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REVIEW

Applying molecular genetic tools to tiger conservation

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Abstract

The utility of molecular genetic approaches in conservation of endangered taxa is now commonly recognized. Over the past decade, conservation genetic analyses based on mitochondrial DNA sequencing and microsatellite genotyping have provided powerful tools to resolve taxonomy uncertainty of tiger subspecies, to define conservation units, to reconstruct phylogeography and demographic history, to examine the genetic ancestry of extinct subspecies, to assess population genetic status non-invasively, and to verify genetic background of captive tigers worldwide. The genetic status of tiger subspecies and populations and implications for developing strategies for the survival of this charismatic species both *in situ* and *ex situ* are discussed.

Key words: conservation genetics, mitochondrial DNA, microsatellite, subspecies, tiger.

INTRODUCTION

Application of molecular techniques to investigate the genetic composition of wildlife species has been considered in nearly all wildlife conservation programs, as it provides essential insights into taxonomic status, phylogeography partitions, conservation management units, demographic history and population profiles of the species of concern. In the past decade, genetic analyses of the critically endangered tiger (*Panthera tigris* Linnaeus, 1758) have been undertaken to address all of these questions and to help develop conservation strategies both *in situ* and *ex situ* (for review, see Luo *et al*. 2010).

There are only between 3000 to 5000 tigers left in the wild, reduced from probably over 100 000 a century ago and occupying only 7% of their historical range. Such a range-wide decline is due to habitat loss and fragmentation, prey base depletion and human persecution (Dinerstein *et al.* 2007; Chundawat *et al.* 2008). The challenge to preserve the existing wild tiger populations has become a major goal of conservation efforts throughout the tiger range (Walston *et al.* 2010).

Traditionally, tigers have been classified into 8 subspecies, 3 of which became extinct in the mid to late 20th century: those from Java, Bali and Caspian regions (Fig. 1). Subspecies are defined as: "geographically defined aggregates of local populations that differ taxonomically from other species subdivisions" (O'Brien & Mayr 1991). In the spatial and temporal context, subspecies represent an assembly of populations, which, with local genetic differences and geographic isolation, have the potential to become a new species or to have accumulated adaptive variations associated with different ecosystems O'Brien & Mayr 1991. Phylogeographic partitions now form the basis of subspecies recognition, species classification, hybridization detection and wildlife forensic applications (Avise 2000). Besides elucidating taxonomic uncertainty, phylogeographic assessments also provide diagnostic characters that assist in the legislative

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protection of endangered species, subspecies and distinct populations.

In light of the dire status of wild tigers, an ecological-based conservation approach has been advocated, in which 76 Tiger Conservation Landscapes (TCLs) have been identified with the ultimate goal of creating habitat corridors that link core areas and allow the ecological requirements of wild tigers to be secured (Sanderson *et al.* 2006; Dinerstein *et al.* 2007). Tigers are clustered by biome (habitat type) and 6 bioregions are recognized: the Indian subcontinent, Indochina, Peninsular Malaysia, Sumatra, the Russian Far East and China/Korea. The bioregion division is congruent with the tiger subspecies distribution range. In addition, an optimal tiger conservation strategy might require conservation interventions, such as establishing corridors, buffer zones and/or implementing reintroduction programs (Tilson & Nyhus 2010). To this end, an assessment of the genetic and evolutionary status of the population of tigers provides a powerful tool for understanding the landscape connectivity among TCLs within different bioregions. Combined with coalescent theory, population genetic measures have promised to reveal patterns of population isolation, gene flow and geographic structure, and allow a more refined view of the timing of historic events (for review, see O'Brien & Johnson 2005).

We provide a review of the current state of molecular genetic markers in the tiger and their application in conservation at the phylogenetic, taxonomic and population level. The implications of genetic findings to both *in situ* and *ex situ* tiger conservation strategy and management are also discussed.

MOLECULAR GENETICS TOOLBOX FOR THE TIGER

Beginnings of conservation genetics for tigers

Phylogeny and taxonomy of tigers in relation to other felid species have been addressed with molecular genetics techniques since the 1980s. Early efforts included comparative karyology, albumin immunological distance and allozyme electrophoresis (O'Brien *et al.* 1987). Later efforts to resolve phylogenetic relationships have focused on partial sequencing of the nuclear and mitochondrial genomes of different felids (Johnson *et al.* 2006). The latest study with supermatrix and species tree phylogenetics methods resolved a sister species relationship between the tiger and the snow leopard, which last shared a common ancestor approximately 2.7–3.7 Ma (Davis *et al.* 2010).

As with many endangered species, tigers have been classified into subspecies based on geographic distribution and morphological characteristics, for purposes of recognition and conservation (Mazak 1981). Five extant subspecies are generally recognized: the South China (*P. t. amoyensis*), Indochinese (*P. t. corbetti*), Amur (*P. t. altaica*), Bengal (*P. t. tigris*) and Sumatran (*P. t. sumatrae*) tigers. However, the validity of the traditional subspecies designations has been questioned with several lines of evidence. First, a wide range of morphological variations have been revealed within the subspecies, overlapping across different subspecies (Herrington 1987; Kitchener & Yamaguchi 2010). Significant morphometric distinctions can be only validated between the mainland Asian tigers and the Sunda Island tigers, but distinctions are mostly clinical in the mainland subspecies (Mazak & Groves 2006; Mazak 2010). Second, early molecular genetic assessments have revealed diminished genetic variation in tigers and little evidence of genetically distinct subspecies, except for a moderate level of monophyly in the island Sumatran tiger. Such studies include an examination of 28 tigers with a short fragment of mitochondrial control region DNA by Wentzel *et al.* (1999), an analysis of 34 tigers with universal mitochondrial *CytB* markers by Cracraft *et al.* (1998), and an investigation of major histocompatibility (MHC) class I loci by Hendrickson *et al.* (2000). In addition, a biogeographic study of historical tiger habitat finds few physical barriers sufficient for subspecies isolation (Kitchener & Dugmore 2000), leading to the suspicion that subspecies designation among modern tigers might require modification.

Several factors complicated the early efforts to fully describe patterns of genetic variation in tigers. Foremost among these was the limited sample size of "voucher specimens," referring to individuals verified as wild-born from a specific geographic locale or descended in captivity directly from parents of known geographic origins. In addition, the presence of 13 kb *Numt* (Lopez *et al.* 1994; Kim *et al.* 2006), nuclear pseudogene insertions of the cytoplasmic mtDNA in tiger autosomes, made it difficult to use universal mammalian primer sets for mitochondrial genes because they will co-amplify *Numt* (Cracraft *et al.* 1998). Furthermore, the paucity of genetic diversity across tigers, especially in mtDNA, have made it necessary to sequence a large portion of the mtDNA genome and to assess genetic variation in multiple rapidly evolving nuclear loci.

Subspecies diagnostic molecular genetic system

In 2004, the conclusions of a 20-year study to charac-

terize differences among the extant tiger populations and subspecies were published based on biological samples from 134 voucher tiger specimens (Luo *et al.* 2004). Several technical hurdles that complicated prior efforts to fully describe patterns of genetic variation in tigers were overcome, primarily by developing better and more extensive molecular genetic markers. The panel of diagnostic markers included: (i) 4 kb of mitochondrial DNA sequences obtained from 10 cytoplasmic-mitochondria (*Cymt*)-specific primers that amplify fragments of 250–600 bp each with 53 variable nucleotide sites in total (Table 1); (ii) a panel of 30 highly variable microsatellite markers (Table 2); and (iii) a highly variable nuclear MHC class II *DRB* gene. Combined phylogeographic analyses resulted in a convincing and robust picture of the tiger's subspecies classification, which supported the traditional classifications for Sumatran, Bengal and Amur tigers, and 1 unique lineage of South China tigers. The traditional Indochinese tiger was divided into 2 groups: the northern Indochinese and a peninsular Malayan subspecies. Each

Table 1 Polymerase chain reaction primers specific for tiger cytoplasmic mitochondrial DNA amplification

Primer ID	mtDNA Segments	Forward (5'-3')	Reverse (5'-3')	Size (bp)†
I. Primers to amplify a total of 4kb tiger mtDNA fragments (Luo *et al*. 2004)				
C53F1/T598R	*ND5*	CCCAGATCCCTATATTAACCAGT	TATATCATTTTGTGTGAGGGCAC	522
C708F/T1300R	*ND5*	CCTTGTCTTCCTGCATATCTG	CCATTGGAAAGTACCCGAGGAGGT	605
C1494F/T1936R	*ND6*	TCTCCTTCATAATCACCCTGA	TGGCTGGTGGTGTTGGTTGCGG	421
C2339F/T2893R	*CytB*	TTGCCGCGACGTAAACCACG	GTTGGCGGGGATGTAGTTATC	533
CR-UPF/CR-R2B	*CR*	TCAAAGCTTACACCAGTCTTGTAAACC	CGTGTTGTGTGTTCTGTAT	219
C-12S-F/NC-12S-R	*12S*	AAAGCCACAGTTAACGTAA	TACGACTTGTCTCCTCTTGTGG	555
C8276F/T8620R	*ND1*	CGAAGCGAGCTCCATTTGATTTA	GTGGAATGCTTGCTGTAATGATGGG	321
T8942F/C9384R	*ND2*	CTTATAGTCTGAATCGGCTTCG	AGCTATGATTTTTCGTACCT	420
C9366F/T9882	*ND2*	GGGGAGTTAACCAAACCGAG	CAAGGACGGATAGTATTGGTG	516
C11020F/T11428R	*COI*	CCAGAAGTCTATATCTTAATCCCG	GCTCCTATTGACAAGACGTAGTGGA	395
II. Primers to amplify short fragments from historical specimens (Driscoll *et al*. 2009). The obtained sequences are subset of those from Luo *et al*. (2004).				
ND5-a-F/R	*ND5*	AAACGACGAGCAAGATATTCG	ATGCGAGGTTCCGATAATA	47
ND6-a-F/R	*ND6*	TAACTATACAGTGCTGCAATTCCT	CTATGGCTACTGAGCCCTACC	184
Cytb-a-F/R	*CytB*	TCACCAACCTCCTGTCAGC	GTTATTGGATCCTGTTTCGTGA	144
Cytb-b-F/R	*CytB*	CCCTCAGGAATGGTGTCC	GGCGGGGATGTAGTTATCA	118
ND2-a-F/R	*ND2*	GGGGAGTTAACCAAACCGAG	TAGGTTTAAAATTATTATTGTGGGGC	66
ND2-b-F/R	*ND2*	TATCACAAACATGAAACAAAACG	GTATAGGTTAAGTAGTGCTGTTATG	136
ND2-c-F/R	*ND2*	GCCATAACAGCACTACTTAACCTA	TGGGAGTAGTATGGTGGACA	123
CO1-a-F/R	*COI*	GCTGATTGGCCACTCTTCAC	ACTCCTATTGACAAGACGTAGTGGA	144
III. Primers to amplify short fragments from scat specimens (Mondol *et al*. 2009). The obtained sequences are subset of those from Luo *et al*. (2004).				
TIGND2-F1/R1	*ND2*	TAGTCTGAATCGGCTTCG	CCGTTATAATGGATGCCA	195
TIGND5-F1/R1	*ND5*	GCCCCTATATTAACCAGT	ATCCTACATCTCCAATAC	195
TIGND5-F2/R2	*ND5*	TATCAGACGCAAACACTG	AATAAAGCGGAGACGGGA	224
TIGND5-F3/R3	*ND5*	ACCTACACCCATGATTGC	TTTTGTGTGAGGGCACAG	187
TIGCYTB-F2/R2	*CytB*	CGTCTGTCTATACATGCA	TACTCTACTAGGTCGGTC	200
TIGCYTB-F3/R3	*CytB*	ATGTCTTTTTGAGGGGCA	GTATTGGATCCTGTTTCG	191
TIGCYTB-F4/R4	*CytB*	TTAACCCTAGCAGCAGTC	TGTAGTTATCAGGGTCTC	184
TIGCR-F1/R1	*CR*	GGGAAGGAGAATATGTAC	CACAGAACGGGTATATGC	142
TIGCR-F2/R2	*CR*	CGAAAACAACCCCATGAC	GCTTCGTGTTGTGTGTTC	137

† Size of the mtDNA fragments is calculated excluding the primer length.

of the subspecies is represented by unique mtDNA haplotypes and signature microsatellite alleles.

These diagnostic molecular genetic markers verified in the voucher specimens can be readily applied to assess genetic ancestry of any tiger with uncertain origins (Luo *et al.* 2008). First, mitochondrial DNA haplotypes were constructed to assign maternal lineage subspecific ancestry based on its phylogenetic relationship to the voucher specimen subspecies group. Second, a Bayesian clustering assignment analysis was applied through the program STRUCTURE (Pritchard *et al.* 2000) based on 30 biparentally inherited tiger microsatellite loci to calculate the likelihood (q) that a tiger could be assigned to 1 of the 6 extant subspecies or, alternatively, the extent of admixture between subspecies. The reference voucher subspecies clusters were used as prior population information in the analysis. Individuals were considered to have a single verified subspecies ancestry (VSA; i.e. they belong to the specific subspecies with high probability) if they were consistently supported by both mitochondrial lineage and microsatellite genotype assignment results (e.g. $q = 0.90$) with a high confidence interval (0.8–1). Individuals with a discrepant subspecies ancestry assignment from mitochondrial and microsatellite data, or those with affiliations (e.g. $0.2 < q < 0.8$) to 2 or more subspecies based on microsatellite assignment test, were classified as admixed tigers. Specimens with only mitochondrial data were considered to have incomplete evidence.

Modified mtDNA marker system for historical tiger specimens

Three subspecies, Bali, Caspian and Javan tigers became extinct from their range in the 20th century, and the South China tiger has not been seen in the wild for over 2 decades. The vast collection of tiger specimens in museums and private collections worldwide has offered invaluable potential to elucidate the historical genetic diversity

Table 2 Nuclear microsatellite markers used in tigers

Reference	No. of loci	Microsatellite loci applied	Utilities	Reference for primer sequence
Luo *et al*. 2004, 2008	30	FCA-5, FCA-161, FCA-91, FCA211, FCA-304†, FCA-32, FCA-126†, FCA-8, FCA-176, FCA-69†, FCA-96, FCA-44, FCA-94, FCA-105, FCA-441†, FCA-310, FCA-212, FCA-90†, FCA-290, FCA-129, FCA-220, FCA-229, FCA-43, FCA-139, FCA-391, FCA-77, FCA-293, FCA-123, FCA-242, FCA-201	Phylogeography of all tiger subspecies; Identification of Verified Subspecies Ancestry (VSA) tigers	Menotti-Raymond *et al*. 1999
Mondol *et al*. 2009a	10	FCA-126†, FCA-69†, FCA-90†, FCA-304†, FCA-441†, FCA-672, FCA-628, FCA-232, FCA-230, FCA-279	Population genetics of Indian tigers	Menotti-Raymond *et al*. 1999
Mondol *et al*. 2009b	10	FCA-453, FCA-391†, FCA-628, FCA-205, FCA-126, F41, FCA-232, FCA-441†, FCA-672, F115	Individual identification of tigers from Indian reserves	Menotti-Raymond *et al*. 1999
Henry *et al*. 2009	12	6HDZ-057, 6HDZ-064, 6HDZ-089, 6HDZ-170, 6HDZ-463, 6HDZ-481, 6HDZ-610, 6HDZ-635, 6HDZ-700, 6HDZ-817, 6HDZ-859, 6HDZ-993	Population genetics of Amur tigers in Russian Far East	Williamson *et al*. 2002

† Microsatellite loci used across different studies.

and the genetic relationships of the extinct tigers to their extant relatives. Advances in ancient DNA techniques have also made possible the retrieval of DNA from degraded historical samples, such as bones, pelt and teeth (for review, see Paabo *et al*. 2004).

Eight mtDNA primers (Table 1) that amplify fragments below 200 bp each for a total of 1140 bp were designed by Driscoll *et al.* (2009) to use in museum Caspian tiger specimens. This modified mtDNA marker system is based on the previously sequenced regions of voucher tigers and encompasses a subset of genetic variation from the 4-kb mtDNA system of Luo *et al.* (2004). The primers are also designed to avoid regions of known *Numt* in tigers and situated in regions conserved across tiger subspecies. However, with limited inclusion of informative sites, this system does not fully resolve the mtDNA phylogeny among tiger subspecies and potential genetic variations that can further elucidate the population structure and the history in tigers might be missed. Therefore it is only recommended for use in historical or degraded tiger specimens from which long DNA fragments are impossible to obtain.

When working with historical samples, ancient DNA procedures need to be taken with great precautions to avoid the contamination from modern DNA. The extraction and preparation of the polymerase chain reaction (PCR) must be done in a laboratory that is rigorously separated from work involving modern DNA. Treatment of the laboratory equipment with bleach, ultraviolet irradiation of the entire facility, protective clothing and independent duplicates of the experiments are routine precautions to ensure the reliability of results (Paabo *et al.* 2004).

Modified genetic system for non-invasively collected tiger specimens

Non-invasively collected samples, such as hair and scat from wildlife, represent an important source of genetic samples that can be relatively easy to collect from the field. Non-invasive sampling also provides great potential for research and management applications in wildlife biology, such as identification of species of rare and cryptic wildlife, individual identification, population size estimation, sex determination and diet analysis (for review, see Waits & Paetkau 2005).

Several studies have been conducted utilizing non-invasively collected fecal samples from wild tigers. Mondol *et al.* (2009a) designed 9 mtDNA primer sets (Table 1) targeting short fragments based on the 4-kb sequences published by Luo *et al.* (2004). These primers are used to amplify a total of 1263 bp sequences from Indian tiger fecal samples (Mondol *et al.* 2009a). An earlier study of Amur tigers in the Russian Far East amplified overlapping PCR fragments from 200 to 350 bp each that spanned the first half of the mitochondrial DNA control region of approximately 700 bp (Kim *et al.* 2001; Russello *et al.* 2004). These sequences are available for the Russian Amur tigers only and, therefore, comparison with tigers from other regions are not possible. Due to the extensive existence of *Numt* in the tiger genome (Kim *et al.* 2006), extreme precautions are required when redesigning short primers from the 4-kb mtDNA marker system of Luo *et al.* (2004) or designing new mtDNA primers.

Primary applications of microsatellite genotyping in fecal samples from natural population include individual identification and characterization of population genetics parameters (Table 2). Criteria for selecting microsatellite markers for reliable individual identification lie in the polymorphic information content of the marker and its consistency in robust amplification from degraded samples. Many microsatellite loci are available for this purpose, such as those from the domestic cat (Menotti-Raymond *et al.* 1999; Menotti-Raymond *et al.* 2003) and the tiger (Williamson *et al.* 2002; Zhang *et al.* 2005; Bhagavatula & Singh 2006). Fifteen microsatellites markers (FCA126, FCA69, FCA90, FCA304, FCA441, FCA672, FCA628, FCA232, FCA230, FCA279, FCA453, FCA391, FCA205, F41 and F115) (Menotti-Raymond *et al.* 1999) were optimized for use in individual identification and genetic structure analysis based on tiger fecal samples from India (Mondol *et al.* 2009a; Mondol *et al.* 2009b). A panel of 12 nuclear microsatellite loci (6HDZ-057, 6HDZ-064, 6HDZ-089, 6HDZ-170, 6HDZ-463, 6HDZ-481, 6HDZ-610, 6HDZ-635, 6HDZ-700, 6HDZ-817, 6HDZ-859 and 6HDZ-993) (Williamson *et al.* 2002) were selected to access the genetic diversity of Amur tigers in the Russian Far East (Henry *et al.* 2009).

Non-invasively collected fecal samples are subject to the degraded nature and low quality of DNA. Strict quality measures must be taken to minimize the possibility of contamination, allele dropout (when a heterozygote individual is genotyped as a homozygote) and false allele typing (when a true homozygote individual is genotyped as a heterozygote). A multiple-tube approach combined with quality index control is a routine procedure when working with fecal samples in order to enhance genotyping accuracy (Taberlet *et al.* 1996). For instance, to obtain a consensus genotype, a minimum of 3 unambiguous amplifications were needed to accept a homozygous genotype and 2 alleles had to be observed at least twice for a heterozygous genotype to be accepted (Henry *et al.* 2009).

APPLICATIONS TO TIGER CONSERVATION: CASE STUDIES

Conservation genetics applies tools of population genetics and molecular evolution to the assessment and management of endangered species. Over the past decade, significant advances have been made in the applications of molecular genetic approaches to answer explicit conservation issues in the tiger, as indicated in Table 3.

Malayan tiger: a novel subspecies *Panthera tigris jacksoni*

As with many endangered species, tigers have been classified into subspecies for purposes of recognition and conservation. The subspecies concept is controversial, but conservation and public awareness of the tiger have been, nevertheless, inextricably tied to its subspecific classification. Several tiger subspecies are considered to be specific units of conservation, which are protected by international treaties and organizations concerned with wildlife species. Therefore, the establishment of formal subspecies definition and recognition and an understanding of the implications of subspecies assignment are critically important for tiger conservation.

Luo *et al.* (2004) provide a comprehensive molecular phylogeographic study on modern tigers and robust statistical support for genetic distinctiveness of 6 extant subspecies, including 4 traditional subspecies (Sumatran, South China, Bengal and Amur tigers), but with 1 exception. The exception is the Indochinese tiger, *P. t. corbetti*, from which 2 distinct groups were resolved: a mainland Indochinese subspecies, *P. t. corbetti*, and a peninsular Malayan subspecies, *P. t. jacksoni*. The pattern was affirmed by monophyly of mtDNA and microsatellite markers and high level of population differentiation by genetic distance measures with both

Table 3 Applications of molecular genetics to conservation of the tiger

Applications in conservation genetics	Tiger subspecies or region of interest	Genetic markers applied	References
Taxonomy/systematics	All extant subspecies	mtDNA (4kb), 30 microsatellite loci, MHC class II	Luo *et al.* 2004, 2008
	Caspian	mtDNA (1kb)	Driscoll *et al*. 2009
Population genetic diversity	Amur	mtDNA (700 bp CR)	Russello *et al*. 2004
	Amur	mtDNA (700 bp CR), 12 microsatellite loci	Henry *et al*. 2009
	Indian	mtDNA (1kb), 10 microsatellite loci	Mondol *et al*. 2009a
	Caspian	mtDNA (1kb)	Driscoll *et al*. 2009
	All extant subspecies	mtDNA (4kb), 30 microsatellite loci, MHC class II	Luo *et al*. 2004
Population genetic structure	Amur	12 microsatellite loci	Henry *et al*. 2009
	Indian	mtDNA (1kb), 10 microsatellite loci	Mondol *et al*. 2009a
Recent demographic history	Amur	mtDNA (700 bp CR), 12 microsatellite loci	Henry *et al*. 2009
	All extant subspecies	mtDNA (4kb), 30 microsatellite loci	Luo *et al*. 2004
	Indian	mtDNA (1kb), 10 microsatellite loci	Mondol *et al*. 2009a
Coalescent dating	All extant subspecies	mtDNA (4kb)	Luo *et al*. 2004
	Indian	mtDNA (1kb), 10 microsatellite loci	Mondol *et al*. 2009a
Population size estimates	Indian	5 microsatellite loci	Mondol *et al*. 2009b
Ex situ conservation	Worldwide	mtDNA (4kb), 30 microsatellite loci	Luo *et al*. 2008
	Amur	mtDNA (4kb), 30 microsatellite loci	Luo *et al*. 2008
	Amur	mtDNA (700 bp CR), 12 microsatellite loci	Henry *et al*. 2009
	Indian, Amur, Sumatran	MHC class I	Hendrickson 2000

microsatellite and mtDNA, and supported by patterns of MHC variation. In addition, each subspecies has a geographically-distinct range and different habitats.

As has been observed in certain other modern felid species (for review, see O'Brien & Johnson 2005), the tiger has a relatively low level of genetic diversity. Coalescence time of the tiger mtDNA genetic diversity dates back to only 72 000–108 000 years ago. It has been suggested that the Toba volcano super-eruption of approximately 72 500 years ago in Sumatra could have contributed to this recent coalescence time for extant tiger populations (Luo *et al.* 2004). The modern tigers subsequently expanded and recolonized Asia, with the return of suitable climate and habitat. The differentiation among subspecies is most likely a result of the combined effects of genetic drift in isolated populations and local adaptation to rapidly changing habitats across tigers' range.

The results have important implications for tiger conservation and management. The Malayan tiger, a newly recognized subspecies found only in the Thai–Malay Peninsula (including Thailand south of the Isthmus of Kra and Peninsular Malaysia), is characterized by 3 unique microsatellite alleles, 5 subspecies-specific mtDNA haplotypes and 3 MHC DRB alleles. The results suggest setting conservation of the Malayan tiger as a high priority both *in situ* in Malaysia and Thailand and in zoos worldwide. Such elevated conservation awareness and commitment towards Malayan tigers has been evident in the Malaysian Government's national campaign for conservation and in the strategic planning of various international and regional non-governmental organizations (Kawanishi *et al.* 2010).

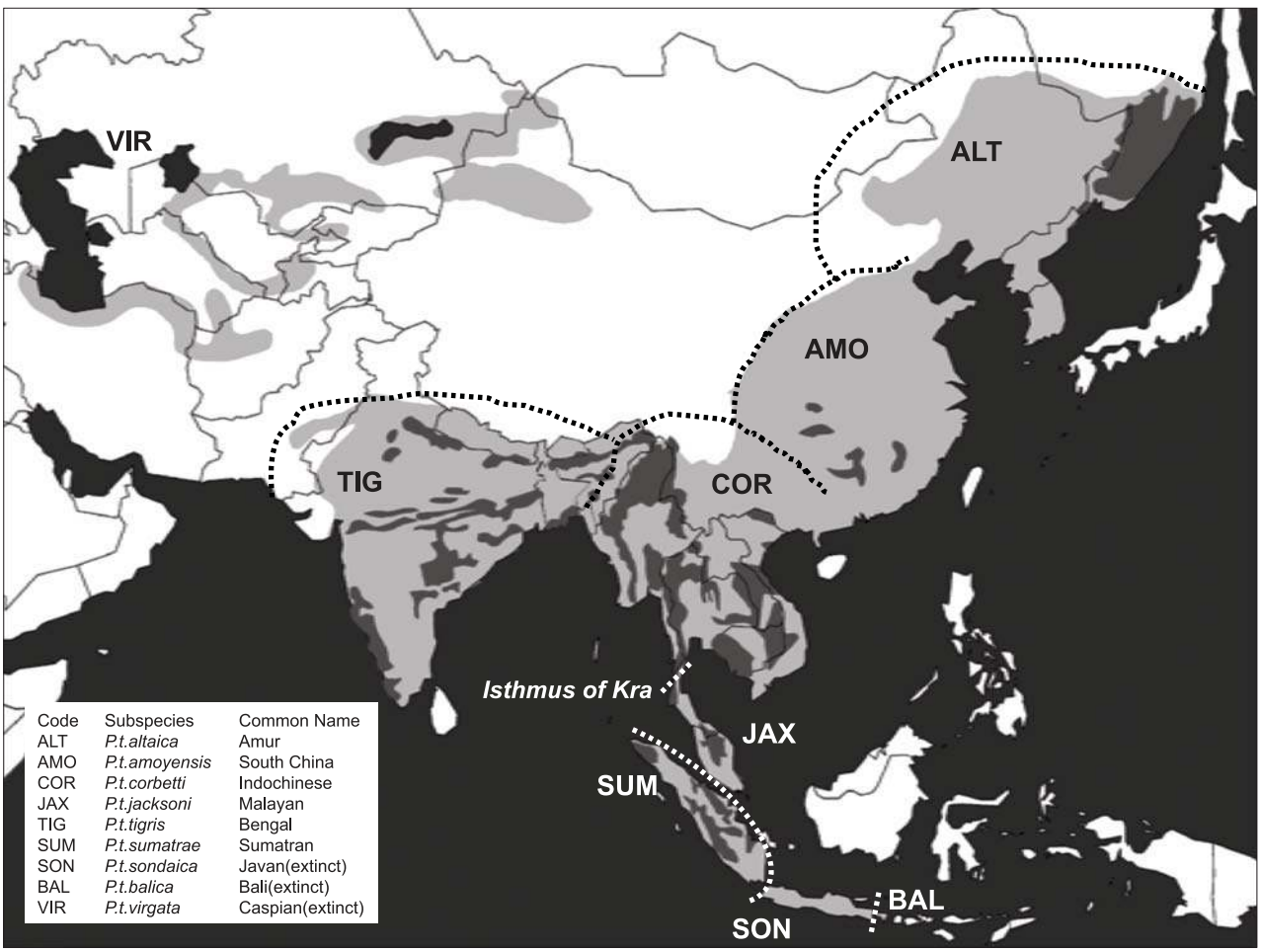

Figure 1 Historic (light grey) and current (dark grey) geographic distribution of tigers corresponding to the redefined subspecies designation (modified after Figure 1 in Luo *et al*. 2004).

An important consequence of the redefined Malayan and Indochinese tiger is that most of the founders in the captive management programs for the subspecies in Europe and North America were originally from the Thai–Malay Peninsula. With the Malayan tiger now managed separately, the northern Indochinese tiger is the least represented in captivity worldwide. Therefore, establishment of a coordinated conservation breeding program for captive management and preservation of Indochinese tigers in the wild, which have been studied little, should be set as a priority to maintain the high genetic diversity and structure harbored in the natural tiger populations from the region.

Captive tigers: Genetic reservoir of wild tigers

Well-managed captive populations of wild animals provide conservation support for their wild relatives in many ways. In addition to their clear role in public education, research and fundraising, they are also justified as the genetic "reservoir" of their wild population counterparts and could serve as stock for reintroduction if their wild brethren were to become extinct (Christie 2010).

In dramatic contrast to the 3000–5000 tigers in the wild (Chundawat *et al.* 2008), there are 15 000–20 000 tigers worldwide in zoos, breeding facilities, circuses and even private homes, outnumbering their wild relatives fivefold to sevenfold (Luo *et al.* 2008). It is estimated that only 1000 of these animals are in managed breeding programs that are designed to preserve genetic diversity for certain subspecies. The other captive tigers are generally considered "generic" tigers of hybrid or unknown origins and are not included in internationally sanctioned conservation programs (Nyhus *et al.* 2010). Debate has persisted over the role of captive tigers in conservation efforts, whether managed captive populations serve as adequate genetic reservoirs for the natural populations, and whether the presumptive "generic" tigers have any conservation value.

The molecular genetic markers by Luo *et al.* (2004) provide a definitive and diagnostic tool to address the dilemma. The genetic background of 105 captive tigers with unknown or unclear origins from 14 countries and regions around the world were verified based on Bayesian clustering analysis and a panel of 134 voucher tigers (Luo *et al.* 2008). A total of 49 VSA tigers were assigned to a certain subspecies (21 Amur, 17 Sumatran, 6 Malayan, 1 Indochinese and 4 Bengal tigers) and 52 to admixed subspecies origins. The tested captive tigers retain appreciable genomic diversity unobserved in their wild counterparts, including 8 new mtDNA haplotypes and 46 new microsatellite alleles. Other studies reveal appreciable genetic variability in the tiger populations *ex situ* (Hendrickson *et al.* 2000; Henry *et al.* 2009). Even after taking into account the sampling bias, it is extrapolated that at least 14–23% of the over 15 000 existing captive tigers might be amenable to verified subspecies ancestry. This new concept in captive animal management, VSA assessment, offers a powerful tool that, if applied to tigers of uncertain background, might increase by thousands the number of purebred tigers suitable for conservation management (Luo *et al.* 2008).

Amur tiger: A successful story both in the wild and captivity

Understanding of the Amur tiger *P. t. altaica* has benefited from long-term science-based conservation programs (Miquelle *et al.* 2010). The *in situ* population of Amur tigers is estimated at approximately 400–500 individuals confined to the temperate forest of the Russian Far East and the border regions of China and North Korea. Its numbers have rebounded from a severe demographic bottleneck of 20–30 individuals in the 1940s due to intense human persecution (Heptner & Sludskii 1972). The *ex situ* population of Amur tigers represents one of the best conservation breeding programs of endangered wildlife, as exemplified by 2 well-managed captive populations in North America (Species Survival Plan) and Europe (European Breeding Program), respectively. Established in the early 1950s, these 2 captive breeding programs are now home to over 420 Amur tiger descended from 57 founders (Traylor-Holzer 2007).

The Amur tiger displays the lowest mtDNA diversity among all tiger subspecies. From 12 unrelated Amur tigers across 6-kb mtDNA sequences, Luo *et al.* (2004) found a single haplotype that is most closely related to a northern Indochinese tiger haplotype. Extensive sampling of the wild Amur tigers and additional sequencing of the highly variable control region revealed 2 more mtDNA haplotypes, which are different from the widespread haplotype (96.4% of the 82 samples tested) by only 1 single nucleotide (Russello *et al.* 2004). Nevertheless, population structural analysis based on microsatellite allele frequency and heterozygosity clearly identified 2 populations isolated by a development and construction barrier in the Russian Far East and estimated the effective population size at 27–35 (Henry *et al.* 2009). The reduced genetic variability in the Amur tiger is consistent with its peripheral distribution of the entire tiger range and might have resulted from a postglacial colonization of the region in the Lower Pleistocene. By contrast, there is no evidence

for a recent population bottleneck associated with the well-documented demographic decline in the 20th century (Henry *et al.* 2009).

The captive breeding programs of Amur tigers have maintained comparable population size and genetic diversity relative to the wild populations in the Russian Far East, yet genetic variants have persisted *ex situ* that were lost *in situ* (Luo *et al.* 2008; Henry *et al.* 2009). In addition, there are fewer pairs of closely related individuals in the *ex situ* population than *in situ*, likely a consequence of the large population size, century-long introduction of new founders, and successful breeding strategies to retain genetic variability in captivity. Overall, the coordinated captive Amur tigers adequately represent the gene pool of the wild populations and might serve as a healthy supplement to *in situ* conservation, if population reinforcements become necessary in the future.

Caspian tiger: Potential for reintroduction of the extinct tigers

Tigers became extinct in Central Asia and the islands of Bali and Java in the mid to late 20th century and the South China tiger has not been seen in the wild for more than 25 years. Elucidation of the phylogeography and evolutionary history of the living tiger subspecies paved the path for exploring the genetic ancestry of the extinct tigers. The Caspian tiger (*P. t. virgata*) inhabited the Central Asian riverine forests and was last seen in the 1970s. Using ancient DNA methodology, genetic analysis of over 20 museum specimens indicate a close relationship with the Amur tiger *P. t. altaica*, with a major mtDNA haplotype differing by only a single nucleotide from the widespread haplotype found across all contemporary Amur tigers (Driscoll *et al.* 2009). Due to their evolutionary proximity, living Amur tigers are likely the closest living genetic stock should reintroductions to the former range of the Caspian tiger be initiated.

Indian tiger: Populations genetics based on non-invasive sampling

Indian tigers *P. t. tigris* are found in Bangladesh, Bhutan, western China, India, western Myanmar and Nepal. The most recent nationwide tiger census estimated the number of tigers in India at 1411, with the lower and higher bounds at 1165 and 1657, respectively (Jhala *et al.* 2008). Despite the significant decline from its 1993 census number of 3750, India is home to the largest number of tigers in a single country and plays a critical role in global tiger conservation (Jhala *et al.* 2008). In captivity, Indian zoos have been breeding tigers since 1880 and currently manage approximately 200 registered individuals. However, Indian tigers have been transported around the world and have been frequently crossed with other subspecies, as reflected by the high frequency (33%) of generic captive tigers that carry partial Indian tiger genetic heritages, according to the test of Luo *et al.* (2008). Mondol *et al.* (2009a) assess genetic variation in 73 wild tigers from 28 nature reserves in the Indian subcontinent and suggest a high genetic diversity and large historic population size for Indian tigers. Simulations reveal a signature of a possibly anthropogenic demographic bottleneck around 200 years ago, and less than 2% of the historical tiger population now persist in peninsular India.

Mondol *et al.* (2009b) improve previous non-invasive genetic sampling methods (e.g. Bhagavatula & Singh 2006) and present a comprehensive study utilizing DNA-based capture–recapture analysis to estimate the size of a wild tiger population in India. Based on 5 highly variable microsatellite loci after extensive validation from 30 candidate loci, individual identification was conducted on 58 non-invasively collected tiger scat samples. Genetic profiling corresponded to 26 unique genotypes in Bandipur National Park, and a total population size of 66 (±12.98) individuals. This was in close agreement with results based on "photographic capture" data from the same site, in which 29 unique individuals were revealed and population size was estimated at 66 (±13.81) individuals. The study demonstrated the feasibility of generating reliable abundance estimation through genetic surveys of scats in an elusive and rare species such as the tiger. It also highlighted the importance of rigorous field survey and laboratory protocols for reliable population size estimation with non-invasive sampling.

CONCLUSIONS AND THE FUTURE OF GENETIC STUDIES

The examples cited in this review represent the major advances of conservation genetics in tigers over the past decade. As genomics technologies evolve, numerous tools for elucidating the origin, variability, divergence, adaptation and survival in free-ranging species are also becoming available. Because all cat species diverged from a common ancestor less than 10 Ma, and because genomic composition is highly conserved in Felidae, the domestic cat genomic resources can be readily applied to nondomestic cat species, including the tiger. For all these reasons, molecular genetics tools are beginning to resolve many conservation questions in tigers that were previously

unachievable: for example, genetic characterization of distinct tiger subspecies, genetic ancestry of the extinct tigers, heritage background of generic captive tigers with unknown origin, population size estimation based on non-invasive sampling and capture–recapture analysis, and demographic history under the influence of geological and human-induced events.

There remain important unanswered questions for tiger systematics, evolution and conservation. What is the extent of population genetic variation, demographic history and population structure among tiger populations across different landscapes? How does the genomic complex in the tiger reflect the species' adaptation and ecological resilience in a variety of habitats? Can forensic applications help trace the geographic origin of illegally traded tiger parts? Are the other recently extinct tiger subspecies as distinctive as the living subspecies? The tiger conservation and research community has the needed expertise and technology at its disposal to address each of these questions, and genetics will play an important role in facilitating informed wild and captive management decisions for the world's conservation icon species.

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