR and TATR populations (relatively higher population size compared to the other areas), the Ne is approximately 20% of the total population (Ne/N ratio of 0.16 and 0.18 for TATR and NNTR, respectively) which is similar to 0.11 across different taxa[56]. Such low values for Ne are not unusual and have been earlier described in social animals with dominance hierarchy (for example, lions[57], African wild dog[58], dwarf mongooses[58]) as well as in endangered species with small population sizes[59]. However, the Ne values obtained from VarEff were comparatively higher and probably realistic than the linkage disequilibrium based approach as this approach can substantially underestimate the Ne in inbred populations[43,57,58,60]. The number of individuals observed in each population could be related to the sampling size which is a limitation in our study due to unavailability of population estimates. We have only used the population size from this study as a surrogate to calculate the Ne/N ratio which is independent of Ne calculation. In addition, we also found out that inbreeding coefficient value (Fis) were different for each population and indicated an inverse relationship with Ne. For example, dhole data from MTR showed the highest Fis value and lowest Ne, whereas STR showed the lowest Fis and high Ne value. This pattern makes sense for a species with social dominance hierarchy where only the dominant member of the pack has highest mating opportnities (thus low Ne) and will have more inbred individuals in a small group or population (high Fis).

Finally, our results from this study also showed that relatively large dhole populations such as NNTR and TATR still retain reasonable high genetic variation despite the severe decline and strong population structure. The genetic variations of NNTR and TATR are comparable with other social canid species such as African wild dog from Kruger National Park[61]. However rest of the populations (need urgent management interventions (possibly in the form of translocations as well as better habitat connectivity) to increase the genetic variation and ensure the future survival of the populations in this landscape as a whole. We acknowledge the chances of bias in our result of individuals identified with low dhole recaptures, which can be correlated with complex interactions between our specific sampling strategy and relatively low amplification success rates from field-collected faeces. Since our field sampling strategy focused on maximum coverage within six protected areas of central Indian dhole distribution and was conducted only once, during which we surveyed all possible latrine sites and collected only fresh samples for DNA analyses. Because of this, we might have missed recapturing the same individuals, thus giving us low recapture rates.

This can be dealt with a genetic capture recapture method in future with more intensive sampling strategy. The high difference in the expected and observed heterozygotes could be a complex combination of species-population marker scenario as also found in other canid (gray wolf, coyote, golden jackal) studies where cross-species dog primers were used[62,63].

Conclusion

Despite sharing most of their current range within India with tiger and leopard, ecological information on dholes are still inadequate for appropriate management planning. Historical information and our quantitative data indicate that the last 300 years has brought drastic reductions in dhole distribution and population size[64] and some cases local extinction[14]. Initiation of relentless tiger conservation efforts since the 1980s has helped the species to survive in most of its existing range[65], but unlike tigers, the dhole population trend continues to decrease globally. Currently, the major strongholds of the species are the Western Ghats (Karnataka) and central India (Maharashtra)[48], where focused conservation efforts are urgently required. With the ongoing habitat fragmentation scenario, dhole-specific threats (habitat loss, prey depletion, disease transmission, human persecution) must be addressed to ensure the long-term persistence of the species. We hope that the results and suggestions from this study will lead to generation of critical information on dhole genetics from Central Indian Landscape which will aid in understanding the effects in the similar landscape across dhole distribution range. We hope the information will help in developing informed strategies for conservation.

Methods

Research permissions and ethical considerations. Permissions for fieldwork and sampling were granted by the Maharashtra Forest Department (Permit No. 09/2016). This work did not require any approval from the ethical committee due to its non-invasive nature.

Study area and sampling. We conducted this study in Maharashtra, which retains one of the major dhole populations in central India. We sampled major known dhole habitats across the state, covering five tiger reserves: Pench Tiger Reserve (PTR), Melghat Tiger Reserve (MTR), Sahyadri Tiger Reserve (STR), Tadoba-Andhari Tiger Reserve (TATR) and Navegaon-Nagzira Tiger Reserve (NNTR) (Fig. 1a). Apart from these areas, we also sampled surrounding regions of Umred-Karandhla Wildlife Sanctuary (UKWLS). This mosaic of the tiger reserves and surrounding regions is already established as tiger corridors[36], making it important to see if obligate forest-dwelling dholes are also using the same corridors. STR is completely disconnected from all other sites (Fig. 1a). The remaining areas form a complex network of habitat patches, where the remaining sites are known to have varying degrees of habitat connectivity[36]. MTR is an exception to this complex as it does not share direct connectivity with the tiger reserves in this complex. All of these areas are characterized by dry deciduous to moist deciduous forests[66].

We sampled the entire region between January 2016 to April 2019, covering PTR (257.3 km^2), MTR (1500.49 km^2), NNTR (152.8 km^2), TATR (627.5 km^2), STR (1166 km^2) and UKWLS (189 km^2), Maharashtra. Each site was sampled intensively once for dhole scats. Through foot and vehicle surveys, extensive scat sampling resulted in 623 scats from 82 latrine sites across all seven study areas. We only collected fresh samples during field surveys, where one bolus/ scat was stored in butter paper following approaches described in Biswas et al.[67]. We also recorded the GPS coordinates and other associated field information (substrate, track marks) for each sample. In the field, the samples were temporarily stored in a large box containing silica gel. In some cases, we sprayed a small amount of absolute ethanol to minimize fungal growth[68]. The samples were kept in the field for a maximum period of 10 days. Once transferred to the laboratory, all the scat samples were stored in a – 20 °C freezer till further processing.

DNA extraction and species identification. We performed DNA extraction from all field-collected scats using already established approaches described in Modi et al.[27]. In brief, we either swabbed twice (samples with no dust) or scraped (samples covered with dust) the top layer of the samples with sterile swabs or blade, respectively. They were lysed overnight in a lysis buffer at 56 °C, and extraction was performed following QIAamp DNA Tissue Kit (Qiagen Inc, Hilden, Germany) protocol. Final elution was performed twice in 100 µl of 1X TE buffer, and the DNA was stored at – 20 °C for long-term use.

We conducted molecular species identification using dhole-specific mitochondrial DholespID-F/R primers described in Modi et al.[27]. PCR reactions were performed in 10 µL volumes with 4 µL of hot-start taq mix (Qiagen Inc, Hilden, Germany), 4 µM BSA, 0.5 µM of primer mix and 3 µL of DNA extract. PCR conditions included an initial denaturation (95 °C for 15 min); 50 cycles of denaturation (94 °C for 30 s), annealing (50 °C for 30 s) and extension (72 °C for 35 s); followed by a final extension (72 °C for 10 min). Negative and extraction controls were included to monitor contaminations. Species ascertainment was done through visualization of dhole-specific bands (236 bp) in 2% agarose gel. All the experiments were conducted in Conservation Genetics Lab in Wildlife Institute of India, Dehradun.

Individual identification. For individual identification from the confirmed dhole scats, we used the earlier validated 12 microsatellite loci panel described in Modi et al.[26] (Supplementary Table 1). We performed PCR reactions in 10 µl reaction volumes containing 4 µl of Multiplex master mix (QIAGEN Inc., Hilden, Germany), 4 µM (2.5 µl) BSA, 0.5 µM of primer mix and 3 µl of DNA extract with PCR conditions including initial denaturation (95 °C for 15 min); 50 cycles of denaturation (94 °C for 30 s), annealing (50 °C for 30 s) and extension (72 °C for 35 s); followed by a final extension (72 °C for 10 min)[26]. Negative and extraction controls were included to monitor contaminations. Amplified products were mixed with HiDi formamide and LIZ 500 size standard (Applied Biosystems, California, United States) and genotyped in an ABI genetic analyzer (Applied Biosystems, California, United States). We scored the fragment lengths manually using the same reference sample and following stringent criteria described in Modi et al.[26]. All samples were genotyped three independent times to ensure good data quality for subsequent analyses. We have also included 101 individual genotypes from our previous study[26] collected from five protected areas (MTR, TATR, PTR, NNTR, UKWLS) along with the newly generated data for further analysis.

Data analyses. To generate the best quality data for analyses, we prepared consensus genotypes of each locus following the multiple tube approach combined with quality index protocol described in Modi et al.[26]. We only considered the genotypes, which produced data for atleast seven out of 12 loci in the consensus[26] The quality index threshold of 0.66 per loci, while the mean quality index of 0.75 across loci was set for the samples to be considered for downstream analyses. We used MICROCHECKER v 2.2.3[69] to determine large allele dropouts as well as genotyping error estimation module of GIMLET[26,70] to calculate overall genotyping error rates (allelic dropout and false alleles). We used FreeNa (Chapuis & Estoup, 2007) to determine the frequency of null alleles (NAs), which estimates the NA frequency using EM algorithm (Dempster, Laird & Rubin, 1977). We removed all genetic recaptures using the identity analyses module of CERVUS[71], allowing up to two mismatches and calculated the cumulative $P_{\mathrm{ID(unbiased)}}$ (probability of identity) and $P_{\mathrm{ID(sibs)}}$ value[72] using GIMLET[73]. We estimated the allelic richness using the rarefaction approach in HP-RARE considering the uneven sample size of populations. We used GENPOP and ARLEQUIN[74] to check deviations from Hardy–Weinberg equilibrium (HWE) and linkage disequilibrium (LD).We also conducted relatedness test using the pairwise relatedness estimators TrioML, QGM, LRM and DyadML incorporated in COANCESTRY v1.0.1.8 to avoid any bias due to related individuals. Both TrioML and Dyad ML considered genotyping errors and had the smallest variance.

Inferring population structure. To infer any possible genetic structure of dholes across the sampled areas, we used a combination of Bayesian clustering and multivariate analyses. These analyses were conducted for only those populations with data from at least ten different individuals.

We implemented the Bayesian clustering approach through program STRUCTURE v.2.3.4[75], where 10 independent runs were performed for a range of population values (K = 1 to 10) with 100,000 burnin and 500,000 iterations. The models were run with admixture models considering correlated allele frequency. The optimal number of clusters was determined by the deltaK approach[76] implemented in STRUCTURE HARVESTER[77]. The admixture proportion of individuals over ten replicates were averaged using CLUMPAK[78]. The ancestry coefficient of the individuals produced by STRUCTURE was interpolated on a map using the R package tess3r[79].

Further, we used the program Discriminant Analysis of Principal Component (DAPC)[80] to identify genetic clusters in our data. This is a multivariate analytical approach where no spatial information is required, and the population does not require to be under Hardy–Weinberg Equilibrium[80,81]. The genetic data is transformed into principal components, followed by clustering using the discriminant function to define a group of individuals with minimum within-group variation and maximum between-group variations. We conducted the analyses using adegenet package 2.1.1 in R studio 1.1.453 (R Development Core Team 2018), where an optimal number of clusters was determined through the Bayesian Information Criterion[80], and number of clusters was assessed using find. clusters *dapc function in R.*

Finally, we used another multivariate method implemented in program spatial Principal Component Analysis (sPCA) that investigates cryptic spatial patterns of genetic variability using georeferenced multilocus genotypes[82]. sPCA incorporates the spatial information along with the genotype data to ascertain local and global patterns of variations[83]. The global pattern (positive autocorrelation) would differentiate between two spatial groups, whereas the local pattern (negative autocorrelation) would determine the genetic differences among neighbours. The analysis was carried out using the nearest neighbour as the connection network. The variance was plotted against spatial autocorrelation (Moran's I)[84] to estimate any spatial structure in the genetic data visually. We used the Monte Carlo test with 10,000 iterations to statistically test global and local spatial structure.

Genetic differentiation among dhole populations. We estimated genetic differentiation through different indices (G'st and Jost D)[85,86] using the R package DiveRsity 1.9[87] in R studio 3.1. We used both the differentiation indices to elucidate the asymmetric migration[88] and differentiation among the sub-populations[89,90].

Assessment of gene flow among different subpopulations. We used a Bayesian approach implemented in BAYESASS ver. 3.0.3[91] to infer the contemporary migration rate (m) among the detected subpopulations. This approach detects recent, low immigration rates in a population based on the genotype disequilibrium relative to the sampled populations without assuming HW equilibrium within the populations. The run parameters included 3×10^6 iterations and 10^6 burn-in with sampling at every 2000 iterations. Delta values were adjusted to maintain an MCMC state change acceptance ratio of 20–40%. We averaged the results of multiple runs for best model fit, as indicated by the Bayesian deviance measure[92].

Effective population size (Ne). We used the program Ne estimator v.2.01[93] to estimate the N_e from genotype data. We used the random mating model and the following critical values (P_{crit}): 0.05, 0.02 and 0.01 and jackknife 95% confidence interval for our analyses. We calculated the Ne for each subpopulation separately based on the number of putative clusters determined with a critical value of 0.02. We further used LDNe[94], which also estimates the effective population size using the linkage disequilibrium approach with bias correction.

Demography analyses. We used qualitative and quantitative approaches to determine past demographic patterns of dhole subpopulations based on population substructure analysis results. For qualitative analysis, we used two different summary statistics-based approaches to detect any signal of population decline in dholes. These approaches are the Ewens, Watterson, Cournet, and Luikart method implemented in program BOTTLENECK ver 1.2.02[95] and the Garza-Williamson index/ M-ratio approach implemented in program ARLEQUIN[74]. For BOTTLENECK, simulations were performed under three mutation models: infinite allele model (IAM), single stepwise model (SMM), and two-phase model (TPM). For the TPM model, 30% of multi-step mutation events were allowed during the simulations. This method detects departures from mutation-drift equilibrium and neutrality, which can be explained by any departure from the null model, including selection, population growth, or decline. The Garza-Williamson index uses data on the frequency and the total number of alleles, and the allele size difference to investigate population decline.

Further, we used R package VarEff 1.2[96] in the R software version 3.1 to quantify dhole demographic patterns. This approach uses a coalescent framework to estimate the variation in effective population size (Ne) from present to ancestral time and determines the time of population decline from genetic data. We performed the analysis assuming the stepwise mutation model (SMM)[97] with a generation time of 5 years for Asiatic wild dogs[14]. We used the SMM model to describe the mutation process for microsatellites in a more wholesome way[98]. We considered a constant mutation rate of 3.5×10^{-3} per generation as described for canid microsatellites[99] over the past 1000 generations. The models were set with parameter DMAXPLUS value of 4 and 6 from the allele frequency histograms (maximum distance observed with a frequency ≥ 0.005 at 4 and 6)[96], along with prior values for Ne (parameter NBAR, range provided by theta), and the variances of the prior log-distributions for Ne (parameter VARP1, value of 3) and time intervals with constant population size (parameter VARP2, value of 3). The prior correlation coefficient between successive population sizes (parameter RHOCORN) was set to zero and Jmax value set at 2. The run parameters included the number batch to 10,000 length and space batch to 10, acceptance rate of 0.25 with a diagonal of 0.5. The demographic analyses were performed for only NNTR and TATR populations as they had adequate sample sizes.

Data availability

The microsatellite dataset used for different analyses in this study is available from the corresponding author on reasonable request.

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Author contributions

B.H. and S.Modi. conceived and designed the experiments. S.Modi. conducted field sampling, performed the experiments. S.Modi. and S.M. analyzed the data. B.H., P.N. and S.M. contributed resources. S. Modi., S.M., P.N B.H. have authored and reviewed draft of paper and approved the final draft.

Competing interests

The authors declare no competing interests.

Additional information

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Correspondence and requests for materials should be addressed to B.H.

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