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Phylogeography of *Panthera tigris* in the mangrove forest of the Sundarbans

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ABSTRACT: Tigers *Panthera tigris* in the Sundarbans represent the only population adapted to living in mangrove forest habitat. Several studies, based on limited morphological and genetic data, have described the population as being differentiated from the Bengal tiger subspecies *P. tigris tigris*. The phylogenetic ancestry of the Sundarbans population has also remained poorly understood. We generated 1263 bp of mtDNA sequences across 4 mtDNA genes for 33 tiger samples from the Bangladesh Sundarbans and compared these with 33 mtDNA haplotypes known from all subspecies of extant tigers. We detected 3 haplotypes within the Sundarbans tigers, of which one is unique to this population and the remaining 2 are shared with tiger populations inhabiting central Indian landscapes. Phylogenetic analyses using maximum likelihood and Bayesian inferences supported the Sundarbans tigers as being paraphyletic, indicating a close phylogenetic relationship with other populations of Bengal tigers, from which the Sundarbans population diverged around 26 000 yr ago. Our phylogenetic analyses, together with evidence of ecological adaptation to the unique mangrove habitat, indicate that the Sundarbans population should be recognised as a separate management unit. We recommend that conservation management must focus on sustaining this representative tiger population adapted to mangrove habitat while at the same time recognising that trans-boundary conservation efforts through reintroduction or exchange of individuals, to enhance genetic diversity, might be needed in the future as a last resort for population recovery.

KEY WORDS: Bangladesh · Haplotype · Management unit · Phylogeny · Tiger

1. INTRODUCTION

Tigers *Panthera tigris* were widely distributed across eastern Asia by the end of the Pliocene and beginning of the Pleistocene (Mazak 1981, Hemmer 1987). They subsequently colonised across a wide variety of landscapes, ranging from taiga and boreal forests to alluvial grasslands and tidal mangrove swamps (Kitchener 1999, Sanderson et al. 2006). The wide distribution of tigers was primarily influenced by environmental changes linked to Pleistocene glaciation events (Kitchener & Dugmore 2000). Subsequently, anthropogenic threats over centuries have turned the once vast tiger range across Asia into

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many fragmented pockets of forested landscapes (Dinerstein et al. 2007, Walston et al. 2010). As a result, the world's remaining wild tigers now survive within 76 tiger conservation landscapes (TCLs), representing only 7% of their ancestral range (Dinerstein et al. 2007). Unfortunately, more than half of the remaining TCLs each hold fewer than 120 individuals, presenting a high risk of localised extinction due to demographic and genetic factors (Smith & McDougal 1991, Dinerstein et al. 1997). The Sundarbans, which straddles Bangladesh and India, represents one of 11 global priority landscapes and is the only habitat where tigers have adapted to surviving on a limited prey species in a mangrove forest environment (Aziz et al. 2020).

Several studies, utilising morphological, ecological and molecular data, have so far identified 2 to 9 subspecies (Luo et al. 2004, Wilting et al. 2015). Tigers of the Sundarbans were designated *P. tigris tigris* among the 6 putative extant subspecies (Chundawat et al. 2011); they were later described as being morphologically smaller (Barlow et al. 2010), and the population was identified as an evolutionarily significant unit (ESU) (Singh et al. 2015). Ideally, ESUs are populations within a species that may not be morphologically distinct but which exhibit a distinct evolutionary history such that they may represent local long-term adaptation to environmental conditions (Kitchener et al. 2017). ESUs are differentiated from management units (MUs), which represent populations within a species that are considered distinctive enough to warrant separate conservation management from that of other populations as a result of more recent genetic differences that cannot be attributed to distinct evolutionary history (Kitchener et al. 2017). Although the identification of ESUs within a species may highlight evolutionary significance, it is recognised as a challenging task that often requires interpretation using natural history information, and morphometric, range and distribution data as well as geographical mapping of nuclear and mtDNA variation (Ryder 1986).

Given the continued debate of tiger subspecies assignment (Luo et al. 2004, Wilting et al. 2015), together with the need to ensure that finite conservation resources conserve as much diversity as possible, accurate diagnosis of population differentiation is of great importance for conservation purposes. More importantly, due to the extreme nature of the population fragmentation of extant tiger populations (Sanderson et al. 2006, Dinerstein et al. 2007, Wikramanayake et al. 2011), conservation actions are needed for every remaining population regardless of subspecies assignment (Wilting et al. 2015). Moreover, future conservation efforts may require the recovery of small populations through the exchange of individuals or reintroduction actions; therefore, separating existing tiger populations into many subspecies without a firm insight into evolutionary distinctiveness may hinder their future recovery (Wilting et al. 2015). Instead, recognising MUs (Waples 1991, Moritz 1994) among such populations regardless of ESU designation or subspecies categorisation (Crandall et al. 2000, Wilting et al. 2015) may benefit the *in situ* conservation of tiger populations that exhibit unique adaptations (Sanderson et al. 2010).

When making informed decisions that may lead to crucial conservation implications, efforts increasingly require in-depth understanding of the systematics of populations, subspecies and species (Ryder 1986). Using mtDNA of tiger samples obtained from across the Bangladesh Sundarbans and equivalent sequence data available on GenBank comprising all putative tiger subspecies so far identified, we aim to shed new light on the phylogenetic history of the tiger population in the Sundarbans to discern its distinctiveness and hence the necessity and level of future conservation actions.

2. MATERIAL AND METHODS

2.1. Study site and sample collection

The Sundarbans is the largest contiguous mangrove forest in the world, encompassing an area of 10 263 km^2 in the Ganges-Brahmaputra delta (Giri et al. 2007). The Bangladesh Sundarbans (21.63° N to 21.49° N and 89.04° E to 89.91° E) covers 6017 km^2, of which 4267 km^2 is forest and the remaining area comprises water bodies (Iftekhar & Islam 2004). The northern and eastern sides of the forest are bounded by dense human settlements and agricultural land and the southern side by the Bay of Bengal (Fig. 1). The Bangladesh Sundarbans is managed as the Sundarbans Reserve Forest (SRF), where 3 isolated areas have been designated as wildlife sanctuaries: Sundarbans West (715 km^2), Sundarbans South (370 km^2) and Sundarbans East (312 km^2) (Fig. 1).

To collect non-invasive tiger samples (scat and hair), 4 areas were selected within the SRF for sampling: East Wildlife Sanctuary with additional areas (383 km^2), West Wildlife Sanctuary (715 km^2), Chandpai block (342 km^2) and Satkhira block (554 km^2). Location, protection status and level of human use of landscapes (e.g. fishing, nypa palm

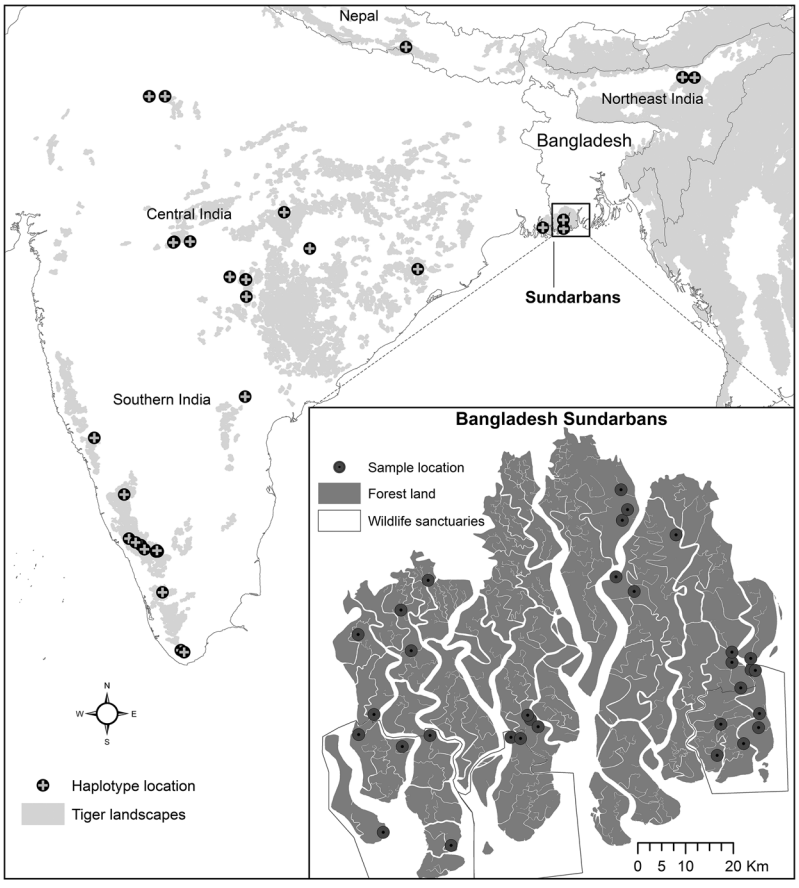

Fig. 1. Bengal tiger haplotype locations (approximate) retrieved from Mondol et al. (2009b) and Luo et al. (2004). Inset shows the location of samples collected from the Bangladesh Sundarbans during this study

harvesting) were considered in selecting these sample areas to ensure representative sampling (Aziz et al. 2017). To select sampling points, each sampling area was divided into 2 × 2 km grid cells, creating 373 grid cells for potential sampling across the 4 areas. Each grid cell was targeted for sampling and walked by a surveying team of 4 trained field staff. Five survey teams comprising 4 observers in each team were used to simultaneously survey a sample area over a short period of time for sample collection.

Winter months were chosen for sampling to avoid extreme weather conditions, with sampling from 20 November 2014 to 26 February 2015. Survey teams collected scat and hair samples (left by tigers in their scratch marks on trees) and recorded the location for each sample using the handheld Garmin GPSMAP 64. In addition to field-collected samples, 1 blood sample (from a rescued tiger), 5 tissue samples (skins confiscated from around the Sundarbans) and 4 hair samples (rescued tigers from the Sundarbans) were also collected. Samples were analysed at the Conservation Genetics Laboratory of the Durrell Institute of Conservation and Ecology, University of Kent, UK, after transporting them from the field under a permit from the Convention of International Trade in Endangered Species (CITES) (permit no. BD 911-8404) and authorisation of the Department for Environment, Food and Rural Affairs, UK (Animal Health and Veterinary Laboratories Agency authorisation TARP/2015/111).

2.2. DNA extraction and amplification

Two separate laboratories were used, one for DNA extraction and another for carrying out all PCR reactions to avoid contamination. Genomic DNA from scat samples was extracted using a QIAamp DNA Stool Mini Kit following the manufacturer's instructions. To extract DNA from each scat, approximately 200 mg of scraped material from the outer surface was incubated overnight with 1.5 ml buffer ASL on a mechanical rotator at 56°C. The DNA supernatant from the sample was lysed with 300 µl buffer AL plus 25 µl proteinase K and incubated at 70°C for 15 min. To increase DNA yield from scat samples, 4 µl of carrier RNA (ThermoFisher Scientific) was added to the buffer AL. To extract DNA from blood, tissue and hair samples, we used a DNeasy Blood and Tissue Kit (QIAGEN); approximately 50 g (or minimum 10 hairs) of each sample was added to 300 µl buffer AL incorporating 20 µl of proteinase K and 20 µl of dithiothreitol (Biotech) and then incubated at 56°C overnight or until the sample was completely digested. The DNA was eluted with 75 µl of buffer solution.

Extracted DNA was screened for species authentication using tiger-specific primers, which have been previously used in non-invasive tiger studies (Mukherjee et al. 2007, Mondol et al. 2009a). All PCR reactions were prepared and carried out under a UV-irradiated fume hood in a separate laboratory. PCR cycling conditions for this screening process consisted of an initial hot start of 95°C for 1 min followed by 45 cycles of 95°C for 15 s, 55°C for 15 s and 72°C for 15 s and a final incubation period of 10 min at

72°C using a G-Storm Thermal Cycler (Labtech). PCR reaction volumes (total 27 µl) contained 3 µl of template DNA, 12.5 µl MyTaq Red Mix (containing dNTPs and $MgCl_2$; Bioline), 0.5 µl of each primer containing 10 pmol $µl^{-1}$, 2.0 µl BSA (New England Biolabs) and 8.5 µl distilled water (dH_2O). All PCR products from each DNA extraction were purified and sequenced by Macrogen using a 3730XL analyser. The sequences were edited using Jalview v2.10.1 (Waterhouse et al. 2009) and then cross-checked with the GenBank (National Center for Biotechnology Information) database to confirm samples were tiger (and not a contaminant prey species).

To generate an mtDNA dataset from tiger-authenticated DNA samples, 9 primer sets (obtained and optimised from Mondol et al. [2009b]) were used to amplify 4 mtDNA gene regions: control region (CR), cytochrome *b* (cyt *b*), NADH dehydrogenase subunit 2 (ND2) and NADH dehydrogenase subunit 5 (ND5) (Table 1). These genes were chosen primarily to compare overlapping gene regions of other tiger populations and subspecies and also because these gene fragments showed sufficient variability across putative tiger subspecies (Luo et al. 2004, Mondol et al. 2009b). PCR reactions were conducted in 27 µl reaction volumes containing 3 µl template DNA, 12.5 µl MyTaq Red Mix (Bioline), 0.5 µl of each primer and 10.5 µl of dH_2O. PCR amplification was performed using a G-Storm Thermal Cycler (Labtech). The PCR profile was comprised of initial denaturation (95°C for 1 min), 45 cycles of denaturation (95°C for 30 s), annealing (annealing temperature [T_a] for 15 s), extension (72°C for 30 s) and final extension (72°C for 10 min) (Table 1). Negative controls were included at both the DNA extraction and PCR amplification stages to ensure no contamination. All amplicons were examined by agarose gel electrophoresis to ensure a clean single band and to check for any signs of contamination. The successful PCR products were purified and amplified by Macrogen.

Table 1. Species-specific mitochondrial primers used in this study. Primers were optimised from Mondol et al. (2009b)

Primer name	Primer sequence	Amplicon size (bp)	Annealing temperature (°C)
TIGND2 F1	TAG TCT GAA TCG GCT TCG	195	52
TIGND2 R1	CCG TTA TAA TGG ATG CCA		
TIGND5 F1	GCC CCT ATA TTA ACC AGT	195	52
TIGND5 R1	ATC CTA CAT CTC CAA TAC		
TIGND5 F2	TAT CAG ACG CAA ACA CTG	224	57
TIGND5 R2	AAT AAA GCG GAG ACG GGA		
TIGND5 F3	ACC TAC ACC CAT GAT TGC	187	57
TIGND5 R3	TTT TGT GTG AGG GCA CAG		
TIGCYT B F2	CGT CTG TCT ATA CAT GCA	200	52
TIGCYT B R2	TAC TCT ACT AGG TCG GTC		
TIGCYT B F3	ATG TCT TTT TGA GGG GCA	191	52
TIGCYT B R3	GTA TTG GAT CCT GTT TCG		
TIGCYT B F4	TTA ACC CTA GCA GCA GTC	184	52
TIGCYT B R4	TGT AGT TAT CAG GGT CTC		
TIGCR F1	GGG AAG GAG AAT ATG TAC	142	52
TIGCR R1	CAC AGA ACG GGT ATA TGC		
TIGCR F2	CGA AAA CAA CCC CAT GAC	137	52
TIGCR R2	GCT TCG TGT TGT GTG TTC		

2.3. Mitochondrial data analysis

MtDNA sequences were edited and aligned with Jalview v2.10.1 (Waterhouse et al. 2009) and concatenated into a complete dataset using SequenceMatrix (Vaidya et al. 2011). For sequence comparison, 2 additional datasets were retrieved from GenBank for Bengal tigers (accession nos. cyt *b* EU661630–EU661650, ND2 EU661651–EU661671, ND5 EU661672–EU661691 and CR EU661609–EU661629) (Mondol et al. 2009b) and for all putative tiger subspecies (accession nos. cyt *b* AY736634–AY736658, CR AY736609–AY736633, ND2 AY736684–AY736708 and ND5 AY736734–AY736758) (Luo et al. 2004).

Haplotypes reported in all other putative tiger subspecies were grouped according to previously assigned tiger subspecies, excluding the tentatively recognised South China tiger (Luo et al. 2004), which survives only in captivity (Seidensticker 2010). The combined dataset contained 33 phylogenetically informative haplotypes (Luo et al. 2004, Mondol et al. 2009b) (Fig. 1) (Table S1 in the Supplement at www.int-res.com/articles/suppl/n048p087_supp.pdf). We have also derived summary statistics for each subspecies and regionally grouped populations.

2.4. Phylogenetic analyses

Phylogenetic tree inferences were computed using Bayesian inference (BI) and maximum likelihood (ML) methods. To identify the best-fit models of nucleotide evolution for each

gene region of the concatenated sequence datasets, PartitionFinder (Lanfear et al. 2012) was used according to Bayesian's information criterion (BIC). BI and ML analyses were implemented in MrBayes v3.2 (Ronquist & Huelsenbeck 2003) and RAxML v7.2.6 (Stamatakis 2006), respectively, on the Cyberinfrastructure for Phylogenetic Research (CIPRES) Science Gateway (Miller et al. 2010). The Bayesian analysis ran for 10 million generations over 4 parallel Monte Carlo Markov chains (MCMCs), under a Hasegawa-Kishino-Yano (HKY) evolutionary model (Felsenstein 1981). Chain convergence was determined using Tracer v1.6 (Rambaut et al. 2014) to ensure sufficiently large effective sample size values (>200). After discarding the first 25%, tree topologies were summarised in a 50% consensus tree. An ML analysis was performed with 1000 bootstrap replications to obtain the best likelihood under a GTAGAMMA model, producing a majority rule consensus tree. All trees were visualised in FigTree v1.4 (Rambaut 2012). A median-joining haplotype network was constructed in Network v4.612 (www.fluxus-engineering.com) to assess the relationships between different tiger subspecies and Bengal tiger populations. Each haplotype was then assigned to a georeferenced sample location to display its spatial distribution across the Indian subcontinent tiger landscape using ArcGIS v10.3.

Finally, we reviewed published evidence of morphological as well behavioural adaptations of tigers in the Sundarbans, drawing upon data on morphology and tiger prey preference to examine the extent of ecological exchangeability within the population.

2.5. Molecular dating

To infer a time-calibrated evolutionary divergence of tigers of the Sundarbans, 2 fossil-based calibration points were used, with (1) a minimum of 3.8 million yr for the earliest *Panthera* lineage from the clouded leopard *Neofelis nebulosa* (Johnson et al. 2006) and (2) 1.6 million yr for the base of the lion *Panthera leo*–jaguar *P. onca* clade (Janczewski et al. 1995). The fossil-calibrated phylogeny was estimated using BEAST v1.8.2 (Drummond & Rambaut 2007) on the CIPRES Science Gateway (Miller et al. 2010) with 10 million generations over 4 parallel MCMCs, under an HKY strict clock model (Felsenstein 1981). A normal distribution was applied by setting the means to 3.8 and 1.6 million yr for the first and second calibrations, respectively, with a common SD of 0.5 million yr at both calibration points. Clouded leopard, lion and jaguar sequences were obtained from GenBank (accession nos. DQ257669 [Wu et al. 2007], AF-006458 [Johnson & O'Brien 1997] and KC834784 [Bagatharia et al. 2013]).

3. RESULTS

A 1263 bp mtDNA sequence was successfully amplified, comprising gene segments of CR (200 bp), cyt *b* (450 bp), ND2 (131 bp) and ND5 (482 bp) for 33 tiger samples from the Bangladesh Sundarbans. The analysis of these concatenated gene sequences revealed 2 haplotypes (TIG29 and TIG23) within the sampled population, of which TIG23 was shared by 36% and TIG29 by 64% of samples. These haplotypes have been submitted to GenBank with accession numbers MH427526 to MH427533. Combining these haplotypes with previously reported haplotypes from the Indian Sundarbans (Mondol et al. 2009b), this study has identified 3 haplotypes (TIG23, TIG30 and TIG29) within the population of the entire Sundarbans (Fig. 2). The TIG29 haplotype is a unique haplotype which distinguishes the tigers of the Sundarbans from other populations of Bengal tigers as well as from other tiger subspecies (Fig. 3). Haplotypes TIG30 and TIG23 were shared among surviving populations across the Indian mainland tiger landscapes. The spatial distribution of haplotypes within Bengal tiger populations showed that TIG30 was shared with tiger populations in Ranthambhore National Park in Rajasthan, India, and the Raipur Zoo in Chattishgarh, India, while TIG23 was observed among the population of Ranthambhore National Park. Notably, these 2 haplotypes were unique to Bengal tigers (Fig. 3).

Analysis of fossil-based time calibration, using sequences of clouded leopard and the lion–jaguar clade, was carried out with concatenated mtDNA gene sequences of tigers of the Sundarbans. The resulting phylogeny (Fig. 4) suggests that the single haplotype unique to the Sundarbans population arose around 26 000 yr ago (95% highest posterior density: 800–62 000 yr). Our haplotype network (Fig. 3) and phylogenetic reconstruction (Fig. 4) indicate that the tiger population of the Sundarbans is paraphyletic (i.e. descended from a common evolutionary ancestor but not including all the descendants) within the Bengal tiger. The lower posterior probability (PP) from the Bayesian phylogeny and the bootstrap support (BS) from the ML inferences (PP 69%, BS 45%; Fig. 4) provide supporting evidence for this conclusion for the Sundarbans popu-

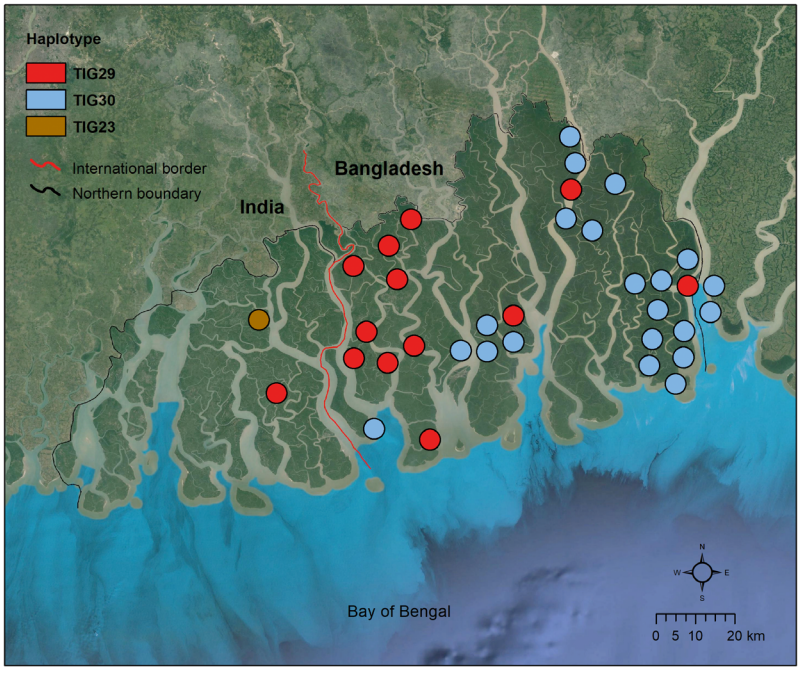

Fig. 2. Spatial distribution of 3 unique haplotypes derived from concatenated 1263 bp mitochondrial gene fragments of the Sundarbans tigers. Dark green areas encompass the entire Sundarbans shared between Bangladesh and India, separated by rivers. Haplotype locations depict the true locations of samples collected from the Bangladesh Sundarbans, while the locations of the 2 samples from the Indian Sundarbans are approximate and derived from Mondol et al. (2009b)

lation. We advocate that the shared phylogenetic relationship with mainland tigers of the Indian subcontinent, together with the ecological adaptation for mangrove habitat, indicate that the tiger population in the Sundarbans should be considered as a separate MU in future conservation efforts.

Tigers adapted to surviving in the Sundarbans mangrove swamps have been disconnected from the nearest tiger populations by 200 to 300 km of human-dominated landscape, suggesting little chance of future population movements by dispersal. A meta-analysis of ecological adaptability derived from skull and body weights demonstrates that Sundarbans tigers are morphologically smaller (Barlow et al. 2010), a trait which has been attributed to adaptation to an island habitat (Kitchener 1999) and also to the absence of larger prey species in the Sundarbans compared to mainland tiger landscapes (Sunquist et al. 1999). This is supported by a recent study by Aziz et al. (2020) which shows that Sundarbans tigers obtain 78% of their diet from spotted deer *Axis axis*, a medium-sized prey species, and 11% from wild boar *Sus scrofa*.

Haplotype diversity (*h*) is lower for the Sundarbans population compared to all regional groups of Bengal tigers, except the Nepal population. At the subspecies level, *h* is higher for the Sundarbans population than for the Amur and Indochinese tiger populations. In terms of nucleotide diversity (π), the Sundarbans tigers exhibit moderate values of π, which is broadly similar to other populations in India but higher than those in Nepal (Table 2).

4. DISCUSSION

The tiger population of the entire Sundarbans has retained 3 informative haplotypes, of which TIG29 is unique to the population and the other 2 are shared with tigers inhabiting central Indian landscapes. Of these 3 haplotypes detected in the Sundarbans population, the TIG30 haplotype was found in a tiger that was wild caught but housed in captivity in the Raipur zoo, Chhattisgarh, and this haplotype was also found in tigers of Ranthambhore National Park, Rajasthan, India. The second shared haplotype, TIG23, was also detected in tigers of Ranthambhore National Park (Mondol et al. 2009b).

4.1. Phylogenetic divergence

Our phylogenetic reconstruction of the Sundarbans haplotypes has revealed a paraphyletic relationship, suggesting that the Sundarbans population has diverged relatively recently from central Indian populations in comparison to the wider population radiations during the late Holocene (Luo et al. 2004). The lower PP and BS support shown in Fig. 4 for the Sundarbans tiger population is likely due to the low resolution afforded by the comparatively modest amount of mtDNA sequence data obtained by this study. The isolation of tigers of the Sundarbans is likely the result of extreme fragmentation of a once continuously distributed tiger population that extended across the Indian subcontinent (Sanderson et al. 2006, Mondol et al. 2009b). The reconstructed tiger distribution models suggest that extreme environmental events during the last glacial maximum (LGM) of ca. 20 000 yr BP might have pushed tigers southwards when the vast continental

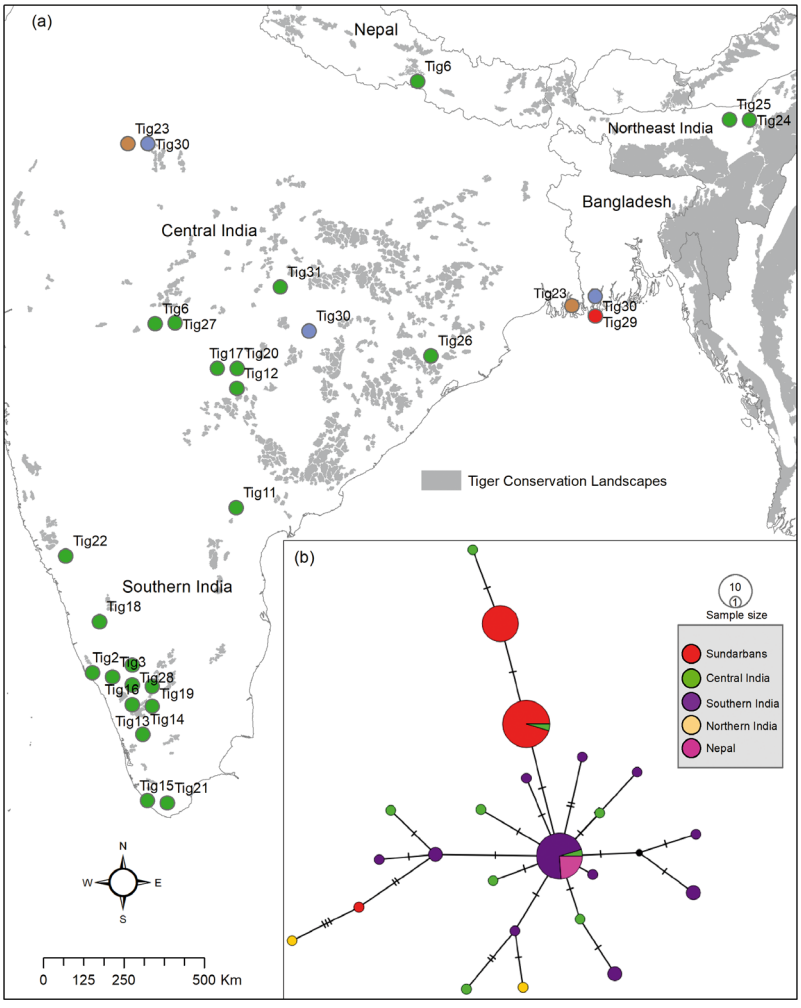

Fig. 3. Distribution and relationship of 20 unique haplotypes detected within Bengal tiger populations, based on 1263 bp across 4 mtDNA genes (details about these haplotypes are given in Table S1). (a) Approximate location of each unique haplotype retrieved from Mondol et al. (2009b) and Luo et al. (2004). Green dots indicate unique haplotypes detected in previous studies, while other colours indicate shared haplotypes detected in the Sundarbans population; TIG29 is unique in the Sundarbans population, while TIG30 and TIG23 are shared with central Indian populations. (b) Median-joining haplotype network, which uses the same 1263 bp mtDNA dataset to visualise the relationship among Bengal tiger populations across the Indian subcontinent, including the Sundarbans. Colours were assigned to each population grouping following Mondol et al. (2009b). The black node indicates an inferred haplotype that has remained undetected. The size of each node is proportional to the haplotype frequency, and the bars on each branch represent the number of mutational steps between haplotypes

shelf was exposed (Kitchener & Dugmore 2000, Siddall et al. 2003, Kitchener & Yamaguchi 2010). During that LGM period, sea levels were approximately 120 m below present-day levels (Siddall et al. 2003), which facilitated the growth of extensive mangroves until the early to mid-Holocene (7000–10 000 yr BP) on the southernmost plain of the Ganges-Brahmaputra delta, extending up to 80–120 km north of the present coastline (Chanda & Mukherjee 1969, Sen & Banerjee 1990). Consequently, during the last few centuries, the markedly increased growth of human activity across the delta — including physical removal of mangroves for wood and as part of reclamation for settlement, agriculture and aquaculture (Naskar 1985, Sikdar & Halt 1997, Verghese 1999, Sarker 2004) — has severed the connectivity of the Sundarbans tigers from the mainland populations (Sanderson et al. 2006, Jhala et al. 2011). Our finding of shared haplotypes between the Sundarbans and central Indian tiger populations corroborates this hypothesis.

4.2. ESU or MU

The Sundarbans tiger population, adapted to unique mangrove habitat, has been geographically isolated from the nearest TCL in Similipal, India, by just ~200 km of landscapes dominated by human settlements and agricultural land. However, this population has ecological traits that show differentiation across different tiger habitats (Sanderson et al. 2006). Across this geographical boundary, we found a close phylogenetic relationship between the Sundarbans population and other populations of Bengal tigers surviving across Indian landscapes. Our finding differs from speculation proposed (Singh et al. 2015) for reciprocal monophyly for the Sundarbans tigers (i.e. coalescence of lineages within each of the 2 taxa before any coalescence events take place between the taxa). Reciprocal monophyly implies that all the members of that population share a more recent common ancestor with each other than with individuals from outside the population (Crandall et al. 2000). However, the processes of population subdivision and speciation are known to produce polyphyletic relationships that slowly progress over time to become paraphyletic and then monophyletic (Neigel & Avise 1986, Takahata & Slatkin 1990, Powell 1991). Thus, applying the crite-

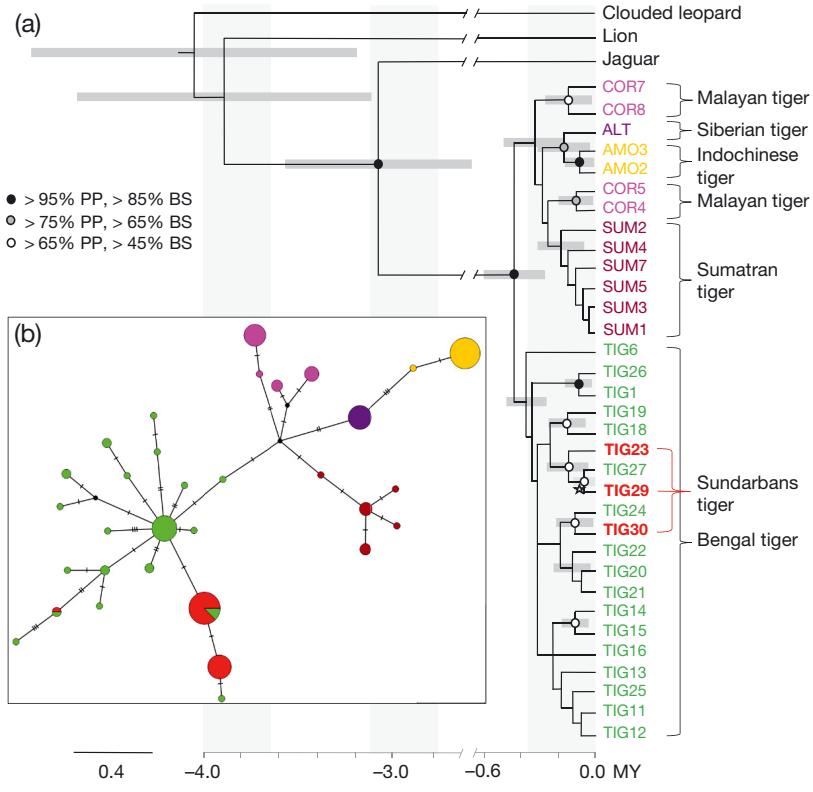

Fig. 4. Phylogenetic position of Sundarbans tigers. (a) Estimated divergence times resolved using BEAST with 33 phylogenetically informative haplotypes (33 sequenced individuals of Bengal tigers together with 33 additional tiger sequences from GenBank) and 2 fossil calibrations. Error bars (grey horizontal bars) display the 95% highest posterior density, and the axis is given in millions of years (MY) before present. PP: Bayesian posterior probability; BS: maximum likelihood bootstrap support. Node values lower than 65% PP and 45% BS were not shown. Node marked with an asterisk indicates the single haplotype TIG29 that is unique to the Sundarbans, with an estimated divergence time of 26 000 yr. Terminal nodes are labelled with names of the unique haplotypes detected in this study, Mondol et al. (2009b) and Luo et al. (2004). Colours represent the different tiger subspecies and the Sundarbans tigers (in red). (b) Median-joining haplotype network comprising 33 mitochondrial haplotypes, with colours representing the 5 tiger subspecies. Yellow: Indochinese tiger; pink: Malayan tiger; maroon: Siberian tiger; fuchsia: Sumatran tiger; green: Bengal tiger. Colours correspond to those in the phylogenetic tree. The size of each node is proportional to the haplotype frequency (detailed sample size information can be found in Table 1), and the bars on each branch indicate the number of mutational steps between haplotypes

rion of reciprocal monophyly to describe the Sundarbans population is not entirely appropriate (Crandall et al. 2000). Furthermore, the monophyletic status presented by Singh et al. (2015) could be misleading because a newly sampled individual can simply overturn a population's reciprocal monophyletic status (Crandall et al. 2000, Fraser & Bernatchez 2001). Indeed, the Singh et al. (2015) study included no samples from the 114 tigers that are estimated from over 60% of the Sundarbans shared by Bangladesh (Aziz et al. 2019). In addition, typically large sample sizes are recommended when assessing the genetic status of any population for ESU designation (Moritz 1994). Furthermore, the previous studies designating the Sundarbans tigers as morphologically distinct or as an ESU are based on relatively limited data, including skull morphological data from 5 individuals from the Bangladesh Sundarbans (Barlow et al. 2010) and mtDNA and nuclear DNA data from 6 and 13 individuals, respectively, from the Indian side of the Sundarbans (Singh et al. 2015). Moreover, a recent detailed study addressing the debate around tiger subspecies concludes that tiger subspecies names are little more than labels for local populations because most of the previous intraspecific taxonomic studies that underpin them often lack a comprehensive analytical approach and frequently used small sample sizes and arbitrary morphological characters (Wilting et al. 2015).

The Sundarbans tiger population may more appropriately be described as a separate MU. An MU designation is more relevant for tigers of the Sundarbans in light of their close phylogenetic connection with mainland tiger populations (Aziz et al. 2018) and ecological differentiation (Legge et al. 1996, Crandall et al. 2000) of global tigers across different habitats (Sanderson et al. 2006). In an ecological context, for instance, tigers in the Sundarbans are particularly adapted to surviving on a limited number of medium-sized prey species, comprising mainly the spotted deer and wild pig, which account for over 89% of the tigers' diet (Aziz et al. 2020), compared to mainland tiger landscapes, where tigers regularly prey on larger ungulates, such as the sambar *Cervus unicolor*, banteng *Bos javanicus*, sika deer *C. nippon* or swamp deer *Rucervus duvaucelii* (Sunquist et al. 1999). Having no such larger prey species in the Sundarbans might have contributed to the relatively smaller skull and body sizes of tigers that survived there (Barlow et al. 2010). Considering the morphological and ecological traits harboured by tigers in the Sundarbans, a less stringent MU status is suited

Table 2. DNA polymorphism within and between all tiger subspecies, and populations of Bengal tigers derived from 33 concatenated mitochondrial haplotypes. N: number of samples *S*: no. of segregating sites; *h:* haplotype diversity; π*:* nucleotide diversity

Tiger subspecies	Population	N	*S*	*h*	π
Bengal					
	Sundarbans	3	5	0.50	0.00266
	Central India	4	11	1.0	0.00266
	Southern India	10	15	0.71	0.00230
	Northeastern India	2	4	0.67	0.00319
	Nepal	1	0	0.0	0.0
Siberian		1	0	0.0	0.0
Indochinese		2	7	0.09	0.00239
Malayan		4	6	0.65	0.00255
Sumatran		6	4	0.68	0.00103

to this population, which is not taxonomically distinct but which is locally adapted and therefore considered worthy of separate conservation management (Legge et al. 1996, Kitchener et al. 2017). Our proposed MU status for the Sundarbans tigers also aligns with recommended taxonomic revisions for *Panthera tigris*. Comprehensive work integrating robust morphological, molecular and ecological data has recognised only 2 tiger subspecies, continental tigers *P. tigris tigris* and Sunda tigers *P. tigris sondaica*, with the former consisting of 2 conservation MUs. These 2 MUs are labelled as northern tigers and southern tigers, where Bengal tigers, including the Sundarbans population, stand together with the South Chinese tiger, Indochinese tiger and Malayan tiger (Wilting et al. 2015). This study rejected the taxonomic division of continental tigers into 6 subspecies (Luo et al. 2004), instead recommending to merge them into a single subspecies (Wilting et al. 2015). Against this backdrop and combining the molecular findings we present here, an ESU designation for the Sundarbans population appears to be less appropriate. Instead, it would seem more appropriate to describe the Sundarbans tiger population as an MU, such that this will not hinder any future efforts of population recovery through reintroduction or exchange of individuals from the mainland tiger populations (Wilting et al. 2015).

4.3. Conservation implications

Maintenance of variability of the different traits that are observed across tiger populations will be necessary for ensuring the long-term viability of global tiger populations, because the remaining variation will be key to their adaptability as environments change (Wilting et al. 2015). The Sundarbans tiger population is important because it is known to be a secured breeding population for the future recovery of tigers (Sanderson et al. 2006, Wilting et al. 2015). However, this uniquely adapted tiger population is at extinction risk due to direct poaching (Aziz et al. 2017), prey population decline (Mohsanin et al. 2013) and human–tiger conflict (Aziz et al. 2019). All these anthropogenic factors have driven the severe decline of this population, from an estimated 300–500 tigers (Barlow 2009) to only 106 (Dey et al. 2015) in only half a decade. In this context, while the ESU designation may highlight the importance of the Sundarbans population, this status may hinder a pragmatic approach to future conservation management of tigers. We recommend that future conservation efforts must focus on sustaining the representative tiger population of mangrove habitat while at the same time recognising that trans-boundary conservation efforts through reintroduction or exchange of individuals might be needed in the future as a last resort for population recovery, to enhance depleted genetic diversity.

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