Title

Using non-invasively collected genetic data to estimate density and population size of tigers in the Bangladesh Sundarbans.

Author names and affiliations

M. Abdul Aziz [a,1,*], Simon Tollington [a,g], Adam Barlow [b], Christina Greenwood [b], John M. Goodrich [c], Olutolani Smith [c,d], Mohammad Shamsuddoha [e], M. Anwarul Islam [f], Jim J. Groombridge [a]

[a] Durrell Institute of Conservation and Ecology, School of Anthropology and Conservation, University of Kent, Canterbury CT2 7NZ, UK

[b] WildTeam, Surfside, St Merryn, Padstow PL28 8NU, Cornwall, UK

[c] Panthera Foundation, 8 West 40th Street, NY 10018, USA

[d] Department of Genetics, Evolution & Environment, University College London, London, UK

[e] Department of Zoology, Jahangirnagar University, Dhaka 1342, Bangladesh

[f] WildTeam, Bangladesh and Department of Zoology, University of Dhaka, Bangladesh

[g] The North of England Zoological Society, Chester Zoo, Caughall Road, Chester, CH2 1LH, UK

* Corresponding author: Department of Zoology, Jahangirnagar University, Savar, Dhaka 1342, Bangladesh; Email: maaziz78@gmail.com; Phone: +88 0176256193

[1] Present address: Department of Zoology, Jahangirnagar University, Dhaka 1342, Bangladesh

Abstract

Population density is a key parameter to monitor endangered carnivores in the wild. The photographic capture-recapture method has been widely used for decades to monitor tigers, *Panthera tigris*, however the application of this method in the Sundarbans tiger landscape is challenging due to logistical difficulties. Therefore, we carried out molecular analyses of DNA contained in non-invasively collected genetic samples to assess the tiger population in the Bangladesh Sundarbans within a spatially explicit capture-recapture (SECR) framework. By surveying four representative sample areas totalling 1,994 km^2 of the Bangladesh Sundarbans, we collected 440 suspected tiger scat and hair samples. Genetic screening of these samples provided 233 authenticated tiger samples, which we attempted to amplify at 10 highly polymorphic microsatellite loci. Of these, 105 samples were successfully amplified, representing 45 unique genotype profiles. The capture-recapture analyses of these unique genotypes within the SECR model provided a density estimate of 2.85 ± SE 0.44 tigers/100 km^2 (95% CI: 1.99-3.71 tigers/100 km^2) for the area sampled, and an estimate of 121 tigers (95% CI: 84-158 tigers) for the total area of the Bangladesh Sundarbans. We demonstrate that this non-invasive genetic surveillance can be an additional approach for monitoring tiger populations in a landscape where camera-trapping is challenging.

Keywords: Bangladesh; Bengal tiger; genetic sampling; population density; Sundarbans.

1. Introduction

Wild tigers now survive within 76 Tiger Conservation Landscapes, representing only seven percent of their ancestral range (Dinerstein et al., 2007). Monitoring changes in the tiger population at each of these landscapes is fundamental in assessing the level of threats and the effectiveness of management actions (Walston et al., 2010). The Indian and Bangladesh Sundarbans, representing 10,236 km^2 of mangrove forest, has been identified as one of 11 Tiger Conservation Landscapes of global priority for long-term conservation in the region (Sanderson et al., 2006). A reliable monitoring approach is, therefore, critical to guide the management of this landscape (Ahmad et al., 2009).

The first tiger population survey in the Sundarbans Reserved Forest of Bangladesh utilised the "pug-mark" method, but this approach was subsequently abandoned due to its methodological shortcomings (Karanth, 2005). Since 2007, a secondary sign survey has been regularly used to monitor changes in relative tiger abundance across the whole forest (Barlow et al., 2008). This type of survey provides reasonable power to detect changes in the relative abundance of tigers over time, but the numerical relationship between the relative abundance index and the actual tiger population size population is not known (Barlow et al., 2008; Hayward et al., 2002). A rough population estimate has also been generated using the estimated home range size of adult female tigers living in the Sundarbans Reserved Forest, but the limitations of this study included a small sample size of only two female tigers (Barlow, 2009). Camera trap surveys carried out in both the Indian and Bangladesh Sundarbans have met with various difficulties, mainly associated with the enormous, daily tidal transformation of this landscape. For example, researchers in India concentrated their efforts around watering holes, because of the difficulty in identifying tiger travel routes in this densely vegetated and muddy habitat, this resulted in very low capture-recapture rates and corresponding density estimates compared to other tiger sites (Karanth and Nichols, 2000). Further camera-trap surveys in both the Indian and Bangladesh Sundarbans attempted to improve capture-recapture rates by using various forms of lures to encourage tigers to come to the camera trap locations (Dey et al., 2015; Jhala et al., 2011). It is not clear, however, if the use of lures in this way has any meaningful effect on the resulting density and population estimates generated from this approach (Dey et al., 2015; Jhala et al., 2011). Moreover, a recent study suffered from the loss of more than half of their camera traps due to suspected theft in the field (Hossain et al., 2016).

Advances in DNA technology have enabled researchers to incorporate non-invasive genetic techniques to survey tiger populations at some sites (Bhagavatula and Singh, 2006; Mondol et al., 2009a), but this approach has never been used before in the Sundarbans. The objective of this study was, therefore, to investigate if this non-invasive genetic approach could be used to provide a reliable density and population estimate for the tiger population in the Sundarbans landscape.

2. Methods

2.1. Study site

The Sundarbans, the largest contiguous mangrove forest of the world, is located on the Ganges-Brahmaputra delta (Giri et al., 2007). The part of the Sundarbans in Bangladesh (21º30′–22º30′ N, 89º00′–89º55′ E) covers 6,017 km^2, of which 4,267 km^2 is forest and the remaining area is comprised of water bodies (Iftekhar and Islam, 2004a). The Sundarbans is bordered on the south by the Bay of Bengal and on the north and east sides by a landmass dominated by human settlements (Hussain and Acharya, 1994). Two rivers, the Raimangal and the Hariabhanga, mark the international boundary between Bangladesh and India, and separate the Indian part of the Sundarbans (Fig. 1).

The Bangladesh Sundarbans is managed as a reserve forest, except three demarcated areas within the forest that were declared wildlife sanctuaries (IUCN Category III and VI) in 1996 for the higher protection of wildlife and their habitat (BFD, 2012). The sanctuaries comprise the Sundarbans West (715 km^2), Sundarbans South (370 km^2), and Sundarbans East (312 km^2), and were collectively declared a UNESCO World Heritage Site in 1997 (BFD, 2012; Iftekhar and Islam, 2004a) (Fig. 1).

The Sundarbans is one of the most biologically diverse mangrove forests in the world, supporting 330 species of plants, more than 400 species of fishes, 35 species of reptiles, over 300 species of birds, and 42 species of mammals (Islam and Wahab, 2005; IUCN–Bangladesh, 2001). The tiger is the only large terrestrial carnivore in the Sundarbans; their major prey species include spotted deer (Axis axis), wild boar (Sus scrofa) and barking deer (Muntiacus muntjak) (Khan, 2008). Several small carnivores found in the forest include fishing cat (Prionailurus viverrinus), jungle cat (Felis chaus) and leopard cat (Prionailurus bengalensis).

The Sundarbans Reserved Forest is mostly comprised of two tree species; Sundri (Heritiera fomes; 39%) and Gewa (Excoecaria agalloch; 39%), with other species constituting only 16% of the forest cover (Iftekhar and Saenger, 2008). The Sundarbans is characterized by a maritime, humid climate with very seasonal weather patterns (Iftekhar and Islam, 2004b). Most of this area is less than one meter above the sea level (Canonizado and Hossain, 1998), and consists of vegetated islands that are inundated regularly by two high and low tides each day with a mean amplitude of 3-4 m (Chaffey et al., 1985; Gopal and Chauhan, 2006).

2.2. Sampling approach

To collect non-invasive genetic samples (scat or hair), four sampling areas (totalling 1,994 km^2) were selected within the Bangladesh Sundarbans: Satkhira block (SB, 554 km^2), West Wildlife Sanctuary (WS, 715 km^2), East Wildlife Sanctuary with additional areas (ES, 383 km^2) and Chandpai block (CB, 342 km^2) (Fig. 1). Location, protection status and level of human use were considered in selecting these sample areas. The ES and WS areas have higher protection status and are situated away from human settlements, whereas the CB and SB areas have lower protection status and are located close to local villages. The Forest Department issues permission to local people for collecting forest and aquatic resources (e.g., nypa palms, honey, fish and crabs) from SB and CB sample areas, but not from the ES and WS (Aziz et al., 2017).

Following standard capture-recapture approaches (Karanth, 1995; Karanth and Nichols, 1998) to select sampling points, each sampling area was divided into 2×2 km grid cells creating a total of 373 grid cells for potential sampling. A survey team of four trained field staff searched each grid cell with three separate transects (using one transect each time). Starting points for each transect were selected by where the grid cell could be easily accessed by boat. From the start point the field team walked each transect roughly in the direction of the opposite side of the grid square. Each transect was walked for a length of 1 km, or until the observers could not continue further because of particularly dense habitat or a large water body obstructing their way. The field team walked in parallel along the line of each transect, with the distance between the first and last observer being maintained at approximately 15 m (5 m between each observer). Five survey field teams, each with four observers, were used to simultaneously survey a sample area over a short (13-22 days) period of time for sample collection.

Field teams managed to survey 10 grid cells with four transects, 297 grid cells with three transects, 7 grid cells with two transects, and 32 grid cells with one transect. A total of 27 (11%) grid cells were not surveyed due to inaccessibility and security issues.

Winter months were chosen for sampling to avoid extreme weather conditions, and to maximise the chance of collecting dry samples. We sampled SB areas from 20 November to 11 December 2014, WS areas from 17 to 30 December in 2014, and areas ES and CB from 4 to 26 February 2015. Survey teams recorded location data for each sample using handheld Global Positioning System (GPS) Garmin GPSMAP 64.

Suspected tiger scat samples, identified by size and associated signs (Johnsingh, 1983; Karanth et al., 1995), were collected in 100ml polypropylene tubes (ThermoFisher Scientific, UK) using twigs to avoid contamination. All scat samples were air-dried before being preserved with silica gel desiccant, and stored at -20 ºC until extraction within a month of collection. Suspected tiger hairs, identified by being associated with territorial scratch marks on trees, were also collected (Sharma et al., 2012). High quality tiger blood and tissue samples were also collected from captive tigers or confiscated tiger products that originated from the Bangladesh Sundarbans to provide reference genotypes for comparison with our field collected samples: one blood sample (from a rescued tiger), five tissue samples (from confiscated skins) and four hair samples (from confiscated and rescued tigers) were collected.

2.3. DNA extraction

All biological samples were transported to the Durrell Institute of Conservation and Ecology, University of Kent, for analyses under permits (Permit No. BD 9118404) from the Convention on International Trade in Endangered Species (CITES), and Department for Environment, Food and Rural Affairs, United Kingdom (Authorization no. AHVLA: TARP/2015/111).

Genomic DNA from scat samples was extracted using QIAamp DNA Stool mini kits (QIAGEN Inc.) following the manufacturer's instructions. Approximately 200 mg of scat material was scraped from the outer surface of each scat sample with a sterilized razor blade and then incubated overnight with 1.5 ml ASL buffer on a mechanical rotator at 56 ºC. The DNA supernatant from the sample was lysed with 300 µl AL buffer plus 25 µl proteinase K and incubated at 70 ºC for 15 min. Four microlitres of carrier RNA (ThermoFisher Scientific, UK) was added to AL buffer to increase DNA yield from scat samples. To extract DNA from blood, tissue, and hair samples, we used DNeasy™ Blood and Tissue Kits (QIAGEN Inc.); approximately 10 hairs of each sample was added to 300 µl AL buffer incorporating 20 µl of proteinase K and 20 µl of DTT (Dithiothreitol, Biotech), and then incubated at 56 ºC overnight or until the sample was completely digested. The elution of DNA was carried out in 75 µl buffer solution. Strict protocols was observed to reduce the chances of contamination including using aerosol barrier pipette tips, separate pre and post PCR rooms and UV PCR hoods for sample preparation. A negative control (with no biological material) was included with each batch of extractions to monitor for possible contamination during the DNA extraction procedure.

2.4. Species authentication

Scat morphology and associated secondary signs have been used to identify scat samples of the study species (Bagchi et al., 2003; Karanth et al., 1995). However, non-target scats can potentially be misidentified and collected when such field protocols are used in isolation (Farrell et al., 2000), and therefore more reliable DNA-based identification of non-invasive scat samples is necessary to avoid inadvertent sampling of scat from non-target species (Bhagavatula and Singh, 2006). A PCR-based assay was used to reliably identify tiger samples (Bhagavatula and Singh, 2006; Davison et al., 2002; Mondol et al., 2009a), so that only genetically authenticated samples were included in further downstream analyses (Mondol et al., 2009a). All field-collected samples were screened using tiger-specific $NADH_5$ gene fragment of 225 base pairs (fwd TTACTAGGACTCCTCCTAGCC; rev GAATAGGGTTGTGATGGCCCC) that has been successfully used in other non-invasive tiger studies (Mukherjee et al., 2007). In this screening process, PCR reaction volumes (total 27 µl) contained 3 µl of template DNA, 12.5 µl MyTaq Redmix (containing dNTPs and $MgCl_2$; Bioline, UK), 5 µM of each forward and reverse primer, 4 µM BSA (Bovine Serum Albumin, New England Biolabs Inc.) and 8.5 µl dH_2O. PCR cycling conditions for this screening process consisted of an initial hot start of 95 ºC 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 France). PCR products were then purified and sequenced using a 3730XL analyser (Macrogen, Amsterdam, Netherlands). Mitochondrial DNA (mtDNA) sequences were edited using Jalview v2 (Waterhouse et al., 2009), and then cross-checked with sequences from the Genbank database (National Center for Biotechnology Information, NCBI) to ensure that positive PCR samples were in fact tiger. This level of rigorous screening process using a tiger-specific gene fragment and the resultant sequence blasting ensured that authenticated samples were not contaminated with prey DNA, and/or not sourced from other wild cats that might have been eaten by tigers. DNA samples that showed poor quality, or no bands in the species-specific PCRs after three independent extraction attempts were removed before microsatellite amplification (Kohn et al., 1999).

2.5. Microsatellite amplification and sex determination

A range of microsatellite primers have been developed in the domestic cat (Menotti-Raymond et al., 1999), and successfully applied in investigating population abundance (Mondol et al., 2009a), genetic structure (Mondol et al., 2009b; Reddy et al., 2012), spatial genetics (Sharma et al., 2012), and connectivity of tiger populations across India (Joshi et al., 2013). Considering the high number of alleles observed in these studies (Bhagavatula and Singh, 2006; Menotti-Raymond et al., 1999; Mondol et al., 2009a), a preliminary set of 14 loci were selected for this study (Table A.1). These loci were then optimised using a subset (n = 10) of field-collected scat samples and reference samples (n = 10). Based on levels of PCR amplification success, allelic richness, and extent of genotyping errors, a set of 10 loci were chosen to genotype all field-collected samples that had been genetically authenticated as being from tiger (Table A.2). A felid specific zinc-finger (Zfx and Zfy) locus was also optimised using samples from known male (n = 1) and female tigers (n = 2) for sex determination (Pilgrim et al., 2005).

Four multiplexes were designed to include the full set of loci. All forward primers were fluorescently labelled for gene-scanning (Table A.2). Each microsatellite PCR reaction volume (10 µl) contained 5 µl Qiagen multiplex PCR buffer mix (Qiagen Inc.), 0.2 µM labelled forward primer (Eurofins Genomics), 0.2 µM unlabelled reverse primer, 2 µM BSA, and 3 µl of DNA template. For all multiplex reactions, the PCR temperature regime included an initial denaturation step of 95 °C for 15 min, 45 cycles of denaturation (94 °C for 30 s), annealing (T_a ranges from 52 °C to 57 °C for 90 s for four multiplexes; Table A.2), extension (72 °C for 90 s), and a final extension of 10 min at 72 °C, using a G-Storm Thermal Cycler. All PCR products were genotyped using an Applied Biosystems 3730 DNA Analyser and ROX 500 ROX™ as the size-standard.

2.6. Genotype data validation

To reduce the possibility of genotyping errors, we discarded any DNA samples that amplified at fewer than three loci at the first PCR attempt; these were re-extracted from source and included in subsequent PCRs thus ensuring that poor quality samples were immediately eliminated (Creel et al., 2003). Furthermore, we employed the comparative genotyping approach (Frantz et al., 2003; Hansen et al., 2008) by ensuring that equivalent heterozygote genotype profiles were scored at least twice and corresponding homozygote genotypes at least three times (up to a maximum of five). This approach ensured a level of rigour in resolving the true genotype of each scat sample and was less laborious and more cost-effective than the multiple tubes approach (Taberlet et al., 1997). A consensus genotype was achieved if genotypes matched 100% at all loci in at least two repeats. If genotype consensus was not reached in five independent scoring attempts the samples were removed from the analysis (Jackson et al., 2016). A negative control was included with each batch of PCR reaction to monitor for possible contamination. Genotyping errors due to stuttering were checked using the program MICROCHECKER v2.2.3 (van Oosterhout et al., 2004). Allele frequencies, observed (Ho) and expected (He) heterozygosity, allelic dropout, false alleles and tests for adherence to the Hardy-Weinberg equilibrium were quantified using GIMLET v1.3.3 (Valiere, 2002). Alleles were identified and scored using GENEMAPPER v3.7 (Applied Biosystems, MA, USA).

2.7. Individual identification

The set of 10 polymorphic loci were used to create consensus genotype profiles for all samples. To distinguish between closely related individuals and to avoid an overestimation of population size (Kohn et al., 1999; Waits et al., 2001), we determined the required number of loci using the probability of identify for siblings, PID(sibs), based on polymorphic information content (PIC) of the loci (Bhagavatula and Singh, 2006; Mondol et al., 2009a; Waits et al., 2001). In addition, three reference samples were sourced from confirmed siblings, which we used to estimate the PID(sibs) in order to determine the required number of loci that could sufficiently distinguish between them. By combining this result with PIC values for the microsatellite loci, we determined a set of five polymorphic loci that were sufficient to distinguish siblings within the population. The program GIMLET v1.3.3 was used to estimate PID(sibs) for the microsatellite loci (Valière, 2002). We then compared consensus genotype profiles in the program CERVUS v3.0 (Marshall et al., 1998) to identify matched genotypes with a minimum of loci criteria. The identity module of CERVUS produced a matrix of pair-wise comparisons that allowed us to separate matched and unmatched individuals based on the criteria of a minimum five loci. While examining the pair-wise matrix, we carefully checked genotypes that differed by fewer than three loci, where we allowed up to two mismatches considering genotyping errors in the dataset (Creel et al., 2003). Matching genotypes based on five or more loci were considered to be sourced from the same individual and classified as a recapture (Budowle, 2004; Mondol et al., 2009a). Incomplete or partial genotype profiles, genotyped at 5 – 10 loci, were also used following approaches used in studies involving tigers (Bhagavatula and Singh, 2006; Mondol et al., 2009a), and badgers (Frantz et al., 2003). When partial profiles were used, we carefully considered samples that had amplified the most informative loci, namely Fca279, Fca232, Fca090, Fca672, and D15. Although it is possible that an incomplete genotype might actually have originated from a new individual (Mondol et al., 2009a) using incomplete genotype profiles in this way provides a conservative population estimate (Bhagavatula and Singh, 2006; Frantz et al., 2003) by minimising the possibility of creating non-existent individuals through genotyping error (Mondol et al., 2009a).

2.8. Density estimation

To estimate tiger population density, we used a likelihood-based spatially explicit capture-recapture (SECR) approach that has become widely used for estimating densities of large carnivores, including tigers (Kalle et al., 2011), leopards, *Panthera pardus* (Kalle et al., 2011), jaguars, *Panthera onca* (Sollmann et al., 2013), and European wildcat, *Felis silvestris silvestris* (Kéry et al., 2010). SECR uses detection locations to fit a spatial likelihood-based model, avoiding the need to estimate *ad hoc* effective sample area. Moreover, the 'area search polygon' (sample area) approach in SECR allows an analysis of all detections (capture-recaptures) of all individuals by pooling them together as a 'single session' (Efford, 2011), avoiding the difficulty of assigning non-invasive samples to predefined sample occasions.

The SECR model assumes that no animal activity centres can occur in non-habitat beyond the animal's range (Efford, 2011; Efford et al., 2009). Therefore, density estimates can potentially be biased if non-habitat is included in the 'sample area polygon' (Efford, 2011; Gerber et al., 2012). Tigers in the Sundarbans are known to navigate water bodies up to but rarely exceeding 1.5 km wide (Barlow, 2009). Therefore, 'non-habitat' of tigers (e.g., water bodies more than 1.5 km wide and human settlements on the northern boundary of the area sampled) were removed from the buffer area; defined as the adjoining area of the sample area polygon ($3 \times \sigma$) where activity centres of sampled tigers can occur (Efford, 2011; Gerber et al., 2012; Mace et al., 1994). For the SB and CB sample areas, tiger movement is restricted on the north side by densely populated human settlements separated by rivers. The WS sample area is bounded on the south side by the Bay of Bengal and on the west side and most of the east sides by rivers >3 km wide. Similarly, tiger movement is restricted on the south side of the ES sample area by the Bay of Bengal and on the east side by rivers >3 km wide

(Fig. 1). Consequently, these areas were also excluded from the overall sample area in the SECR analysis.

Two matrices of spatiotemporal detection history and spatiotemporal search area polygons were used in the SECR analysis for estimating density parameters. The spatiotemporal detection history included capture-recapture locations for each individual tiger and the spatiotemporal search area polygon contained geographic coordinates defining the area sampled. Using these two input datasets, a detection model was fitted by maximum likelihood, with parameters, g0 (detection probability at the activity centre of the animal's home range), and σ (the spatial movement parameter away from the centre of the animal's home range). Using the detection function as half-normal, g0 and σ were modelled as constant to estimate overall and sample area-wise tiger density (Borchers and Efford, 2008; Efford, 2011). The SECR analysis was carried out in the R package SECR v2.10.3, and ArcGIS v10.3 was used for creating polygons of areas sampled.

3. Results

3.1. Species and individual identification

A total of 440 samples of putative tiger faeces and hair were collected. Molecular identification using tiger-specific $NADH_5$ primers confirmed the existence of 233 (53%) tiger samples after replicate extraction and amplification procedures. The remaining samples were discarded from further analysis because they failed to produce quality, identifiable tiger DNA. A final set of 105 separate scat and hair samples were genotyped at 5-10 loci (see Table 1 for full sample information).

A higher level of amplification success was obtained for the reference samples (13 loci showed 100% amplification) than the field collected samples (78 - 100%) (Table A.1). Using the set of 10 microsatellite loci, we were able to derive consensus genotypes, based on a minimum of five loci, for 105 scat and hair samples (45% of the tiger-positive samples). A higher genotyping success rate was obtained for samples from the CB sample area (58%) compared to the SB area (39%).

The CERVUS analysis yielded high proportions of pairwise matrix with zero difference (ranging from 49% to 74% for sample areas) as well as pairwise matrix that differed by more than 7 loci (ranging from 15% to 26% for sample areas) of the final sample genotypes. Using a minimum of five loci criteria, a total of 45 individual tigers comprising six from SB, 15 from WS, 14 from CB and 10 from the ES sample area was identified from 105 (capture and recaptures) genotype profiles (Table 2). Sexing of individuals was attempted for these 45 individuals resulting in a total of 11 males and 24 females. The sex of the remaining 10 individuals could not be determined due to inconclusive genotypes.

All loci were polymorphic with a mean number of alleles of 5.50 ± SD 1.65 per locus. The marker set revealed a level of polymorphism sufficient to distinguish between individuals, with a mean PIC of 0.58. Several loci showed allelic dropout and false alleles in the dataset. Deviations from Hardy-Weinberg equilibrium were also detected for loci FCA304, FCA279 in ES; D15 in SB; and FCA230, FCA279 for samples from the CB area (Table 3).

3.2. Estimating tiger density

The estimated probabilities of detections of 45 tigers ranged from 0.02 to 0.04 across the four sample areas, with the highest in the ES area and lowest in the SB area (Table 2). The null model, D(.)g0(.)σ(.), yielded an overall tiger density of 2.85 ± 0.44 SE tigers/100 km^2 (95% CI: 1.99-3.71). The highest density of tigers was estimated for the CB area (3.18 ± SE 0.90) followed by the ES (3.17 ± SE 1.04), WS (2.99 ± SE 0.80) and SB (1.86 ± SE 0.81) (Table 2). By extrapolating the overall tiger density of 2.85± SE 0.44 tigers/100 km^2 to the total of 4,247 km^2 occupied by tigers (Dey et al., 2015), we estimate that the Bangladesh Sundarbans may currently support approximately 121 tigers (95% CI: 84-158).

4. Discussion

4.1. Identifying species and individual identity of tigers

Although there are no large carnivores in the Sundarbans except tigers, DNA-based screening to genetically confirm species ensures that samples from non-target species are removed prior to downstream analysis (Mondol et al., 2009a; Mukherjee et al., 2007). The low PCR amplification rate (53%) in this study compared to higher success rates reported from drier areas in India (e.g. 93% in Bandipur National Park, India; Mondol et al., 2009a), may be a consequence of inferior sample quality due to the humid and wet mangrove habitat in the Sundarbans.

Each microsatellite locus used in this study amplified a region less than 160 base pairs, so they were appropriately-sized to amplify low quality, potentially highly fragmented faecal DNA (Bhagavatula and Singh, 2006; Frantzen et al., 1998). The overall genotyping success rate of all samples (46%) was relatively low because of the rigorous screening process undertaken to reduce genotyping errors. Although no genotyping errors were detected in the reference samples, field samples produced 5-26% genotyping errors for five loci (Table A.1; Table 3). These error rates, however, are reasonably low when compared to other non-invasive genetic studies of tigers (2-65%) (Bhagavatula and Singh, 2006), and other carnivore species such as wolves (3-33%) (Lucchini et al., 2002).

The five most informative loci with a PID(sibs) value of 0.0186 (0.0003 for full microsatellite panel) and mean PIC value of 0.58 demonstrated that together these loci could successfully distinguish even siblings with 99% certainty. We also note that the PID(sibs) for the five least informative loci accounted to be 0.0193, which is close to the value of the five most informative loci, might be due to the fact that the number of alleles for our loci set ranged from 4 to 7, mostly with 5 alleles (see the Table 3 for details). These PID(sibs) estimates closely aligned with the suggested value of approximately 0.01 for studies intended to estimating population density following mark-recapture approach (Waits et al., 2001). With this level of statistical rigour, we determined that a minimum of five loci of the set used in this study were sufficient to distinguish unique genotype profile from the pool of pair-wise genotype matrix. Using the conservative cumulative PID (sibs) attained for the five loci, and with suggestions made in similar tiger studies (Mondol et al., 2009a), we therefore avoided an overestimation of population size by reducing the incidence of false individuals due to genotyping error (Creel et al., 2003; Bhagavatula and Singh, 2006). There is a general consensus that genotyping error might not be completely eliminated from the dataset demonstrated in non-invasive genetic studies (Bhagavatula and Singh, 2006; Creel et al., 2003; Mondol et al., 2009a), it is therefore reasonable to allow genotypes with one or more mismatches to be scored as identical to avoid an overestimate (Creel et al., 2003). Moreover, it is plausible that our scored individuals represent an underestimate of the true population abundance, however on the management perspective this would be an impetus for protected area managers to intensify monitoring and law enforcement for this important tiger landscape.

4.2. Estimating tiger density

Our estimates of tiger density and population size most likely accounts for adult and sub-adult tigers, which gives a sex ratio of 2.18 females : 1 male, similar to sex ratios recorded in other sites for adult tigers (Barlow et al., 2009). It is also important to note that the strict methodological procedures followed in our study might have excluded an unknown number of tigers. It is possible that our sampling approach was not representative of the entire Bangladesh Sundarbans population or that it did not account for tigers from all demographic groups. For example, tiger scats from juveniles may not have been detected because this group tends to have more limited, clumped movement patterns, (Smith, 1978), or they may not have been collected due to the similarity in size to the scats of other species (e.g. fishing cat or leopard cat). Moreover, survey teams did not collect suspected tiger scats that were degraded due to being submerged by tidal waters in areas of low elevation. Therefore the overall lower probability of detections in our study may be due to the sampling approach and/or subsequent screening of samples.

However, despite these methodological limitations, our density estimates (2.85 tigers/100 km^2) are comparable to the 2.17 tigers/100 km^2 estimated in a recent camera-trap survey in the Bangladesh Sundarbans (Dey et al., 2015). Our extrapolated population size estimates (95% CI: 84-158 tigers) are also in line with the camera trap results (SE interval: 84-130 tigers) produced by Dey et al. (2015). It is relevant to mention that, due to the overlapping sampling period between these studies (Dey et al. sampled between 2013-2015; this study sampled between 2014-2015), we extrapolated our density estimates for the entire Sundarbans in order for meaningful comparisons to be made. Both studies used SECR modelling in density estimates. Furthermore, our density estimates for SB (1.86 ± SE 0.81 tigers/100 km^2) and ES sample areas (3.17 ± SE 1.04 tigers/100 km^2) were also similar to estimates from camera-trap data in Block III (2.77 ± SE 0.78 tigers/100 km^2), and Block I (3.70 ± SE 0.91 tigers/100 km^2) (Table 4). These two areas overlap with our study area and that of the camera-trap study by Dey et al. (2015).

In contrast, a study by Barlow (2009) estimated much higher tiger density (9.33 tigers/100 km^2) but similar population size (133-200 adult and sub-adult individuals) in the Bangladesh Sundarbans using telemetry. The difference in the estimate of tiger density is most likely due to differences in sampling method (telemetry versus DNA sampling) or changes in the tiger population in the time that separated the two surveys. Although current tiger densities in the

Bangladesh Sundarbans may also be lower than densities estimated in Nepal and Bhutan (e.g., Karki et al., 2015; Thinley and Curtis, 2015), combining our study's estimates with the estimated 4.3 ± SE 0.3 tigers/100 km^2 for the Indian Sundarbans (Jhala et al., 2015, 2011), supports previous assertions (Barlow, 2009) that the entire Sundarbans has the capacity to support one of the largest tiger populations, up to 197 tigers (95% CI: 146-254), in the world.

4.3. Conservation implications

We have demonstrated the utility of noninvasive genetic sampling to assess the tiger population of the Bangladesh Sundarbans, complementing camera trap and secondary sign surveys already employed in this landscape (Barlow et al., 2008; Dey et al., 2015). For assessing population parameters in conventional camera trap studies, it is critically important to place camera trap on routes regularly travelled by tigers (Karanth and Nichols, 1998) in order to obtain improved detections for precise estimates. The topography of the Sundarbans mangrove habitat is only few meters above the sea level, therefore, most of the forest land is regularly washed by tidal waters twice daily, leaving few recognizable tiger signs that could be used for camera placements. As a result, previous camera trap studies were able to obtain limited detections both in Bangladesh (Khan, 2012) and the Indian Sundarbans (Karanth and Nichols, 2000), except the one that used lures and baits (Dey et al., 2015). Moreover, a recent study could not recover more than half of their camera traps from the Bangladesh Sundarbans due to suspected theft (Hossain et al., 2016). Given these challenges with camera trapping, we have demonstrated that non-invasive genetic sampling approach could overcome these constraints with considerable success. Additionally, potential statistical biases related to using various types of lures to bring tigers to camera-traps sites (Kéry et al., 2010; Mowat and Strobeck, 2000; Noyce et al., 2001) or disease transmission (Thiry et al., 1988) using some forms of lures can be overcome by using the non-invasive genetic technique.

The limitations of camera trap studies include the requirement to follow typical field designs for installation, they need to be maintained in often challenging conditions in which they are prone to failure and even theft, and the logistics for vast survey areas such as the Sundarbans are considerable. Conversely, a non-invasive genetic sampling approach is much easier to implement where all the genetic samples collected over a short period of time could be pooled together without assigning them into different sampling sessions, and can be analysed adopting the 'area search' SECR approach (Efford, 2011).

Estimates of population density and size of our study are almost similar to camera trap surveys. One of the major challenges of non-invasive genetic technique is to ensure good quality DNA extraction for successful amplification and individual detection. However, we note that genetic sampling can provide additional demographic and population-level information which can be useful for detailed monitoring of these populations. For example, genetic status, sex ratios, family size, effective population size, patterns of dispersal etc.

Finally, non-invasive genetic sampling can be advantageous over camera trapping for other low density and secretive carnivores (e.g., fishing cat) which cannot be detected and identified by camera trapping using their natural markings.

We conclude that non-invasive genetic sampling is an appropriate method for assessing tiger population in the Sundarbans mangrove habitat where camera trapping techniques face a range of constraints in relation to limited detections due to unsuitable habitat condition. Therefore, the future monitoring of tigers to determine long-term patterns of population demography and genetic health in this habitat could be carried out by non-invasive genetic sampling.

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Table 1. Summary of scat samples (plus number of hair samples in parentheses) collected, screened and genotyped from each of the sample areas of the Bangladesh Sundarbans between November 2014 and February 2015.

Sampling area	Area* (km^2)	Protection status	Sampling duration (days)	Samples collected from field	Samples screened as tiger	Samples genotyped for at least 5 loci	Samples sexed
Satkhira Block (SB)	342	Reserve forest	21	62(15)	23(10)	10(5)	6(5)
West Wildlife Sanctuary (WS)	715	Protected area	13	124(28)	61(21)	21(12)	16(12)
Chandpai Block (CB)	554	Reserve forest	21	91(36)	57(17)	27(6)	19(6)
East Wildlife Sanctuary (ES)	383	Protected area	21	62(22)	29(15)	18(6)	17(6)
Totals	1,994	--	**76**	**440**	**233**	**105**	**87**

* Area included forest land and waterbodies

Table 2. Sample area (forest land only), capture-recaptures and density parameter estimates with spatially explicit capture-recapture (SECR) model for area-wise and overall estimates of tigers using non-invasively collected DNA data from the Bangladesh Sundarbans between November 2014 and February 2015.

Name of sample area	Area* (km^2)	No. of individuals detected	No. of total detections	Tiger density (D ± SE per 100 km^2)	Probability of detection (g0 ± SE)	Spatial distance moved (σ ± SE km)
Satkhira Block (SB)	275	6	15	1.86 ± 0.81	0.0226 ± 0.0098	3.989 ± 0.825
West Wildlife Sanctuary (WS)	414	15	33	2.99 ± 0.85	0.0185 ± 0.0057	3.920 ± 0.506
Chandpai Block (CB)	418	14	33	3.18 ± 0.90	0.0224 ± 0.0071	3.088 ± 0.438
East Wildlife Sanctuary (ES)	290	10	24	3.17 ± 1.04	0.0361 ± 0.0128	2.918 ± 0.416
Overall (all sampled areas)	1,397	45	105	2.85 ± 0.44	0.0223 ± 0.0038	3.478 ± 0.262

* Area estimated excluding waterbodies.

Table 3. Genetic variability at 10 microsatellite loci for field samples (n = 105*) collected from the Bangladesh Sundarbans between November 2014 and February 2015.

Locus	Allele size range (bp)	No. of alleles/locus	Dropout rate	False allele rate	H_E	H_O	$P_{ID(sibs)}$
FCA279	97-107	7	0	0.19	0.78	0.5	8.14E-02
FCA232	99-113	5	0	0	0.78	0.42	6.79E-03
FCA090	107-117	5	0	0	0.77	0.38	6.61E-04
FCA672	93-105	6	0	0	0.67	0.24	1.45E-05
D15	119-139	5	0	0.12	0.68	0.39	9.61E-05
FCA304	121-129	4	0.26	0	0.67	0.34	2.44E-06
FCA126	138-144	4	0	0	0.68	0.15	4.17E-07
F41	111-135	6	0.05	0	0.63	0.59	7.61E-08
FCA230	103-115	7	0	0	0.54	0.14	1.19E-09
E7	137-151	5	0	0	0.56	0.28	4.61E-09

*Sample area-wise amplified samples: SB (n=15), WS (n=33), CB (n=33), ES (n=24)
He: Expected heterozygosity, Ho: Observed heterozygosity.

Table 4. Sample area-wise comparison of tiger density estimates between this study and a camera-trap survey by Dey et al. (2015) in the Bangladesh Sundarbans.

Sampling area	Study method	No. of individuals detected	SECR model	Tiger density (D±SE per 100 km^2)	Probability of detection (g0 ± SE)	Spatial distance moved (σ ± SE km)
Area-wise						
Satkhira Block[a]	DNA study	6	D(.)g0(.)σ(.)	1.86 ± 0.81	0.0226 ± 0.0098	3.989 ± 0.825
Block III (Satkhira)[a]	Camera traps	13	D(.)g0(bk)σ(.)	2.77 ± 0.78	0.0100 ± 0.0020	4.270 ± 0.050
East Wildlife Sanctuary[b]	DNA study	10	D(.)g0(.)σ(.)	3.17 ± 1.04	0.0361 ± 0.0127	2.918 ± 0.416
Block I (Sarankhola)[b]	Camera traps	18	D(.)g0(bk)σ(.)	3.70 ± 0.91	0.0100 ± 0.0030	3.370 ± 0.350
Overall						
Sampling area (1,397 km^2)	DNA study	48	D(.)g0(.)σ(.)	2.85 ± 0.44	0.0231 ± 0.0038	3.478 ± 0.262
Sampling area (1,265 km^2)	Camera traps	38	D(.)g0(bk)σ(.)	2.17 (1.73-2.68)	Not available	Not available

Note: [a]Satkhira Block completely overlapped with Block III (Satkhira), and [b]East Wildlife Santuary with Block I (Sarankhola) of camera-trap study (Dey et al., 2015).

Table A.1. Characteristics of 14 microsatellite loci optimized for reference samples (RS, n = 10) and field-collected samples (FS, n = 10).

Locus*	Allele size range (bp)	Amplification success (%)		Allelic dropout		False alleles		No. of alleles/locus		Expected heterozygosity, H_E		Observed heterozygosity, H_O		Probability of identity, $P_{ID(sibs)}$	
		RS	FS	RS	FS	RS	FS	RS	FS	RS	FS	RS	FS	RS	FS
FCA090	111-113	90	78	0	0	0	0	4	5	0.69	0.81	0.63	0.63	1.86E-02	1.64E-01
FCA672	93-105	100	100	0	0	0	0	4	5	0.76	0.81	0.5	0.8	4.23E-01	3.99E-01
FCA232	99-103	100	89	0	0	0	0	5	5	0.73	0.71	0.3	0.33	1.87E-01	7.62E-02
D15	119-139	100	89	0	0	0	0	4	4	0.71	0.71	0.67	0.9	8.54E-02	3.71E-02
FCA279	99-107	100	100	0	0	0	0	3	3	0.62	0.66	0.44	0.6	9.15E-03	1.86E-02
FCA304	121-129	100	89	0	0	0	0	4	3	0.5	0.66	0.3	0.44	8.49E-04	4.88E-03
F41	111-133	100	89	0	0	0	0.14	5	5	0.55	0.66	0.33	0.4	1.44E-03	9.44E-03
FCA126	140-144	100	89	0	0.11	0	0	3	3	0.71	0.49	0.22	0.1	3.96E-02	1.53E-03
FCA309	98-100	100	89	0	0.11	0	0	2	2	0.5	0.48	0.11	0.1	3.07E-04	2.58E-03
FCA230	105-115	100	100	0	0	0	0	6	3	0.57	0.29	0.5	0.2	2.58E-03	2.74E-04
E7	138-151	100	100	0	0	0	0	3	3	0.43	0.27	0.3	0.1	1.23E-04	2.74E-04
FCA043	120-130	100	89	0	0	0	0	2	2	0.48	0.52	0.3	0.44	1.91E-04	5.40E-04
FCA052	108-114	100	100	0	0	0	0	3	2	0.61	0.39	0.4	0.3	4.81E-03	3.35E-04
FCA164	80-90	100	100	0	0	0	0	2	2	0.53	0.53	0.78	0.78	5.04E-04	9.09E-04

*All loci optimized from Menotti-Raymond et al. (1999), except E7 and D15 (Bhagavatula and Singh, 2006).

Table A.2. Locus name, primer sequences, annealing temperature (AT), forward primer fluorescent dye (FD), and PCR multiplexes (PM) used in this study.

Locus name	Forward sequence	Reverse sequence	AT (°C)	FD	PM
F41	GTCTGCATCTTCAAATAGGA	GTACCTGAGTTGGCTGTTGA	56	FAM	Set 1
D15	TGTGACCTTTCTCTAGTTTC	GCACAAAACATTCAGTCTCC	55	FAM	Set 1
Fca232	ATGACCATCTCAAACTTCATGG	AGCTGAGTTTGCGTTTATCATG	56	HEX	Set 1
Fca304	TCATTGGCTACCACAAAGTAGG	CTGCATGCCATTGGGTAAC	56	FAM	Set 2
E7	GCCCCAAAGCCCTAAAATAA	GCATGTCGGACAGTAAAGCA	55	NED	Set 2
ZN (ZFx/Zfy)	AAGTTTACACAACCACCTGG	CACAGAATTTACACTTGTGCA	55	NED	Set 2
Fca126	GCCCCTGATACCCTGAATG	CTATCCTTGCTGGCTGAAGG	56	HEX	Set 3
Fca672	AAGTTGCTTGCACACACTGC	TCCAAGAGCCTTTTCAGTTAGG	56	HEX	Set 3
Fca090	ATCAAAAGTCTTGAAGAGCATGG	TGTTAGCTCATGTTCATGTGTCC	52	HEX	Set 4
Fca230	AAGAATGGACTTGGGAAATGG	AAACCACAACAGGCAAAAGG	52	NED	Set 4
Fca279	AGCCAAGTAATATTCCTCTGTG	GTCCATCCGCAGATGAATG	52	FAM	Set 4

All loci optimized from Menotti-Raymond et al. (1999), except D15, E7 (Bhagavatula and Singh, 2006), and ZN (Pilgrim et al., 2005).

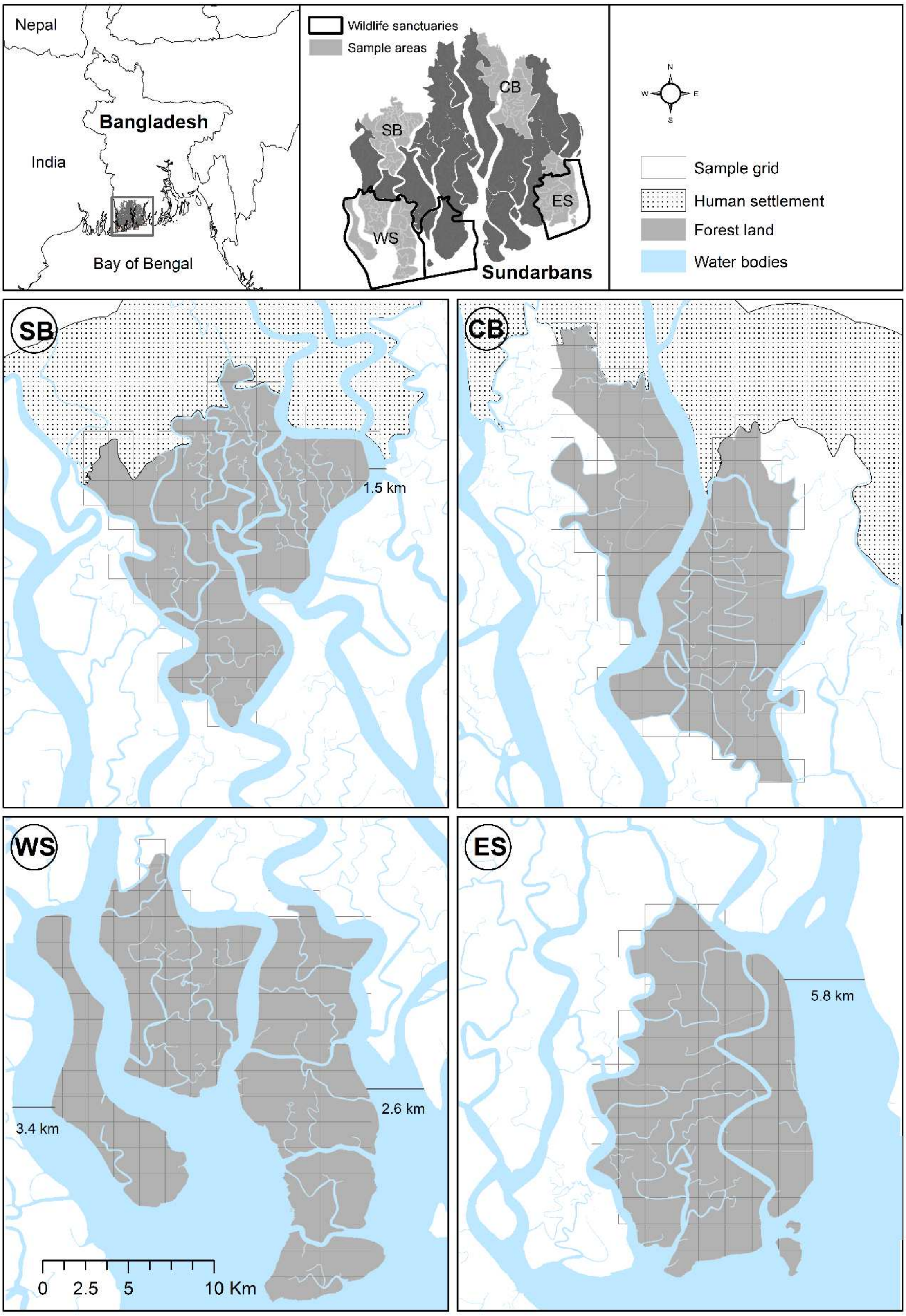

Fig. 1. Location of Bangladesh, and Sundarbans with wildlife sanctuaries, sample areas and sample grids. Sample area: SB – Satkhira Block, CB – Chandpai Block, WS – West Wildlife Sanctuary, ES – East Wildlife Sanctuary.