Molecular Tools For Biodiversity Conservation

Unravelling Cat Mysteries

Shomita Mukherjee and Uma Ramakrishnan

Molecular techniques are gaining importance in biodiversity conservation in India. They are especially beneficial in the case of rare species with cryptic habits that make them difficult study subjects when using conventional field techniques. Molecular tools can be used to decipher distributions and population connectedness in fragmented habitats and identify populations of immediate conservation concern. We discuss these with case studies on some cat species in India.

Introduction

In the current global scenario with a burgeoning human population, the fight for space is reaching critical proportions in many regions of the world. Natural habitats are rapidly being converted to make way for human requirements resulting in an unprecedented loss of biodiversity. Additionally, developmental activity, especially since the industrial revolution, is pushing species extinctions to catastrophic levels and threatening the productivity of ecosystems [1, 2]. Humans are not invulnerable to this fallout since vital resources such as clean air, water, and food are intricately linked to the health of ecosystems. As a realization, climate change has become a much-dreaded catchword, and biodiversity conservation is regaining importance. The field of ecology/biodiversity/natural history has come a long way in India and worldwide during the last several decades and has been transformed from a largely observational, field related exercise to a blend of various subjects. Conservation biology has gained immensely from major technological advancements ranging from molecular genetic tools to space technology.

Information on ecological aspects of several species that could

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not be obtained earlier due to their cryptic nature or rarity is now easily accessible through these modern techniques. Such information is useful in conservation planning with respect to current actions required and future directions to be taken. In this article, we discuss molecular ecology and conservation genetics that have contributed immensely to unraveling patterns and understanding the processes behind species histories and geographic distributions, with a focus on cats in India.

Conservation Genetics: An Overview of Possibilities

Species distributions are fundamental to understanding their ecologies and planning their conservation. With molecular markers (such as stretches of DNA, RNA or amino acids), it is now possible to determine the distributions of species that are difficult to study and to also refine the existing knowledge about other species. Aspects of ecology such as diets can also be determined. Apart from this, evolutionary relationships among species (phylogeny or evolutionary tree), which were earlier determined using morphological features such as body form and shape, can now be constructed using genetic data. Such phylogenetic trees, along with fossil data, can be used to estimate the time when species diverged from each other. Combining data on distributions (biogeography) and evolutionary relationships between species with research in the field of palaeontology can enable us to infer the climatic conditions and habitat characteristics during some past period that have led to current species distributions. With information from past and current distributions and prevailing climatic conditions, one can predict how the species would fare in various climate change scenarios in the future, allowing future conditions to be considered during conservation planning to avoid catastrophic extinctions.

Moving from species to populations, genetic information can be very useful in managing different populations of a species. One can find out whether different populations are genetically connected or isolated from each other, and whether the lack of con-

nectivity if observed, arose from specific natural or human-induced barriers. This field of study known as 'phylogeography', examines genetic variation over geographical space. Patterns are observed from current genetic samples and processes are inferred, given the backdrop of ecology and evolution, and also considering the geography of the regions over which populations are distributed. At the level of populations within species, one can also examine the genetic diversity amongst populations and find out whether there are unique or critical populations that require special conservation. The field of population genetics can inform us about the extent of inbreeding, migration between populations, and expected impacts of reduced population size on genetic diversity.

Several analytical tools are common to these various spatial and temporal scales of focus but the molecular markers used are different, depending on the information they provide, and how resistant or amenable they are to mutation. For instance, some DNA sequences are the same within species but differ between species, and these can be used to study relationships among species – phylogeny – whereas, some stretches are variable within species and hence provide access to information on populations within species – phylogeography. Yet other regions are useful for determining the differences between individuals in a population and are used in population genetic studies.

In order to carry out studies using molecular markers, samples have to be collected from the organism that is being studied. The sample collected may be leaves in the case of plants, entire specimens in the case of small organisms such as invertebrates or non-endangered larger organisms (e.g., fish species), or pieces of tissue from larger dead animals. When endangered or cryptic animals are the focus of study, one often relies on fecal samples (dung/scat/pellets) or hair or feather samples. Fecal samples are easier to locate than cryptic species and are a treasure trove for ecologists studying such species. The outermost layer of fecal samples contains cells that are shed from the colon of the individual. These cells contain DNA which can be used for identifying

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the species and determining the sex of the individual. Further, it can be used to determine relatedness of this individual to others in that population. If the study organism is a carnivore, the inner part of scat (fecal sample of a carnivore) contains remains of the prey that the organism has consumed. Therefore, the profile of the carnivore's diet can be determined by extracting DNA from the remains of the prey in scats.

Using Molecular Genetic Techniques to Study Cats

We will now discuss how molecular tools have been used to study distributions, phylogeography, and population genetics of one family – Felidae (Cats), which is extremely well represented in India, with 15 of the 41 globally distributed cat species found here. Molecular tools have allowed for a phylogeny (evolutionary tree) of cats to be constructed [3, 4, 5]. Reconstructing the phylogeny of cats has been a challenging task because morphological features are extremely similar across cat species, and most species diverged over a very short time duration of approximately one to three million years ago [4, 6].

All cat species are rare and cryptic, and most are small and nocturnal. This makes them very difficult study subjects. But, since all are severely impacted by habitat loss, it is imperative to gauge and monitor their conservation status. Hence, ecologists have resorted to studying them using indirect methods such as placing camera traps for determining their distributions and densities (in some species) and collecting their scats to study diets. The former is extremely labor intensive and hence covering large areas at a time is logistically challenging. Scats of several similar sized cat species (or even of cat and some canid species) are not possible to differentiate visually but can be differentiated using molecular tools.

In the following sections, we will describe the methods we used for studying distributions, phylogeography, and population ecology of some cats in India using scats. Scats were collected in alcohol to prevent any degradation, and depending on the ques-

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tions in the study, either a portion of the scat or the entire scat (comprising of several pieces) was picked. For addressing questions related to diets, the entire scat was picked while for phylogeographic and population genetic studies, a small portion was picked. Since scats are used as a means of communication in cats, an intensive collection of entire scats could potentially disrupt their behavior and hence collection should be regulated (e.g., collect samples from an area for a week and then move to another area) to minimize disturbance. On encountering a scat, the collection vial was prepared with appropriate records of date, sample ID, and GPS coordinates. Photographs of the scat with the GPS showing coordinates and a scale (*Figure* 1) were taken. The scat was then gently nudged into the vial using a stick and later, all scats were coated with 70–100% Ethanol. In the laboratory, DNA was extracted using commercially available stool kits and PCR[1].

Scats had to be first assigned to a species. This was done by amplifying[2] a specific portion of DNA (called 16s rRNA region of mitochondrial DNA) that is present in all species of cats. Although this segment of DNA is present across species, there are different mutations in different species. By sequencing the regions, one can determine which cat species the scat belongs to.

[1] Polymerase Chain Reaction: used to make multiple copies of target DNA primers were designed according to the requirement.

[2] Making multiple copies of DNA using PCR.

Distribution of Cat Species in India

We assigned the scats we collected to different species. *Table* 1 shows the results of several years of scat collections across India and the outcome of such scat assignment analysis. This is only a demonstration of how the technique can inform us about distributions of rare taxa. For example, a couple of scats collected in Kanha Tiger Reserve in Madhya Pradesh and BR Hills in Karnataka were found to be of Rusty-spotted Cat, a species not reported from these areas earlier. On the other hand, there were unsubstantiated reports of Fishing Cat from coastal Kerala. A survey in this region including scat analysis and interviews with local people suggested that there is no current evidence of the species in the region, and it perhaps does not exist there. In Eaglenest Wildlife Sanctuary, in Arunachal Pradesh, scat analysis supported evidence from camera trap data of Leopard Cat being the most abundant cat species. If carried out systematically with the objective to determine distributions, this technique could provide important information to inform conservation.

Comparative Phylogeography of Two Small Cats in India

We also compared the geographic distributions and genetic variations in two widespread and similar sized species of wild cats – Jungle Cat and Leopard Cat – in India. Their comparative phylogeography showed some unexpected results [7]. The Jungle Cat, weighing on average 5 kg in India, is a short-tailed (tail measuring less than 40% of its head and body length), long-limbed and long-eared cat with a uniform coat except on the legs and tail, which are stripped. The Leopard Cat, in India, weighs on average 3 kg and has a medium sized tail (measuring about 50 to 60% of its head and body length) with a strongly patterned (rosettes and spots) coat and relatively shorter limbs than the Jungle Cat (*Figure* 2). These two species are the most widespread among small wild cats in India. At the time of the study, known Jungle Cat distribution was contiguous across India, and the known Leopard Cat distribution though widespread had a hole in its distribution in

	Arid	Semi-arid	Western Ghats	Himalayas (Eastern and Western)	Mangroves /Inland wetlands	Total
DNA extraction	66	21	27	468	114	696
*Success in PCR amplification (%)	33 (50)	14 (67)	14 (52)	304 (65)	37 (33)	402 (58)
Jungle cat	12	10	3	0	11	36
Fishing cat	0	0	0	0	19	19
House cat/Asiatic wild cat	8	0	3	1	0	12
Leopard cat	0	0	4	295	3	302
Rusty-spotted cat	0	0	1	0	0	1
Snow leopard	0	0	0	1	0	1
Clouded leopard	0	0	0	1	0	1
Golden cat	0	0	0	5	0	5
Unresolved	4	1	3	0	4	12

Table 1. A summary of species assignments from DNA analysis of scats collected across various biogeographic regions in India.

* *Not all scats have good quality DNA that can be amplified and since some scats could belong to other carnivores like canids and the cat specific primers will not amplify the DNA from them. Note that the scats from mangroves/wetlands have low success. This is because the outer layer of scats that contain the predator DNA get washed away in the wet conditions in these regions.*

Central India, where its occurrence was ambiguous [8, 9]. However, the population was contiguous. Going by this distribution, we expected similar genetic patterns for both species. Despite the contiguous distribution for the two cats, various subspecies have been described based on morphological features (e.g., coat color, pattern). For the Jungle Cat, four subspecies are described, one each from the Himalayas, Northern Peninsular India, Southern
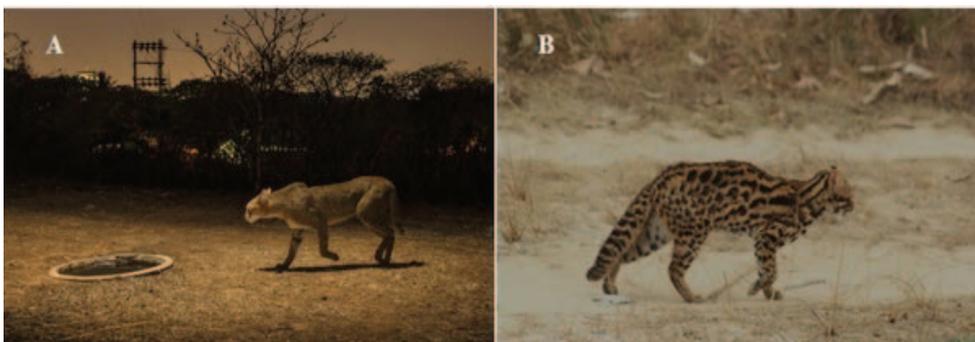
Figure 2. **(A)** Jungle Cat **(B)** Leopard Cat. Note the difference in size of tails of the two species. (Image credits: Jungle Cat: Nayan Khanolkar; Leopard Cat: Prafull Choudhary)

India, and the Thar Desert, while two have been described for the Leopard Cat, with one occurring in the western Himalayas and the other through the rest of the country [10]. We also wanted to explore how the subspecies compared with genetic subdivisions.

Scats were collected from various biogeographic zones in India since both species were believed to be distributed across the country. We found that the Jungle Cat showed high genetic diversity and genetic continuity with relatively low genetic differences between biogeographic zones. The same level of genetic differences was seen among the subspecies as well. Genetic continuity or the lack of it (called genetic differentiation) can be inferred by how much difference there is between the genetic compositions of individuals from different areas. If individuals from different areas are not similar genetically, the areas are said to show genetic differentiation. We found that the Leopard Cat had low genetic diversity, and the population in the Western Ghats was highly genetically differentiated from the populations in the Western and Eastern Himalayas and Eastern India, and the subspecies classification did not match the genetic data. This huge genetic differentiation between the Western Ghats population and the rest was surprising, given that they were supposed to be distributed almost across India. This indicated that perhaps, in reality, they were not continuously distributed. We did not find any Leopard Cat scat in Central India, even from areas where they were shown to be distributed. Was our sampling not adequate or were they really not found there at all? To confirm, we conducted an-

other analysis that can predict where a species could occur, based on the environmental conditions that prevail in the places where they do occur. This revealed that the climatic conditions in Central India were not suitable for the Leopard Cat which is perhaps limited by high summer temperatures. Regions with highest temperatures exceeding 40° Celsius had an extremely low probability of harboring Leopard Cat. This is just a model that needs to be tested by surveying the regions with high summer temperatures for the presence of Leopard Cat. Additionally, we searched through information from museum collections across the world that had Leopard Cat specimens, and there was no specimen from Central India. Hence, we concluded that the Leopard Cat likely does not occur through much of Peninsular India. Thus, it was the genetic data that first pointed to the break in distribution. The Western Ghats population is disconnected from all other Leopard Cat populations and hence requires special conservation attention. This information has been updated in the current IUCN (International Union for the Conservation of Nature) Red List of Threatened species [11].

Box 1. What Do You Think?

Can you predict what types of genetic patterns we might expect for species with different distributions? Look at the distributions of two cat species (Cat 1 and Cat 2) below. Which would be more genetically differentiated? Why?
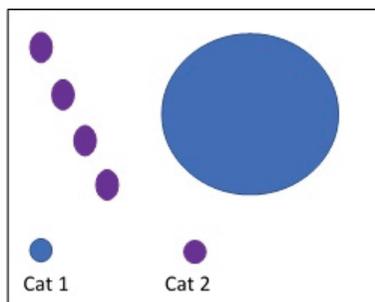
The Phylogeography of World's Largest Cat: Indian Tigers

Wild Tigers historically occurred across 70 degrees of latitude and 100 degrees of longitude, spanning 30 present-day nations ranging from Armenia to Indonesia, the Russian Far East to the Southern tip of India. This geographical range included a variety of habitats, including taiga and boreal forests, tropical evergreen, moist and dry deciduous forests, alluvial grasslands and mangroves. In historical times, we have witnessed a dramatic range collapse of 93% for wild Tigers due to habitat loss, prey depletion, and direct hunting. Current global estimates of wild Tiger populations range between 3500 − 4000 individuals. Of these, the Indian subcontinent is estimated to harbor around 2500 Tigers or about 60% of the global wild population. We wanted to understand the phylogeography or the distribution of Tiger genetic variation across different parts of India. Like with the small cats, our starting material for Tiger DNA was scat. Between 2006 − 2008, we collected Tiger scat samples from across the Indian subcontinent and found that Indian Tigers retain 60 − 70% of the global species' genetic variation [12] (*Figure* 3). Further, our results revealed that Tigers are most probably not originally 'Indian'. Our data suggested (other researchers have also suggested this [13]) that Tigers probably evolved in South China, and colonized India as part of a secondary range expansion. Despite this, Indian Tigers retain a large proportion of the species variation, possibly because they live in very varied habitats here.

Our analyses also revealed something interesting about relatively recent history. We discerned a signature of a population bottleneck or a population crash. Very interestingly, the timing of this crash corresponds well with when the British introduced bounty hunting. The number of Tigers before the crash (around 200 years ago) size was ten times higher than numbers today. That Tiger numbers have declined in the last 200 years is no surprise. However, it is quite interesting that we could detect this purely based on genetic data from scat! But in the last 200 years, have only Tiger numbers decreased? Have we lost any specific genetic variants of Tigers during this decline? The only way to compare his-
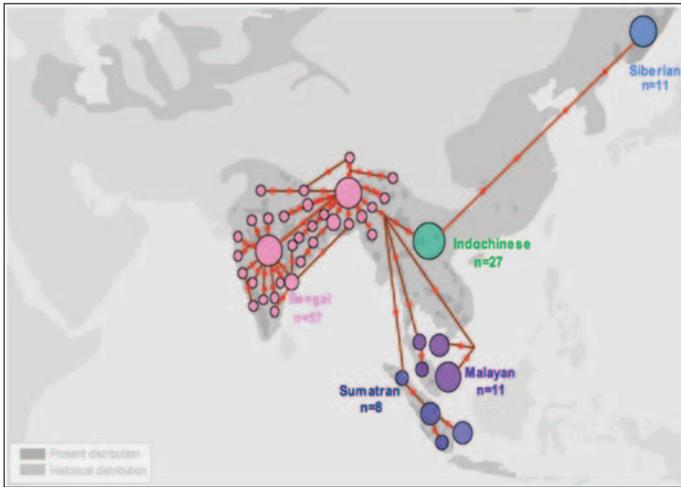
Figure 3. Genetic variation in tigers. Each circle is a genetic variant of mitochondrial DNA, called a haplotype. The number of red dots on the lines connecting the genetic variants shows how many mutations are there between the haplotypes, i.e., how different the haplotypes are. Locations of each haplotype within India correspond only approximately to the site of sample collection. The larger the circle size, the greater the number of tigers that had that genetic variant. The different colors show different tiger subspecies. The sample size (*n*) is the numbers of each subspecies sampled.

torical Tigers to those that we have among us today is to go back in time. We did this using museum skins of Tigers from the London museum of natural history. Comparing Tigers alive today to those that lived in the past revealed that genetic variation has been lost in the last 200 years [14], but, more significantly, the connections between Tiger populations have changed. What used to be two relatively large Tiger populations have become three populations (*Figure* 4). In the past, the semi-arid populations appeared to be connected to the Terai population, and all of Peninsular India appeared connected. Now, the semi-arid population seems to be cut off from the Terai. It appears that loss of habitats over the last 200 years has resulted in the loss of Tigers that inhabited those spaces. Such a loss could have then resulted in the loss of the genetic variants associated with those areas, culminating in a breakdown of connections between populations, populations that now become different from each other.

It surprised us that all Tigers south of Madhya Pradesh appeared to be a single population. Was this true? Were Tigers in the Western Ghats, for example, able to mate with those in Madhya Pradesh? To be absolutely sure, we needed to have more information. So far, in all the research above, we had looked at segments in the DNA that were 1000 – 2000 base pairs long. New technolo-

Figure 4. Genetic data reveals that there are three 'genetic clusters' of Tigers in India. The three clusters: purple, orange, and blue are found as shown in the map. The orange cluster is geographically the most widespread. We earlier thought that the orange and purple clusters were connected. (Image credit: Tiger from Ranthambore Tiger Reserve by Kaushal Patel)
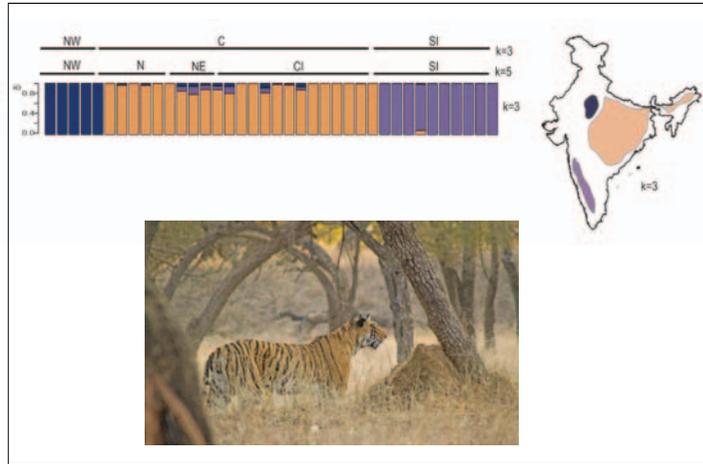
gies allowed us to sequence the whole Tiger genome (it is 2400 million base pairs long!), but we needed good biological sources of DNA, like tissue or blood. While Tiger scats do provide us DNA, sometimes the DNA from scats is not of very good quality and it is difficult to get good information from it. We used next-generation sequencing to read 10,000 positions (SNP's) in the Tiger genome across 38 wild individuals. This immediately revealed that Tigers in the Western Ghats were not the same population as those in Central India [15]. Again, this much more extensive genetic dataset reiterated that semi-arid Tigers are isolated and have low genome-wide variation. This makes them a population of conservation concern. Isolated populations with low genetic variation have higher probabilities of extinction.

Our research on Tiger phylogeography over the last decade also exemplifies the process of science; our understanding of nature deepens over time, every study refined our understanding a little bit. Technology changes, allowing us to get novel insights, and these insights help prioritize conservation efforts.

Through the years and studies, Tigers in Central India always appeared to have high variation. Was this because the Tiger reserves here were better connected? We used landscape genetics and tried to zoom into the landscape to understand what types of

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habitats are good or bad for Tiger movement here. Back to scat, we walked through several protected areas, collected scat, generated genetic data, and investigated how similar Tigers were across protected areas, and how the habitats between protected areas affected this [16, 17]. Our results revealed that high traffic roads and big cities were impediments to Tiger movement. In the same landscape, genetic data from the much smaller Jungle Cat reveals that even small roads can be barriers for them to move!

Conclusion: The Way Forward for Endangered Species in India

Around 4% of India's land area is protected for large cats like the Tiger and small cats like the Leopard Cat and Jungle Cat. On the

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Box 2. What Do You Think?

Ranthambore Tiger Reserve, in Rajasthan, is the only semi-arid Tiger population left. Based on what you read above, this population is isolated and has low genetic variation. If you wanted to increase genetic variation in Ranthambore what would you do?

(a) Re-establish a corridor between Ranthambore and a close-by Tiger reserve where Tigers are breeding.

(b) Catch Tigers from Kanha Tiger Reserve in Madhya Pradesh and introduce them in Ranthambore.

(c) Not do anything.

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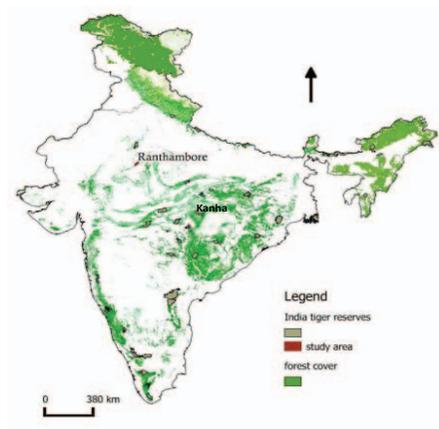
other hand, projections suggest that in the next 40 years, 60% of India will be urban. Will cats in India be able to navigate these increasingly urban landscapes? What will happen when they come out? Some species, like the Jungle Cat, may co-exist (and do) around human settlements like villages. But might they hybridize with domestic cats? Is hybridization really an issue, since closely related small cats are known to hybridize naturally, and new hybrid species have been described [18, 19]? Hybridization may also preserve some genes that may otherwise be totally lost with the species – after all, biodiversity conservation also includes genetic diversity. What about big cats like Tigers? They might kill livestock and conflict with people. To study the effects of fragmentation, conflict, and hybridization, the tools of genetics will be invaluable! We are moving towards a future where technology may be available to sequence Tiger genomes in the field! Maybe one of you will design a mobile lab that helps us understand what is happening with India's wildlife!

An understanding of the natural history of a species as well as of the history and geography of the region is fundamental to designing field sampling protocols and interpreting results from molecular data.

The most important point to note is that field craft and laboratory skills should work in harmony. Molecular tools are not very useful in the absence of field data. An understanding of the natural history of a species as well as of the history and geography of the region is fundamental to designing field sampling protocols and interpreting results from molecular data. Patterns can have various processes leading to them, and a sound knowledge of natural history is indispensable to biodiversity conservation.

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