

Population status, threats, and evolutionary conservation genetics of Bengal tigers in the Sundarbans of Bangladesh



A dissertation submitted for the degree of
Doctor of Philosophy in Biodiversity Management
By

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Declaration of originality

I declare that this PhD dissertation entitled *Population status, threats, and evolutionary conservation genetics of Bengal tigers in the Sundarbans of Bangladesh* is my own research work and contains no material that has been submitted in whole or part elsewhere for the award of any academic degree or diploma. I conceived the idea of this research project, arranged and trained survey teams, collected all samples from wild and captivity, conducted the genetic laboratory work and carried out the genetic analyses, and wrote all the chapters with editorial suggestions by my PhD supervisor Jim Groombridge. Chapters 3-6 include co-authors from other research and conservation organizations, and who contributed to data analyses and provided editorial suggestions for improvement of the content. Their specific contributions have been cited in the acknowledgement section.

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Field survey team, from left to right *standing* Faraji, Sekander, Alam, Alamin, Taher, Monirul, Yasin, Ahad, Ripon, Nanha, Motiar, Robiul, Khalil, Author, Rahim, Shahid, Nirmal, Chairman, Chunnun, Shobhan, Jahid, Saddam, Shahinur, Shikhul, Alam, Manik, Rubel, Sirajul, Zenarul *sitting* Jahangir, Jafar, Dulal, Panu, Jamal, Nur, Manik, Jasim, Habibur, Sohel, Shohag, Ismail.

Abstract

The Sundarbans is a Tiger Conservation Landscape of global priority that supports one of the most important tiger populations across their current range. In Bangladesh, Sundarbans is the last stronghold of the critically endangered tiger, therefore conserving this flagship species will help to ensure the long-term future of the Sundarbans which has been providing significant economic and ecosystem services to human communities for centuries. However, scientific information is lacking on many aspects of the Sundarbans tigers, including population and genetic status, and detailed patterns of tiger and prey poaching. The objectives of this study were therefore to improve the knowledge base to help design better management strategies for long-term persistence of the Sundarbans tigers. As a consequence of challenges faced in applying conventional census methods in the Sundarbans mangrove habitat, a non-invasive genetic approach was applied to collect samples that were then screened using polymorphic microsatellite markers to estimate density and population size of tigers within the spatially explicit capture-recapture model. DNA analyses provided reasonable population estimates, indicating that a non-invasive genetic approach is a viable method for monitoring tigers and can be applied to monitor tiger populations elsewhere. Bayesian and Maximum likelihood inferences using mitochondrial DNA sequences supported a polyphyletic relationship between tiger population in the Sundarbans and the populations in central India. Together, microsatellite and mitochondrial DNA analyses revealed a signal of fine-scale genetic structure and significant genetic differentiation on a spatial scale which is probably the consequence of limited tiger dispersal due to the presence of wide rivers in the Sundarbans landscape. Systematic field survey across sample areas detected a range of snaring methods used to catch tiger prey and evidence of killing tigers by poisoning prey carcasses with the Carbofuran pesticide. Spatial analysis showed that poachers selected sites that tended to be further away from guard posts, and close to river banks. Based on these results, a range of future management interventions were recommended including the reduction of water-based commercial and resource collection activities to allow tiger dispersal, and regulation of Carbofuran and snare materials to better tackle tiger and prey poaching in the Sundarbans.

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Chapter 1
General introduction

The tiger, *Panthera tigris*

Tiger population

Tigers were declared as an endangered species in 1969 by IUCN, but their range and number in the wild have collapsed despite a long history of concern for their conservation (Seidensticker, 2010; Walston et al., 2010a). Global populations have declined to fewer than 4,000 tigers from an estimated 100,000 tigers in just 100 years ago (Morell, 2007; Seidensticker, 2010). Tigers have already lost 93% of their ancestral range across the globe (Sanderson et al., 2010). Accelerated urbanization, habitat degradation, increasing demand for natural resources, large-scale infrastructural expansion, and the effects of human-induced climate change have placed unprecedented pressure on biodiversity in general, and on the dwindling tiger population in particular (McNeely, 1997; Shahabuddin, 2010; Sodhi et al., 2004). Except in the Russian Far East, tigers are now restricted to relatively small regions mostly as small remnant populations in isolated protected areas (Walston et al., 2010b). Although it has been speculated that tigers may not go extinct within the next two decades, the current trajectory will certainly cause wild populations to disappear in many ranges, or to shrink to the point of “ecological extinction” – where their numbers are too few to sustain their role as a top predator in their ecosystem (Sanderson et al., 2006). This state of population decline of wild tigers and massive destruction of their range exemplifies the wider global biodiversity crisis.

Tigers now live in only 13 range countries (Tiger Range Countries: TRCs): Bangladesh, Bhutan, Cambodia, China, India, Indonesia, Lao PDR, Malaysia, Myanmar, Nepal, Russia, Thailand and Vietnam (Seidensticker, 2010). The recent estimates from these TRCs include approximately 2,000 Bengal tigers (*P. t. tigris*) living in the Indian subcontinent, fewer than 400 Sumatran tigers (*P. t. sumatrae*) in Sumatra, *ca.* 500 Malayan tigers (*P. t. jacksoni*) in Peninsular Malaysia, *ca.* 300 Indochinese tigers (*P. t. corbetti*) in Cambodia, China, Lao PDR, Myanmar and Thailand, and *ca.* 400 Amur tigers (*P. t. altaica*) in northeast China, and the Russian Far East (Seidensticker, 2010). However, it is feared that Cambodia, China, and Vietnam might have already lost their breeding population of tigers (Walston et al., 2010b). Tigers have gone extinct from their extreme ranges of the Caspian regions and

the islands of Java and Bali, and were probably already extirpated in southern China (Tilson et al., 2004) (Fig. 1).

To reverse the decline of tigers in their remaining landscapes, a range of conservation management efforts have been undertaken by government and non-government agencies in association with national and international donors and conservationists. As part of the conservation efforts, Sanderson et al. (2006) have identified a total of 76 Tiger Conservation Landscapes (TCLs) which cover 1,185,000 km² of occupied and potential global tiger habitat (7% of their historic range) in order to help recover the remaining populations. These TCLs were defined based on sufficient coverage of habitat for at least 5 tigers, with confirmation of tigers occurring there within the past 10 years (Fig. 2). Only 16 of the 76 TCLs were ranked as Class I, having sufficient habitat to support at least 100 tigers, with evidence of breeding, minimal to moderate levels of threat, and effective conservation measures in place. Approximately half of all TCLs are large enough to support 100 or more tigers, with the seven largest TCLs offering the potential to support 500 or more tigers (Sanderson et al., 2006). Moreover, 42 “source sites” containing a majority of the world’s remaining tigers have been recognised as having the potential to maintain >25 breeding females, being embedded in a larger landscapes with the potential to contain >50 breeding females, conservation infrastructure and legal mandate for protection. These 42 sites contain almost 70% of all remaining wild tigers (Walston et al., 2010a).

Acknowledging the need for concerted and collaborative conservation action to reverse the tiger decline, heads of the state and representatives from the governments of all TRCs met in St Petersburg, Russia, in 2010 and formed an unprecedented commitment to saving wild tigers. During the summit, the St. Petersburg Declaration was made by setting an ambitious goal of doubling the population of wild tigers by 2022, and endorsed the Global Tiger Recovery Program (GTRP) (Wikramanayake et al., 2011).

The Indian Subcontinent is estimated to support approximately 60% of the global tiger populations, within only an estimated 8–25% of remaining global habitat (Jhala et al., 2008; Sanderson et al., 2006). Unfortunately, the Indian Subcontinent has already lost 98% of their wild tigers over the past 200 years (Mondol et al., 2009b). The Sundarbans mangrove forest supports one of largest populations of Bengal tigers,

which has been ranked as a Class III TCL of global priority (Sanderson et al., 2006), and included amongst the ‘source sites’ for tiger recovery (Walston et al., 2010a) (Fig. 2). Several studies were carried out to assess tiger population of the Sundarbans (Barlow et al., 2011; BFD, 2004; Dey et al., 2015; Khan, 2012), but consistent population estimates are still lacking. Although non-invasive genetic sampling approach was recommended to assess the Sundarbans tiger population due to difficulties of applying camera-trapping method (Jhala et al., 2011; Mondol et al., 2009a), no such genetic approaches have ever been attempted.

Tiger phylogeography

Tigers, probably originated in east Asia, were well established throughout their historical range approximately two million years (MY) ago (Hemmer, 1987; Kitchener, 1999). Studies suggest that the evolution of large-bodied forest ungulates created a niche for a large-bodied, forest-edge predator (Sunquist et al., 1999), thereby the divergence of the current *tigris* line from the *Panthera* stock likely followed the Pleistocene radiation of cervids and bovids in Southeast Asia (Kitchener, 1999). Consequently, tigers adapted to a wide range of ecological conditions, from temperate forests to mangroves during their evolutionary history (Kitchener, 1999).

The oldest tiger fossils, approximately two MY old, were discovered from northern China and Java (Hemmer, 1987). From the discovery of fossil remains of tigers in the extreme north of Siberia in the Pleistocene and the survival of the species in Manchuria and Amurland, it is believed that the tiger is of northern origin and migrated southwards to south-western Asia on the side of the Tibetan Plateau and through China to Burma and ultimately to the Sunda islands (Pocock, 1939). By the late Pliocene and early Pleistocene tigers were distributed in eastern Asia (Luo et al., 2004); however, Pleistocene glacial and interglacial fluctuations and other geological events probably caused repeated geographic restrictions and expansions (Hemmer, 1987; Kitchener, 1999; Kitchener and Dugmore, 2000).

Carolus Linnaeus first formally described tigers as *Felis tigris* in 1758; since then eight subspecies of tigers have been recognised based on physical features including body size, skull morphology, pelage colouration, and stripe patterns (Herrington, 1987; Mazak, 1981). Of the recognised tiger subspecies, the populations of Bali tiger

(*P. t. balica*), Caspian tiger (*P. t. vigrata*) and Javan tiger (*P. t. sondaica*) were decimated by the 1940s, 1970s, and 1980s, respectively (Nowell and Jackson, 1996). Multivariate craniometric analysis, and morphological and palaeontological analyses have revealed a wide range of morphological variations, with some levels of overlaps within subspecies (Herrington, 1987; Kitchener, 1999; Kitchener and Dugmore, 2000). However, a molecular genetic study by Wentzel et al. (1999) indicated a low level of genetic variation, suggesting little evidence for subspecies distinctiveness. Recently, Luo et al. (2004) using mitochondrial and microsatellite data, identified six subspecies of tigers: Amur tiger (*P. t. altaica*), Northern Indochinese tiger (*P. t. corbetti*), South China tiger (*P. t. amoyensis*), Malayan tiger (*P. t. jacksoni*), Sumatran tiger (*P. t. sumatrae*), and Bengal tiger (*P. t. tigris*) in addition to three extinct subspecies. However, this classification was disputed on morphological, genetic, and biogeographical aspects of the proposed tiger subspecies (Cracraft et al., 1998; Kitchener and Dugmore, 2000; Mazák, 2010). A comprehensive analyses by Wilting et al. (2015) using molecular, morphological (craniodental and pelage data) and ecological (climate, habitat and prey data) characteristics of all nine putative tiger subspecies acknowledged only two subspecies: the Sunda tiger (*P. t. sondaica*) and the Continental tiger (*P. t. tigris*) with the latter consisting of two management units.

Tigers are the largest of the living cats, with an average Bengal tiger of about 3 m in length from the tip of the nose to the end of the tail. Adult females are slightly smaller and lighter, weighing about 100-160 kg whilst males weigh 200-260 kg (Sunquist and Sunquist, 2002). It was generally believed that the largest tigers occur in the Russian Far East, and the smallest are found in the Sunda Islands (Luo et al., 2004). However, measurements of tigers from the Russian Far East show that they are currently no larger than the Bengal tigers (Sunquist and Sunquist, 2002). Variations in body sizes of tigers are attributed to weather patterns, with individuals in the southern latitudes having smaller body size due to an adaptation to higher temperatures, as well as providing a way to reduce energy needs in an environment where large ungulate prey are not readily available (McNab, 2005).

Tigers living in the Sundarbans were traditionally assigned as being Bengal tigers. The Sundarbans are currently isolated from the nearest other tiger habitat by approximately 200 km of landscapes dominated by human settlements and agriculture (Fig. 3), so there is no opportunity of gene flow between tiger populations via normal

dispersal events (Barlow et al., 2010). Tigers surviving in the Sundarbans were inferred as distinct from other Bengal tiger populations based on their skull morphology and weight measurements obtained from Bangladesh Sundarbans (Barlow et al., 2010), but this inference has been debated by molecular studies (Singh et al., 2015). To date, no molecular studies have been carried out using samples from the Bangladesh Sundarbans which may provide improved understanding about genetic and phylogenetic status of this globally significant tiger population, and may highlight its conservation importance.

Tiger conservation genetics

Genetic diversity is the raw material for evolutionary changes within any natural population (Frankel and Soulé, 1981). High levels of genetic diversity have potential benefits to conservation because genetic variation is critical for fitness, viability and evolutionary responsiveness of endangered populations in rapidly changing landscapes (Wilson et al., 2008). Therefore, preservation of genetic diversity is a fundamental principle in conservation genetics (Frankel and Soulé, 1981).

Small isolated populations, often resulting from habitat fragmentation, are critically susceptible to the loss of genetic diversity due to random genetic drift and genetic bottlenecks which together can increase the risk of population extinction (Frankham and Briscoe, 2002). Habitat fragmentation and formation of barriers to a species' dispersal can limit the opportunities for gene flow and therefore may have significant consequences for the genetic diversity within isolated populations (Milton et al., 2008). Tigers, for example, experienced severe population collapse over the past several decades, and with a range contraction of more than 50% during the last three generations (Sanderson et al., 2010; Walston et al., 2010b). As a result, most of the remaining Bengal tigers, for instance, now survive in relatively small populations ranging between 20 and 120 individuals within the geographically isolated protected areas (Ranganathan et al., 2008). Although historically there was a much higher degree of connectivity between tiger habitats across their range (Henry et al., 2009), loss of habitat connectivity has induced demographic isolation of tigers in modern times (Mondol et al., 2013). A historical population size bottleneck, due to a severe demographic decline in the 1940s, has been detected in Amur tiger populations in the Russian Far East (Alasaad et al., 2011; Miquelle et al., 2007). Meanwhile, Bengal

tigers have lost a substantial genetic variation as a consequence of their population decline (Mondol et al., 2009b; Sharma et al., 2008).

Apart from human-induced habitat fragmentation, natural barriers such as valleys, rivers, and mountains can have significant impact on a species' dispersal which in turn can create population genetic structure (Frankham and Briscoe, 2002; Segelbacher et al., 2010; Trizio et al., 2005). The human footprint such as roads and settlements is well known to affect connectivity between many carnivore populations (Dickson et al., 2005; Frantz et al., 2010; Riley, 2006). The Sundarbans mangrove forests are dissected into many isolated forest fragments by a number of wide river systems, and therefore it is very likely that some of these major river systems might act as potential barriers to tiger dispersal (Fig. 3). No molecular studies have yet been conducted to assess the impact of habitat elements such as rivers on fine-scale genetic structure of the Sundarbans tigers, which may help guide future conservation efforts.

Tiger conservation threats

Tiger populations continue to decline across their range (Dinerstein et al., 2007), due to illegal killing of tigers and their prey, in addition to massive loss and fragmentation of supporting landscapes (Jhala et al., 2008; Karanth and Stith, 1999; Linkie et al., 2006). Poaching of tigers, driven by the demand for tiger parts in Asian traditional medicine has decimated many of the tiger populations across Asia (Walston et al., 2010b). Two Indian tigers reserves, the Sariska and Panna, have lost their last tigers in 2004 and 2010 respectively, largely due to the intense poaching of tiger and their prey (Dinerstein et al., 2007). In the Russian Far East, declines in tiger numbers have been associated with a decline in law enforcement (Goodrich et al., 2008) while a similar pattern has been observed in Nepal due to lack of effective protection (Karki et al., 2009). Prey depletion is the second most pressing threat to tiger populations (Damania et al., 2003; Karanth and Stith, 1999). Tiger prey are being mainly depleted due to illegal hunting primarily driven by local hunting for human consumption (Madhusudan and Karanth, 2002; Mohsanin et al., 2013). Therefore, the scarcity of prey populations may adversely affect tiger populations because tiger numbers are sensitive to the depletion of their prey animals (Karanth and Stith, 1999). The Sundarbans is no exception, Aziz et al. (2013) identified a total of 23 threats; four were linked to tigers, two to prey and 17 to habitat (Table 1). Of the identified threats,

the highest ranked threats included tiger poaching, prey poaching, sea level rise, upstream water extraction/divergence, wood collection, and fishing and harvesting aquatic resources (Aziz et al., 2013). However, field-based tiger and prey poaching techniques and their spatial intensity as well as socio-economic characteristics of these threats were largely lacking (Ahmad et al., 2009).

The Sundarbans

Tigers were once found throughout the Bengal region – the current location of Bangladesh and parts of India (Ahmad et al., 2009). Bangladesh is bordered to the west, north and east by India, to the south-east by Myanmar, and to the south by the Bay of Bengal. Most of Bangladesh is low-lying land comprising mainly the delta of the Ganges and the Brahmaputra rivers. Floodplains occupy 80% of the country (Rashid, 1991). The northeast and southeast portions of the country are hilly, with some tertiary hills over 1,000 m above mean sea level. Bangladesh is a very densely populated country, with a population of over 133 million, where 75% of the population lives in rural areas (Huq and Asaduzzaman, 1999). The Sundarbans is part of the world's largest delta (100,000 km²) formed from sediments deposited by three great rivers, the Ganges, the Brahmaputra, and the Meghna, which converge on the Bengal Basin (Seidensticker and Hai, 1983). The part of the Sundarbans within the territory of Bangladesh is located in the south-west corner of country, between 21°30' and 22°30' N and 89°00' and 89°55' E, extended over parts of Khulna, Satkhira and Bagerhat districts (Iftekhar and Islam, 2004a).

In Bangladesh, tiger is the national animal, and categorised as critically endangered (IUCN Bangladesh, 2015). Tigers were once distributed across the country, but widespread hunting and habitat loss has depleted both their range and numbers (Ahmad et al., 2009). Although there are reports of vagrant tigers in the Chittagong Hill Tracts of Bangladesh (Khan, 2004; Reza et al., 2004), the Sundarbans is known to hold the remaining viable population of tigers (Ahmad et al., 2009). The forests of the Kassalong-Sajek and Sangu-Matamuhuri valleys of the Chittagong Hill Tracts have been identified as Tiger Restoration Landscapes within the greater Northern Forest Complex-Namdapha-Royal Manas Global Priority TCL (Sanderson et al., 2006) (Fig. 3). However, no study has yet been undertaken to assess the population status of tigers in these landscapes of Chittagong Hill Tracts.

The Bangladesh Sundarbans currently covers an area of 6,017 km² (Iftekhar and Islam, 2004a), whereas the Indian Sundarbans encompasses 4,000 km² (Chaudhuri and Choudhury, 1994). Despite the highest human population density in the world in its immediate vicinity, the extent of the mangrove forest in the Sundarbans has not been changed significantly in the last 25 to 30 years (Giri et al., 2007). The mangrove is one of the three major forest types, and one of the most important features of the coastal areas of Bangladesh (Islam and Wahab, 2005). The Bangladesh Sundarbans, representing almost half of the remaining forest within the country (Hussain and Acharya, 1994), is the last stronghold of tigers in Bangladesh (Ahmad et al., 2009).

Brief account of Sundarbans management

The entire Bengal regions were once covered with dense forests and wilderness, but over centuries enormous deforestation has occurred, driven predominantly by humans (Eaton, 1990). The forests came under state supervision for the first time during the Muryan period (321-226 BC), when forests were divided into *Gaja-vanas* (meaning elephant forests), and *Angireya-vana*, the forests located in the North and South Bengal including the Sundarbans. The Muryans introduced the first formally constituted Forest Department which was headed by a *kupyadhyaksta*, the superintendent and the administration was assisted by *vanapalas*, the forest guards. During the Bengal Sultanate period (1204-1575), land reclamation and human settlement in the Sundarbans regions were encouraged by Islamic religious leaders while the clearance of the Sundarbans forests gained state recognition during the Mughal Empire (1575-1765) (Eaton, 1990). The British administration (1757-1947) stepped up the process by introducing a revenue system in 1781 when the Sundarbans had undergone massive conversion into rice fields. The first ‘Sundarbans Plan’ developed by Henckell in 1787 was considered a ‘great success’ for cultivating Sundarbans forest lands. Although reclamation of the Sundarbans forests under the colonial rule began in the 1770s, much of control even until 1785 was under the *zamindars* (landholders) who continued collecting “large sums” as *ban-kar* (forest tax) and *noon-kar* (salt tax). In 1828, the British administration assumed property rights to the Sundarbans and began leasing out forests to invest capital and labour into clearing operations. The following 25 years witnessed widespread destruction until 1855 when the first Forest Act was formulated. In 1862, the Conservator of Forests of Burma (now Myanmar) put forward a convincing argument in favour of preserving the

Sundarbans which subsequently stopped the leasing process. As a result, portions of the Sundarbans were declared 'reserved' and 'protected' in 1878 (Bhattacharya, 1990). Until 1872, a cumulative area of about 1,997 km² of the Sundarbans was cleared for rice cultivation. By 1873, the total area under cultivation from the whole Sundarbans forest had increased to approximately 2,815 km² (Hunter, 1875). In 1875, the government included unleased forest reserves in the Forest Act and placed these areas under the jurisdiction of the Forest Department (Richards and Flint, 1990). The entire Sundarbans has been reduced to half of its former size in the last two or three centuries, and by 1,500 km² in the last 100 years (Blair, 1990).

The first 'Management Plan' for the Sundarbans was prepared in 1875 for regulative extraction of *Sundri* (*Heritiera fomes*) based on the diameter classes. Since then, the Sundarbans was categorised as 'production forest' and subsequent management plans were formulated to generate state revenue from forest resources (Canonizado and Hossain, 1998; Chaffey et al., 1985; Chowdhury, 1968; Curtis, 1933; Heinig, 1892; Trafford, 1911). The first 'Bangladesh Forest Policy' was formulated in 1979 after independence from Pakistan in 1971 with an aim of careful preservation and scientific management of the Sundarbans. However, timber extraction of *Sundri* continued until 1989, and extraction was suspended afterwards due to the spread of 'top dying' disease in the *Sundri*. In 1998, 'Forest Resources Management Plan' was prepared for the period of 1998-2010 with emphasis on the regulation of timber harvesting to allow regeneration of forests, and tourism potential of the Sundarbans (Canonizado and Hossain, 1998). The latest 'Integrated Resource Management Plan' was developed for the period of 2010-2020 by the Bangladesh Forest Department with support from the Integrated Protected Area Co-management project. Over decades, several conservation projects have been implemented and get underway in the Bangladesh Sundarbans with particular emphases on tiger conservation, including the Sundarbans Biodiversity Conservation Project, Sustainable Environmental and Livelihood Security project, and the Bengal tiger conservation activity (Bagh) project.

The Bangladesh Sundarbans is managed as a reserved forest (SRF). In 1996, three isolated areas on the southeast, south and southwest corners of the SRF were delineated as wildlife sanctuaries; the Sundarbans West (715 km²), Sundarbans South (370 km²), and Sundarbans East (312 km²) for higher protection of wildlife and their habitats (BFD, 2012; Iftekhar and Islam, 2004b). These wildlife sanctuaries were

collectively declared a UNESCO World Heritage Site in 1997 (Iftekhar and Islam, 2004b). In 1999, a 10-km strip along the SRF boundary was declared as Ecologically Critical Area (ECA) under the Bangladesh Environment Protection Act 1995. These ECA zones are spread over 17 *upazila* under Satkhira, Khulna, Bagerhat, Pirojpur and Barguna districts, where approximately eight million people live (Hussain, 2014; Iftekhar and Islam, 2004b). In 2012, three river-based wildlife sanctuaries, Chandpai (5.6 km²), Dudhmukhi (1.7 km²), and Dhangmari (3.4 km²) have been established in the eastern part of the SRF for the protection of cetaceans (BFD, 2012).

Tiger hunting was encouraged in Bangladesh until 1973 but tigers' received legal protection when the Bangladesh Wildlife (Preservation) Order 1973 was sanctioned. In 1974, this order was revised and enacted as the Bangladesh Wildlife (Preservation) (Amendment) Act 1974, and revised again as the Wildlife (Protection and Security) Act, 2012. According to Article 24 of this act, collection, possession, and export of meat, bones or other body parts including any trophies, and farming of species listed in the Appendix IV including tigers require obtaining permits from the Chief Conservator of Forest (CCF). A person shall get a maximum of 12 years in prison and a fine of BDT 15 lacs (US\$ 17,000) for poaching a tiger according to Article 34 of this act, but tigers can be killed in a situation of threat to human lives, with permission from the CCF.

The overall management of the SRF is administered by the two Divisional Forest Officers under a Conservator of Forest stationed the under the Khulna circle, and implemented by 17 stations and 72 guard posts deployed across the SRF (Khan, 2011). The management authority of the Bangladesh Forest Department generally issues permits for limited collection of forest (e.g., *golpata*, honey) and aquatic (e.g., fishes, crabs) resources from areas of reserve forest. However, article 14 of the Wildlife (Protection and Security) Act, 2012 prohibits cultivation, establishing industry, collection or damage of plants and animals, setting fire, water pollution, carrying firearms or chemicals, introducing livestock and alien species, etc. within the sanctuaries (Fig. 4).

Topography and river systems

The SRF is located in south of the Tropic of Cancer at the lower moribund end of the Delta where it meets the Bay of Bengal (Iftekhar and Islam, 2004b). As a result, a complex network of rivers and streams of varying width and length intersects the entire SRF (Siddiqi, 2001). Generally, all major rivers pass from north to south direction, but are connected with each other by smaller east-west channels (Islam and Wahab, 2005). The total length of all small and large rivers within forest is about 12,000 km, which clearly reflects the intensity of river networks. The SRF may therefore be described as a tangled region of estuaries, rivers and watercourses, enclosing a vast number of low-lying swampy forest islands of various shapes and sizes. The elevation of the SRF generally varies from only 0.5 m to 4.0 m, with mean elevation for most of the SRF is less than 1 m above the mean sea level (Canonizado and Hossain, 1998; Prain, 1979). As a result, most parts of the SRF remain under water at every high tide during monsoon, but many of the forest islands remain quite dry at winter months (Prain, 1979).

The major rivers that pass through the SRF include the Baleswar, Passur, Sibsa, Arpangassia, Raimangal and Hariabhanga. The Raimangal (upstream) and Hariabhanga rivers (sea face) mark the international boundary between Bangladesh and Indian parts of the Sundarbans whereas the Baleswar forms the eastern boundary of the SRF. The river Arpangassia, formed by the junction of the Kholpetua and the Kobadak rivers near Burigoalini, flows southward for about 64 km between the forest ranges of Satkhira and Khulna. The river Sibsa is one of the two widest rivers of the SRF flows a course of about 60 km starting at Nalian on the north of the SRF to the sea. It has several distributaries such as the Morjat and Hangsharaj, and connected with the Arpangassia by the Hansura and the Batlagang rivers, and with the Passur by several east-west channels. The river Passur, one of widest rivers of the SRF, is an effluent of the Bhirab at Khulna; from this point it flows about 136 km to the sea. About 30 km from its mouth the Passur gives off several distributaries and, and receives the Kaga, and the Shella rivers. The Arpangassia is about 1.2-3.1 km wide along its 64 km course, while the Sibsa, one of the widest rivers of the SRF at more than 1.5 km wide (ranging from 1.3 to 3.1 km) for most its 60 km course. The Passur river varies in width from 1.4 to 3.1 km, with the width of its major portion greater

than 1.5 km along its 136 km course, and divides the remaining eastern half of the SRF into two large fragments (Prain, 1979) (Fig. 5).

Physical and climatic features

The SRF is surrounded by human habitations entirely on the north, and to some extent on the east side. There is no permanent human settlement within the SRF, except camps of the Forest Department, Navy and Coast Guards. However, hundreds of temporary fishing camps can be found on several islands within the southern border of the South Wildlife Sanctuary, where *ca.* 8,000 people congregate for 6-8 winter months every year for fishing activities (Huda and Haque, 2001).

The geophysical formations and structures of the Sundarbans have been shaped by the tonnes of sediments carried by the distributaries of the Ganges (Allison et al., 2003), which governs the complex drainage systems across the Ganges deltaic regions including the Sundarbans. Therefore, the characteristics of riverine systems, salinity, and tidal level of the SRF are heavily influenced by seasonal rainfall, upstream freshwater flow and tidal effects of the Bay of Bengal (Chaudhuri and Choudhury, 1994; Hussain and Acharya, 1994; Karim, 2004).

Salinity is an important abiotic factor for the Sundarbans ecosystem, which influences survival, distribution, growth, reproduction and zonation of the mangroves. The salinity generally increases from east to west and north to south, but remains less than 6 dS/m (desiSiemens per metre) even in the driest month. Soil salinity in April-May ranges from 2 to 4.5 dS/m for most parts of the SRF. Based on the level of soil salinity distribution, three distinct salinity zones— oligohaline (salinity >2 dS/m), mesohaline (salinity 2–4 dS/m) and polyhaline (4 dS/m) zones can be recognised across the SRF (Siddiqi, 2001). There is a strong link between salinity and diversity of plants and animals in the SRF (Karim, 2004). The *Sundri* is the climax species under low salinity and within a primary succession condition in the eastern part while increased saline areas in the south and west parts are dominated largely by *Gewa* (*Excoecaria agallocha*) and *Goran* (*Ceriops decandra*) (Karim, 2004). Relationship between the level of salinity and human-tiger conflict had been proposed but no empirical evidence was provided to support such argument (Barlow, 2009; Hendrichs, 1975).

Tides in the SRF are semi-diurnal with a tidal period of about 12 hours. The average variation between low and high tides is about 3 m, fluctuating between 1.5 m and 2.5 m. Approximately 70% of the forest land lies between 1.5 m to 3.0 m elevations, which go under water during high tide twice a day. However, almost 85% of the forest lands are flooded during the high tide in the monsoon season (CEGIS, 2006). Based on the tidal amplitude, the SRF can be divided into four tidal zones—inundated by all tides (new accretions), inundated by normal high tide (covers most of the area), inundated only by spring high tide (mostly in the northern part), and inundated by monsoon high tide (north-eastern part) (Siddiqi, 2001). Tides and storm surges result in the low lying forests being regularly flooded (Iftekhhar and Islam, 2004a). Therefore, the daily and seasonal tidal magnitudes in different areas of the SRF leave different amount of un-inundated forest land available for tigers and other wild animals (Fig. 6).

The SRF climate can be described as maritime, humid, and tropical, with marked seasonal weather patterns. The major four seasons can be identified as dry (December-February), pre-monsoon (March-May), monsoon (June-September), and post-monsoon (October-November) (Iftekhhar and Islam, 2004a). Average annual rainfall ranges from about 1,800 mm in Khulna near the north of the SRF to 2,790 mm on the coast, with the majority of the rainfall (70-80%) occurring during the monsoon. Daily temperatures range from 2°C in January to 43°C in March (Gopal and Chauhan, 2006). Tropical storms and cyclones produce large water level rises, with tidal waves up to 7.5 m recorded (Seidensticker and Hai, 1983).

Biodiversity status

The Sundarbans has a high level of species richness compared to other mangroves of the world (Robertson and Blaber, 1992). A total of 334 species of plants belonging to 245 genera and 75 families have been recorded from the Sundarbans and adjacent areas (Prain, 1979). The floral diversity is comparatively higher in the Bangladesh side (123 species) of the Sundarbans than in the Indian side (71 species) (Chaudhuri and Choudhury, 1994; Hussain and Acharya, 1994). The vegetation structure of the SRF is dominated by two tree species, *Sundri* and *Gewa* (Siddiqi, 2001). The former species constitute about 65% of the total merchantable timber in the forest. The commonly found tree species are *Keora* (*Sonneratia apetala*), *Kankra* (*Bruguiera*

gymnorrhiza), *Baen* (*Avicennia officinalis*), *Passur* (*Xylocarpus mekongensis*), and *Jhanna* (*Xylocarpus granatum*). The *Golpata* (*Nypa fructicans*) grows profusely along river banks, with higher occurrence across eastern parts of the forest.

The latest forest inventory conducted during 1995-1997 had identified 13 major forest types in the SRF, of which five are monospecific, six contain two species and two consist of three species. The *sundri-gewa* forest type occupied the largest area, followed by *gewa-sundri* composition. The non-vegetated categories include grass and bare ground, sandbar, and tree plantation (Revilla et al., 1998).

The forest canopy is more or less open and hardly exceeds 10 m in height. A survey carried out in 1985 showed 65% of the SRF as having a canopy closure of 70% or more. Generally, the forest is more closed in the east region than in the west parts of the SRF (FAO, 1994).

A total of 425 species of wildlife were identified from the SRF which includes 42 species of mammals, 315 species of birds, 35 species of reptiles, and eight species of amphibians (Blower, 1985; Hussain and Acharya, 1994; Iftekhar and Islam, 2004a). Conversely, Indian Sundarbans was known to harbour 40 species of mammals, 161 species of birds, 57 species of reptiles and eight species of amphibians (Chaudhuri and Choudhury, 1994). The mammalian diversity is relatively high compared to other mangrove forests in the region (Gopal and Chauhan, 2006; Hussain and Acharya, 1994; Iftekhar and Islam, 2004a).

The tiger is the supreme predator in the SRF; no other large carnivore such as the Leopard (*Panthera pardus*) which commonly inhabits as a sympatric species to tiger across the Asian tiger landscapes, is mysteriously absent in the SRF. Small felids occurring in the SRF include Leopard cat (*Prionailurus bengalensis*), Fishing cat (*Prionailurus viverrinus*), and Jungle cat (*Felis chaus*) (Seidensticker and Hai, 1983). Although poorly known, the Small-clawed otter (*Aonyx cinerea*) is notable in the SRF. Asiatic golden jackal (*Canis aureus*), and Common palm civet (*Paradoxurus hermaphroditus*) have their restrictive distribution across the north and eastern boundary areas (personal observation).

The Spotted deer is the most abundant ungulate in the SRF, with relatively low density Wild boar, and Barking deer. Rhesus macaque (*Macaca mulatta*) is the only nonhuman primate found in this forest (Hussain and Acharya, 1994).

Several large terrestrial mammals, for instance, the Javan rhinoceros (*Rhinoceros sondaicus*) last reported in the Imperial Gazette in 1909, became extinct in the Sundarbans. The Swamp deer (*Cervus duvauceli*), Hog deer (*Axis porcinus*), and Wild buffalo (*Bubalus bubalis*) were known to occur in the SRF but disappeared in the last century (Blower, 1985; Chaudhuri and Choudhury, 1994; Curtis, 1933; Hendrichs, 1975). Notably, the Barking deer has also been reported to be extinct in the Indian Sundarbans (Chaudhuri and Choudhury, 1994; Sahgal et al., 2007). In the context of disappearance of these mammals, the Bengal District Gazetteer (1908) noted,

“... the one-horned rhinoceros has become rare and is only found within the southern portion of the reserved forests. Buffaloes are also fast disappearing and at present are only found in the waste lands of the Backergunge portion of the Sundarbans. Barking deer and hog deer are not uncommon, but, being very shy, are seldom seen along the banks of streams. They are found in the reserve forests and uncultivated parts of the northern side of the Sundarbans” (Hendrichs, 1975).

Massive clearings of Sundarbans during the past centuries and subsequent habitat degradation along with indiscriminate killing by humans are believed to have pushed these species towards extirpation (Chaudhuri and Choudhury, 1994).

The Sundarbans waters support a diverse cetaceans such as the Ganges river dolphin (*Platanista gangetica*), Irrawaddy dolphin (*Orcaella brevirostris*), Indo-pacific hump-backed dolphin (*Sousa chinensis*), and Finless porpoise (*Neophocaena phocaenoides*) (Smith et al., 2008, 2006).

The diverse avifauna include 95 species of waterfowls (Scott, 1989), and 38 species of raptors (Sarker, 1985). The most notable bird species include the endangered Masked finfoot (*Heliopais personata*), endangered White-bellied sea eagle (*Haliaeetus leucogaster*), Lesser adjutant (*Leptoptilos javanicus*), and Osprey (*Pandion haliaetus*). The endangered Grey-headed fish eagle (*Ichthyophaga ichthyaetus*), and Pallas’s fish eagle (*Haliaeetus leucoryphus*) are found but relatively rare (Hussain and Acharya, 1994; Seidensticker and Hai, 1983). The high assemblage of kingfisher species (nine

species) with the notable Brown-winged kingfisher (*Pelargopsis amauropterus*), and relatively rare Ruddy kingfisher (*Halcyon coromanda*) are found in the eastern parts of the SRF (personal observation).

A total of 53 species of reptiles were recorded from the SRF. The most notable are the Saltwater crocodile (*Crocodylus porosus*), King cobra (*Ophiophagus hannah*), Indian spectacled cobra (*Naja naja*), and Indian python (*Python molurus*). Turtles and tortoises include 14 species, of which the endangered Olive ridley (*Lepidochelys olivacea*) is known to occur on several coastal islands (e.g., *Dublar char*), while the endangered River terrapin (*Batagur baska*) are now very rare (Gopal and Chauhan, 2006; Hussain and Acharya, 1994). The Marsh crocodile (*Crocodylus palustris*) was extirpated in the SRF during 1980s (Mountfort, 1969).

Eight species of amphibians are known to occur in the SRF (Hussain and Acharya, 1994). The most commonly encountering species across forests is the Crab-eating frog (*Fejervarya cancrivora*), while the notable Green frog (*Euphlyctis hexadactylus*) is usually found in freshwater ponds within the SRF (personal observation).

The fish fauna of the SRF includes 53 pelagic and 124 demersal species (Sarker, 1989). Of these, over 120 species have been recorded in commercial catches (Seidensticker and Hai, 1983). Conversely, 250 species of fish have been reported from the Indian Sundarbans (Chaudhuri and Choudhury, 1994).

Human relationship with the Sundarbans

The SRF provides a range of ecological, economic, and protective services which are fundamental to the wellbeing for millions of people across the south western coastal regions of the country. The major ecological services provided by the SRF include timber and non-timber forest products such as honey, and *golpata*; protecting human communities from cyclones and tidal surges; breeding and nursery grounds for aquatic and terrestrial organisms; sediment deposition and land formation; ecosystem support through producing organic detritus; water recycling; oxygen production; and acting as a carbon sink for the environment (Biswas et al., 2008; Islam and Peterson, 2008). The SRF represents approximately 44% of the forest coverage of the country, and contributes about half of the total revenues generated from the national forestry sector (Tamang, 1993).

Approximately 3.5 million people earn their livelihood from the SRF while about 10 million people are benefiting from a variety of related economic and subsistence activities (Hoq, 2007; Islam and Wahab, 2005). A value chain analysis revealed that about 740,000 people are involved with resource extraction activities in the SRF, where 80% are collectors, and the remaining are traders relating to such activities (IPAC, 2010).

The SRF has been the largest sources of timber, fuelwood and other minor forest products in the past, but now only seasonal harvest of *golpata* and honey collection are permitted. An estimated 67,000 metric tonnes of *golpata* leaves, used primarily as thatching material, are annually harvested by “*Bawali*” (meaning wood cutter) (Hussain, 2014). Forest Department continues to issue permit to local traders for harvesting *golpata* during winter months each year (Fig. 6).

The diverse and abundant populations of fish and fisheries make the Sundarbans an important economic and subsistence activity centre in the region. At present, fishing and harvesting aquatic resources (e.g., crabs, shrimp fry) are the major livelihood activities for local communities living next to the SRF. Annual production of fisheries from the SRF accounts for about 12,000 metric tonnes, where about 200,000 local people were engaged. An estimated 14% people (both male and female across all age groups) living inside a 10-km buffer of the SRF were involved in shrimp fry (*Penaeus monodon*) collection (MARC, 1995). These activities are generally operated in the rivers of upper part of the SRF (Fig. 6).

One of the important resources of the Sundarbans is the honey collected by thousands of local “*mawali*” (meaning honey collector) since centuries. The swarms of the honey bee (*Apis dorsata*) starts to migrate to the vast mangrove forests of the Sundarbans from March to June every year, and build hives preferably in the *henthal-gewa* vegetation communities of the Sundarbans (Chaudhuri and Choudhury, 1994). Honey collection starts on 1st April every year with permits issued by the Forest Department. Large number of local people comprising 6-12 people team depart by hand-driven country boat for searching hives within the forest. An estimated annual collection of 200 metric tonnes of honey and 55 metric tonnes of wax are harvested annually from the SRF (Das and Siddiqi, 1985).

Tourism has been an important economic activity in the SRF, where several tour operators regularly provide touring services to national and international visitors. An estimated 96,000 tourists visited the SRF during the year 2006-07, and the figure has increased to 208,000 in 2010-2011. The major attractive locations for tourists included the Karamjal, Harbaria, Katka, Kochikhali, Dubla Island, and Nilkamal (Hussain, 2014). The Katka and Kochikhali in the East Wildlife Sanctuary on the south-east corner of the Sundarbans has been the principal attraction for tourists due to extended meadows, and sandy beaches (personal observation).

Apart from the forest and security (navy, coast guard) staffs of the SRF, including permitted resource collectors, a number of local pirates called “*dacoit*” or “*party*” also live in the forests, who primarily make earnings by collecting “fees” from resource collectors, and sometimes by kidnapping if resource collectors deny or avoid them during their work. These illegal miscreants usually live in the remote areas of the forest by making temporary shelters (“*machan*”), who often carry weapons and preferably move at night (personal observation).

Over centuries, the Ganges deltaic regions have been the rich source of natural resources, but inhabited by poor human communities. The early settlers around the Sundarbans were the migrants of tribal origin and a small number of indigenous people, whose mainstay were the wood cutting, honey gathering and fishing. Therefore, the culture of the local communities had been deeply influenced by animistic and totemistic (plant worshipping) beliefs in relation to forests and animals in the region. Even today, local people follow those early embedded religious and spiritual customs before entering into the Sundarbans, so that they remain safe during their work. Seeking blessings from local spiritual and religious leaders are also common, particularly before setting off for the honey collection. Offerings are also made to a number of deities, of which notable are the *Dakshin Rai* and *Banbibi*. *Dakshin Rai* is the main folk deity, and the god of the tiger, often depicted as a warrior seated on a tiger with a bow and arrows in his hands. *Banbibi* is considered as the presiding female deity and guardian of the Sundarbans forests (Fig. 6). All community people irrespective of religious beliefs pay respect to these deities before entering the forests (Chaudhuri and Choudhury, 1994; Eaton, 1990).

Previous tiger research in the Sundarbans

Tigers in the SRF have been studied less than other tiger populations, yet a number of ecological studies have been carried out in the recent years. Previous studies have assessed tiger population by interviewing local people (Hendrichs, 1975; Tamang, 1993), and using pugmark method (BFD, 2004). In 2004, a joint study covering both Bangladesh and Indian Sundarbans was severely criticised (Karanth, 2005), and no longer been used due to methodological limitations. Tiger home range has been investigated using radio-collar on two female tigers (Barlow et al., 2011), in addition to tiger monitoring using pugmark index (Barlow et al., 2008). Khan (2012) conducted a camera-trap study in the East Wildlife Sanctuary, while Dey et al. (2015) carried out camera-trapping using a range of baits and lures.

Two studies (Khan, 2004; Reza et al., 2001) investigated food habits of tigers by analysing scat remains, while Khan and Chivers (2007) assessed habit selection by tiger; all these studies were conducted in the East Wildlife Sanctuary.

A large number of studies have assessed human-tiger conflicts (Curtis, 1933; Gani, 2002; Islam et al., 2007; Khan, 2004; Reza et al., 2002), human-tiger conflict mitigation framework (Barlow et al., 2009), social context in such conflict (Inskip et al., 2013), and human tiger coexistence (Inskip et al., 2016).

Several studies investigated threats to tigers and their prey animals including threat prioritisation (Aziz et al., 2013), consumption of deer meat by local communities (Mohsanin et al., 2013), local use of tiger parts (Saif et al., 2015), people involved in tiger killing (Saif et al., 2016) and detecting illegal human activities within wildlife sanctuaries (Hossain et al., 2016).

However, information on many aspects of tigers and their prey are still lacking, therefore research needs have been identified for future research activities in order to guide science-based tiger management in the SRF (Ahmad et al., 2009; Aziz et al., 2013). Very limited or no substantial information exist on consistent population estimates, genetic ancestry and phylogeny, fine-scale landscape genetics, and patterns of tiger and their prey poaching in the SRF. Nonetheless, information is extremely lacking on the population density and abundance of tiger prey populations that are vital to long-term tiger conservation in the Sundarbans.

Objectives of this research

With the goal of increasing the knowledge base to guide scientific management of Bengal tigers in the SRF, this study was designed to achieve the following specific objectives:

1. To estimate density and population size of tigers of the Bangladesh Sundarbans.
2. To investigate genetic ancestry and phylogeny of tigers of the Sundarbans.
3. To assess impact of rivers on the fine-scale genetic structure of tigers of the Sundarbans.
4. To investigate patterns of tiger and prey poaching in the Bangladesh Sundarbans.

The Bangladesh Tiger Action Plan (BTAP) has been developed to guide tiger conservation activities with an aim to increase or stabilize the tiger population in the Sundarbans (Ahmad et al., 2009). This PhD research, aligning with BTAP's specific aims, addressed three highly ranked research needs, namely, (i) to determine population size, density and distribution of Bengal tigers in the SRF, (ii) to determine the nature and scale of tiger and tiger's prey poaching, and (iii) to assess genetic and taxonomic status of tigers of the Sundarbans. Therefore, results of my PhD research will of direct benefit to fulfilling BTAP objectives to guide tiger conservation in the Bangladesh Sundarbans (Fig. 7).

Thesis structure

This thesis is constructed around the following six chapters. Chapter 2 provides a concise review on relevant population census approaches used in tiger studies, and non-invasive genetic methods. Chapter 3 estimates density and population size of tigers of the Bangladesh Sundarbans using non-invasively collected genetic samples under the spatially explicit capture recapture modelling. Chapter 4 assesses the genetic ancestry and phylogenetic relationship of tigers of the Sundarbans by comparative analyses of mitochondrial DNA data from all other tiger subspecies. Chapter 5 investigates the population genetic structure and examines the spatial genetic structure of tiger population of the Sundarbans. Chapter 6 investigates the patterns of tiger and prey poaching in the Bangladesh Sundarbans, and assesses the probability of poaching activities across the Bangladesh Sundarbans. Chapter 7 discusses research findings and future conservation directions.

Table

Table 1

Prioritised threats to tiger, prey, and the Sundarbans (adapted from Aziz et al., 2013).

Target	Threat	Ranking*			Priority
		Scope	Severity	Irreversibility	
Tiger	Tiger poaching	High	High	Medium	High
	Stray tiger killing	Medium	Medium	Medium	Medium
	Tiger disease	High	Medium	Medium	Medium
	Inbreeding depression	Low	Low	Medium	Low
Prey	Prey poaching	High	High	Medium	High
	Prey disease	Medium	Medium	Medium	Medium
Habitat	Sea level rise	High	High	High	High
	Upstream water extraction/divergence	High	High	High	High
	Wood collection	High	High	High	High
	Fishing and harvesting aquatic resources	High	High	High	High
	Invasive species	Medium	Medium	High	Medium
	River pollution	Medium	Medium	Medium	Medium
	Mineral and gas extraction	Medium	Medium	Medium	Medium
	Storm and tidal surge	Low	Low	Very High	Medium
	Melting Himalayan glaciers	Low	Low	Very High	Medium
	Temperature change	Low	Low	Very High	Medium
	Sea acidification	Medium	Medium	Very High	Medium
	Commercial infrastructure	Low	Low	High	Low
	Plant disease	Low	Medium	High	Low
	Housing infrastructure	Low	Low	Medium	Low
	Livestock grazing	Low	Low	Low	Low
	Fire	Low	Low	Low	Low
	NTFP ¹ collection	Low	Low	Low	Low

¹NTFP – Non-timber Forest Products

**Definition of ranking criteria*

Scope: The geographical scope of impact on the biological target that can reasonably be expected within 10 years under current circumstances; *Severity*: The level of damage to the biological target that can reasonably be expected within 50 years under current circumstances; *Irreversibility*: The degree to which the effects of a source of stress can be reversed.

Figures

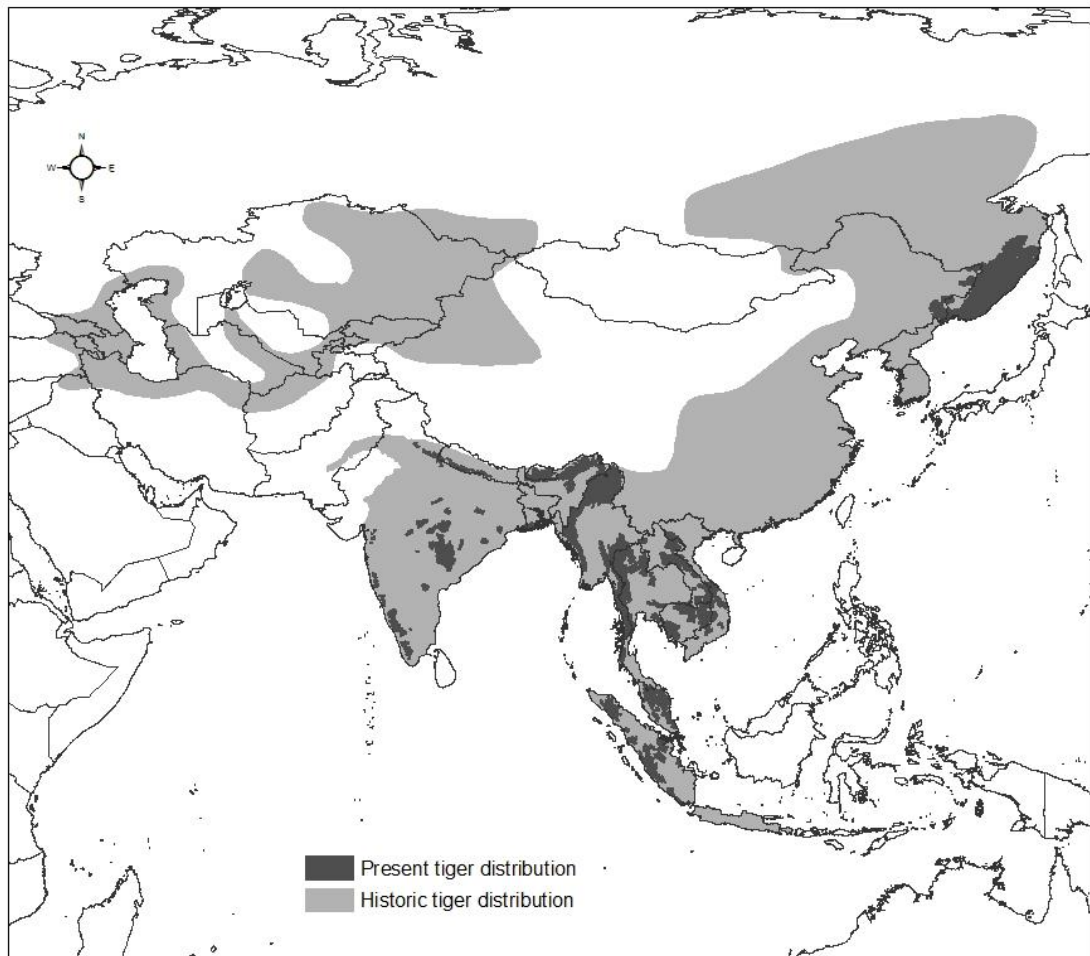


Fig. 1. Historical and current global range of tigers (Sanderson et al., 2006). Notably, tigers occurred across the entire Bangladesh in the historic times.

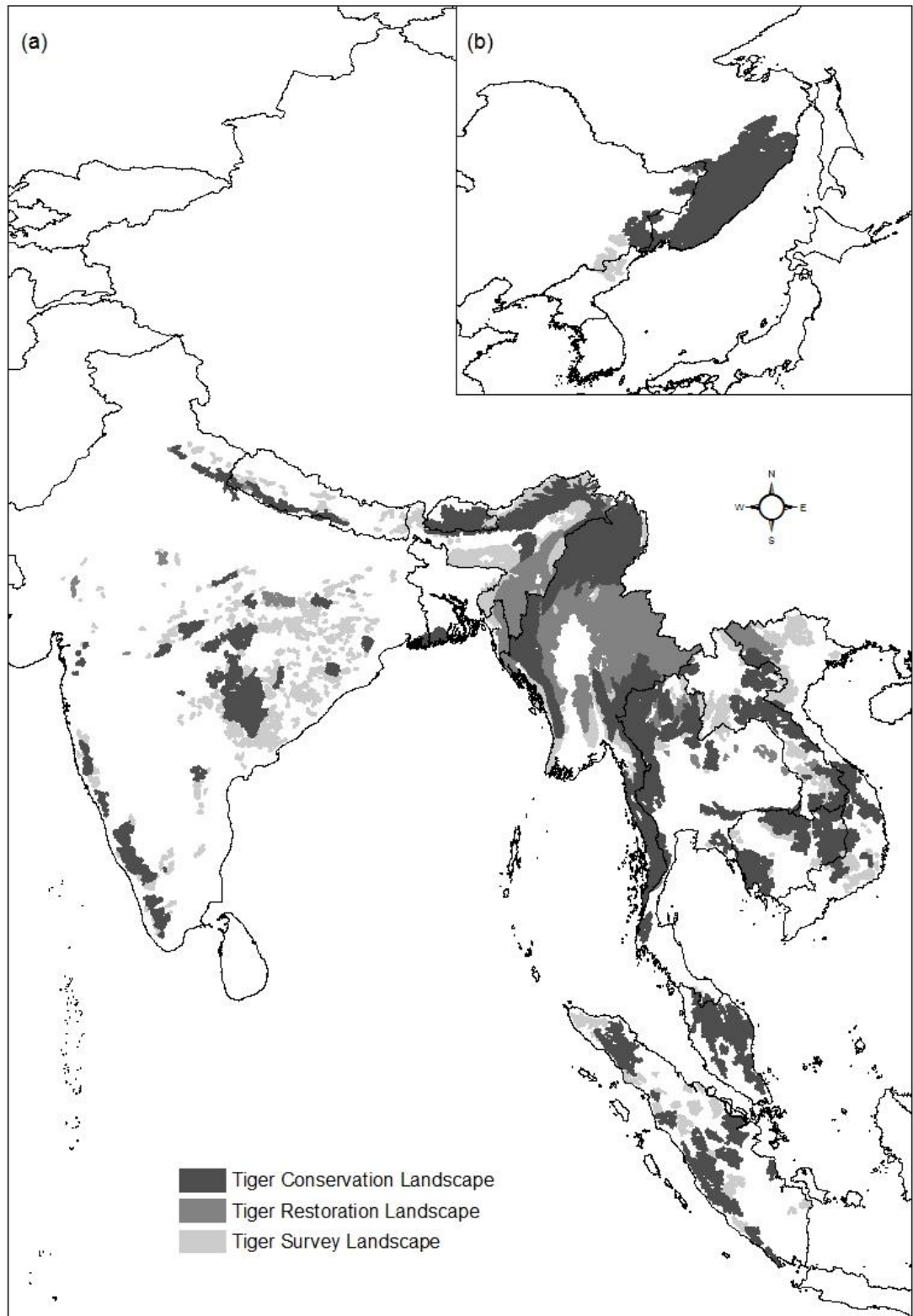


Fig. 2. Tiger landscapes (a) across Asia and (b) the Russian Far East (Sanderson et al., 2006).

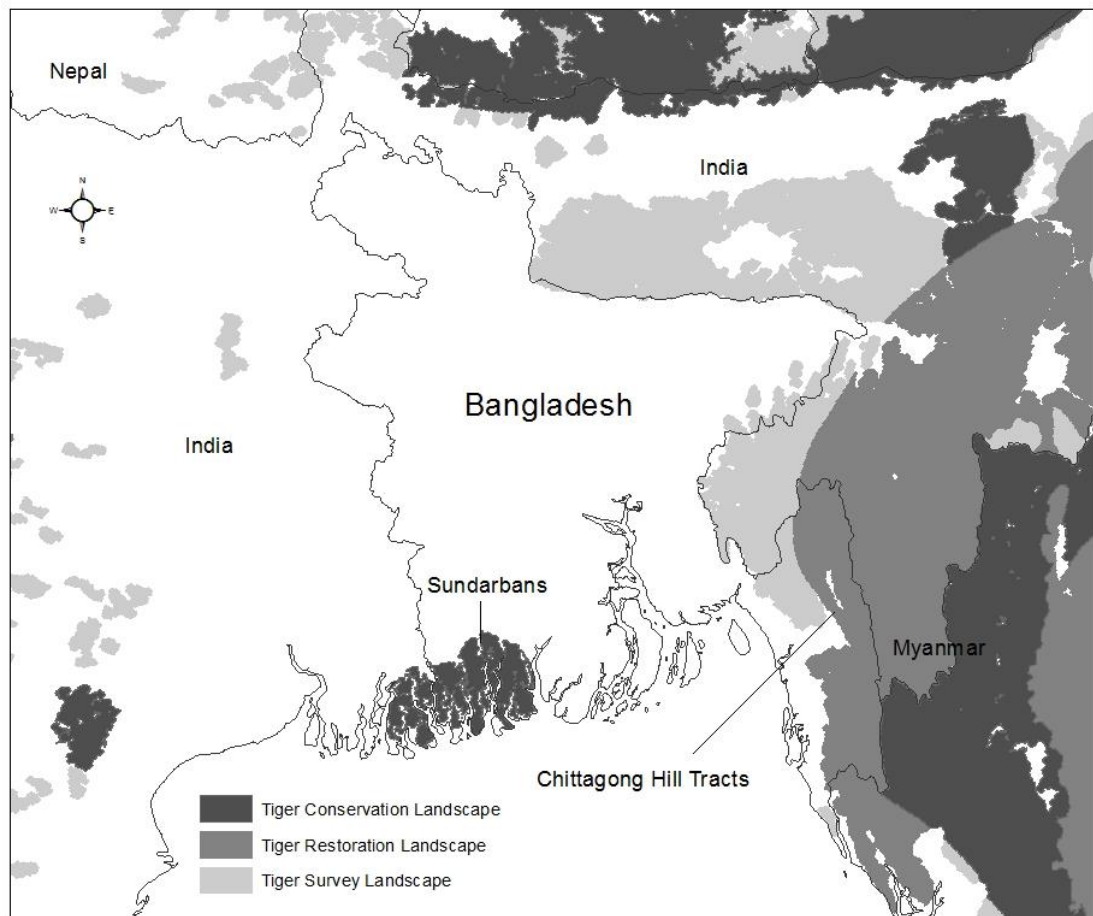


Fig. 3. Tiger Conservation, Restoration, and Survey Landscapes in and around Bangladesh (Sanderson et al., 2006).

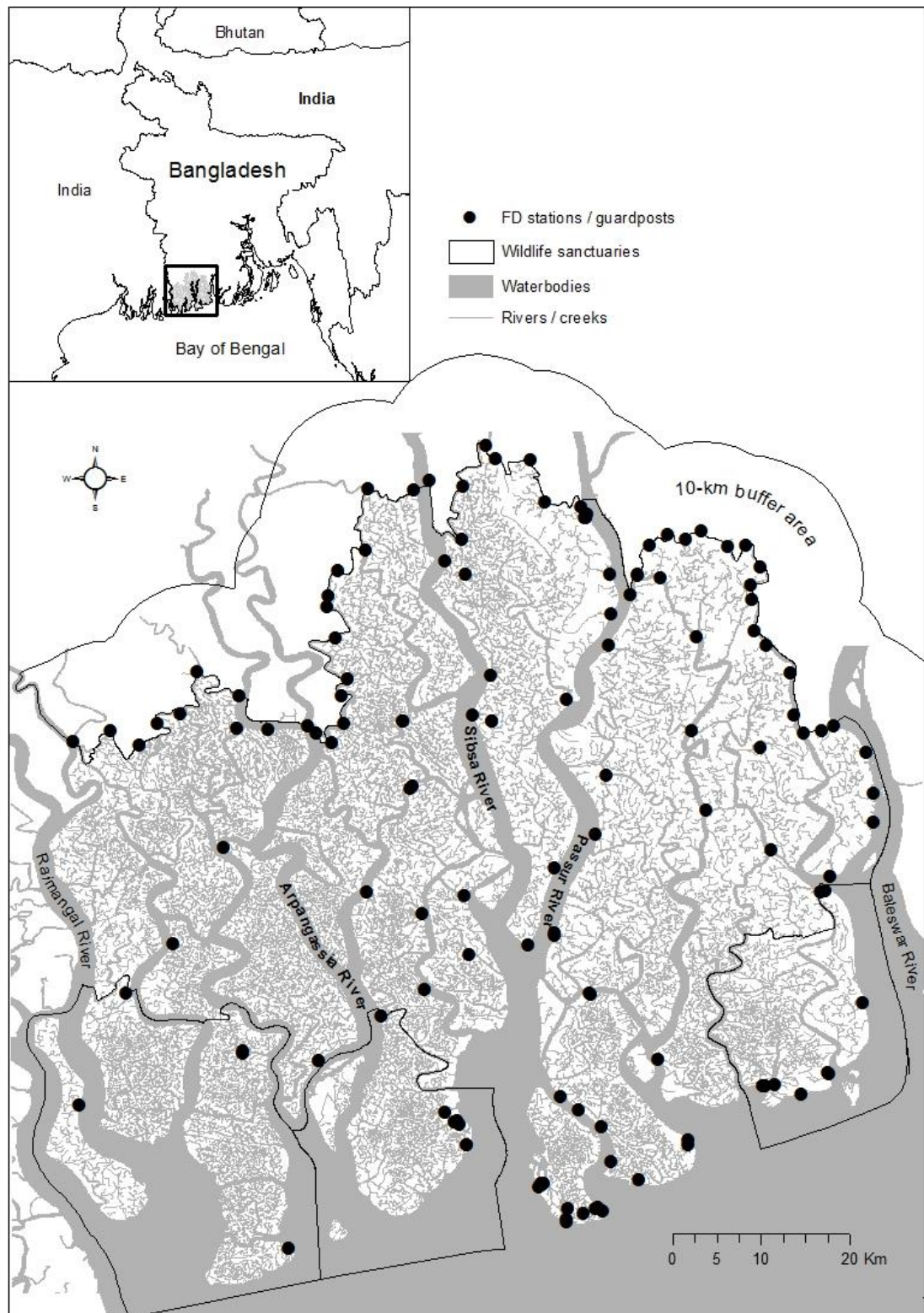


Fig. 4. The Bangladesh Sundarbans showing wildlife sanctuaries and Forest Department stations and guard posts.

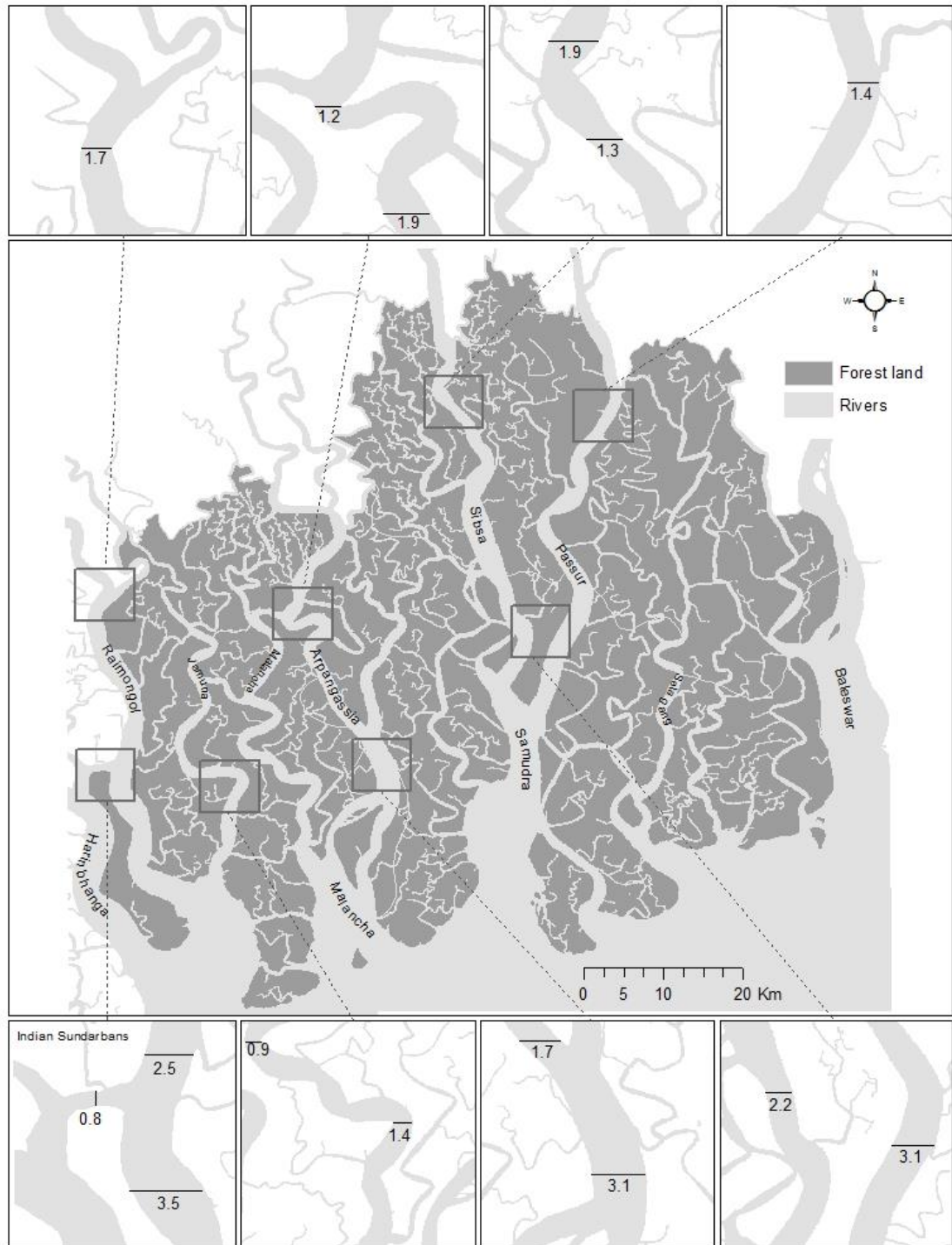


Fig. 5. The Bangladesh Sundarbans showing major river systems. Numbers in the top and bottom maps showing the width in kilometres of major rivers at different segments of their courses.

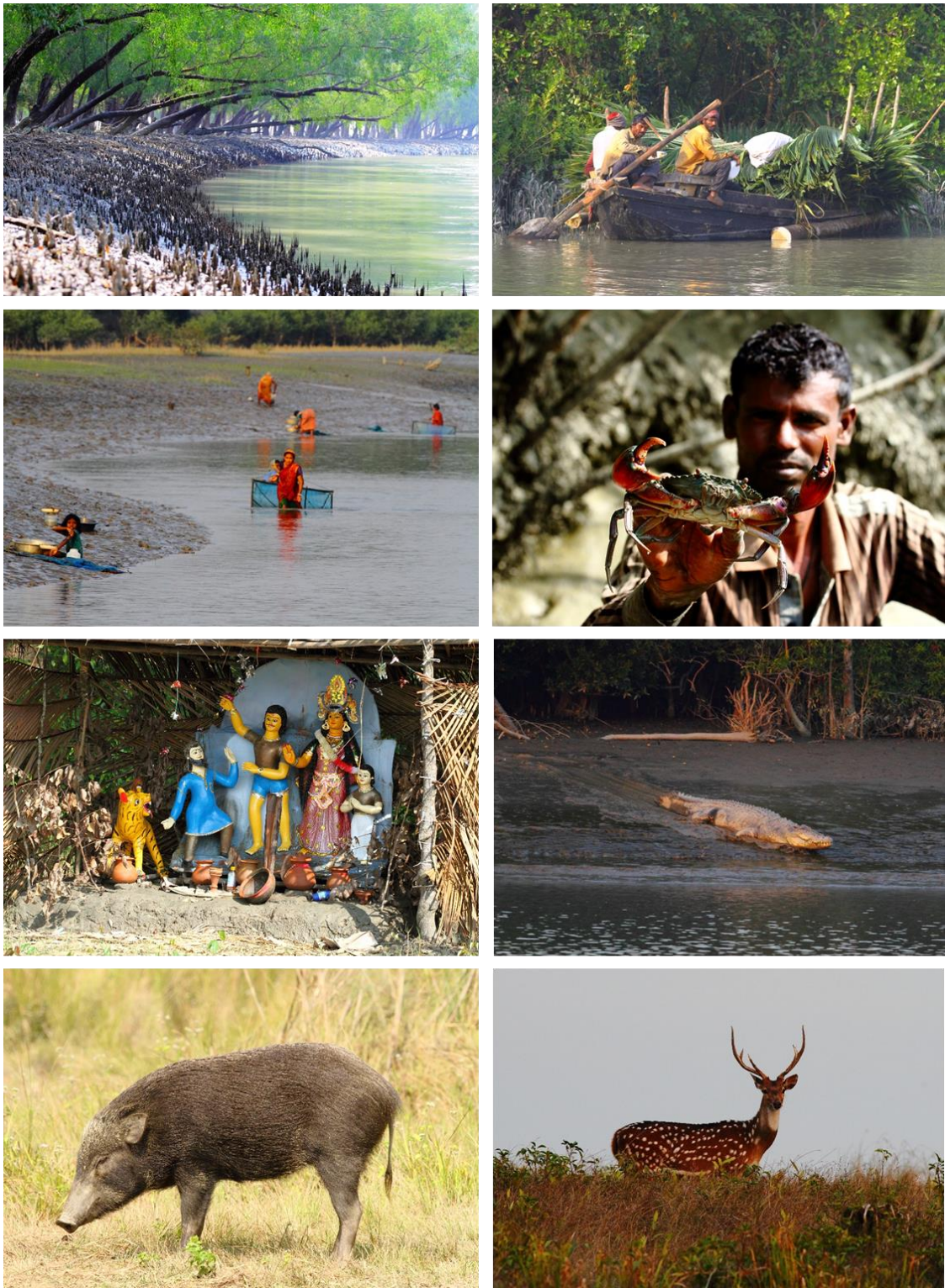


Fig. 6. Habitat features of the Bangladesh Sundarbans (reading from top to bottom, left to right): typical topography and habitat condition; local people collecting *golpata* leaves; female and children collecting shrimp fry; crab fisherman showing his catch; deities erected in the forest; Saltwater crocodile sliding from its basking spot; two major prey species of tiger - Wild boar and Spotted deer in the Sundarbans.

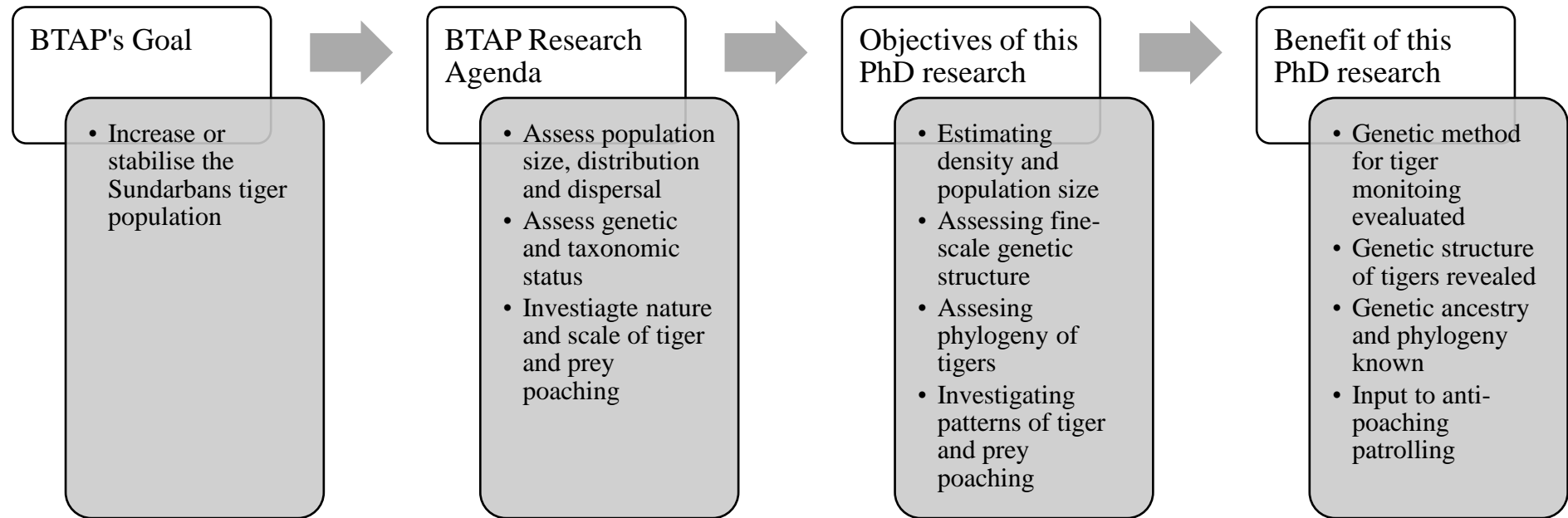


Fig. 7. Schematic relationship between the Bangladesh Tiger Action Plan (BTAP) (2009-2017) and objectives of this PhD research benefiting tiger conservation in the Bangladesh Sundarbans.

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Chapter 2

**Why carry out non-invasive genetic sampling? A
concise review of field methods and laboratory
procedures**

Methodological approaches

Conventional survey methods for carnivores

Tigers are elusive and nocturnal carnivores (Karanth and Sunquist, 2000; Sunquist, 1981), and occupy large home ranges typically with low densities (Kawanishi and Sunquist, 2004). This elusiveness often makes it very difficult to accurately assess the size of their populations (Karanth and Nichols, 2010). Moreover, census methods may require surveying hundreds or even thousands of square kilometres across rugged and inhospitable landscapes to obtain reliable information on tiger populations (Karanth and Nichols, 2010). As a result, distance sampling protocols that require visual detection (Buckland et al., 2001) are largely unsuitable for investigating tiger populations (Karanth and Nichols, 2010).

A variety of field methods have evolved to ‘capture’ individual tigers for demographic and population studies which can be summarized as: (i) physical trapping and radio-tagging; (ii) recognition of tiger tracks using experts or statistical methods of pattern recognition; (iii) scat identification using trained scenting dogs; (iv) photographic identification using camera traps; (v) DNA analysis of field-collected scat samples. However, all these methods have their own advantages and limitations when they are applied to studying tigers (Karanth and Nichols, 2010).

Radio-telemetry has been widely used to study secretive carnivores including tigers since the 1960s (Karanth and Sunquist, 2000; Smith, 1993; Sunquist, 1981), which provided a substantial body of information on tiger predatory behaviour, home range, social structure, movement patterns and dispersal behaviour (Karanth and Nichols, 2010; Smith et al., 1987). However, high financial costs and logistical constraints of radio-telemetry often limit the number of animals that can be monitored, and can introduce issues relating to statistical analysis and sampling coverage (Karanth and Nichols, 2010).

In the Russian Far East, an ecological model established the correlation between the expected number of tiger track sets produced by an individual per day, and then field counts of track sets were used for estimating wild tiger numbers. Although the widespread substrate of snow in the habitat where this approach was used has produced ‘standard track sets’ that can be used to produce reasonable results; its

underlying assumptions, require further validation for wide field application (Stephens et al., 2006).

Pugmark-based tiger population assessment methods that rely on counting ‘individually identified tracks’ have been extensively used in India for decades but this approach produced unreliable results in different field conditions (Karanth and Nichols, 2010). Consequently, ‘pugmark census’ approaches have been abandoned after a quarter of a century of field applications in India and elsewhere (Karanth, 2005).

Karanth and Sunquist (2000) used an *ad hoc* prey-tiger ratio to assess carrying capacity, and population number as an indirect index for tiger monitoring. The assumption was that an ‘average tiger’ requires about 50 ungulate prey animals per year, with about ‘10% annual cropping’ of available prey numbers by tigers. Within this ecological assumption, tiger numbers can be related to prey numbers using the simple relationship of one tiger for every 500 prey animals. Since the usual ungulate prey of tigers can be counted with reliable detection by using distance sampling methods (Karanth and Sunquist, 2000), this prey-tiger ratio can be useful in estimating potential carrying capacity of tiger habitats, but is unsuitable for accurately estimating tiger populations (Karanth and Nichols, 2010).

The non-invasive photographic capture-recapture (CR) method, also known as ‘mark-recapture’ or ‘capture-mark-recapture’, has been extensively applied over decades for investigating populations of tigers (Karanth, 1995; Karanth and Nichols, 2002, 1998), jaguars (Silver et al., 2004), and ocelots (Trolle and Kéry, 2003). The underlying principle of this method is that several ‘samples’ consisting of individually identifiable tigers are obtained from a population of an unknown size (N = abundance). This sampled ‘population’ consists of ‘individual tigers’ which are counted uniquely from ‘tags’ applied at initial capture or from natural marks of the animal. The ‘detection probability’ is then estimated from the frequencies at which such individuals are caught in subsequent samples. Finally, the unknown tiger abundance can be estimated by simple general estimator that relates the field counts (C) of tigers to the real number of tigers (N) in the population with, $N = C/p$, where N = abundance estimate, C = count statistic, and p = estimated proportionality constant

(in other words, detection probability) relating the count statistic and abundance (Karanth and Nichols, 2010).

During field application, camera traps are generally installed on regular tiger trails to increase the detection probability of capturing individual tigers for a precise estimate (Karanth and Nichols, 1998). The Sundarbans mangrove habitat, the only swamp forest in the world supporting tigers, has a substantial lack of regular tiger trails that are appropriate for setting camera-traps. As a result, a previous camera-trapping study in the Bangladesh Sundarbans was only able to obtain limited detections (Khan, 2012), while a more recent study had to use range of bait and lure in order to increase detection rate (Dey et al., 2015).

Low detection rate is prone to introduce imprecise population estimates (Karanth et al., 2004), in addition to several other potential disadvantages of this method in field applications. For example, large numbers of camera traps are required to cover a large geographic range for low density carnivore species such as tigers. Moreover, vulnerability of cameras to theft when deployed in the field, vandalism, adverse weather, and lack of tiger tracks in the field are among the potential constraints for their field application (Mondol et al., 2009a).

Over decades, non-invasive genetic sampling, using scats or hairs left behind by the animal, has become a powerful approach (Adams et al., 2003; Piggott and Taylor, 2003; Prugh et al., 2005; Wasser et al., 2004) to answer a wide range of research questions relating to population abundance, geographic distribution, genetic diversity, phylogeny, hybridization, kinship, sex ratio, movement, and home range size (Adams et al., 2003; Creel et al., 2003; Piggott and Taylor, 2003; Wilson et al., 2003), and examining population dynamics over a longer period of time (Prugh et al., 2005). Scat material offers numerous advantages over live- or camera-trapping including larger sample size, surveillance over larger areas, and with possibly less-biased data since all animals defecate regularly (Fernando et al., 2003; Smith et al., 2005; Taberlet and Luikart, 1999).

Non-invasive genotyping of scat-based DNA has been used as an alternative approach to estimate abundance of cryptic or endangered species following CR models (Marucco et al., 2011; Waits, 2004). In the recent past, ‘tiger capture-

recapture' studies based on scat DNA have been shown to provide reliable population estimates of tigers (Bhagavatula and Singh, 2006; Mondol et al., 2009a).

Non-invasive genetic sampling strategies

Sampling design is a crucial step in non-invasive genetic studies. Standard sampling protocols are required to adhere to assumptions of conventional CR analytical approaches (Karanth, 1995; Karanth and Nichols, 1998), in order to investigate population parameters such genetic samples (Mondol et al., 2009a). In particular, Mondol et al. (2009a) demonstrated a trade-off between scat-based genetic and photography-based capture-recapture population methods in determining population abundance of tigers in India (Karanth, 1995; Karanth and Nichols, 1998; Mondol et al., 2009a). Following the basic principle of a CR approach, Mondol et al. (2009a) surveyed 18 'search routes' as transects for collecting tiger scat, covering an area of 671 km² of Bandipur tiger reserve in India. The assumption was that the selected search routes were spatially distributed in a way that each individual tiger within the reserve would have an equal probability of being detected. The distribution of 'search routes' was based on the design of established photographic capture-recapture studies to avoid large 'gaps' in which an individual tiger had no exposure of detection. The entire study area was surveyed by three teams over six successive days, and repeated over six consecutive weeks (Mondol et al., 2009a). The individual CR dataset from this study were then analysed using standard CR analysis (Mondol et al., 2009a). Other studies simply estimated the minimum number of tigers from random collection of scat samples over the study area (Bhagavatula and Singh, 2006; Borthakur et al., 2013). However, recent advances in statistical analyses such as Spatially Explicit Capture Recapture (SECR) in relation to CR sampling relaxed several core assumptions of typical CR sampling protocol, where geographic closure and sampling session are not mandatory to be met (Efford, 2011; Efford et al., 2009). Therefore, non-invasive genetic sampling using SECR models can now be more easily applied to estimating robust population parameters of elusive carnivores (Efford, 2011).

Sample collection techniques

Scat-based non-invasive genetic studies have shown that there are substantial variations in DNA extraction and amplification success due to a number of inherent

attributes incurred during sampling (Broquet et al., 2007). Preservation techniques (Murphy et al., 2002), type of preservatives and seasonality of sampling (Maudet et al., 2004), age or condition of scats (Piggott, 2004), environmental or habitat conditions (Nsubuga et al., 2004), the species of interest and its diet (Murphy et al., 2007), and extraction protocols (Piggott and Taylor, 2003) can all play a significant role in successful extraction and amplification of scat DNA. Therefore, sampling technique should be selected based on sample types, study species and objectives.

A wide range of methods to obtain non-invasive samples from wild animal has already been developed and evaluated. Non-invasive sample type ranges from menstrual fluid to mucus trails or whatever is left by an animal (Beja-Pereira et al., 2009). For example, a non-invasive study detected a frog species in natural wetlands by PCR testing for mtDNA in water samples (Ficetola et al., 2008). Saliva is a good source of DNA, and has been useful in forensics and criminal case analysis (Beja-Pereira et al., 2009), identifying canids that attacked domestic sheep (Williams et al., 2003), identifying predators of coyote (Lampa et al., 2013) or in solving the cases of livestock attacks in which wolves and dogs were the main suspects (Sundqvist et al., 2008). Saliva of the target animal is often collected with swab stick (Blejwas et al., 2006; Inoue et al., 2007; Sastre et al., 2009; Sundqvist et al., 2008), while urine is sampled either using disposable plastic tools or as a frozen snow-urine mixture (Hayakawa and Takenaka, 1999; Inoue et al., 2007). However, animal scats are the most common non-invasive samples that are easy to find in habitats and have been useful in providing more information than other sample types (Beja-Pereira et al., 2009).

For non-invasive genetic studies, scats are either sampled entirely (Kohn et al., 1999; Solberg et al., 2006), partially (Bellemain et al., 2005; Hajkova et al., 2011) or only the surface materials for extracting DNA (Frantz et al., 2003; Wilson et al., 2003). Several studies demonstrated that a surface-wash of the entire scat or cut off parts can increase amplification success, and can reduce genotyping errors compared to homogenization of the entire sample (Flagstad et al., 1999; Palomares et al., 2002; Piggott and Taylor, 2003). Higher amplification success of DNA samples derived from the outer scrapings of scat can be obtained; because the outer layer of scat contains higher quality DNA originating from the intestinal tract of the target species while the probability of containing foreign DNA and PCR inhibitors is comparatively

higher inside the scat (Fernando et al., 2003; Flagstad et al., 1999; Maudet et al., 2004). Furthermore, collecting whole scats may influence the marking behaviour of the target species if scat deposits are used by the target species for intraspecific communication (Lampa et al., 2008). In these instances, scraping off the surface of the entire scat with disposable collecting tools such as toothpicks or cotton swabs (prior to replacement of the scat) can be an alternative option to reduce behavioural responses (Lampa et al., 2013). Sharma et al. (2012) sampled the outermost layer of scats weighing about 5-10 gm, supplemented with hair and claw samples collected opportunistically from trees marked by tigers and from the kill sites that were encountered during the study. To avoid cross contamination, only hairs that were found in a single clump were collected (Sharma et al., 2012).

Collecting target species' scat is another crucial step in non-invasive genetic studies. In particular, it is often difficult to identify the target animal's scat based only on physical characteristics particularly when there is more than one sympatric predator species exists in the area. Scat of tigers, for instance, can be easily misidentified with scat of sympatric carnivores such as leopards (Mondol et al., 2015).

The quality of scat samples may influence the quality of DNA in it, therefore collection of relatively fresh samples have been recommended (Mondol et al., 2009a). The physical appearance and the amount of moisture content in scat can be useful clues to judge the freshness of the samples (Andheria, 2006; Mondol et al., 2009a).

Most scat-based studies have collected samples during transect surveys (Banks et al., 2002; Brinkman et al., 2010; Jacob et al., 2010; Kohn et al., 1999), using trails (Cullingham et al., 2010; Curteanu, 2007; Eggert et al., 2003; Flagstad et al., 2004; Mondol et al., 2009a), or using latrine sites (Frantz et al., 2003; Wilson et al., 2003), marking sites (Ruibal et al., 2009) and resting points of the species (Piggott et al., 2006; Puechmaille and Petit, 2007). In a tiger study, Mondol et al. (2009a) followed dirt roads and trails, known to be the regular travel routes and which are marked by scat deposits. While Joshi et al. (2013) used existing roads and trails across six tiger reserves in central India to search for fresh scats.

Sample preservation methods

Sample preservation is a cornerstone in scat-based genetic studies in order to obtain high quality DNA from samples. The principle of preserving scat samples can be

described by three main approaches: (i) deactivation of enzymatic activities that degrade the sample via removal of water, (ii) deactivation of nucleases via the elimination of cations (e.g., MgCl_2) from the sample, and (iii) inhibition of nuclease activity via the storage of samples at low temperature (Beja-Pereira et al., 2009). Drying agents (e.g., silica gel or ethanol) and drying techniques (e.g., vacuum spinning, lyophilization, over-heating) are the most commonly used preservation methods to remove moisture from scat samples. Removal of cations that potentially degrade the DNA in scat samples is commonly achieved by using chelators (e.g., EDTA or resin, as chelating agents). Numerous methods have been developed and used for the preservation of scat sample in non-invasive genetic studies (Beja-Pereira et al., 2009). Methodological advancements in preservation of non-invasive scat samples have provided a choice for researchers, but it is often difficult to select the most reliable method for specific field conditions (Beja-Pereira et al., 2009).

Hard genetic materials (e.g., hairs, feathers, or egg shells) are relatively easy to preserve with conventional methods by using either refrigeration or storing at room temperature with silica gel. However, preservation of moist samples, such as urine, saliva and scat, is often a challenge during field surveys, since DNA degradation in the samples caused by bacteria, enzymes (e.g. nucleases), oxidation or hydrolysis needs to be reduced by using appropriate preservatives (Beja-Pereira et al., 2009; Lampa et al., 2013). Freezing samples is common in many scat-based genetic studies in a wide range of wild animals. In estimating Wolf (*Canis lupus*) populations, Creel et al. (2003) stored scat at below -20°C for several weeks without any preservatives. A similar approach was followed to preserve scats of Canadian Swift fox (*Vulpes velox*) for testing the feasibility of scat sampling as a non-invasive population survey technique (Curteanu, 2007). While Piggott and Taylor (2003) preserved samples of Red fox (*Vulpes vulpes*) in Australia by air-drying that proved to be the most effective technique of sample preservation.

A number of preservation protocols has demonstrated varying degree of successes in DNA extraction and subsequent amplification. For example, scat samples of Eurasian badger (*Meles meles*) preserved at 70% ethanol produced a higher amplification success than samples preserved in a buffer solution or as frozen (Wilson et al., 2003). Prugh et al. (2005) obtained high DNA amplification success from Coyote (*Canis latrans*) samples preserved at -80°C in buffer solution than samples preserved without

buffer at same temperature. Bhagavatula and Singh (2006) examined the efficiency of different scat preservation methods, where each scat sample was divided into two parts and preserved separately in 90% ethanol and in silica gel pouches. All samples were then preserved at room temperature for about a week until transported to the laboratory for analysis. The results showed no significant difference between the two sample preservation methods ($p > 0.05$, two tailed-test) in subsequent DNA extraction and PCR amplification success. Tiger scat preservation using air-tight plastic bags with silica gel (Borthakur et al., 2013), and absolute ethanol (Joshi et al., 2013; Sharma et al., 2012) have also been used. Zhang et al. (2009) explored the potential of 'scat hairs' as a DNA source for genetic analysis in South China captive tigers, and preserved fresh scats at 100% ethanol at normal temperature (22°C). The combined use of silica and ethanol has been repeatedly tested in studies involving Western gorilla (*Gorilla gorilla gorilla*) and Chimpanzee (*Pan troglodytes verus*), and this mixed method yielded more DNA from scats than those were preserved separately (Nsubuga et al., 2004). However, scat samples preserved at 90% ethanol alone provided similar results in cases of lower quality scats (Roeder et al., 2004). The correct amount of preservatives is critical to inhibiting DNA degradation in the sample, because insufficient volume of drying agents or failure to freeze samples can often lead to rapid DNA degradation (Beja-Pereira et al., 2009).

There are several advantages of using ethanol over silica for the preservation of scat samples. Ethanol prevents formation of scat powders that may reduce the risk of cross-contamination by aerosol. Ethanol also maintains the external mucous layers containing animal cells packed against the scat material, whereas silica can remove outer cell layers of scat during transportation. However, being highly flammable and potentially dangerous, air transportation of ethanol-preserved scat is often problematic and expensive. Therefore, silica may be the best alternative preservative that has been tested and used widely in scat preservation (Beja-Pereira et al., 2009). It is suggested that any sample preservation method should be easy to execute in the field, and that the method should have no adverse effect on the subsequent DNA extraction and amplification (Lampa et al., 2013).

Laboratory procedures

DNA extraction from non-invasive genetic samples

DNA extraction is one of the most important steps in non-invasive genetic studies because performance of all downstream analysis relies on the extraction success (Beja-Pereira et al., 2009). Therefore, the extraction step should aim to obtain the greatest possible amount of DNA but avoiding PCR inhibitors and non-target DNA (Frantzen et al., 1998; Kohn and Wayne, 1997; Reed et al., 1997). Moreover, an extraction technique should be fast, cost-effective and easy to accomplish because most genetic projects often deal with large number of samples (Reed et al., 1997). Several DNA extraction methods are commonly used for non-invasive samples, which can be summarised as follows:

Phenol-chloroform extraction: This method had been widely used over the last 10-15 years. However, it is hardly used today mainly because the chemicals are hazardous, the approach is time-consuming, and sometimes PCR inhibitors remain even after DNA extraction (Beja-Pereira et al., 2009).

Resin-based extraction: Resin-based (e.g., Chelex, Sigma-Aldrich, Germany) extraction methods are often used for hair samples, because Chelex is particularly useful for extracting DNA from hairs and formalin-fixed archived tissues (Chakraborty et al., 2006). This method is quick and low-cost. However, extracted DNA may not always be very pure and DNA can become degraded after several months of extraction. Furthermore, Chelex itself is a PCR inhibitor (Beja-Pereira et al., 2009).

Silica-based extraction: This is the most frequently used method for scat-based DNA extraction in genetics studies. The QIAamp DNA Stool Mini Kit (Qiagen, Germany) is often used, and this technique has been proved to be highly efficient in dealing with PCR inhibitors and yielding sufficient amount of high quality target DNA (Bhagavatula and Singh, 2006; Lampa et al., 2013; Piggott and Taylor, 2003).

DNA extraction methods may vary in relation to types and forms of non-invasive genetic samples, so a study may require its own adjustment or modification. For example, surface-wash technique combined with commercial extraction kits (e.g., DNeasy Blood Kit) were suitable for pellet-form scat samples. In this technique, a

scat pellet is incubated in a buffer solution followed by extraction of DNA from the buffer using the extraction kit (Luikart et al., 2008). Besides, Wan et al. (2006) described a 'cell enrichment' method which dissolved large quantity of scat into large volumes of buffer and that this approach yielded high amount of high molecular weight DNA. This method is however very expensive and has reduced capability in DNA quantification, as well as target DNA separation from microbial or other non-target DNA (Beja-Pereira et al., 2009).

Bhagavatula and Singh (2006) evaluated five different methods for DNA extraction from tiger scat samples: (i) Chelex-100 method (Walsh et al., 1991); (ii) the Digest buffer/phenol chloroform method (Reed et al., 1997); (iii) the Lysis buffer/column purification method (Fernando et al., 2003); (iv) Guanidinium thiocyanate-silica method (Reed et al., 1997); and (v) Qiagen Stool DNA extraction method. Amplification success rate was 38% for the Chelex-100 method; 75% for the Digest buffer/phenol chloroform method; 25% for the Lysis buffer/column purification method; 88% for Guanidinium thiocyanate-silica method; and 100% for the Qiagen Stool DNA extraction kit (Bhagavatula and Singh, 2006).

Mondol et al. (2009a) extracted DNA from field-collected scat samples using the QIAamp DNA Stool mini kit (Qiagen Inc.), using approximately 180-200 mg of sample from the outer parts of the scat. To increase potential DNA yield, four micrograms of carrier RNA (Poly-A from NEB) were added to the sample (Kishore et al., 2006). A number of scat-based studies have used Qiagen extraction kit to identify wild tiger species from non-invasive samples (Mukherjee et al., 2007), to assess genetic connectivity across fragmented landscapes (Reddy et al., 2012), and to investigate spatial genetics between tiger populations in India (Sharma et al., 2012).

Genetic markers used in non-invasive tiger studies

Improved and extensive molecular genetic markers have been increasingly used in a wide range of non-invasive studies involving large carnivores such as tigers. The diagnostic genetic markers may include mitochondrial DNA (mtDNA) sequence, a panel of highly polymorphic microsatellite markers, and a highly variable nuclear MHC (major histocompatibility complex) class II DRB gene (Luo et al., 2010a). The mtDNA markers have been used to assess population genetic structure, resolving taxonomic uncertainties of tigers, and to detect illegal hunting or poaching of

endangered species (Arif et al., 2011). The multiple copies of mitochondria in most cells allow to obtain genetic information from a very tiny amount of samples (Khan et al., 2008).

The mtDNA markers were useful for screening scat samples to identify target species in non-invasive genetic studies (Bhagavatula and Singh, 2006; Joshi et al., 2013; Luo et al., 2010; Mondol et al., 2009a; Sharma et al., 2012). In particular, a range of mtDNA markers have been developed to amplify different gene fragments of mtDNA for assessing genetic status of Bengal (Mondol et al., 2013, 2009b) and Amur tigers (Russello et al., 2004), genetic ancestry of extinct tigers (Xue et al., 2015), and phylogeography and genetic ancestry of all tiger subspecies (Luo et al., 2004; Wilting et al., 2015).

Alongside mtDNA markers, a range of nuclear markers have been frequently used for DNA fingerprinting in genetics studies. The most commonly used markers are the Random Amplified Polymorphic DNA (RAPD), Amplified Fragment Length Polymorphism (AFLP), and microsatellites or Short Sequence Repeats (SSRs). The RAPD and AFLP are considered as dominant markers as they contain two alleles per locus which can be identified as 'absence' or 'presence' of a band in sequenced data. While the microsatellites are a co-dominant markers that recognises both dominant and recessive alleles, so they are useful to differentiate homozygotes and heterozygotes in the population (Arif et al., 2011). There are some other types of genetic markers such as variable number of tandem repeats (VNTRs), consisting of 10-64 nucleotides (minisatellites) which are useful for long-term genetic studies in understanding genetic fitness of animal populations (Mishra et al., 2014). Nonetheless, the Single Nucleotide Polymorphisms (SNPs) has been utilised as genetic markers that are very useful to perform rapid, large-scale and cost-effective genotyping (Brumfield et al., 2003; Chen and Sullivan, 2003; Vignal et al., 2002).

Microsatellite markers are made up of short repeat sequence of genome that are usually 2-8 nucleotides in length (Pompanon et al., 2005), which are highly polymorphic, co-dominant in nature, and follow the Mendelian inheritance, making them suitable for traditional as well as conservation genetic studies (Mills, 2013; Pompanon et al., 2005). A quite good number of studies has used a wide range of microsatellite markers in non-invasive genetic studies involving tigers (Henry et al.,

2009; Luo et al., 2010b; Mondol et al., 2009a; Williamson et al., 2002). Microsatellite markers developed for domestic cat (Menotti-Raymond et al., 1999) were widely used for investigating genetic status and diversity of tiger subspecies (Alasaad et al., 2011; Henry et al., 2009; Liu et al., 2006; Luo et al., 2008, 2004; Mishra et al., 2014; Sharma et al., 2008; Williamson et al., 2002). A diverse panel of microsatellite primers has already been developed and screened in domestic cat (*Felis catus*) (Menotti-Raymond et al., 1999), and used in Bengal tigers to estimate tiger population (Mondol et al., 2009a), and to investigate genetic structure (Mondol et al., 2009b; Reddy et al., 2012b), spatial genetics (Sharma et al., 2012), and genetic connectivity between tiger populations across Indian landscapes (Joshi et al., 2013). A list of microsatellite markers optimised and commonly used in tiger studies can be found in the Table 1.

Molecular species identification

Identifying scat samples by size, shape or moisture content can be inconsistent and unreliable; because body size can vary greatly within species, and an individual animal can leave scat in a broad range of sizes (Farrell et al., 2000). Therefore, non-invasive molecular assays were increasingly used to determine donor species of interest (Deagle et al., 2005; Farrell et al., 2000; Jarman et al., 2002; Purcell et al., 2004).

Molecular identification of species from scat samples can be determined with PCR-based assay using species-specific mtDNA markers, which has been a regular practice to reliably identify target species (Bhagavatula and Singh, 2006; Mondol et al., 2009a). Determining the identity of scat sample is crucial in non-invasive studies to ensure that only the correct samples receive further downstream analysis (Mondol et al., 2009a). The mtDNA markers containing genes from NADH sub-unit and cytochrome *b* regions were used for screening scat samples in tiger studies (Bhagavatula and Singh, 2006; Mondol et al., 2009a; Mukherjee et al., 2007).

DNA amplification

Majority of non-invasive tiger studies have optimised microsatellite markers (Bhagavatula and Singh, 2006; Sharma et al., 2008; Williamson et al., 2002; Zhang et al., 2006) from previously developed markers in domestic cat (Menotti-Raymond et al., 1999). For instance, Mondol et al. (2009a) optimised a panel of 33 microsatellite

loci, previously developed in domestic cat (Menotti-Raymond et al., 1999) to investigate population abundance of Bengal tigers.

Standardising markers using good quality samples alongside scats has been common to almost all non-invasive genetic studies involving tigers. This standardisation allowed to compare genotypes obtained from low-quality samples with that of good quality samples to assess the performance of markers (Bhagavatula and Singh, 2006; Mondol et al., 2009a). While other studies obtained captive scat sample to compare with field-collected samples for such standardisation (Bhagavatula and Singh, 2006; Borthakur et al., 2013).

Replication of microsatellite genotyping is common to most scat-based studies to obtain reliable genotypes. The “comparative multiple tube approach” is one to generate consistent genotypes from low quality samples (Bhagavatula and Singh, 2006; Mondol et al., 2009a; Sharma et al., 2012; Taberlet et al., 1996). In this approach, multiple PCR reactions were performed at a time from each sample and then amplified genotypes were compared for obtaining consensus genotypes. The another approach, called “comparative genotyping” is also used in contrast to the multiple tube approach because it is more cost-effective and less laborious, where each sample is genotyped consecutively until consistent genotypes are obtained (Hansen et al., 2008; Jackson et al., 2016).

Challenges in genotyping

The success of PCR amplification in non-invasive genetic studies can be challenging due to a number of issues in genotyping process (Lampa et al., 2013). Potential PCR inhibitors, low quality DNA in degraded samples, and contaminations are common in non-invasive studies, which may result in low amplification success and high amount of genotyping errors (Beja-Pereira et al., 2009; Lampa et al., 2013). However, approaches have been developed to overcome these impediments, which may include choosing suitable microsatellite markers, using improved PCR reagents, and standardization of PCR protocols (Pompanon et al., 2005). Major challenges and approaches for successful genotyping can be described as follows:

Overcoming PCR inhibitors: PCR inhibitors can cause low amplification, even for samples which apparently yield good amounts of DNA (Kontanis and Reed, 2006). Scat samples were known to carry compounds which are potential PCR inhibitors,

such as complex polysaccharides, products from food degradation (e.g., acids, enzymes, lipids, proteins, etc.), RNA, and microorganisms (Lampa et al., 2013). To remove these inhibitors, DNA extractions can be combined with washes for DNA purification, which can be done simply by dilution (Lampa et al., 2013; Palomares et al., 2002). However, genotyping errors can be caused due to low quantity of target DNA after dilution, therefore a balance between dilution and amount of DNA in the extract must be established (Lampa et al., 2013). Precipitation of DNA also removes inhibitors which can be carried out ethanol wash before re-dissolving the DNA precipitant in water or buffer. Additionally, PCR adjuvants (e.g., BSA - bovine serum albumin), or non-ionic detergents (e.g., Tween 20 and Triton X-100) are often used to bind inhibitors to improve amplification specificity (Lampa et al., 2013).

Overcoming DNA degradation: To overcome difficulties of amplifying degraded DNA, it was suggested to amplify only very short fragments (e.g., mini-STRs, SNPs) (Campbell and Narum, 2009). Several non-invasive studies have revealed that large DNA amplicons (>200-300 bp) generated significantly higher allelic dropout rates than shorter amplicons (Broquet and Petit, 2004; Buchan et al., 2005). Other studies demonstrated that single nucleotide polymorphism (SNP) studies can achieve higher amplification success and lower error rate than microsatellites, because SNP amplicons are generally shorter (<100 bp) than microsatellites (100-300 bp) (Musgrave-Brown et al., 2007). However, the bi-allelic nature of SNPs (compared to the multi-allelic nature of microsatellite markers) must be compensated for by typing a larger number of SNP loci (Morin et al., 2009, 2004).

Overcoming low DNA quantity: Pre-amplification (i.e., products from a first amplification are used as templates for a subsequent PCR) is an efficient procedure to overcome problems associated with low-quantity DNA, which can increase the number of low copy template DNA (Lau et al., 2003). However, this pre-amplification may require additional PCR optimisation before genotyping.

Overcoming non-specific amplification and contamination: Co-amplification of non-specific products and contamination in genotyping process can be major problems in non-invasive genetic studies (Beja-Pereira et al., 2009). Studies have showed that the contamination can produce up to 7% errors in genotyping (Buchan et al., 2005; Navidi et al., 1992; Pompanon et al., 2005). However, hot start PCR can significantly

improve specificity, fidelity and sensitivity of DNA amplifications (Beja-Pereira et al., 2009). A number of widely used *Taq* polymerases such as AmpliTaq Gold (Applied Biosystems), Fast-Start *Taq* DNA polymerase (Roche), Platinum *Taq* DNA polymerase (Invitrogen), TrueStart *Taq* DNA polymerase (Fermentas), AccuSure DNA polymerase (Bioline), Phusion High-Fidelity DNA polymerase (Finnzymes) are known to perform better in genotyping of low quality DNA sample (Beja-Pereira et al., 2009).

Dealing with genotyping errors

The most subtle problem in non-invasive genetic studies is that of genotyping errors (Luikart et al., 2008; Pompanon et al., 2005). Genotyping error can occur when observed genotype of an individual does not correspond to the true genotype (Bonin et al., 2004). Genotyping errors have been known to affect genetic data, thereby profoundly influence the biological inferences (Pompanon et al., 2005). Genotyping errors can be encountered as: (i) *Allelic dropout* - stochastic detection of false homozygotes at heterozygous loci because of failure of one allele to amplify; (ii) *False allele* - creation of new alleles caused by slippage of *Taq* polymerase during early cycles of PCR; and (iii) *Human error* - incorrect identification of alleles as a result of cross-contamination in the field or in the laboratory or database manipulation errors (Hoffman and Amos, 2005; Pompanon et al., 2005). Besides, occurrence of null alleles is the most common error in microsatellite genotyping (Callen et al., 1993; Paetkau and Strobeck, 1995). Occurrence of genotyping errors and their effect can be limited by following procedures in non-invasive studies:

Repeated genotyping: This is the most common approach to limit genotyping errors (Navidi et al., 1992; Taberlet et al., 1996), where each sample at each locus is amplified multiple times to determine an individual as homozygous or heterozygous. However, multi-tubing does not mean to prove error free database. Also, this approach is expensive, and may increase errors as samples are handled more often (including human error), and there are more chances of producing false allele (Beja-Pereira et al., 2009).

Quantification of target DNA: The quantification of amplifiable DNA in the sample allows to determining the approximate number of multi-tube re-runs to be conducted (Morin et al., 2001). For example, if a sample has <25 picogram (amplifiable DNA)

per reaction, it should be discarded; if it has 101-200 picogram per reaction, then four repeats can be performed (Morin et al., 2001).

Using computer algorithms: Various computer-based algorithms are used to detect genotyping errors depending on the data and study objective (McKelvey and Schwartz, 2005; Miller et al., 2002). The way of using algorithms is to examine deviations from Hardy-Weinberg proportions, use pedigree information to detect errors, use the number of mismatches (i.e., genotypes identified more than once and differing by only one or two alleles) as an error signal (Beja-Pereira et al., 2009). Sample-specific errors (only a few poor quality individual samples) can cause significant deviations from Hardy-Weinberg proportions, and such samples should be discarded (Miquel et al., 2006).

Incorporating errors in statistical analysis: Numerous models have been developed to incorporate genotyping errors in statistical analysis (Beja-Pereira et al., 2009). For example, Johnson and Haydon (2007) developed a maximum likelihood-based approach, which is accurate, robust and implemented in a computer program that estimates allelic dropout and false allele error rates with statistical significance.

The most common and universal metric for quantifying genotyping errors is the error rate per locus, providing an idea of the reliability of laboratory protocol, and of the experimental procedure, which allow comparisons between studies and microsatellite markers. Estimating error rate per multi-locus genotype is useful for individual identification, population assignment, kinship and census studies, because it reflects the reliability of genotypes obtained (Waits and Leberg, 2000).

The challenges of studying elusive tigers in the unique Sundarbans mangrove habitat were enormous compared to other tiger landscapes in the Indian Subcontinent and elsewhere. As a result, the Sundarbans tigers remained largely unknown on the aspects of precise population status including threats to tigers and their prey animals while literally no information exist on the genetic status and phylogenetic ancestry of this uniquely adapted population. This non-invasive genetic study was therefore designed to investigate a range of research questions reflecting the research needs outlined in the Bangladesh Tiger Action Plan (BTAP) 2009-2017 (Ahmed et al., 2009) as presented in the introductory chapter. Following the exhaustive literature review carried out in this chapter, the best available approaches of non-invasive

genetic sampling (e.g., SECR) and preservation (e.g., ethanol-mediated air-dry) for the collected genetic samples have been chosen. Extraction of DNA and genotyping, validation and analyses of genetic data also followed the standard protocols described in this chapter, all of which have been clearly explained in the following data chapters.

Table

Table 1

Microsatellite markers optimised and applied in some major genetic studies in tigers.

Microsatellite loci used	Study objectives	References
FCA5, FCA161, FCA91, FCA211, FCA304†, FCA32, FCA126†, FCA8, FCA176, FCA69, FCA96, FCA44, FCA94, FCA105, FCA441, FCA310, FCA212, FCA90†, FCA290, FCA129, FCA220, FCA229, FCA43, FCA139, FCA391, FCA77, FCA293, FCA123, FCA242, FCA201	Phylogeography of tiger subspecies; identification of verified subspecies ancestry of tigers	Luo et al. (2008; 2004)
FCA126, FCA69, FCA90, FCA304†, FCA441, FCA672†, FCA628, FCA232†, FCA230†, FCA279†	Population genetics of Bengal tigers	Mondol et al. (2009b)
FCA453, FCA391, FCA628, FCA205, FCA126, FCA41, FCA232, FCA232, FCA441, FCA672†, FCA115	Individual identification of Bengal tigers	Mondol et al. (2009a)
6HDZ057, 6HDZ064, 6HDZ089, 6HDZ170, 6HDZ463, 6HDZ481, 6HDZ610, 6HDZ635, 6HDZ700, 6HDZ817, 6HDZ859, 6HDZ993	Population genetics of Amur tigers in the Russian Far East	Henry et al. (2009)
F42, F42, FCA-279, FCA441, FCA628, FCA672, E7†**	Spatial genetic structure of populations	Sharma et al. (2012)
F37, F42, F53, F96, F115, F124, F141, FCA-391, FCA-424, FCA-441, E6**, E7	Population structure and genetic connectivity	Reddy et al. (2012a)

All makers identified with F and FCA were optimised from Menotti-Raymond et al. (1999); and with HDZ markers from Williamson et al. (2002), except **Bhagavatula and Singh (2006).

† Markers applied in this study.

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Chapter 3

Estimating density and population size of Bengal tigers in the Bangladesh Sundarbans using non-invasively collected genetic data

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Abstract

A population parameter is crucial to monitor endangered animals that are the focus of conservation management efforts. Typical photographic capture-recapture methods were widely used for decades to monitor tigers (*Panthera tigris*) but the application of this technique was challenging due to poor levels of detections in the Sundarbans tiger populations. Advances in molecular analyses of DNA contained in non-invasively collected genetic samples can be used to assess tiger population within a spatially explicit capture-recapture (SECR) framework. A total of 440 non-invasive putative tiger samples were collected from four representative sample areas covering 1,994 km² of the Bangladesh Sundarbans. Genetic screening of these samples provided 230 authenticated tiger samples, which we attempted to amplify at 10 highly polymorphic microsatellite loci. Of these, a total of 105 samples was successfully amplified, representing 45 unique genotype profiles of tigers. Analyses of the capture-recapture history of these tigers using the SECR model provided a density estimate of $2.85 \pm \text{SE } 0.44$ tigers/100 km² (95% CI: 2.11-3.85 tigers/100 km²) for the area sampled, and an estimate of 121 tigers (95% CI: 90-164 tigers) for the total area of the Bangladesh Sundarbans. We demonstrate the utility of non-invasive genetic surveillance as a viable method for monitoring tiger populations in a landscape where traditional camera-trapping were challenging.

Introduction

Density is a fundamental biological parameter for monitoring animal populations in the wild (Kohn et al., 1999; O'Brien and Kinnaird, 2011). Reliable population estimates as a function of environmental and habitat changes are important for predicting long-term persistence of endangered species (Sutherland, 1996), or to evaluate management responses for rapidly declining tiger (*Panthera tigris*) populations (Dinerstein et al., 2007; Walston et al., 2010). Extant tiger populations now survive within the globally identified 76 Tiger Conservation Landscapes (TCLs), representing only seven percent of their ancestral range (Dinerstein et al., 2007). The Sundarbans, representing 10,236 km² of mangrove forest, was prioritised within 11 global priority TCLs for long-term conservation significance in the region (Sanderson et al., 2006). A reliable monitoring technique is therefore critical to monitor this important tiger population to guide conservation management activities (Ahmad et al., 2009). However, monitoring wide ranging elusive carnivore such as tiger is often difficult, because they occur at low densities over extensive geographic range (Eisenberg, 1981; Karanth and Nichols, 1998; Schipper et al., 2008).

A common approach to estimating population parameters is to capture, mark, and recapture animals within a capture-recapture (CR) framework (White et al., 1982). This approach has been tailored to photographic CR technique that used unique coat patterns to monitor tigers over decades (Jhala et al., 2011; Karanth, 1995; Karanth and Nichols, 1998). However, the low capture-recapture rates, logistical constraints and unsuitable habitats can significantly limit the application of photographic CR technique in many tiger landscapes (Karanth et al., 2004; Mondol et al., 2009a; O'Brien and Kinnaird, 2011). For example, the substantial lack of regular tiger tracks (typically used to set camera-trap for higher detection rate) in the Sundarbans mangrove habitat resulted in low detection rate (Karanth and Nichols, 2000; Khan, 2012), thereby limiting the value of camera-trapping in the Sundarbans (Karanth and Nichols, 2000).

An alternative method of monitoring tigers is the sign survey that uses index of track set to detect changes in the tiger population (Barlow et al., 2008; Hayward et al., 2002) that provided useful information needed for species management (Caughley, 1977). In the Bangladesh Sundarbans, Barlow et al. (2008) applied this sign survey

that provided reasonable statistical power in detecting changes in the tiger populations. However, direct relationship between the index and population abundance remained unknown, therefore this critical assumption requires careful consideration during field application (Barlow et al., 2008; Hayward et al., 2002).

Advances in DNA technology have enabled researchers to use non-invasive genetic techniques to survey populations of a range of species including coyotes (Kohn et al., 1999), bears (Boersen et al., 2003; Creel et al., 2003; Kindberg et al., 2011; Woods et al., 1999), leopards (Janecka et al., 2008), jaguars (Sollmann et al., 2013), and tigers (Bhagavatula and Singh, 2006; Mondol et al., 2009a). Molecular genetic markers such as microsatellite loci allow individual identification from non-invasively collected samples, eliminating the need to recognise individual animals by capture or physical markers (Kohn et al., 1999; Lucchini et al., 2002). Microsatellite loci are short repeat motifs of DNA sequence (Mills, 2013), and can be highly polymorphic between individuals and populations, making them widely used as a tool to estimate parameters for many animal populations (Pompanon et al., 2005; Sawaya et al., 2011). A suite of microsatellite loci have been developed in domestic cats (Menotti-Raymond et al., 1999), and subsequently used to identify individuals from non-invasive DNA samples in a wide range of carnivores including tigers (Bhagavatula and Singh, 2006; Creel et al., 2003; Kindberg et al., 2011; Mondol et al., 2009a). Although this non-invasive genetic sampling has been recommended for assessing the Sundarbans tiger populations (Jhala et al., 2011; Karanth and Nichols, 2000), this technique has never been applied either to the Bangladesh or Indian Sundarbans.

The Sundarbans, shared between Bangladesh and India (Giri et al., 2007), supports one of the most globally important tiger populations in the region, and the only one which is adapted to living entirely in a mangrove ecosystem (Dinerstein et al., 2007; Gopal and Chauhan, 2006; Sanderson et al., 2006). The Bangladesh Sundarbans is the last stronghold for critically endangered tigers, representing nearly half of the remaining forest in the country (Hussain and Acharya, 1994), and providing wide range economic and ecosystem services to communities (Biswas et al., 2008; Islam and Peterson, 2008).

Several studies have investigated tiger populations in the Sundarbans using a number of methods, including pugmark survey (BFD, 2004), now abandoned owing to

methodological shortcomings (Karanth, 2005), index-based tiger monitoring (Barlow et al., 2008), radio-collaring of two female tigers (Barlow, 2009), and camera-trapping (Karanth and Nichols, 2000; Khan, 2012). Although a recent study managed to increase detection rate with range of lure and baits in camera-traps (Dey et al., 2015), non-invasive genetic technique can be a valuable alternative method to monitor Sundarbans tigers to evaluate management responses (Ahmad et al., 2009) in a situation when poaching of tigers and their prey (Aziz et al., 2017) might have contributed to the continued population decline (Rahman et al., 2012).

The objectives of this study were therefore to evaluate the non-invasive molecular technique as a viable method to estimate density and abundance of tigers in the Bangladesh Sundarbans within a spatially explicit capture-recapture framework, and also to demonstrate that this non-invasive technique may be useful for monitoring other carnivores including tigers elsewhere.

Methods

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 Sundarbans in Bangladesh (21°30'–22°30' N, 89°00'–89°55' E) covers 6,017 km², of which 4,267 km² is forest and the remaining area is comprised of waterbodies (Iftekhara and Islam, 2004a). The Sundarbans is bordered on the south by the Bay of Bengal and on the north and east sides by landmass dominated with human settlements (Hussain and Acharya, 1994). Two rivers, the Raimangal and the Hariabhangha, have separated the Indian part of the Sundarbans and mark the international boundary between Bangladesh and India (Fig. 1).

The Bangladesh Sundarbans is managed as a reserve forest (SRF), except three isolated areas within the forest that have been declared wildlife sanctuaries in 1996 for higher protection of wildlife and their habitat (BFD, 2012). The sanctuaries comprise of the Sundarbans West (715 km²), Sundarbans South (370 km²) and Sundarbans East (312 km²), and were collectively declared a UNESCO World Heritage Site in 1997 (BFD, 2012; Iftekhara and Islam, 2004a) (Fig. 1). The SRF is divided into the Sundarbans East and Sundarbans West Forest Divisions, and administered by two

separate Divisional Forest Officers (DFO) under a Conservator of Forest within Khulna circle of the Bangladesh Forest Department. An additional DFO in the Wildlife Management and Nature Conservation Division within the same circle looks after the management activities specific to the wildlife of the Sundarbans. The regular activities are executed by field management staffs, stationed across 17 stations and 72 guard posts within the SRF (Khan, 2011).

The SRF 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 SRF include Fishing cat (*Prionailurus viverrinus*), Jungle cat (*Felis chaus*) and Leopard cat (*Prionailurus bengalensis*).

The SRF forest is mostly comprised of two timber species; *Sundri* (*Heritiera fomes*; 39%) and *Gewa* (*Excoecaria agalloch*; 39%), with other timber 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 the SRF 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).

Sampling strategy and coverage

To collect non-invasive genetic samples, four sampling areas (totalling 1,994 km²) were selected within the SRF: East Wildlife Sanctuary with additional areas (ES, 383 km²), West Wildlife Sanctuary (WS, 715 km²), Satkhira Block (SB, 342 km²), and Chandpai Block (CB, 554 km²) (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. Forest Department issues permission to local people for collecting forest and

aquatic resources (e.g., *golpata*, honey, fish and crabs) from SB and CB sample areas, but not from the ES and WS.

Following standard CR approaches (Karanth, 1995; Karanth and Nichols, 1998), and in order 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. Each grid cell was targeted for sampling with three separate transects (using one transect each time), searched by a surveying team of four trained field staffs for collecting samples. 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.

Population sampling methods generally deal with two important statistical issues of spatial sampling and observability (Thompson et al., 1998; Williams et al., 2002). Due to the vastness of the Sundarbans and given the typical inability of animal survey methods to cover the entire area of interest (Karanth et al., 2003), we assumed that our four spatial sample areas were representatively subset of the entire SRF. Secondly, despite our intensive sampling effort over four sample areas, it is very likely that we sampled a proportion of tiger populations due to inability to collect all scat samples sourced from all tiger populations residing within the survey area (Karanth et al., 2003). Moreover, small-sized scats deposited by cubs or juveniles were probably not collected by survey teams as if they were sourced from other small cats. Also, adult transient and sub-adult tigers having generally more wide ranging behaviour with

widespread distribution of their scats thereby would have different probability of detection than tigers having stable territory.

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

Scat samples were collected in polypropylene tubes (ThermoFisher Scientific, UK) using twigs to avoid contamination. All scat samples were air-dried before being preserved with silica gel desiccant. Tiger hairs deposited in territorial markings (e.g. scratched marks on trees) were also collected.

Tiger blood and tissue samples sourced from the SRF were also collected to provide genotype standards (“reference”) 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.

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).

DNA extraction

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 scrapped 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 °C. The DNA supernatant from the sample was lysed with 300 µl AL buffer plus 25 µl proteinase K and incubated at 70 °C for 15 min. Four microlitre 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 50 g (or

minimum 10 hairs) of each sample was added to 300 μ l AL buffer incorporating 20 μ l of proteinase K and 20 μ l of DTT (Dithiothreitol, Biotech) and then incubated at 56 °C overnight or until the sample was completely digested. The elution of DNA was carried out in 75 μ l buffer solution. A negative control was included with each batch of extractions to monitor for possible contamination during the DNA extraction procedure.

Species authentication

Morphological features of scat and associated signs of species were commonly used to identify scat samples of the study species (Bagchi et al., 2003; Karanth et al., 1995). However, non-target scat can potentially be 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 target species (Bhagavatula and Singh, 2006; Davison et al., 2002; Mondol et al., 2009a), so that only genetically authenticated samples from the target species are included for further downstream analysis (Mondol et al., 2009a). Therefore, all field-collected samples were screened for species authentication using tiger-specific primers that have been successfully used in other non-invasive tiger studies (Mondol et al., 2009a; Mukherjee et al., 2007). 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 France). PCR reaction volumes (total 27 μ l) contained 3 μ l of template DNA, 12.5 μ l MyTaq redmix (containing dNTPs and MgCl₂; Biorline, UK), 0.5 μ l of each primer, 2.0 μ l BSA (Bovine Serum Albumin, New England Biolabs Inc.) and 8.5 μ l dH₂O. All PCR products from each of the extracted samples were 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 (National Center for Biotechnology Information, NCBI) database to confirm species identity of each sample.

Microsatellite amplification and genotyping

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 high numbers 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 S1). These loci were then optimised using a subset (n=10) of field-collected scat samples and reference samples (n=10). Based on the levels of PCR amplification success, allelic richness, and the extent of genotyping errors, a set of 10 loci was chosen for amplifying all field-collected samples that had been genetically authenticated as being from tiger (Table S2). A felid specific zinc-finger (*Zfx* and *Zfy*) locus was also optimised using samples comprising known male (n=1) and female tigers (n=2) from reference samples 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 S2). Each microsatellite PCR reaction volume (10µl) contained 5 µl Qiagen multiplex PCR buffer mix (Qiagen Inc.), 0.2 µl labelled forward primer (Eurofins Genomics), 0.2 µl unlabelled reverse primer, 2 µl BSA and 3 µl of DNA template. For all multiplex reactions, the PCR temperature regime included an initial denaturation step for 15 min at 95 °C with 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 S2), extension (72 °C for 90 s), and a final extension of 10 m 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. Alleles were identified and scored using GENEMAPPER v3.7 (Applied Biosystems, MA, USA).

Genotype data validation

Multiple screening processes were followed to minimise errors in microsatellite genotyping. Firstly, low quality samples that showed poor quality bands in species-specific PCRs were removed before microsatellite amplification (Kohn et al., 1999). Secondly, samples that amplified successfully for fewer than three loci at the first

PCR attempt were removed from the set of samples to be included in a second genotyping PCR. This screening process ensured that further poor quality samples were eliminated in order to minimize genotyping errors (Creel et al., 2003). For the final set of samples, a comparative genotyping approach was followed, with each sample independently genotyped at least twice (Hansen et al., 2008) to ensure a level of rigour in resolving the true genotype of each sample; this approach was less laborious and more cost-effective than the multiple tube approach (Taberlet et al., 1997). Thirdly, samples that could not be scored consistently in the repeated genotype profiles were removed from the analysis (Jackson et al., 2016). Genotyping errors due to stuttering were checked using the program MICROCHECKER v2.2.3 (van Oosterhout et al., 2004). Allele frequencies, observed (H_o) and expected (H_e) heterozygosity, allelic dropout, false alleles and tests for adherence to the Hardy-Weinberg equilibrium were quantified using GIMLET v1.3.3 (Valière, 2002).

Individual identification

The set of 10 polymorphic loci was used to create consensus genotype profiles for all samples (Table S2). A minimum number of loci were required to distinguish between closely related individuals to avoid overestimation of population (Kohn et al., 1999; Waits et al., 2001). We therefore 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 sourced from siblings provided a unique opportunity to estimate 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 even siblings within the populations (Fig. 2). The program GIMLET v1.3.3 was used for PID(sibs) analysis (Valière, 2002). We then compared consensus genotype profiles in the program CERVUS v3.0 (Marshall et al., 1998) for identification of matched genotypes for a minimum of five loci criteria. Matching genotypes based on five or more loci were considered to be sourced from the same individual and classified as a capture and/or recapture (Budowle, 2004; Mondol et al., 2009a). Incomplete or partial genotype profiles, genotyped less than 10 but at minimum of five loci, were also used following the approaches in other studies involving tigers (Bhagavatula and Singh, 2006; Mondol et al., 2009a), and badgers

(Frantz et al., 2003). 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).

Density estimation

To estimate population abundance from genotype data, non-invasive genetic studies apply either a rarefaction curve (Eggert et al., 2003; Frantz et al., 2003; Kohn et al., 1999; Wilson et al., 2003) or a Jackknife estimator (Flagstad et al., 2004; Mondol et al., 2009a) that follows a photographic CR framework (Karanth and Nichols, 1998). However, abundance estimate using these approaches can be biased by edge effect, and *ad hoc* estimation of effective sample area (Gardner et al., 2010; Obbard et al., 2010). Nonetheless, the choice of estimator for deriving abundance is likely to strongly affect density estimates (Boulanger et al., 2002; Gray and Prum, 2012). Alternately, a likelihood-based spatially explicit capture-recapture (SECR) approach can avoid these limitations; because SECR analyses are unbiased by edge effects, allow incomplete detection or heterogeneous capture probabilities (Borchers and Efford, 2008; Royle et al., 2009), and do not require the assumption of geographic closure to be met (Efford et al., 2009). Importantly, SECR uses detected 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 (Mondol et al., 2009a). Meanwhile, the SECR approach has become widely used for estimating densities of large carnivores including tigers (Kalle et al., 2011), leopard (*Panthera pardus*) (Kalle et al., 2011), jaguar (*Panthera onca*) (Sollmann et al., 2013), and European wildcat (*Felis silvestris silvestris*) (Kéry et al., 2010). We therefore applied the SECR approach to our data to estimate population density and size of the Sundarbans tigers.

The SECR model assumes that no activity centres of animals can occur in non-habitat beyond the animal’s range (Efford, 2011; Efford et al., 2009), therefore, density estimate can be potentially biased if non-habitat is included in the sample area

(Efford, 2011; Gerber et al., 2012). Tigers in the SRF were 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, human settlement on northern boundary of the area sampled) was removed from the buffer area using the habitat mask when a sample area was bounded by water bodies >1.5 km wide and surrounded by human settlement (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 settlement 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. 3). Consequently, these areas were excluded from the SECR analysis.

Two sets of data matrices of spatiotemporal detection history and spatiotemporal search area polygon were used in SECR for estimating density parameters. The spatiotemporal detection history for each individual tiger comprised initial capture and recapture(s) with their spatial locations (e.g., geographic coordinates), and the corresponding sample area with geographic coordinates (Fig. S2). Using these two input matrices, a detection model was fitted by maximum likelihood, with the parameter, g_0 , 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, g_0 and σ were modelled as constant to estimate overall and sample area-wise tiger density parameters (Borchers and Efford, 2008; Efford, 2011). The SECR analysis was carried out in R package SECR v2.10.3, and ArcGIS v10.3 was used for creating polygons of areas sampled.

Results

Identifying species and individual tigers

From a total of 440 suspected tiger genetic samples, tiger-specific mtDNA cytochrome *b* gene sequence was obtained successfully from 230 (52%) samples (Table 1). The remaining samples were discarded because they either failed to produce identifiable DNA or were sourced from other felid species (e.g., Fishing cat).

A higher level of amplification success was obtained for the reference samples (13 loci showed 100% amplification) than the field collected samples (78- to 100%) (Table S1). Using the set of 10 microsatellite loci, 105 samples (46% of the tiger-positive samples) were amplified successfully resulting in consensus genotypes for five to 10 loci per sample. A higher success rate of genotyping was obtained for samples from the CB area (58%) compared to the SB area (39%). The marker set revealed a level of polymorphism sufficient to distinguish between individuals, with a mean PIC of 0.58 and a mean number of allele per locus of 5.5. Several loci showed allelic dropout and false alleles in the dataset. Deviation from Hardy-Weinberg equilibrium was also detected for loci FCA304, FCA279 for ES; D15 for SB; and FCA230, FCA279 for samples from the CB area (Table 2).

The probability of identity, PID(sibs), for the microsatellite loci set was approximately 0.0003 for both reference and field samples (Fig. 2). The five most informative of the 10 loci with a PID(sibs) value of 0.0186 demonstrated that together these loci could successfully distinguish between closely related individuals with 99% certainty. Using a minimum of these five loci as the critical set, a total of 45 tiger individuals 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 3, Fig. 3). No closely related individuals were detected in the genotyped samples.

Estimating tiger density

The estimated probabilities of detections (CR) 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 3). The null model, $D(.)g_0(.)\sigma(.)$, g_0 and σ as constant, fitted with half-normal detection function yielded an overall density of 2.85 ± 0.44 SE tigers/100 km² (95% CI: 2.11-3.85). The highest density of tigers was estimated for the CB area ($3.18 \pm SE 0.90$) followed by the ES ($3.17 \pm SE 1.04$), WS ($2.99 \pm SE 0.80$) and SB ($1.86 \pm SE 0.81$) (Table 3). By extrapolating the overall tiger density of $2.85 \pm SE 0.44$ tigers/100 km² to the total tiger occupied area of 4,247 km², the Bangladesh Sundarbans may currently support a population of 121 tigers (95% CI: 90-164 individuals).

Sexing of individuals was attempted for 78% (n=45) of identified tigers, resulting in a total of 11 males and 24 females. The sex of the remaining 10 individuals remained

unknown due to inconclusive genotypes. The geographic distribution of the genotyped tigers and their gender were shown in the Figure 3.

Discussion

Identifying species and individual tigers

Although there were no large carnivores in the Sundarbans except tiger, the DNA-based screening to genetically confirm tiger source ensured that samples from non-target species removed prior to downstream analysis (Mondol et al., 2009a; Mukherjee et al., 2007). In a separate analysis (data not shown), we found that scat samples of Fishing cat could be easily picked up as tiger samples in the Sundarbans. The low PCR amplification rate (52%) of tiger versus non-tiger samples in this study may be a consequence of inferior sample quality due to the humid and wet mangrove habitat compared to higher success rates reported from India (e.g. 93% in Bandipur National Park; Mondol et al., 2009a).

The microsatellite marker set amplified less than 160 base pairs, so they were appropriately-sized to amplify low quality, potentially highly fragmented scat DNA (Bhagavatula and Singh, 2006; Frantzen et al., 1998). The overall genotyping success rate (46%) was relatively low because of rigorous screening processes undertaken in order to reduce genotyping errors. Although no genotyping errors were detected in the reference samples, field samples produced 5- to 26% genotyping errors for five loci (Table S1). These error rates however are reasonably low when compared to other non-invasive studies of tigers (2-65%) (Bhagavatula and Singh, 2006), and wolves (3-33%) (Lucchini et al., 2002).

The microsatellite loci set demonstrated sufficient power to distinguish between siblings (Bhagavatula and Singh, 2006; Mondol et al., 2009a) and could therefore avoid overestimation of population by reducing artificial individuals due to genotyping errors (Creel et al., 2003). Other non-invasive tiger studies that have used scat samples have used sets of three to seven polymorphic loci for individual identification (Bhagavatula and Singh, 2006; Mondol et al., 2009a). Our study was therefore conservative to identify closely related individual using an optimum number of loci recommended in previous studies (Mondol et al., 2009a).

Estimating tiger density

The overall tiger density estimate of 2.85 tigers/100 km² for the SRF is higher than a recent density estimate derived from camera-trap data which yielded a density of 2.17 tigers/100 km² (Dey et al., 2015). The difference could be due to methodological approaches used, choice of areas sampled, or likely changes in the population between the surveys. However, our density estimates for SB (1.86± SE 0.81 tigers/100 km²) and ES sample areas (3.17± SE 1.04 tigers/100 km²) were significantly lower than those estimates from camera-trap sampled Block III (2.77± SE 0.78 tigers/100 km²), and Block I (3.70± SE 0.91 tigers/100 km²), respectively (Table 4). Notably, these two sample areas completely overlapped between the present study and the previous camera-trap study (Dey et al., 2015). So the decrease of density estimates between these areas could be simply be an effect of using genetic versus camera-trapping, or might be due to extensive tiger poaching incidents between the two surveys (Aziz et al., 2017).

For the Indian Sundarbans, the tiger density estimates from combined camera-trap and satellite telemetry data provided 4.3± SE 0.3 tigers/100 km² (Jhala et al., 2011), which is higher than our estimate in the Bangladesh Sundarbans. Comparing to other tiger landscape, density estimates in this study are lower than the camera-trapping estimates of Chitwan National Park in Nepal (4.3± SE 0.3 tigers/100 km²) (Karki et al., 2015), and Jigme Dorji National Park in Bhutan (3.7± SE 1.1 tigers/100 km²) (Thinley and Curtis, 2015).

The estimated tiger abundance (95% CI: 90-164 individuals) is higher than the recent estimate of camera-trapping study (95% CI: 84-130 tigers) (Dey et al., 2015), but relatively lower than previous studies using telemetry (100-150 adult females or 300-500 tigers in total; Barlow, 2009), and camera-trap (200 tigers; Khan, 2012).

For the Indian Sundarbans, the combined camera-trap and satellite telemetry data provided a population of 70 tigers (95% CI: 64 and 90 tigers) (Jhala et al., 2011). Together this estimate (Jhala et al., 2011) with the present study, the entire Sundarbans appears to support one of the largest tiger populations in the world, with an estimated 191 tigers (95% CI: 154-254 tigers).

Sex determination success rate (78%) was relatively higher in this study than previously reported from similar studies (57%) (Mondol et al., 2015). The higher

success rate in our study might be due to rigorous screening that left with only good quality samples for sex determination, or it may be because we used single sex marker compared to multiplex molecular sexing (using two sex markers) approach (Mondol et al., 2015).

Conservation implications

We demonstrated the utility of non-invasive genetic sampling as a potential method to monitor tiger populations in the landscape, where application of camera-trapping was challenging due to a range of constraints (Karanth and Nichols, 2010; Mondol et al., 2009a). This technique of non-invasive genetic sampling can be very useful to monitor very low density carnivore populations including tigers dispersed over larger geographic landscapes such as the Russian Far East and other rainforests of South-eastern Asia (Mondol et al., 2009a). Moreover, genetic sampling can provide additional demographic and population levels information which can be useful for detailed monitoring of these populations. Importantly, statistical bias related to baiting or luring camera-traps (Kéry et al., 2010; Mowat and Strobeck, 2000), responses of tigers to these baited devices (Noyce et al., 2001), and ethical issues of using such baiting to wild tigers can be overcome with non-invasive genetic surveys as a supplementary method (Mondol et al., 2009a).

Tables

Table 1

Summary of samples collected, screened and genotyped from each of the sample areas of the Bangladesh Sundarbans.

Sampling area	Area* (km ²)	Protection status	Sampling duration (day)	Samples collected	Samples screened	Samples genotyped
Satkhira Block	342	Reserve forest	21	77	30	15
West Wildlife Sanctuary	715	Protected area	13	152	82	33
Chandpai Block	544	Reserve forest	21	127	74	33
East Wildlife Sanctuary	383	Protected area	21	84	44	24

* Area included forest land and waterbodies

Table 2

Genetic variability at 10 microsatellite loci for field samples (n=105*) collected from the Bangladesh Sundarbans.

Locus	Allele size range (bp)	No. of allele	Dropout	False allele	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); H_E: Expected heterozygosity, H_O: Observed heterozygosity.

Table 3

Sample area (forest land only), capture-recapture(s) 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.

Name of sample area	Area* (km ²)	No. of individual detected	No. of total detections	Tiger density, $D \pm SE$ (in 100 km ²)	Probability of detection, $g_0 \pm SE$	Spatial distance moved, $\sigma \pm SE$ (in 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 4

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

Sampling area	Study method	No. of Individual detected	SECR model	Tiger density, $D \pm SE$ (in 100 km ²)	Probability of detection, $g_0 \pm SE$	Spatial distance moved, $\sigma \pm SE$ (in km)
<i>Area-wise</i>						
Satkhira Block ^a	DNA study	6	$D(.)g_0(.)\sigma(.)$	1.86±0.81	0.0226±0.0098	3.989± 0.825
Block III (Satkhira) ^a	Camera trap	13	$D(.)g_0(bk)\sigma(.)$	2.77±0.78	0.0100± 0.0020	4.270± 0.050
East Wildlife Sanctuary ^b	DNA study	10	$D(.)g_0(.)\sigma(.)$	3.17±1.04	0.0361± 0.0127	2.918± 0.416
Block I (Sarankhola) ^b	Camera trap	18	$D(.)g_0(bk)\sigma(.)$	3.70±0.91	0.0100± 0.0030	3.370± 0.350
<i>Overall</i>						
Sampling area (1,397 km ²)	DNA study	48	$D(.)g_0(.)\sigma(.)$	2.85±0.44	0.0231± 0.0038	3.478± 0.262
Sampling area (1,265 km ²)	Camera trap	38	$D(.)g_0(bk)\sigma(.)$	2.17±(1.73-2.68)	Not available	Not available

Note: ^aSatkhira Block completely overlapped with Block III (Satkhira), and ^bEast Wildlife Sanctuary with Block I (Sarankhola) of camera-trap study (Dey et al., 2015).

Table S1

Characteristics of 14 microsatellites optimised for reference sample (RS, n=10) and field-collected sample (FS, n=10).

Locus*	Allele size range (bp)	Amplification success (%)		Allelic dropout		False allele		No. of allele		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 optimised from Menotti-Raymond et al. (1999), except E7 and D15 (Bhagavatula and Singh, 2006).

Table S2

Locus name, primer sequences, annealing temperature (AT, °C), fluorescent dye (FD), and PCR multiplexes (PM) used in this study.

Locus name	Forward sequence	Reverse sequence	AT	FD	PM
F41	GTCTGCATCTTCAAATAGGA	GTACCTGAGTTGGCTGTTGA	56	FAM	Set 1
D15	TGTGACCTTTCTCTAGTTTC	GCACAAAACATTCAGTCTCC	55	FAM	Set 1
Fca232	ATGACCATCTCAAACCTTCATGG	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	TGTTAGCTCATGTTTCATGTGTCC	52	HEX	Set 4
Fca230	AAGAATGGACTTGGGAAATGG	AAACCACAACAGGCAAAGG	52	NED	Set 4
Fca279	AGCCAAGTAATATTCCTCTGTG	GTCCATCCGCAGATGAATG	52	FAM	Set 4

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

Table S3

Genotyped tigers of the Sundarbans of Bangladesh.

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(Table removed intentionally)

Table S4

Sample area-wise and overall detailed parameter estimates of tigers of the Bangladesh Sundarbans under the spatially explicit capture-recapture (SECR) model.

Sample area-wise density					
<i>Satkhira Block</i>					
	link	estimate	SE.estimate	lcl	ucl
D	log	1.86E-04	8.06E-05	8.26E-05	4.20E-04
g0	log	2.23E-02	9.84E-03	9.73E-03	5.09E-02
sigma	log	3.99E+03	8.26E+02	2.67E+03	5.96E+03
<i>West Wildlife Sanctuary</i>					
	link	estimate	SE.estimate	lcl	ucl
D	log	2.99E-04	8.09E-05	1.78E-04	5.04E-04
g0	log	1.83E-02	5.72E-03	1.00E-02	3.33E-02
sigma	log	3.92E+03	5.07E+02	3.05E+03	5.04E+03
<i>Chandpai Block</i>					
	link	estimate	SE.estimate	lcl	ucl
D	log	3.18E-04	9.00E-05	1.84E-04	5.48E-04
g0	log	2.24E-02	7.14E-03	1.21E-02	4.12E-02
sigma	log	3.09E+03	4.38E+02	2.34E+03	4.07E+03
<i>East Wildlife Sanctuary</i>					
	link	estimate	SE.estimate	lcl	ucl
D	log	3.17E-04	1.04E-04	1.69E-04	5.94E-04
g0	log	3.61E-02	1.28E-02	1.84E-02	7.08E-02
sigma	log	2.92E+03	4.16E+02	2.21E+03	3.85E+03
Overall density for four sample areas					
	link	estimate	SE.estimate	lcl	ucl
D	log	2.85E-04	4.39E-05	2.11E-04	3.85E-04
g0	log	2.23E-02	3.80E-03	1.60E-02	3.11E-02
sigma	log	3.48E+03	2.62E+02	3.00E+03	4.03E+03

Note: D = density, g0 = detection probability, sigma = spatial movement by tigers, SE = standard error, lcl = lower confidence interval, ucl = upper confidence interval.

Figures

(Figure removed intentionally)

Fig. 1. Sample areas and grid squares with location of tiger-positive samples in the Bangladesh Sundarbans. Sample area: SB – Satkhira block, CB – Chandpai block, WS – West Wildlife Sanctuary, ES – East Wildlife Sanctuary.

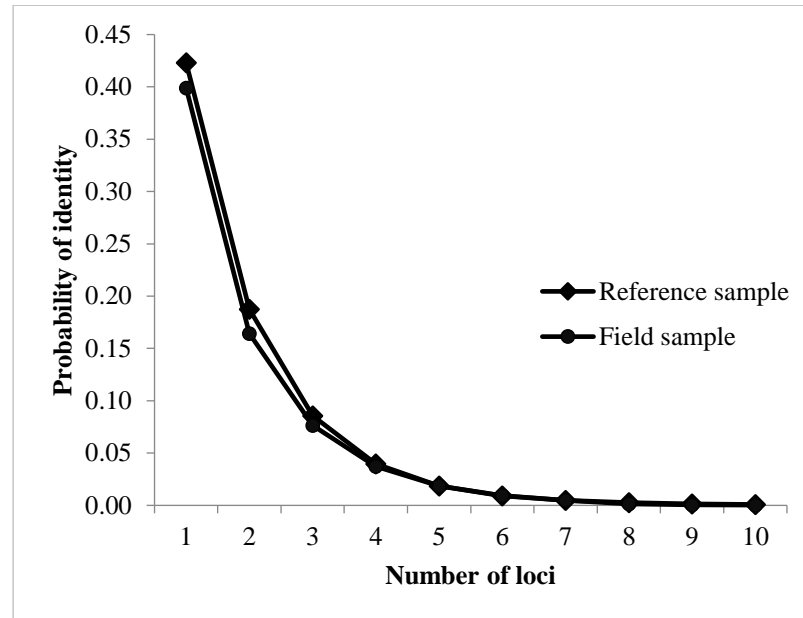


Fig. 2. The plot shows the probability of identity, $P_{ID(sibs)}$ for reference and field-collected samples for the 10 most informative microsatellite loci taken in the order of decreasing polymorphic information content. The cumulative $P_{ID(sibs)}$ for the first five (from left to right) loci is near to zero and thus can be used with a high degree of certainty to distinguish between samples collected from even closely related individuals. The first locus has the highest power to discriminate among individuals, and the order of power decreases from left to right.

(Figure removed intentionally)

Fig. 3. Location of genotyped tigers and boundary characteristics of sample areas in the Bangladesh Sundarbans. A random single location of multiple recaptures of an individual was used for plotting the sex status of tigers.



Fig. S1. A composite snapshot of fieldwork for collecting non-invasive tiger samples from the Sundarbans of Bangladesh, showing team training, field logistics, transect walking, collection and processing of samples and meeting with Forest Department officials.

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Fig. S2. Habitat mask and detection history of tigers generated in SECR modelling.

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Chapter 4
Genetic ancestry and phylogeny of the Sundarbans
tigers

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Aziz, M.A., Smith, T., Jackson, H.A., Barlow, A., Goodrich, J., Tollington, S., Darlow, S., Islam, M.A., and Groombridge, J.J. Genetic ancestry and phylogeny of the Sundarbans tigers.

Abstract

The Sundarbans tiger (*Panthera tigris*) is the only tiger population that is adapted to inhabit mangrove forest in what is the world's largest mangrove forest shared between Bangladesh and India. Taxonomic assignment of this population in relation to the full suite of tiger subspecies has been limited by low sampling effort which has meant that the genetic ancestry of the Sundarbans population has remained poorly known. We generated 1,263 base pairs of DNA sequence across four mitochondrial DNA (mtDNA) fragments for 39 tiger individuals from the Bangladesh Sundarbans, and compared these with 33 mtDNA haplotypes known across all subspecies of extant tigers. The results showed that the Sundarbans tigers contain three haplotypes, of which one is unique and distinctly separated the population from all other tigers, while the remaining two are shared with tiger populations from central India. Maximum likelihood and Bayesian inferences supported Sundarbans tigers as polyphyletic, indicating a close phylogenetic affinity with the Bengal tigers. An estimated time of divergence that broadly supports the tigers' probable colonisation in the Sundarbans during the mid-Holocene (7000-10,000 years before present) when the southernmost plain of the Ganges-Brahmaputra delta had been covered by the extensive growth of mangroves, up to 80-120 km north of the present-day coastline. In light of ecological, demographic and phylogenetic evidence, we argue the Sundarbans tigers should be managed as a 'Management Unit'. Such focused management will ensure the population remains genetically viable and is able to adapt to rapidly changing environments for long-term persistence.

Introduction

The historical global range of tigers encompassed a wide variety of landscapes, ranging from taiga and boreal forests to alluvial grasslands and tidal mangrove swamps (Sanderson et al., 2006). Unfortunately, 93% of the tiger's historical range has been lost due to habitat loss, prey depletion and tiger poaching (Dinerstein et al., 2007; Sanderson et al., 2006). Moreover, the remaining tiger landscapes have been heavily impacted by a variety of anthropogenic threats (Sanderson et al., 2006; Wikramanayake et al., 2010), forcing the once widely distributed tigers in to isolated pockets of protected areas (Dinerstein et al., 2007; Walston et al., 2010). Currently, most of the tiger populations comprise less than 120 individuals, increasing the risk of local extinction due to demographic and genetic factors (Dinerstein et al., 1997; Smith and McDougal, 1991). Therefore, global tiger management and conservation approaches emphasise the protection of all remaining tiger populations regardless of tiger subspecies assignment (Sanderson et al., 2006; Wilting et al., 2015).

Although tiger taxonomy has been studied for centuries since the first formal description by Linnaeus in 1758 (Herrington, 1987; Luo et al., 2004; Mazak, 1981; Wilting et al., 2015), subspecies designations are still debated (Kitchener, 1999; Kitchener and Dugmore, 2000; Wentzel et al., 1999; Wilting et al., 2015). Based on morphology, distribution and genetic analysis (Herrington, 1987; Luo et al., 2004; Mazak, 1981), up to nine subspecies are currently recognised (Chundawat et al., 2011): the Bengal (*P. t. tigris*; Linnaeus, 1758), Caspian (*P. t. virgate*; Illiger, 1815), Siberian (*P. t. altaica*; Temminck, 1844), Javan (*P. t. sondaica*; Temminck, 1844), South China (*P. t. amoyensis*; Hilzheimer, 1905), Bali (*P. t. balica*; Schwarz, 1929), Sumatran (*P. t. sumatrae*; Pocock, 1929), Indochinese (*P. t. corbetti*; Mazak, 1968), Malayan (*P. t. jacksoni*; Luo et al., 2004). Of them, the Javan, Bali and Caspian tigers became extinct in the 20th century (Chundawat et al., 2011; Nowell and Jackson, 1996), and the South China tiger survives only in captivity (Seidensticker et al., 2010). More recently, a study by Wilting et al. (2015) recognised just two tiger subspecies: the Sunda tiger (*P. t. sondaica*), and the continental tiger (*P. t. tigris*).

Given the continued decline of tiger populations across their range (Dinerstein et al., 2007; Seidensticker et al., 1999), an ecology-based conservation approach has been proposed for the protection of about 160 habitat patches currently supporting tigers

(Dinerstein et al., 1997). Consequently, a total of 76 Tiger Conservation Landscapes (TCL) have been identified based on the representation of a suite of adaptations, and their contribution to the population's long-term persistence (Sanderson et al., 2006). The Sundarbans has been classified as a global priority TCL, because it represents a population adapted to a unique mangrove habitat, and its contribution to the conservation of tigers across the bioregion (Sanderson et al., 2006). However, a widespread phylogenetic survey that encompasses the Sundarbans has not, until now, been possible in order to confirm if such ecological adaptation is reflected in the evolutionary history of the group.

A prerequisite for managing biodiversity is the identification of populations with independent evolutionary histories (Moritz, 1994). Given the extreme nature of population fragmentation of the remaining tiger populations (Dinerstein et al., 2007; Sanderson et al., 2006; Wikramanayake et al., 2011), and to benefit from management of populations below species level (Moritz, 1994; Waples, 1991), future tiger conservation efforts must determine the presence of 'evolutionarily significant unit' (ESU) for focused management (Moritz, 1994; Wilting et al., 2015). An ESU is a subset of a population segment that is substantially reproductively isolated from other conspecific populations, and possesses rare genetic attributes significant for present and future generations of the species (Ryder, 1986; Waples, 1991). An alternative hypothesis is to define Management Unit (MU) where populations will be genetically distinct as well as morphologically independent due to exchange of a few migrants between the conspecific populations (Moritz, 1994).

Molecular genetic markers have been increasingly applied to assess genetic structure and viability of geographically isolated populations, and to identify populations that require conservation management as an ESU and MU (Avice and Ball, 1990; Fraser and Bernatchez, 2001; Moritz, 1994). Previously, mitochondrial (mtDNA) and microsatellite DNA markers (Luo et al., 2010; O'Brien and Johnson, 2005) have been used to assess taxonomic status of wild (Luo et al., 2004) and captive (Luo et al., 2008) tigers, and to evaluate tiger restoration priorities in the Caspian region (Driscoll et al., 2011). In particular, advances in DNA technologies have made it possible to retrieve DNA from fragile museum specimens to assess genetic ancestry of the extinct Javan and Bali tigers (Xue et al., 2015), and from scat samples to investigate phylogeography and demographic history of Bengal tigers (Mondol et al., 2009b,

2013). Such mtDNA makers were increasingly used because nuclear autosomal, X-linked, and Y-chromosome markers showed no variation within tigers (Luo et al., 2014), and even major histocompatibility complex and autosomal microsatellite variability were low (Luo et al., 2004). A range of taxonomic and population genetic investigations in tigers have utilised mtDNA markers to assess population genetic diversity of Caspian and Siberian tigers (Driscoll et al., 2009; Russello et al., 2004), determining the taxonomic status of Caspian tigers (Driscoll et al., 2009), coalescent dating of all tiger subspecies (Luo et al., 2004), and to investigating intraspecific variation of tigers (Wilting et al., 2015). All these studies substantially contributed to the understanding of tigers, highlighting conservation importance for long-term persistence of the species (Barlow et al., 2010; Cracraft et al., 1998; Kitchener, 1999; Kitchener and Dugmore, 2000; Luo et al., 2004; Mazak and Groves, 2006; Sanderson et al., 2006; Wilting et al., 2015). However, the Sundarbans is a global priority TCL (Sanderson et al., 2006), it supports one of the top five largest populations of tigers in the world (Dey et al., 2015), and this unique habitat has a huge potential for contributing to the long-term survival of tigers in the region (Sanderson et al., 2006) and yet a comprehensive phylogenetic survey of Sundarbans tiger population has not been carried out.

This study aims to survey phylogenetic diversity across the Sundarbans tiger population and to assess the genetic ancestry and phylogenetic relationships of this population to other extant tiger subspecies. Given the limited sampling experienced by other studies (Barlow et al., 2010; Singh et al., 2015), we aim to use a larger, more robust dataset that samples across the population's vast range. Using tiger scat samples from across the Bangladesh Sundarbans, we combine mtDNA sequence data with data from across all tigers subspecies (Luo et al., 2004; Mondol et al., 2009b) to answer the following specific questions: (i) is the Sundarbans tiger population genetically distinct from Bengal tiger subspecies? (ii) do the presence/absence of haplotypes from other populations improve our interpretation of the ancestry of the Sundarbans tiger population, and (iii) is there evidence from mtDNA that the Sundarbans tiger population should be managed as an ESU?

Methods

Study site and sample collection

The Sundarbans is the largest contiguous mangrove forest in the world encompassing an area of 10,263 km² located in the Ganges-Brahmaputra delta (Giri et al., 2007). The Bangladesh Sundarbans covers 6,017 km², of which 4,267 km² is forest and the remaining area is comprised of water bodies (Iftekhar and Islam, 2004). The north and east sides of the forest are bounded by dense human settlements and agriculture land, and the south by the Bay of Bengal (Fig. 1). The Bangladesh Sundarbans is managed as Sundarbans Reserve Forest (SRF), where three isolated areas have been designated as wildlife sanctuaries: Sundarbans West (715 km²), Sundarbans South (370 km²) and Sundarbans East (312 km²) (Fig. 1).

To collect non-invasive tiger samples (scat and hair) four intensive sample areas were selected within the SRF: East Wildlife Sanctuary (ES, 383 km²), West Wildlife Sanctuary (WS, 715 km²), Chandpai block (SB, 342 km²), and Satkhira block (CB, 554 km²). Location, protection status and level of human use (e.g., fishing, nypa palm harvesting) were considered in selecting these sample areas (Aziz et al., 2017). In order 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 across the four areas. Each grid cell was targeted for sampling with three separate transects (using one transect each time), walked by a surveying team of four trained field staff. Starting points for each transect were selected by where the grid cell could be easily accessed by boat. 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. Apart from intensive sampling in these grid cells, samples were also collected from remaining part of the SRF opportunistically. 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 (from scratched marks by tigers in trees) and recorded location for each sample using handheld Global Positioning System (GPS) *Garmin GPSMAP 64*. In addition to field collected samples, one blood sample (from a rescued tiger), five tissue samples (skins confiscated from around the SRF) and four hair samples (rescued tigers from SRF) were also collected.

Samples were analysed at Conservation Genetics Laboratory of the Durrell Institute of Conservation and Ecology (DICE), University of Kent, UK after transporting from the field under permits from the Convention of International Trade in Endangered Species (CITES) (Permit No. BD 9118404), and the authorisation of the Department for Environment, Food and Rural Affairs, United Kingdom (AHVLA authorization: TARP/2015/111).

DNA extraction and amplification

Two isolated laboratory spaces were used for analyses of all biological samples in order to prevent possible contamination. Samples were prepared for DNA extraction under a pre-sterilized fume hood in batches of 10 samples. Workstations were sterilized before and after each use by irradiation from UV light and with 10% bleach. Genomic DNA from scat samples was extracted using QIAamp DNA Stool mini kits following the manufacturer's instructions with minor modifications. During the scat DNA extraction process, approximately 200 mg of material was scrapped from the outer surface of each sample with a sterilized razor blade, and then incubated overnight with 1.5 ml ASL buffer on a mechanical rotator at 56 °C. The DNA supernatant from the sample was lysed with 300 µl AL buffer 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, UK) was added with AL buffer. To extract DNA from blood, tissue, and hair samples, we used DNeasy Blood and Tissue Kits (QIAGEN Inc.); approximately 50 g (or minimum 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 °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 (Mondol et al., 2009a; Mukherjee et al., 2007). 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 m at 72 °C using a G-Storm Thermal Cycler (Labtech France). PCR reaction volumes (total 27 µl) contained 3 µl of template DNA, 12.5 µl MyTaq redmix

(containing dNTPs and MgCl₂; Bioline, UK), 0.5 µl of each primer, 2.0 µl BSA (Bovine Serum Albumin, New England Biolabs Inc.) and 8.5 µl dH₂O. All PCR products from each DNA extraction were purified and sequenced using a 3730XL analyser (Macrogen, Amsterdam, Netherlands). The sequences were edited using Jalview v2.10.1 (Waterhouse et al., 2009) and then cross-checked with GenBank (National Center for Biotechnology Information, NCBI) database to confirm the sample was tiger (and not a contaminant prey species).

To generate mtDNA dataset from tiger-authenticated DNA samples, a total of nine primer sets (obtained and optimised from Mondol et al., 2009b) were used to amplify four mtDNA gene regions: control region (CR), cytochrome *b* (*cyt b*), NADH dehydrogenase subunits 2 (ND2), and NADH dehydrogenase subunits 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 tiger subspecies (Luo et al., 2004; Mondol et al., 2009b). PCR reactions were conducted in 27 µl reaction volume containing 3 µl template DNA, 12.5 µl MyTaq redmix (Bioline), 0.5 µl of each primer, and 10.5 µl of dH₂O. PCR amplification was performed using a G-Storm Thermal Cycler (Labtech France). The PCR profile comprised of initial denaturation (95 °C for 1 m); 45 cycles of denaturation (95 °C for 30 s), annealing (T_a for 15 s), extension (72 °C for 30 s) and a final extension phase (72 °C for 10 m) (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 clean single band and to check for any signs of contamination. The successful PCR products were purified and amplified using a 3730xl analyzer (Macrogen, Amsterdam, Netherlands).

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

Genetic analyses

To compare levels of genetic diversity between Sundarbans tiger population and Bengal tigers, all haplotypes identified in Bengal tiger populations were grouped into southern India ($n = 12$), central India ($n = 7$), northeast India ($n = 2$) and Nepal ($n = 1$) following the approach of Mondol et al. (2009b). The Sundarbans samples were analysed separately for population- and subspecies-wise comparisons. Haplotypes reported in all other tiger subspecies were grouped according to previously assigned tiger subspecies, excluding tentatively recognized South China tiger (Luo et al., 2004), surviving only in captivity (Seidensticker et al., 2010). The combined datasets contained 33 phylogenetically informative haplotypes (Luo et al., 2004; Mondol et al., 2009b) used in these analyses (Table 2).

Measures of haplotype diversity, nucleotide diversity, and segregating sites were estimated using DnaSP v5.10.01 (Rozas and Rozas, 1995). Average evolutionary divergence of concatenated mtDNA sequences within and between populations and subspecies were calculated using MEGA v7.0.14 (Kumar et al., 2015) by the Maximum Composite Likelihood model with 1,000 bootstrap resampling (Tamura et al., 2004).

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 information criteria (BIC). The BI and ML analyses were implemented in MrBayes v3.2 (Ronquist and Huelsenbeck, 2003) and RAxML v7.2.6 (Stamatakis, 2006), respectively on the CIPRES Science Gateway (Miller et al., 2010). The Bayesian analysis ran for 10 million generations over four parallel Monte Carlo Markov chains (MCMCs), under an HKY evolutionary model (Felsenstein, 1981). Chain convergence was determined using TRACER v1.6 (Rambaut et al., 2014) to ensure sufficiently large ESS values (>200). After discarding the first 25%, tree topologies were summarised in a 50% consensus tree. A ML analysis was performed with 1000 bootstrap replications to obtain the best likelihood under a GTR+GAMMA model, producing a majority rule consensus tree. All trees were visualized 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 georeferenced sample location to display their spatial distribution across the Indian subcontinent tiger landscape using ArcGIS v10.3.

Molecular dating

To infer a time calibrated evolutionary divergence of the Sundarbans tigers, two fossil-based calibration points were applied; (i) a minimum of 3.8 million years for the earliest *Panthera* lineage from the clouded leopard (*Neofelis nebulosa*) (Johnson et al., 2006), and (ii) 1.6 million years for the base of lion (*Panthera leo*)-jaguar (*Panthera onca*) clade (Janczewski et al., 1995). A fossil-calibrated phylogeny was estimated using BEAST v1.8.2 (Drummond and Rambaut, 2007) on the CIPRES Science Gateway (Miller et al., 2010) with 10 million generations over four parallel Monte Carlo Markov chains (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 years in the first and second calibrations, respectively with a common standard deviation to 0.5 million years at both calibration points. Clouded leopard, lion and jaguar sequences were obtained from Genbank (accession numbers DQ257669 (Wu et al., 2007), AF006458 (Johnson and O'Brien, 1997), and KC834784 (Bagatharia et al., 2013)).

Results

Evolutionary history

A total of 1,263 bp mtDNA sequence has been successfully amplified for CR (200 bp), *cyt b* (450 bp), ND2 (131 bp) and ND5 (482 bp) for 39 tiger samples from the Bangladesh Sundarbans. The analysis of concatenated gene sequences revealed two haplotypes (SBT1 and SBT2) within the Bangladesh Sundarbans population, of which SBT1 was shared by 36% and SBT2 by 64% samples. Combining these haplotypes with previously reported haplotypes (TIG23 and TIG29) from the Indian Sundarbans (Mondol et al., 2009b) revealed three haplotypes within the entire Sundarbans population. Of these, haplotype SBT2 was detected in the Bangladesh population for the first time in this study. The haplotype SBT1 matched with TIG29, indicating that

this haplotype is common to both Bangladesh and Indian Sundarbans. Importantly, this haplotype (SBT1/TIG29) distinguished the Sundarbans tigers from all other populations of Bengal as well as other tiger subspecies (Fig. 2). The spatial distribution of haplotypes within the Bengal tiger populations showed that SBT2 was shared among the populations in the Ranthambhore national park of Rajasthan and Raipur Zoo of Chattishgarh, India, while the haplotype TIG23 was observed among the population of Ranthambhore national park of Rajasthan, India. These two haplotypes were unique to the Bengal tiger subspecies. The haplotype networks placed the Sundarbans tiger as a polyphyletic lineage within Bengal tiger subspecies (Fig. 3).

Posterior probabilities (PP) from Bayesian inference and bootstrap support (BS) from Maximum likelihood trees produced congruent topologies broadly corresponding to major geographic and subspecies partitions. Two major groupings of extant tigers (Bengal tigers and all other subspecies) were highly supported (PP/BS 100%), whilst the Sundarbans tiger population was placed as a polyphyletic group within the Bengal tigers (less strongly supported, PP 69%, BS 45%; Fig. 4). Time calibrated phylogenetic analysis suggests that modern tigers diverged from the most recent common ancestor between 3.02 million years ago (Mya) (95% Highest Posterior Density, HPD: 2.10-3.93 Mya). Likewise, the most recent molecular divergence time of the Sundarbans tigers from the central Indian tiger populations was 26,000 years ago (95% HPD: 800-62,000 years).

Genetic diversity and differentiation

Haplotype diversity (h) and nucleotide diversity (π) varied between populations of Bengal tigers, ranging from 6.5 to 1.0 and from 0.001 to 0.003, respectively. The Sundarbans tigers exhibited moderate values of h and π which is almost similar to other populations in India but higher than the Nepal populations (Table. 3). Notably, the Sundarbans tigers have almost similar h and π to Malayan and Sumatran tigers but higher than found in the Siberian and Indochinese tigers (Table. 3). The evolutionary divergence estimates showed that the Sundarbans tiger population differed by 0.3% from other populations of Bengal tigers and by 0.5-0.6% from all other tiger subspecies (Table 4).

Discussion

Evolutionary history

Haplotype analysis has revealed that the Sundarbans tiger population retained three informative haplotypes, two of which are identical to haplotypes previously found elsewhere in India, and one is unique to the Sundarbans tiger population. The phylogenetic reconstruction using these haplotypes has revealed polyphyletic position of the Sundarbans tiger population within the Bengal tigers, indicating that tigers diverged into the Sundarbans from central India approximately 26,000 years ago (95% HPD: 800-62,000 years). The retention of a unique haplotype by the Sundarbans tiger population suggests that gene flow with neighbouring populations in India has not been so recent as to erase signs of genetic distinctiveness. However, the large variance of the divergence time estimate might have been due to the shared haplotypes, reflecting the polyphyletic relationship between the Sundarbans population and the tiger populations in Central Indian landscape.

The finding of a polyphyletic relationship between the Sundarabans population and the Bengal tigers across India however contrasts with a previous study (using mtDNA data of six specimens from the Indian Sundarbans) that found reciprocal monophyly for the Sundarbans tigers (Singh et al., 2015). Although Singh et al. (2015) utilised the Mondol et al. (2009b) mtDNA datasets in their analysis that clearly showed shared haplotypes between Sundarbans and central Indian populations, it remains unclear how reciprocal monophyly was achieved for the Sundarbans tigers. Alternately, these contrasting results could be explained by the hypothesis that monophyletic relationships as a criterion for phylogenetic distinctiveness may not always hold in the population, therefore such a stringent conclusion can be problematic because a single individual in a new sample can simply overturn a population's reciprocal monophyletic status (Crandall et al., 2000; Fraser and Bernatchez, 2001). Nonetheless, the reciprocal monophyletic relationships may not always infer historical isolation of the population (Crandall et al., 2000). Currently, the Sundarbans tiger population is demographically isolated from other tiger populations (Singh et al., 2015), and surviving on relatively small-sized prey species (Khan, 2008; Reza et al., 2001), which might have profound influence on distinct morphological traits (Barlow et al., 2010) and unique genetic structure of the population (Singh et al., 2015). Together, all

these results suggest that the Sundarbans tigers have independent separate evolutionary trajectory and historical fate (Simpson, 1961), and therefore, ensuring long-term persistence through conservation management may allow the demographically isolated and uniquely adapted Sundarbans population to evolve and differentiate further via the mechanism of allopatric speciation (Barlow et al., 2010). Also, the lower PP and BS support for the Sundarbans tiger population is likely due to the fact that the Sundarbans tiger population is a polyphyletic group within the Bengal tiger populations. The Sundarbans tiger population is therefore clearly marrying the conditions of demographic isolation (Singh et al., 2015) and morphological independence (Barlow et al., 2010) in conforming to an MU (Moritz, 1994).

The isolation of the Sundarbans tigers is a result of an extreme fragmentation of a once continuously distributed tiger population that extended across the Indian subcontinent (Mondol et al., 2009b; Sanderson et al., 2006). Reconstructed tiger distribution models suggest that extreme environmental events during the last glacial maximum (LGM) of ca. 20,000 years before present (ypb) heavily pushed tigers southwards when the vast continental shelves were exposed (Kitchener and Dugmore, 2000; Kitchener and Yamaguchi, 2010; Siddall et al., 2003). Sea levels were approximately 120 m below present-day levels during that LGM period (Siddall et al., 2003), which facilitated growth of extensive mangroves until early to mid-Holocene (7,000-10,000 ybp) on the southernmost plain of the Ganges-Brahmaputra delta, extending up to 80 - 120 km north of the present coastline (Chanda and Mukherjee, 1969; Sen and Banerjee, 1990). However, during the last few centuries 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; Sarker, 2004; Sikdar and Halt, 1997; Verghese, 1999) have severed the connectivity of the Sundarbans tigers from other tiger populations surviving today in the Indian subcontinent (Jhala et al., 2011; Sanderson et al., 2006).

Conservation implications

The Sundarbans tiger population, adapted to unique mangrove habitat, has been isolated from the nearest TCL in Similipal, India by approximately 200 km of landscapes dominated by human settlements and agriculture (Sanderson et al., 2006), preventing any opportunity for gene flow between these TCLs. Consequently,

ecological, demographic, and historic biogeographical factors have influenced genetic subdivisions within Bengal tigers (Luo et al., 2004), and collectively played a role in producing a unique genetic signature (Singh et al., 2015), leading to morphologically distinct Sundarbans tigers (Barlow et al., 2010). Therefore the approach of identifying the population as an MU for prioritized conservation proposed by Moritz (1994) is more relevant for the Sundarbans tiger population that integrate diverse biological traits (e.g., life history patterns, population genetic structure), and unique adaptations of the species to their landscape (Mundy et al., 1995; Sanderson et al., 2006; Wilting et al., 2015). Based on our findings in combination with unique morphological (Barlow et al., 2010) and ecological (Sanderson et al., 2006) adaptations, we argue that the Sundarbans tigers should therefore be managed as an MU.

Tables

Table 1

Species-specific mitochondrial primers used in this study*

Primer name	Primer sequence	Amplicon size (bp)	Annealing temperature (°C)
TIGND2 F1	TAGTCTGAATCGGCTTCG	195	52
TIGND2 R1	CCGTTATAATGGATGCCA		
TIGND5 F1	GCCCCTATATTAACCAGT	195	52
TIGND5 R1	ATCCTACATCTCCAATAC		
TIGND5 F2	TATCAGACGCAAACACTG	224	57
TIGND5 R2	AATAAAGCGGAGACGGGA		
TIGND5 F3	ACCTACACCCATGATTGC	187	57
TIGND5 R3	TTTTGTGTGAGGGCACAG		
TIGCYT B F2	CGTCTGTCTATACATGCA	200	52
TIGCYT B R2	TACTCTACTAGGTCGGTC		
TIGCYT B F3	ATGTCTTTTTGAGGGGCA	191	52
TIGCYT B R3	GTATTGGATCCTGTTTCG		
TIGCYT B F4	TTAACCCTAGCAGCAGTC	184	52
TIGCYT B R4	TGTAGTTATCAGGGTCTC		
TIGCR F1	GGGAAGGAGAATATGTAC	142	52
TIGCR R1	CACAGAACGGGTATATGC		
TIGCR F2	CGAAAACAACCCCATGAC	137	52
TIGCR R2	GCTTCGTGTTGTGTGTTTC		

* Primers were optimised from Mondol et al., (2009b).

Table 2

Detailed information of samples used in this study; populations were grouped according to Mondol et al. (2009b) and Luo et al. (2004).

Haplotype	Number of sample	Sample location	Tiger population	Tiger subspecies	Ref.
SBT1*	15	Bangladesh Sundarbans	Sundarbans	Bengal tiger	1
SBT2**	26	Bangladesh Sundarbans	Sundarbans	Bengal tiger	1
TIG11	15	Tamil Nadu, Karnataka, Maharastra, Andhra Pradesh	Southern India	Bengal tiger	2
TIG12	1	Maharastra	Central India	Bengal tiger	2
TIG13	1	Karnataka	Southern India	Bengal tiger	2
TIG14	1	Kerala	Southern India	Bengal tiger	2
TIG15	2	Kerala	Southern India	Bengal tiger	2
TIG16	1	Karnataka	Southern India	Bengal tiger	2
TIG18	2	Karnataka	Southern India	Bengal tiger	2
TIG19	1	Karnataka	Southern India	Bengal tiger	2
TIG20	1	Maharastra	Central India	Bengal tiger	2
TIG21	1	Kerala	Southern India	Bengal tiger	2
TIG22	2	Karnataka	Southern India	Bengal tiger	2
TIG23	1	Indian Sundarbans	Sundarbans	Bengal tiger	2
TIG24	1	Assam	Northeast India	Bengal tiger	2
TIG25	2	Assam	Northeast India	Bengal tiger	2
TIG26	1	Orissa	Central India	Bengal tiger	2
TIG27	1	Maharastra	Central India	Bengal tiger	2
TIG1	1	Chitwan National Park	Nepal	Bengal tiger	3
TIG6	1	Karnataka	Southern India	Bengal tiger	3
ALT	13	Russia, Estonia	Siberian tiger	Siberian tiger	3
AMO2	1	China	Indochinese tiger	Indochinese tiger	3
AMO3/ COR1	22	Thailand, Vietnam, Cambodia	Indochinese tiger	Indochinese tiger	3
COR4	11	Malaysia	Malayan tiger	Malayan tiger	3
COR5	1	Malaysia	Malayan tiger	Malayan tiger	3
COR7	2	Thailand & Malaysia	Malayan tiger	Malayan tiger	3
COR8	3	Malaysia	Malayan tiger	Malayan tiger	3
SUM1	4	Indonesia	Sumatran tiger	Sumatran tiger	3
SUM2	1	San Diego Zoo, USA	Sumatran tiger	Sumatran tiger	3
SUM3	1	Phoenix Zoo, USA	Sumatran tiger	Sumatran tiger	3
SUM4	1	Indonesia	Sumatran tiger	Sumatran tiger	3
SUM5	1	Indonesia	Sumatran tiger	Sumatran tiger	3
SUM7	3	Indonesia	Sumatran tiger	Sumatran tiger	3

Ref. Samples used from, 1 = This study, 2 = Mondol et al. (2009b), and 3 = Luo et al. (2004).

* Haplotype matched with TIG29, and ** with TIG30 (Mondol et al., 2009b).

Table 3

DNA polymorphism within and between all tiger subspecies, and populations of Bengal tigers derived from 33 concatenated mitochondrial haplotypes.

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

N: number of samples, *S*: number of segregating sites, *h*: haplotype diversity, and π : nucleotide diversity, *n/a*: not applicable due to single haplotype detected in the population.

Table 4

Mean evolutionary divergence over 33 informative haplotype sequence pairs within and between subspecies of tigers and populations.

	Interspecific variations (between groups)					Intraspecific variations (within groups)
	1	2	3	4	5	
1. Sundarbans tiger						0.003
2. Bengal tiger	0.003					0.003
3. Siberian tiger	0.005	0.005				n/a
4. Indochinese tiger	0.006	0.006	0.003			0.004
5. Malayan tiger	0.005	0.005	0.003	0.005		0.003
6. Sumatran tiger	0.005	0.005	0.004	0.005	0.004	0.001

The number of base substitutions per site from averaging all sequence pairs between groups;

n/a – not calculated due to single haplotype within the population of this subspecies.

Figures

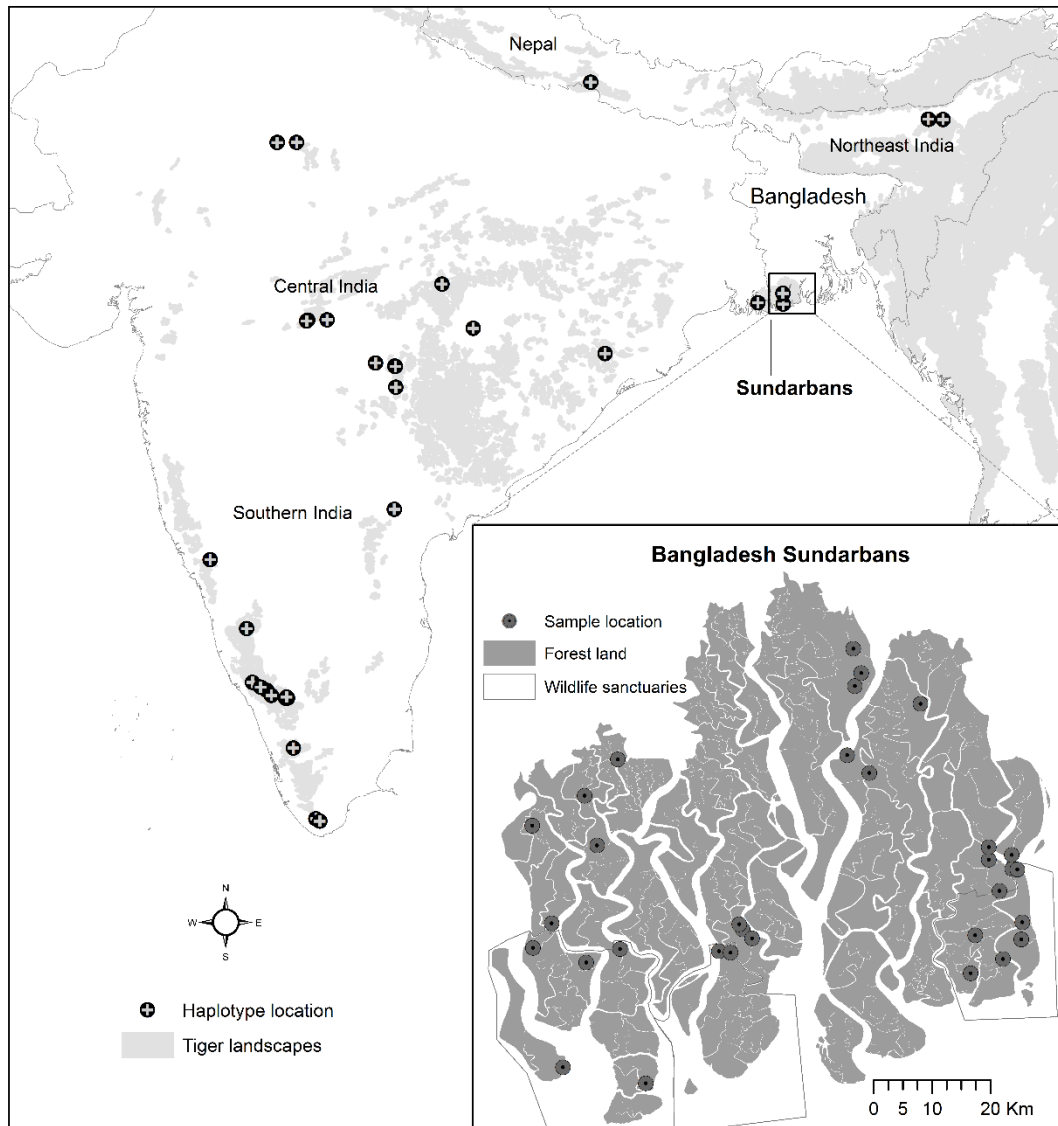


Fig .1. Bengal tiger haplotype locations (approximately) were retrieved from Mondol et al. (2009) and Luo et al. (2004). The inset figure showing the location of samples collected from the Bangladesh Sundarbans during this study.

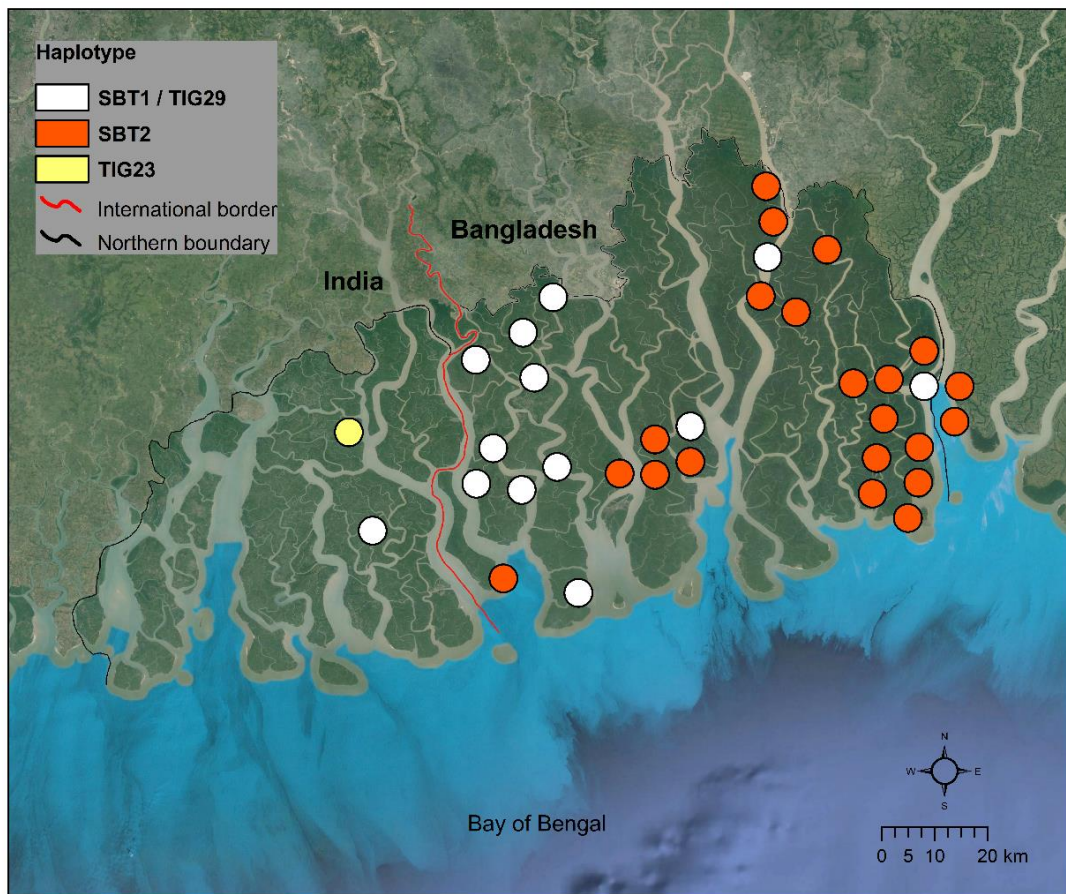


Fig. 2. The spatial distribution of three unique haplotypes derived from concatenated 1,263 mitochondrial gene fragments of the Sundarbans tigers. The dark green areas in the Google Earth image encompass the entire Sundarbans shared between Bangladesh and India, separated by rivers. Location of haplotypes depict the actual location of samples collected from the Bangladesh, while approximate location of two samples of the Indian Sundarbans were derived from Mondol et al. (2009b).

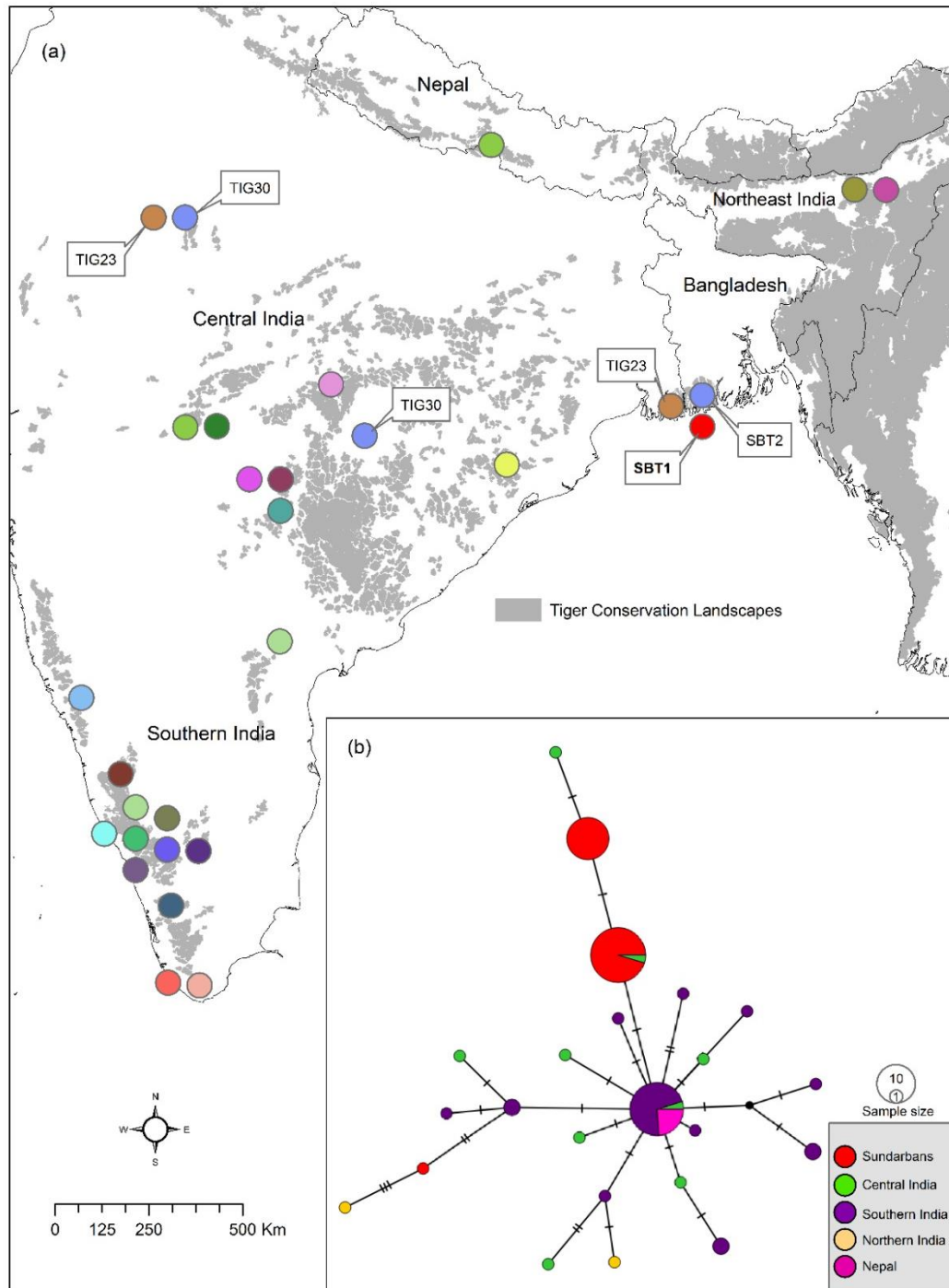


Fig. 3. Distribution and relationship of 20 unique haplotypes detected within the Bengal tiger populations, based on 1263 bp across four mitochondrial DNA (mtDNA). The pie chart (a) represents the approximate location of each unique haplotype retrieved from Mondol et al., (2009) and Luo et al., (2004). The colour scheme is coded for each unique haplotype. The callout text shows the distribution of haplotypes detected in the Sundarbans population, where SBT1 is unique in the population, while SBT2, TIG23 are shared with central Indian populations. Median-joining haplotype network (b) using the same *mtDNA* sequence dataset visualises the relationship among populations of Bengal tigers across the Indian subcontinent, including the Sundarbans. The colour schemes were assigned to each population grouped following Mondol et al., (2009). The clade coloured with red indicates the population of the Sundarbans. The size of the pie is proportional to the haplotype frequency, while black dot indicating inferred haplotype remained undetected. The bar between circles indicates the mutational steps between haplotypes.

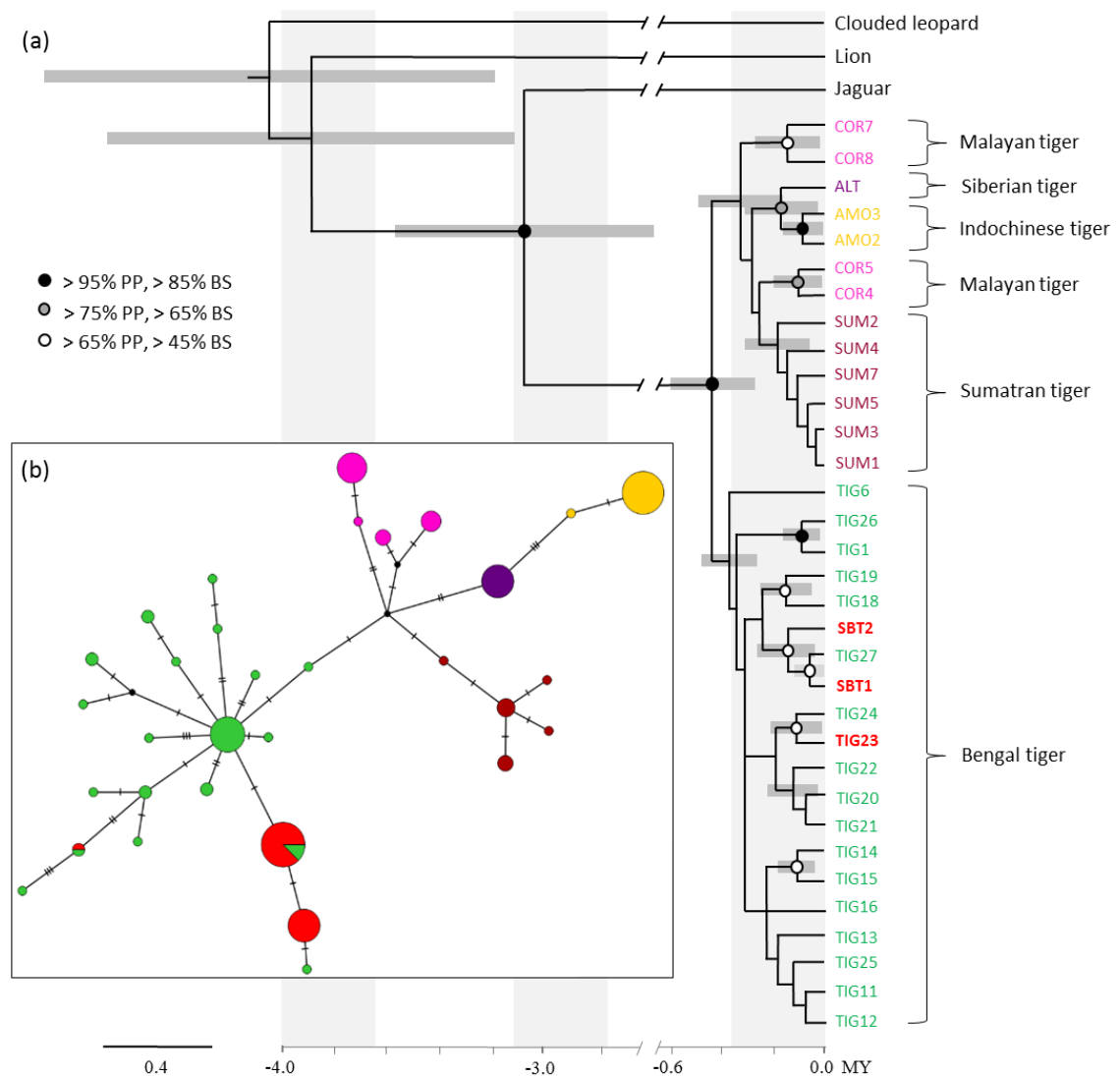


Fig. 4. Phylogenetic position of Sundarbans tigers. (a) Estimated divergence times resolved using BEAST with using 33 phylogenetically informative haplotypes and with two fossil-calibrations. Error bars display the 95% highest posterior density, and the axis is given in millions of years (MY) before present. Black dots indicate nodes with the Bayesian posterior probabilities (PP) > 95% and the maximum likelihood bootstrap support (BS) > 85%, grey dots indicate > 75% PP and > 65% BS, and white dots indicate > 65% PP and > 45% BS. Node values lower than 65% PP and 45% BS were not shown. Terminal nodes are labelled with names of unique haplotype detected in this study, Mondol et al. (2009), and Luo et al. (2004). Colours identify the tiger subspecies, except the Sundarbans tigers, which are in red. Inset (b): Median-joining haplotype network comprising 33 mitochondrial haplotypes, with unique colour representing the five tiger subspecies (pink = Indochinese tiger, purple = Siberian tiger, maroon = Sumatran tiger, fuchsia = Malayan tiger, green = Bengal tiger), corresponding to the colours coded in phylogenetic tree. The Sundarbans tigers were shown in red within the Bengal tigers. The size of the circle is proportional to the haplotype frequency (detailed sample size information can be found in the Table 1). The bar between circles indicates the mutational steps between haplotypes.

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Chapter 5

Do rivers influence fine-scale genetic structure of tigers in the Sundarbans?

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Abstract

The current tiger (*Panthera tigris*) populations are mostly confined to geographically isolated forest patches across their range, with limited genetic exchange between populations due to a range of landscape barriers. Assessing genetic structure of such populations can reveal the effects of dispersal barriers in the habitat and provide critical insight for guiding future conservation management efforts. Using non-invasively collected genetic samples, we investigated genetic structure of tigers in the Sundarbans, a vast homogeneous landscape of mangroves dissected by large river systems, and which holds one of the five top global tiger populations. We genotyped 52 individuals for a suit of 10 highly polymorphic microsatellite loci and sequenced 33 of them for a total of 1,263 base-pairs across four mitochondrial (mtDNA) genes. Microsatellite analyses revealed a signal of fine-scale genetic structure, which is likely be the consequence of limited tiger dispersal due to the presence of wide rivers. The distribution of mtDNA haplotypes showed a close phylogenetic affinity of tigers in the western Bangladesh Sundarbans region with that of the Indian Sundarbans, reflecting the nuclear pattern of genetic structure across the western part of the Bangladesh population. Spatial autocorrelation analyses using microsatellite data demonstrated a significant genetic differentiation as an effect of geographic distances, suggesting that genetic exchange within the population might have been limited by wide river systems of the Sundarbans. For long-term persistence of the population, future management approaches should aim to stabilising the tiger populations up to the carrying capacity of the Sundarbans in order to maintain sufficient genetic variation, in addition to reducing commercial and human activities within the forest to prevent further genetic structuring and erosion within the population.

Introduction

Human-induced historic deforestation in the tropics has led to the transformation of large continuous forested areas into a series of isolated patches, threatening the survival of many forest-dependent species (Loxterman, 2011; Walker et al., 2000). Tigers (*Panthera tigris*) are globally threatened forest carnivores, and their populations have collapsed to fewer than 4,000 from an estimated 100,000 in just 100 years (Morell, 2007; Seidensticker, 2010). Habitat destruction and fragmentation, hunting and the demand for body parts for traditional medicine, and depletion of their prey have been cited as some of the main causes of this dramatic decline in population size and range (Check, 2006; Clark et al., 1996; Damania et al., 2003; Woodroffe, 2000). At present, the remaining tiger populations survive mostly within isolated forest patches (Walston et al., 2010) across just 7% of their historic range (Sanderson et al., 2006). Increased fragmentation of these forest patches may prevent tiger dispersal between populations (Ewers and Didham, 2005; Joshi et al., 2013), and in turn promote loss of genetic diversity, inbreeding depression (Liberg et al., 2005; O'Brien et al., 1985; Vila et al., 2003), thus increasing the likelihood of extinction (Woodroffe, 2000).

The Sundarbans, a vast region of mangrove forests shared between Bangladesh and India, is one of the six regional Tiger Conservation Landscapes (TCLs) of global priority (Sanderson et al., 2006), and supports one of largest populations of Bengal tigers (Barlow, 2009; Dey et al., 2015). Unfortunately, Sundarbans tigers have long been isolated from other TCLs across the region (Sanderson et al., 2006). In particular, the Sundarbans is isolated from the nearest Simlipal TCL of India by approximately 200 km agricultural lands and human settlements, completely limiting opportunity for gene flow between populations. Studies have demonstrated that dispersal is a key mechanism of gene flow (Dieckmann et al., 1999; Johnson and Gaines, 1990) in maintaining genetic connectivity and preventing differentiation between populations (Cullingham et al., 2009). Importantly, dispersal plays a key role in long-term viability of tiger populations (Chapron et al., 2008), but normal dispersal between TCLs in India has been negatively affected by extensive habitat fragmentation and isolation (Jhala et al., 2011; Karanth and Gopal, 2005; Sharma et al., 2012).

Although the development of fine-scale spatial genetic structure is unlikely in widely dispersing animal taxa such as tigers (Smouse and Peakall, 1999) due to having large home range and high dispersal capability (Sunquist and Sunquist, 2001), the genetic connectivity between tiger populations has been documented to have been adversely affected by landscape elements such as road density and human settlements in central India (Joshi et al., 2013). Nonetheless, natural populations often mate non-randomly with greater chance between neighbouring individuals (Guillot et al., 2005), which may create genetic ‘isolation by distance’ (IBD) in the population (Wright, 1943). As a result, individuals living nearby tend to be genetically more similar than those living further apart (Wright, 1946, 1943). Such populations are prone to develop IBD if the normal dispersal is influenced by landscape barriers (Pritchard et al., 2000).

The Sundarbans is a river-dominated forest landscape containing a large number of rivers of varying width which has transformed the forest into mosaics of numerous swampy forest islands. Although Sundarbans tigers could normally cross considerably wide rivers (Barlow, 2009), it is entirely possible that these wide rivers influence tiger dispersal, leading to genetic structure within the population. Therefore, understanding the impact of river-dominated Sundarbans landscape on the genetic structure of tigers can help guide management activities for long-term persistence of the population (Joshi et al., 2013).

Several studies have investigated the ecology (Khan, 2004; Reza et al., 2001), home range (Barlow et al., 2011), population density (Dey et al., 2015), and genetic status of Sundarbans tigers (Singh et al., 2015), but none have examined the role of rivers as potential barriers to dispersal and their consequences on the fine-scale patterns of genetic structure of the Sundarbans tigers.

The objectives of this study were therefore to assess the effects of landscape barriers on the genetic structure of tigers in the Bangladesh Sundarbans. Using non-invasively collected genetic samples, we applied a comprehensive set of microsatellite and mitochondrial (mtDNA) markers to investigate fine-scale genetic structure, and to assess the role of rivers in genetic architecture of the Sundarbans tigers. We interpreted our findings to guide long-term conservation management of the tiger populations in the Sundarbans.

Methods

Study site

Of the 10,263 km² of the world largest mangrove forest shared between Bangladesh and India (Giri et al., 2007), the Bangladesh part covers 6,017 km², of which 4,267 km² is forest and the remaining area is comprised of water bodies (Iftekhar and Islam, 2004). The north and east sides of the forest are bounded by dense human settlements and agriculture land, and to the south by the Bay of Bengal (Fig. 1). Most of the Sundarbans topography is less than one metre above sea level (Canonizado and Hossain, 1998), and consists of vegetated islands that are inundated by intermitted high and low tides each day with a mean amplitude of 3-4 metres (Chaffey et al., 1985; Gopal and Chauhan, 2006). The Bangladesh Sundarbans is managed as Reserve Forest (SRF), except three isolated areas that have been designated as wildlife sanctuaries: Sundarbans West (715 km²), Sundarbans South (370 km²) and Sundarbans East (312 km²).

A complex network of rivers and streams with varying widths, giving a cumulative distance of 12,000 km, intersects the entire SRF (Hussain, 2014; Siddiqi, 2001). The SRF may therefore be described as a tangled region of estuaries, rivers and watercourses, enclosing a vast number of low-lying swampy forest islands of various shapes and sizes (Prain, 1979). Generally, all the major rivers flow from north to south, but are interconnected by the smaller rivers and creeks (Islam and Wahab, 2005). Three major rivers namely, the Arpangassia, the Passur, and the Sibsa passing north-south direction, divide the SRF into four major isolated regions (marked as *A*, *B*, *C*, *D*) (Fig. 1). The Arpangassia is about 1.2-3.1 km wide along its 64 km course and separates the western part (*A*) from the rest of the SRF. The Sibsa, one of the widest rivers of the SRF at more than 1.5 km wide (ranging from 1.3 to 3.1 km) for most its 60 km course, isolates a large portion (*B*) between the Arpangassia and the Passur rivers. The Passur river varies in width from 1.4 to 3.1 km, with the width of its major portion greater than 1.5 km along its 136 km course, and divides the remaining eastern half of the SRF into two large fragments (*C* and *D*) (Prain, 1979). Most of these major rivers have been used as cargo channels for centuries (Fig. 1).

Sampling strategy and sample collection

To collect non-invasive tiger samples, four intensive sample areas were selected within the SRF: East Wildlife Sanctuary with additional areas (ES, 383 km²), West Wildlife Sanctuary (WS, 715 km²), Chandpai block (SB, 342 km²), and Satkhira block (CB, 554 km²) (Fig. 1; Table 1). Location, protection status and level of human use (e.g., fishing, *golpata* collection) were considered in selecting these sample areas in order to sample across a range of areas with different status. In order to ensure intensive sampling, each sample area was divided into 2×2 km grid cells to create a total of 373 grid cells. Each grid cell was targeted for collecting samples with three separate transects (using one transect each time), walked by a surveying team of four trained field staff. Starting points for each transect were selected by where the grid cell could be easily accessed by boat. 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 period of time (13-22 days) for sample collection. Alongside intensive sampling in these grid cells, samples were also collected opportunistically from the remaining regions of the SRF. 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 (i.e. deposited scats, and hairs left on scratched marks on trees) and recorded the location of each sample using a handheld Global Positioning System (GPS) *Garmin GPSMAP 64*.

All biological samples, including scat samples were transported from Bangladesh to United Kingdom under the Convention on International Trade in Endangered Species (CITES) (Permit No. BD 9118404), and authorisation of the Department for Environment, Food and Rural Affairs, United Kingdom (AHVLA authorization: TARP/2015/111). DNA was then extracted from these samples and analysed at the Conservation Genetics Laboratory of the Durrell Institute of Conservation and Ecology (DICE), University of Kent, UK.

DNA extraction and sample screening

To prevent potential contamination, two isolated laboratory spaces were used for analyses of all biological samples. All scat samples were prepared for DNA extraction

under pre-sterilized fume hood conditions in batches of 10 samples. The workstation was sterilized before and after each use by irradiation using UV light and cleaning using 10% bleach. All PCR reactions were carried out in a separate laboratory under a fume hood pre-irradiated using UV light.

Genomic DNA from scat and hair samples was extracted using QIAamp DNA Stool mini kits and QIAamp DNA Blood and Tissue kits (QIAGEN Inc.), respectively following the manufacturer's instructions. Approximately 200 mg of scat material was scrapped 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 °C. The DNA supernatant from the sample was lysed with 300 µl AL buffer plus 25 µl proteinase K and incubated at 70 °C for 15 min. Four microlitre 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 50 g (or minimum 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 °C overnight or until the sample was completely digested. The DNA was eluted in 75 µl of buffer solution. A negative control was included with each batch of extractions to monitor for possible contamination during the DNA extraction procedure. To confirm that scats had been deposited by tigers rather than non-target wild cat species, extracted DNA was screened using tiger-specific primers to amplify a 245 base-pair fragment of the tiger cytochrome *b* gene (Mondol et al., 2009a; Mukherjee et al., 2007). 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 m at 72 °C using a G-Storm Thermal Cycler (Labtech France). PCR reaction volumes (total 27 µl) contained 3 µl of template DNA, 12.5 µl MyTaq redmix (containing dNTPs and MgCl₂; Bioline, UK), 0.5 µl of each primer, 2.0 µl BSA (Bovine Serum Albumin, New England Biolabs Inc.) and 8.5 µl dH₂O. PCR products were purified and sequenced using a 3730XL analyser (Macrogen, Amsterdam, Netherlands). Sequences were edited with Jalview v2 (Waterhouse et al., 2009) and then cross-checked and aligned with sequences from GenBank (National Center for Biotechnology Information, NCBI) to confirm species identity for each sample prior to inclusion of each sample for subsequent downstream genotyping.

Microsatellite genotyping

A suit of 10 microsatellites, from a set 16 optimised polymorphic loci, was used to amplify tiger-authenticated DNA samples (Table S1). Four multiplexes were designed to include the full set of loci. All forward primers were fluorescently labelled for gene-scanning (Table S1). Each microsatellite PCR reaction volume (10 µl) contained 3 µl of DNA template, 5 µl Qiagen multiplex PCR buffer mix (Qiagen Inc.), 0.2 µl forward primer, 0.2 µl reverse primer (Eurofins Genomics), and 2 µl BSA. Each microsatellite PCR reaction volume (10µl) contained 5 µl Qiagen multiplex PCR buffer mix (Qiagen Inc.), 0.2 µl labelled forward primer (Eurofins Genomics), 0.2 µl unlabelled reverse primer, 2 µl BSA and 3 µl of DNA template. For all multiplex reactions, the PCR temperature regime included an initial denaturation step for 15 min at 95 °C with 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; details in Table 2), extension (72 °C for 90 s), and a final extension of 10 m 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. Alleles were identified and scored using GENEMAPPER v3.7 (Applied Biosystems, MA, USA). To ensure a level of rigour in resolving the true genotype of each sample a comparative approach was followed, with each sample independently genotyped at least twice (Hansen et al., 2008); this approach was less laborious and more cost-effective than the multiple tube approach (Taberlet et al., 1997). Any samples that could not be scored consistently across amplifiable loci in the repeated genotype profiles were removed from the analysis (Jackson et al., 2016).

mtDNA sequencing

Nine primer sets were used to amplify four mtDNA gene regions: control region (CR), cytochrome *b* (Cyt *b*), NADH dehydrogenase subunit 2 (ND2), and NADH dehydrogenase subunit 5 (ND5) (Table S2; Mondol et al., 2009b). These gene regions were considered to be sufficiently variable for phylogenetic and genetic differentiation analyses in other tiger studies (Luo et al., 2004; Mondol et al., 2009b). PCR reactions were conducted in 27 µl reaction volumes which contained 3 µl template DNA, 12.5 µl MyTaq redmix (Bioline), 0.5 µl of each primer, and 10.5 µl of dH₂O. PCR amplification was performed using a G-Storm Thermal Cycler (Labtech France). The

PCR profile comprised of initial denaturation (95 °C for 1 m); 45 cycles of denaturation (95 °C for 30 s), annealing (T_a for 15 s), extension (72 °C for 30 s) and a final extension phase (72 °C for 10 m). All amplicons were examined by agarose gel electrophoresis to check for a clean single band and to check for any signs of contamination. PCR products were purified and amplified using a 3730xl analyser (Macrogen, Amsterdam, Netherlands).

Descriptive statistics and individual identification

MICROCHECKER v2.2.3 (van Oosterhout et al., 2004) was used to check genotyping errors due to stuttering. Allele frequencies, observed (H_o) and expected (H_e) heterozygosity, allelic dropout, false alleles, and probability of identify for siblings, PID(sibs) were estimated using GIMLET v1.3.3 (Valière, 2002). Linkage disequilibrium and departure from Hardy-Weinberg equilibrium (HWE) was tested using GENEPOP v4.2 (Raymond and Rousset, 1995). CERVUS v3.0 (Marshall et al., 1998) was used to identify unique or recaptured genotypes from pooled samples. Matching genotypes based on five or more loci were considered to be sourced from the same individual (Budowle, 2004; Mondol et al., 2009a). Incomplete or partial genotype profiles amplified at a minimum of five of the 10 loci set were also used to identify unique and recapture (s) individuals following the approaches of previous studies in tigers (Bhagavatula and Singh, 2006; Mondol et al., 2009a), and badgers (Frantz et al., 2003).

Fine-scale genetic structure analysis

A Bayesian clustering approach was used in the program STRUCTURE v2.3.4 (Pritchard et al., 2000) to assess the fine-scale genetic structure using microsatellite data. STRUCTURE determines the most likely number of genetic clusters (K) by assigning each individual tiger to the most likely clusters using multiple Markov Chain Monte Carlo (MCMC) algorithms for multilocus genotypes. STRUCTURE was run with admixture and correlated allele frequency model (Falush et al., 2003), using an initial burn-in length of 20,000 followed by a total run length of 500,000 iterations (Pritchard et al., 2000). The admixture model assumes that individuals can be of mixed ancestry, and is thus more suitable to studying populations that are harder to split into arbitrary predefined populations. Ten independent runs were performed, each time inferring the number of genetically distinct clusters ($K=1-10$), in order to

verify that the estimates are consistent across the runs (Pritchard et al., 2000). Posterior likelihood values for the most likely K and ΔK were evaluated following Evanno method (Earl and vonHoldt, 2012) in STRUCTURE HARVESTER (Earl and vonHoldt, 2012; Evanno et al., 2005), and averaged the proportions of individual assignment for 10 independent runs using CLUMPP v1.1.2 (Jakobsson and Rosenberg, 2007). The proportions of individual assignment was graphically displayed using POPHELPER (Francis, 2017) and geographically plotted using ArcGIS v10.3.

The spatial autocorrelation analyses were conducted in order to investigate the variation of genetic distance as a function of geographic distance (isolation by distance), which is robust to sampling variance, and a widely used descriptor of spatial genetic structure (Guillot et al., 2005; Peakall et al., 2003; Peakall and Smouse, 2012). This multivariate spatial analysis allows understanding fine-scale spatial genetic signal generated by multiple genetic loci in space (Peakall et al., 2003). First, a linear pairwise geographic matrix was calculated as the Euclidean geographic distance between geographic locations (latitudes and longitudes at UTM) of all sample tigers. Then, a pairwise squared genetic distance matrix were calculated using microsatellite loci dataset typed in tiger samples. These two matrices were then used to estimate spatial autocorrelation coefficient (r , bounded by -1, +1), a measure of the genetic similarity between pairs of individuals whose geographic separation falls within the specified distance classes (Smouse and Peakall, 1999). The distance classes are set of geographic distances in relation to sample location created following GenAlEx v6.5 documentation (Peakall and Smouse, 2012; Smouse and Peakall, 1999) which created 11 geographic distance classes each with 8 km apart between minimum and maximum sample distance within the SRF geographic extent. For example, first geographic distance class is less than or equal to 8 km, the second one from 8 to less than or equal to 16 km, and so on up to 88 km (Fig. 5). Estimates of r were then plotted at the endpoint of each distance class. Test for statistical significance was conducted by 9,999 random permutations of data to create a 95% confidence interval around a null hypothesis of no spatial genetic structure ($r = 0$), and 9,999 bootstraps resampling to create a 95% confidence interval around the mean estimate of r .

To complement the spatial autocorrelation analyses, Mantel test of matrix correspondence (Mantel, 1967; Smouse and Peakall, 1999) was performed on the

respective pairwise geographic and genetic distance matrices, with test of significance. The Mantel test was carried out with 9,999 permutations to achieve statistical significance of matrix correlations. The test returns a *P*-value for empirical correlation coefficient (*r*) between the geographic and genetic distance matrices, with a significant correlation being indicative of spatial genetic structure in the sample (Mantel, 1967; Smouse et al., 1986). The spatial genetic autocorrelation, and Mantel text analyses were performed using the software package, GenAlEx v6.5 (Peakall and Smouse, 2012).

Phylogenetic structure analysis

Four mtDNA gene fragments of CR, Cyt *b*, ND2, and ND5 were edited and aligned with Jalview v2 (Waterhouse et al., 2009), and concatenated into a complete dataset using SEQUENCEMATRIX (Vaidya et al., 2011). To assess phylogenetic relationships between tiger populations of Bangladesh and Indian Sundarbans, previously reported haplotype data (TIG23 and TIG 29; Mondol et al., 2009b) were obtained from GenBank (<https://www.ncbi.nlm.nih.gov/genbank/>; accession number for TIG23: EU661642 and TIG29: EU661648), and added to our dataset. To infer evolutionary relationships among tiger haplotypes, a median joining haplotype network was constructed in the program PopART v1.7 (<http://popart.otago.ac.nz>). To investigate spatial distribution across the Sundarbans landscape, each haplotype was then assigned to a georeferenced sample location and plotted using ArcGIS v10.3.

Results

Genetic diversity

Out of 512 putative tiger samples, 265 samples were successfully amplified using the felid specific mtDNA cytochrome *b* primers, and were therefore considered to be have been genuinely sourced from tiger individuals. From the 265 tiger samples, only 125 samples yielded genotype data for a minimum of five to 10 microsatellite loci, giving a microsatellite genotype dataset comprising 53 individuals. Sex was determined for 57% individuals, resulting in 12 males and 18 females. The mean proportion of loci typed was 87% across the dataset, with a mean polymorphic information content (PIC) of 0.64.

Significant deviations from Hardy-Weinberg equilibrium (HWE) after Bonferroni correction were detected at loci Fca304, Fca126, Fca230, Fca90, and Fca672, but no linkage disequilibrium was found between loci pairs (Table 2). Estimated frequencies of allelic dropout ranged from 0.11 to 0.34 for three loci. Estimated frequency of null alleles ranged between 0.11 and 0.48 per locus. All loci were polymorphic with a mean number of alleles of $5.50 \pm \text{SD } 1.65$ per locus. The overall expected heterozygosity (H_e) and observed heterozygosity (H_o) were $0.68 \pm \text{SD } 0.04$ and $0.37 \pm \text{SD } 0.02$, respectively.

Fine-scale population genetic structure

STRUCTURE analysis using microsatellite data consistently displayed three genetic clusters ($\Delta K = 3$) across ten independent runs. An additional peak at $\Delta K = 7$ indicated the possibility of further genetic structure within the populations (Figs. 2, 3). At $K = 3$, the individual assignment to inferred clusters was higher for cluster 1 (40%) than clusters 2 and cluster 3, where number of individuals were nearly equally divided between these clusters (32% and 28%, respectively). The estimated probability of individual membership to cluster 1 ($q = 0.495\text{-}0.969$, with 71% individual membership assignment, $q > 0.817$) demonstrated a signal of substructure in the region A across the south-western part of the SRF (Fig. 4a). However, two individuals ($q = 0.375, 0.587$) from region B and one individual ($q = 0.430$) from region C were also assigned to this cluster (Fig. 4a). The estimated probability of individual membership to cluster 2 ($q = 0.429\text{-}0.712$) and cluster 3 ($q = 0.372\text{-}0.716$) showed an overall admixture among the individuals distributed between regions B, C, and D. Conversely, individual assignment when assuming $K = 7$ suggested a high level of admixture, with almost half of the individuals assigned to cluster 3 (25%), and cluster 7 (21%), and the remaining individuals were split over five clusters, ranging from 3 to 7 individuals (Fig. S1). At $K = 7$, the estimated probability of individual membership to cluster 3 ($q = 0.353\text{-}0.534$), and cluster 7 ($q = 0.251\text{-}0.675$) were comparatively lower than the membership probabilities at $K = 3$. The spatial distribution of the individual membership probabilities at $K = 7$ also displayed a high level of admixture across spatial regions B, C, and D of the SRF, with a lower level of admixture in region A (Fig. S1).

A total of 1263 base-pairs from four mtDNA gene fragments across 33 tiger samples revealed two haplotypes (SBT1 and SBT2) within the SRF (Fig. 4b). The results across four gene fragments showed that only the cytochrome *b* gene region contained a single segregating site, while the remaining three gene regions were identical across all samples. Sixty four percent of the total sample population shared the haplotype SBT2 while 36% was the second haplotype (SBT1). Interestingly, 90% of all tiger individuals in the region A contained the second haplotype (SBT1), displaying a clear pattern distinguishing the tigers sampled in this region from the remaining B, C, and D regions. Conversely, 80%, 67%, and 94% of individuals of B, C, and D regions respectively shared the second haplotype (SBT2). The overall haplotype distribution clearly revealed a pattern of mtDNA haplotypes that distinguishes region A from the combined regions B, C, and D (Fig. 4b). Subsequent inclusion of haplotypes reported from the Indian part of the Sundarbans (Mondol et al., 2009b) revealed three haplotypes within the entire tiger population of the Sundarbans. Haplotype SBT1 was identical to haplotype TIG29 reported by Mondol et al., (2009b) from the Indian Sundarbans. Haplotype SBT2 appears to be unique to the Bangladesh Sundarbans, and haplotype TIG23 in the Indian Sundarbans (Fig. 4c).

Genetic isolation by distance

The results of spatial genetic autocorrelation showed that genetic differentiation varied over geographic distances (Fig. 5). The correlograms showed the genetic correlation as a function of distance between genotypes, with r values remaining positive and significant from zero to 8 km ($r = 0.068$, $p = 0.001$) until 24 km ($r = 0.038$, $p = 0.002$) geographic distance, although there were little changes between 8 km and 16 km. With the larger distance classes, the r values were significantly negative at 72 km onwards ($r = -0.044$, $p = 0.002$), indicating significant genetic erosion beyond this distance (Fig. 5). The Mantel test between the pairwise geographic and pairwise genetic distance matrices showed a significant positive relationship ($r = 0.161$, $p = 0.01$), indicating the presence of spatial genetic structure in the tiger populations (Fig. 6). Although providing a less powerful test than the autocorrelation analysis, Mantel test results are also consistent with the autocorrelation results. Both of these analyses revealed a significant decline in genetic similarity at larger geographic distances, which is unlikely for tigers given their long-dispersal capability.

Discussion

Genetic diversity

Approximately half of the samples (52%) were screened successfully, of which 47% provided consistent genotypes. These success rates are relatively lower than reported in the previous non-invasive genetic studies in tigers (Bhagavatula and Singh, 2006; Mondol et al., 2009a), which might be a consequence of inferior quality of samples collected from humid and wet mangrove forest of the Sundarbans. Overall, low level of genetic diversity was found in the population compared to previous tiger studies in the Indian landscapes (Borthakur et al., 2013; Joshi et al., 2013). The mean number of alleles observed in this study (5.50) is higher than population in Indian Sundarbans (3.33; Singh et al., 2015), but lower than Central (11.71; Joshi et al., 2013) and Northeast India (6.61; Borthakur et al., 2013). The observed heterozygosity in the population of Bangladesh Sundarbans ($H_o = 0.37$) is lower than the population of the Indian Sundarbans ($H_o = 0.49$; Singh et al., 2015), Central India ($H_o = 0.54$; Joshi et al., 2013) and Northeast India ($H_o = 0.47$; Borthakur et al., 2013). However, these variations across tiger populations could not be directly compared due to differences in marker sets and size of samples used. Half of the loci showed violation of HWE due to heterozygote deficiency, which simply could be due to allelic dropout. The deviated loci were retained in subsequent analyses, because genetic assignment analyses were typically robust to potential causes of heterozygote deficiency (Lonsinger et al., 2015; Pilot et al., 2006).

Fine-scale population genetic structure

STRUCTURE revealed the greatest support for three clusters ($K = 3$) within the tiger populations of the SRF, with a possibility of subtle substructure at $\Delta K = 7$. The probability of individual memberships to each cluster as illustrated in the Figure 3 showed that tigers in the region A might have limited level of genetic exchange with other populations in the B, C and D regions, where a relatively weak genetic structure was detected. Interestingly, distribution of individual membership probabilities in the region A further signalled a relatively greater level of gene flow at north-south direction, implying that north-south laid wider rivers might have been the potential barriers to tiger dispersal. With regard to the cluster at $K = 7$, the distribution of individual membership probabilities in the region A further showed a distinct pattern

in the two remote southeast islands, lending additional support for structure in the tiger populations. However, higher level of admixture membership in the remaining regional samples (*B*, *C*, and *D*) suggests considerable contiguity of gene flow, even though tigers in these regions have been also split over by several major rivers.

The population structure at $K = 3$ appears to be the most likely for the tiger population, reflecting the landscape realities of the Sundarbans. Also, the lowest ΔK is usually the most reliable one for the population in question when STRUCTURE simulated multiple values of K with similar probabilities (Pritchard et al., 2000). Besides, over-estimation of the true K could be due to the presence of related individuals in the sample population (Pritchard et al., 2010), which is common for natural populations (Pusey and Packer, 1987; Spong and Creel, 2001). Moreover, many animal populations in the real world may not simply conform precisely to the Bayesian clustering methods due to presence of IBD or inbreeding (Blair et al., 2012; Frantz et al., 2009; Pritchard et al., 2000).

The genetic connectivity between populations across Indian tiger landscapes has been hampered by human-induced landscape features (Joshi et al., 2013; Sharma et al., 2012; Yumnam et al., 2014), fortunately no such landscape barriers exist in the Sundarbans mangrove forests. However, the entire Sundarbans landscapes has long been sliced (by >1.5 km wide rivers for most of their courses) into east-west forest islands by the Passur, Sibsa, and Arpangassia rivers. These rivers might have been potential barriers to tiger dispersal, because tigers could normally cross rivers between 0.6 and 0.7 km wide, with up to a record of 1.5 km (Barlow, 2009). Therefore, in the absence of such landscape barriers in the Sundarbans, the wider rivers might have been the effective barrier to tiger dispersal, impeding genetic exchange between locally colonised populations within the forest islands, as revealed in further southwest islands in the region A. Therefore, it cannot be ruled out the possibility of the population structure even at $K = 7$, where it might be revealing cryptic population structure surviving in the mosaic of Sundarbans mangrove forests.

Several case studies have detected profound influence of rivers on genetic structure of a range of species in the Amazon where rivers are particularly wide enough to be an efficient barrier (Hayes and Sewlal, 2004; Lougheed et al., 1999; Moritz et al., 2000).

For example, rivers have influenced fine-scale genetic structure in the mammalian carnivore of Stone marten (*Martes foina*) (Basto et al., 2016). Similarly, rivers strictly limited the population distribution and gene flow in primates (Gehring et al., 2012; Goodman and Ganzhorn, 2004; Pastorini et al., 2003), Raccoon (*Procyon lotor*) (Cullingham et al., 2009), and White-tailed deer (*Odocoileus virginianus*) (Blanchong et al., 2008). Therefore, it is not unlikely that wide rivers might have been potential barrier to tiger dispersal, and have influenced genetic architecture of the Sundarbans tiger populations.

Secondly, haplotype distribution shows a clear segregating trend within the population, particularly between regions separated by the Arpangassia, which is congruent with structuring signature detected in nuclear data. This distinct pattern also suggests that population in this region (A) might have been historically isolated from the remaining populations. Nonetheless, spatial distribution of three unique haplotypes in tiger populations of Bangladesh and Indian Sundarbans displayed an interesting pattern, where the shared haplotype between Bangladesh and Indian populations is the one (SBT1) that detected in the region A. While the unique haplotype in the Bangladesh Sundarbans was predominantly distributed in the regions B, C, and D, this clearly indicates that tiger populations in the region A are phylogenetically more related with tiger populations in the Indian Sundarbans. This result also suggests that tiger populations living further eastern regions of B, C, and D in the Bangladesh Sundarbans are relatively distant phylogenetically from Indian ones, might be due to an effect of north-south directed wide rivers including the Raimangal and Haringhata rivers across the International border between countries. This finding is supported by a satellite telemetry study that found frequent movement of tigers between the Bangladesh and Indian parts of the Sundarbans (Jhala et al., 2011).

Genetic isolation by distance

The combined spatial genetic analyses showed a consistent pattern of genetic isolation in regard to the increasing geographic distances. In particular, the autocorrelation analysis detected a significant pattern of fine-scale genetic structure within tiger population with positive at smaller geographic distances, while significantly negative beyond 72 km. This result is aligned with the hypothesis that if IBD occurs in a continuously distributed population, genetic distance among individuals is expected to

be increased linearly with the geographic distance (Rousset, 2000). However, significantly negative genetic relatedness beyond 72 km is unexpected given the dispersal capability of tigers. For instance, genetic connectivity was detected up to 650 km of geographic distance in the Indian tiger landscapes (Joshi et al., 2013), despite having strong barriers of human settlement and roads to tiger dispersal (Joshi et al., 2013; Sharma et al., 2012). This implies that dispersal ability of tigers might have been impaired in the Sundarbans due to wider rivers, in absence of other landscape barriers. This can be attributed to the fact that spatial genetic structure can be developed quickly in animal populations under restrictive gene flow (Epperson, 1990; Sokal and Wartenberg, 1983). Several studies have found similar relationship between geographical and genetic distances in a range of cetaceans (Fontaine et al., 2007), Sitka black-tailed deer (*Odocoileus hemionus sitkensis*) (Colson et al., 2013), and Red foxes (*Vulpes vulpes*) (Kirschning et al., 2007), where rivers and watersheds affected IBD trends within the populations.

Conservation implications

Habitat connectivity is fundamental to sustaining regional populations of tigers because they need contiguous forest connectivity for dispersal and genetic exchange (Smith, 1993). Unfortunately, the Sundarbans tigers have been completely isolated from nearby tiger populations by settlement and agriculture landscapes, removing any chance of future genetic exchange that is vital to long-term persistence of the population. So the immediate conservation effort should focus on stabilising the existing tiger population up to the carrying capacity of the Sundarbans to maintain sufficient genetic variation within the population because the amount of genetic diversity is significantly positively correlated with the population size (Frankham, 1996). Secondly, the Sundarbans landscape itself has been bisected by large number of rivers for centuries, forcing tigers to colonise into small and isolated populations within mosaic forest islands of the Sundarbans. Given that natural geographical setting of these rivers across the Sundarbans, future management should aim to reduce the intensity of commercial cargo movement, and resource collection activities across wider rivers such as the Passur, Sibsa and Arpangassia. Because human activities significantly limited tiger dispersal across Indian landscapes (Yumnam et al., 2014), reduction of human disturbance created through commercial and resource collection activities will allow tigers to disperse between forest patches more frequently,

preventing further genetic structuring and erosion within the population. Nonetheless, tiger populations between Bangladesh and Indian Sundarbans are phylogenetically closely related, therefore tigers across the entire Sundarbans should be managed as a single population through bilateral cooperation between countries.

Tables

Table 1

Sample area and number of samples used in this study.

Sample area	Area (Km ²)	Protection status	Samples collected	Samples screened	Samples genotyped
Satkhira block	342	Reserve forest	77	30	15
West wildlife sanctuary	715	Protected area	152	82	33
Chandpai block	544	Reserve forest	127	74	33
East wildlife sanctuary	383	Protected area	84	44	24
Opportunistic sample	--	Both types	72	35	20

Table 2

Characteristics of microsatellite loci used: locus name, number of allele (A), observed heterozygosity (Ho), expected heterozygosity (He), polymorphic information content (PIC), null allele (NA), allelic dropout (AD), False allele (FA), Hardy-Weinberg Equilibrium (HWE), and the probability of identity for siblings (PID_{sibs}) at 10 microsatellites for 53 individuals.

Locus name	A	Ho	He	PIC	NA	AD	FA	HWE	PID(sibs)
Fca304	4	0.35	0.71	0.65	0.33	0.00	0.11	No	9.77E-03
Fca126	5	0.18	0.69	0.62	0.48	0.00	0.02	No	1.91E-03
F41	5	0.61	0.65	0.58	0.02	0.00	0.40	Yes	9.10E-04
Fca230	9	0.13	0.70	0.66	0.37	0.00	0.14	No	4.26E-03
E7	4	0.30	0.45	0.38	0.19	0.00	0.10	Yes	3.00E-04
Fca279	7	0.62	0.81	0.77	0.13	0.16	0.00	Yes	3.67E-01
D15	4	0.49	0.74	0.68	0.20	0.11	0.00	Yes	2.25E-02
Fca43	5	0.44	0.58	0.50	0.11	0.00	0.13	Yes	4.80E-04
Fca90	5	0.34	0.79	0.75	0.39	0.00	0.12	No	1.40E-01
Fca672	7	0.28	0.78	0.74	0.47	0.34	0.00	No	5.43E-02

Table S1

Microsatellite locus name, forward and reverse sequences, annealing temperature (AT), fluorescent dye (FD) and PCR multiplexes (PM) used in this study.

Locus name	Forward sequence	Reverse sequence	TA	FD	PM
Fca043	GAGCCACCCTAGCACATATACC	AGACGGGATTGCATGAAAAG	55	NED	Set 1
F41	GTCTGCATCTTCAAATAGGA	GTACCTGAGTTGGCTGTTGA	56	FAM	Set 1
D15 ^a	TGTGACCTTTCTCTAGTTTC	GCACAAAACATTCAGTCTCC	55	FAM	Set 1
Fca232	ATGACCATCTCAAACCTTCATGG	AGCTGAGTTTGGCTTATCATG	56	HEX	Set 1
Fca304	TCATTGGCTACCACAAAGTAGG	CTGCATGCCATTGGGTAAC	56	FAM	Set 2
Fca309	AGAGATGGGCTCAGTTGCAT	CTGGTTACCCCGAATTCTCA	55	NED	Set 2
E7 ^a	GCCCCAAAGCCCTAAAATAA	GCATGTCGGACAGTAAAGCA	55	NED	Set 2
ZN (ZF _x /Zfy) ^b	AAGTTTACACAACCACCTGG	CACAGAATTTACACTTGTGCA	55	NED	Set 2
Fca052	TGTATCCTCTGCTCCTGAAACA	ACCTGTCCCAGTGCTTGTG	59	FAM	Set 3
Fca126	GCCCCTGATACCCTGAATG	CTATCCTTGCTGGCTGAAGG	56	HEX	Set 3
Fca164	TCATGGCTGAGTAATAGTCGTG	GCAGCCCAAATGTCCATC	59	NED	Set 3
Fca672	AAGTTGCTTGCACACACTGC	TCCAAGAGCCTTTTCAGTTAGG	56	HEX	Set 3
Fca090	ATCAAAGTCTTGAAGAGCATGG	TGTTAGCTCATGTTTCATGTGTCC	52	HEX	Set 4
Fca230	AAGAATGGACTTGGGAAATGG	AAACCACAACAGGCAAAGG	52	NED	Set 4
Fca279	AGCCAAGTAATATTCCTCTGTG	GTCCATCCGCAGATGAATG	52	FAM	Set 4

All loci optimised from Menotti-Raymond et al. (1999), except ^aBhagavatula and Singh (2006) and ^bPilgrim et al. (2005).

Table S2

Mitochondrial primers sets across four gene fragments of DNA used in this study.

Primer name	Primer sequence	Amplicon size (bp)
TIGND2 F1	TAGTCTGAATCGGCTTCG	195
TIGND2 R1	CCGTTATAATGGATGCCA	
TIGND5 F1	GCCCCTATATTAACCAGT	195
TIGND5 R1	ATCCTACATCTCCAATAC	
TIGND5 F2	TATCAGACGCAAACACTG	224
TIGND5 R2	AATAAAGCGGAGACGGGA	
TIGND5 F3	ACCTACACCCATGATTGC	187
TIGND5 R3	TTTTGTGTGAGGGCACAG	
TIGCYT B F2	CGTCTGTCTATACATGCA	200
TIGCYT B R2	TACTCTACTAGGTCGGTC	
TIGCYT B F3	ATGTCTTTTTGAGGGGCA	191
TIGCYT B R3	GTATTGGATCCTGTTTCG	
TIGCYT B F4	TTAACCCCTAGCAGCAGTC	184
TIGCYT B R4	TGTAGTTATCAGGGTCTC	
TIGCR F1	GGGAAGGAGAATATGTAC	142
TIGCR R1	CACAGAACGGGTATATGC	
TIGCR F2	CGAAAACAACCCCATGAC	137
TIGCR R2	GCTTCGTGTTGTGTGTTT	

Note: These primer sets were optimised from Mondol et al. (2009b).

Figures

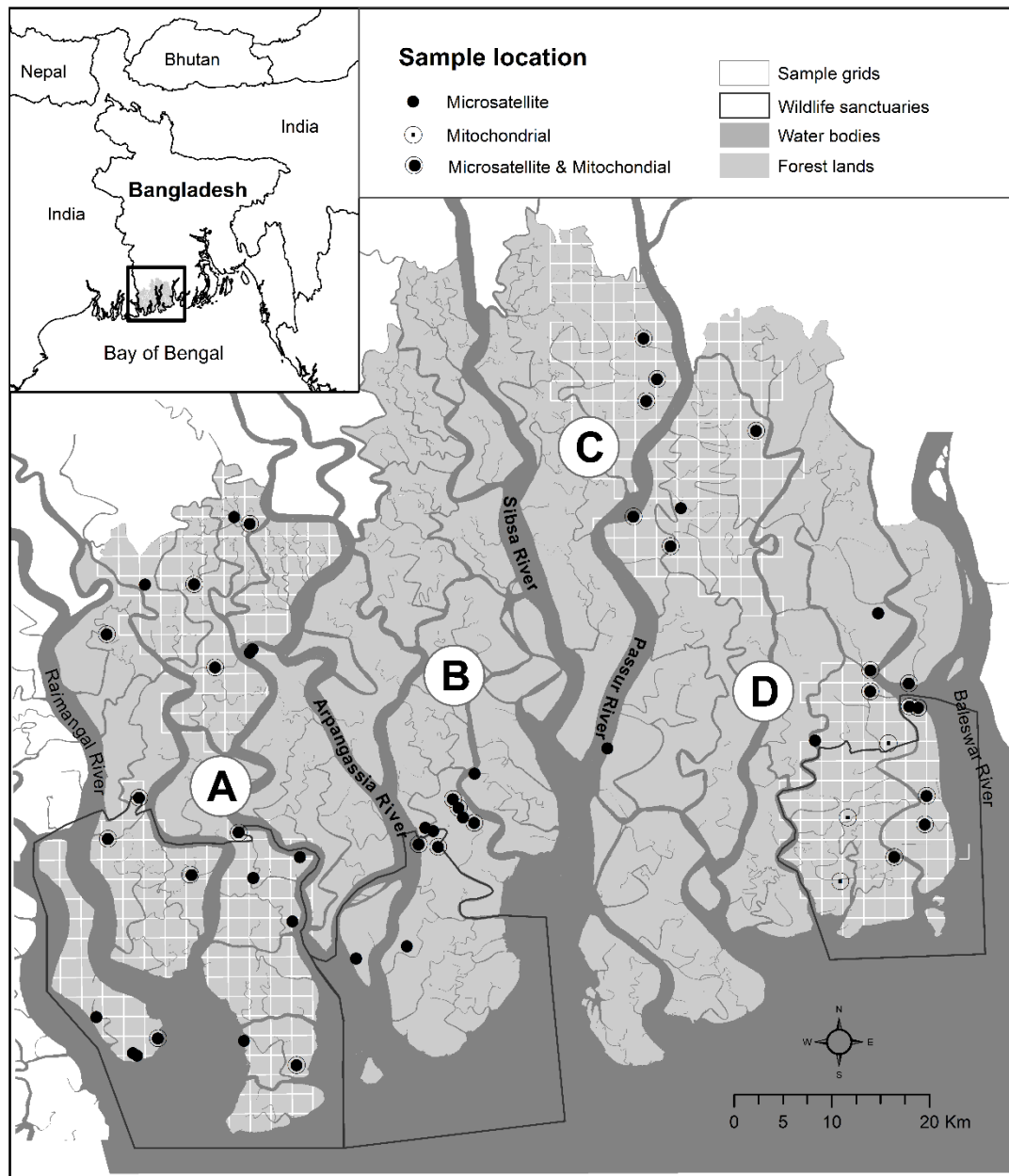


Fig. 1. The Bangladesh Sundarbans showing sampling locations and regions separated by the major rivers.

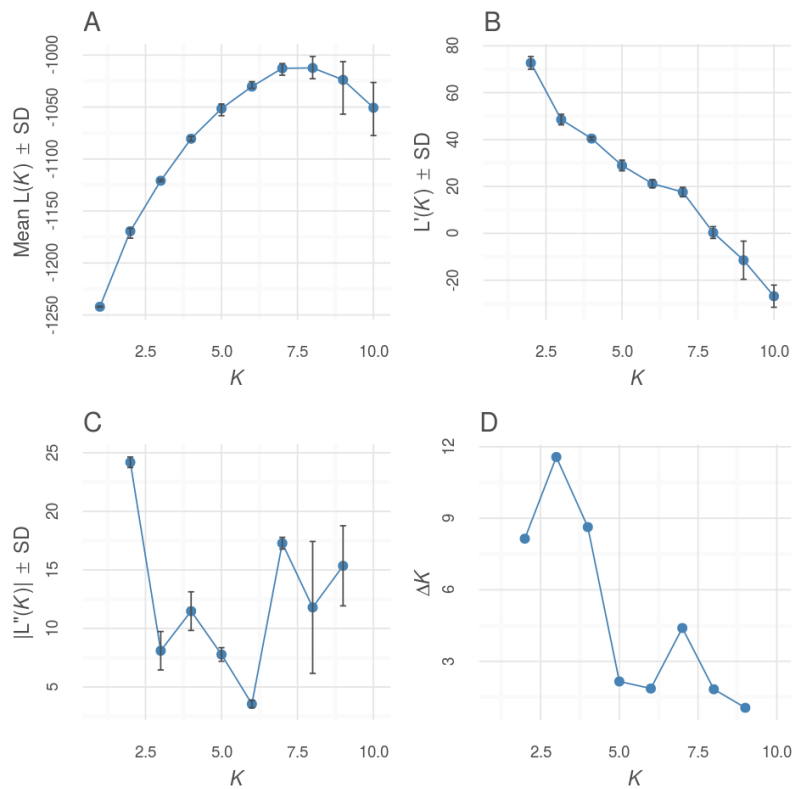


Fig. 2. Plot of (A) mean likelihood $L(K)$; (B) rate of change of the likelihood distribution of mean; (C) absolute value of second order rate of change of the likelihood distribution of mean, with variance per K value from the STRUCTURE on a dataset containing 53 individuals genotyped at 10 microsatellite loci. Evanno method (D) detected ΔK values indicating the higher probability of number of K , that best fit the data. Here, three clusters are clearly detected, with the next higher ΔK at $K=7$.

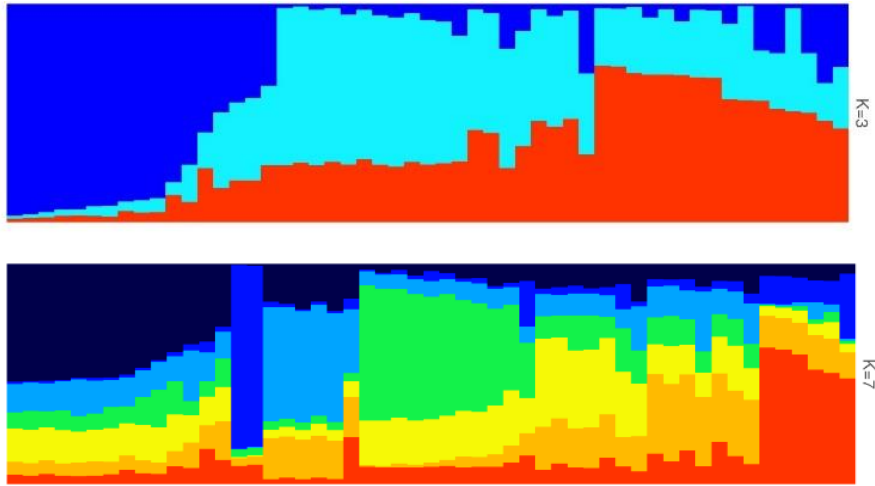


Fig. 3. Genetic structure of tiger population showing in the bar plot from STRUCTURE at $K = 3$ and $K=7$ for 53 individuals typed at 10 microsatellite loci. Each bar represents one individual, where colouration corresponds to the percentage of genotype shared with the respective cluster.

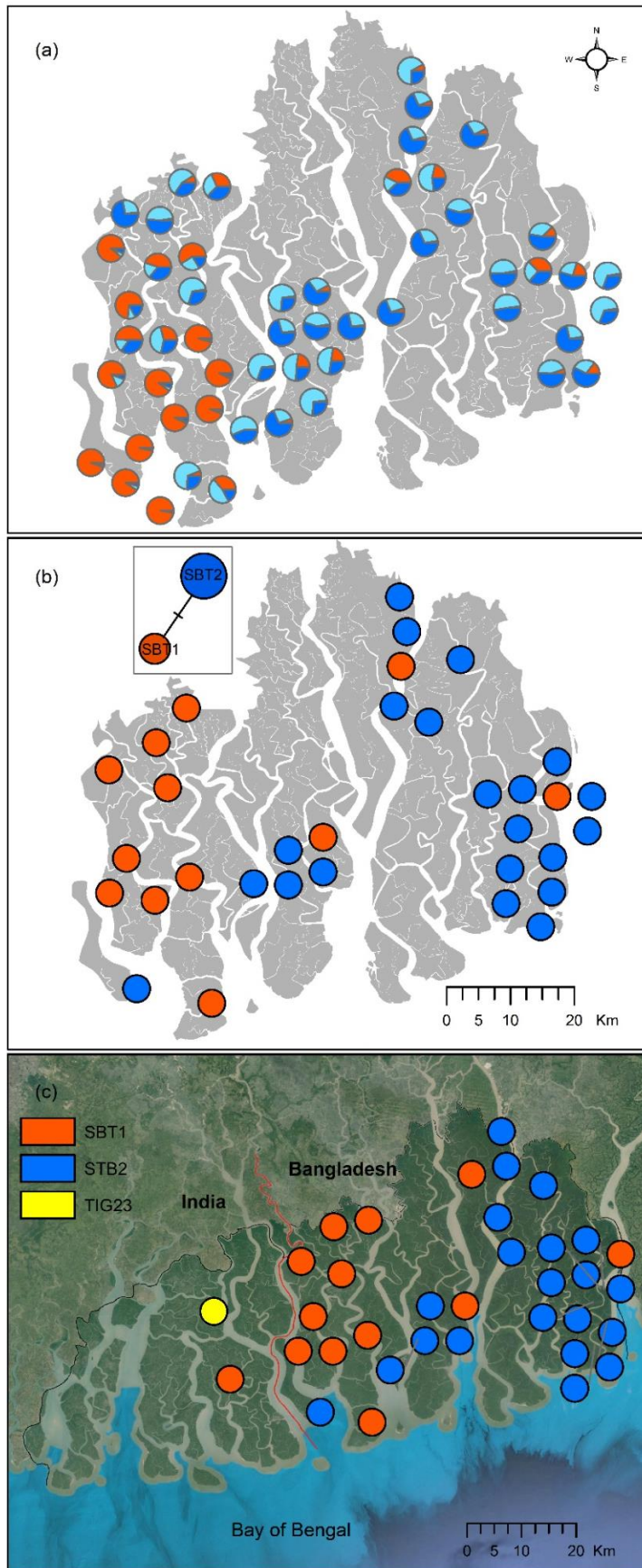


Fig. 4. Geographical representation of the assignment probabilities for 53 tiger samples typed at 10 microsatellite loci to each of the $K = 3$ (a) clusters, proportional to the colour of each pie chart. The placement of each pie chart indicates the sampling location of individual tiger sample. Distribution of haplotypes identified in 33 tiger samples for 1,263 base pairs sequences across Bangladesh Sundarbans (b), and the entire Sundarbans (c) using data from Mondol et al. (2009b) for Indian Sundarbans. Two haplotypes were shown in inset (b) with sample sizes of SBT1 ($n = 12$) and SBT2 ($n = 21$).

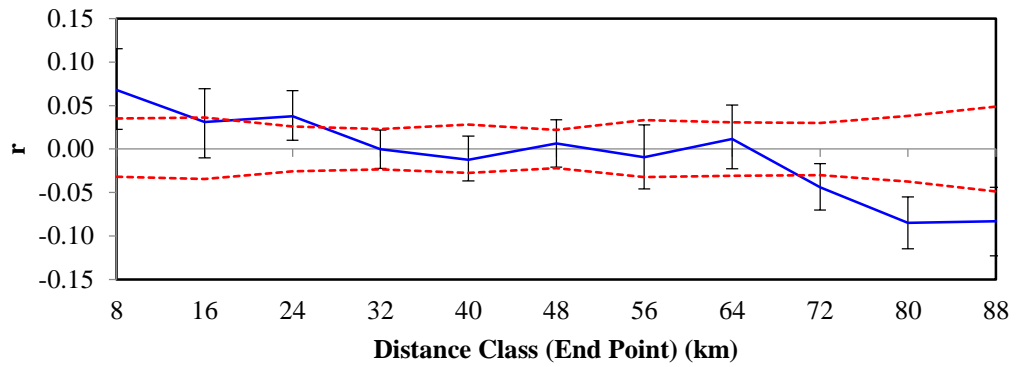


Fig. 5. The spatial autocorrelation at various distance classes for 53 tiger samples of the Sundarbans genotyped at 10 microsatellite loci. The correlograms showing genetic correlation, r as a function of distance, with 95% CI about the null hypothesis of a random distribution of genotypes, and 95% confidence error bars around r as determined by bootstrap resampling.

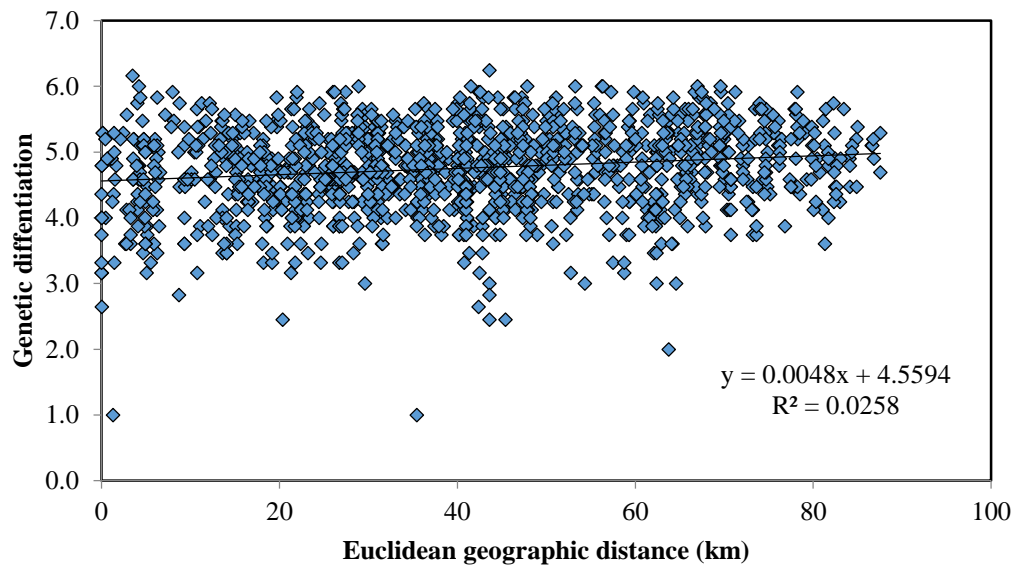


Fig. 6. The diagram showing the results of Mantel test between pairwise geographic and genetic distance matrices, with test of significance by permutation. The dots represent the permuted tiger samples, with regression line indicating the level of genetic differentiation over geographic distances.

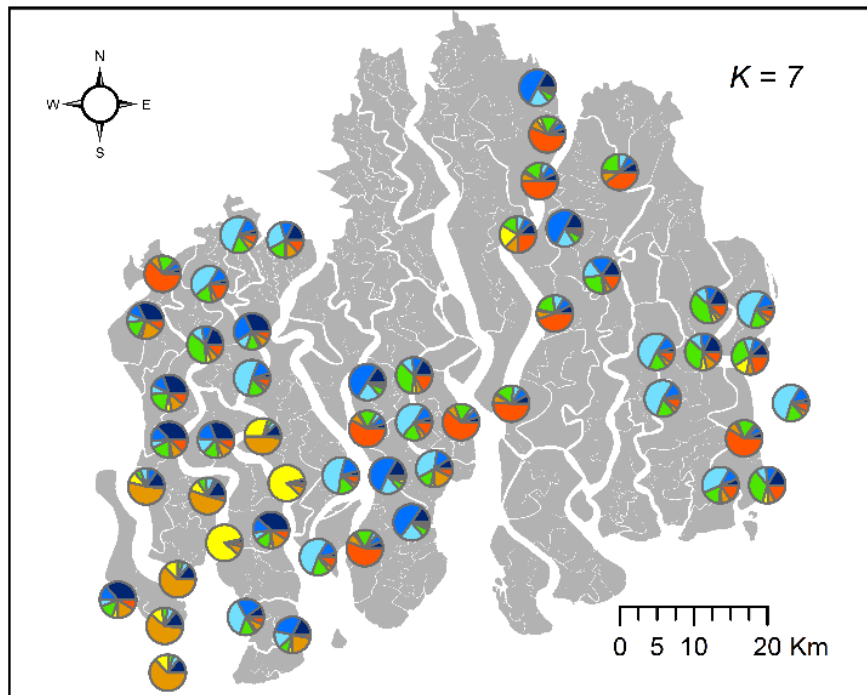


Fig. S1. Geographical representation of the assignment probabilities for 53 tiger samples typed at 10 microsatellite loci to each of the $K = 7$ clusters, proportional to the colour of each pie chart. The placement of each pie chart indicating the sampling location of individual tiger sample.

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Chapter 6
Investigating patterns of tiger and prey poaching in
the Bangladesh Sundarbans: implications for
improved management

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Abstract

Poaching of tigers and their key prey threatens the survival of tigers across their range. This study investigated the methods, intensity, and driving factors of tiger and prey poaching in the Sundarbans Reserved Forest of Bangladesh, to help better design and direct future management interventions. The study identified a range of snaring methods used to catch prey and an approach to killing tigers by poisoning prey carcasses with a Carbofuran pesticide. We recorded six poisoned baits set to kill tigers and 1,427 snare loops in 56 snare sets to kill tiger prey. With an average of 23 snare loops/snare set, this is equivalent to an estimated 6,268 snare loops across the Sundarbans or 147 snare loops/100 km². Poachers selected sites that tended to be away from guard posts, and close to river banks, but were not influenced by protected area status or distance to the forest boundary. The current poaching pressure is likely to have contributed to a recent decline in relative tiger abundance. We recommend using better regulation of Carbofuran use across tiger range countries, and using remote camera traps set up around snares and poisoned baits to help authorities identify poachers for arrest. This study demonstrates a simple approach to investigating the methods, intensity and distribution of poaching, that could be replicated across all tiger landscapes to better direct mitigating actions and monitor changes in threat levels over time.

Introduction

Global tiger (*Panthera tigris*) populations have collapsed from an estimated 100,000 to 3,500 tigers in just 100 years (Morell, 2007; Sanderson et al., 2006), and now occupy less than 7% of their historic range (Sanderson et al., 2006). The remaining tigers are mostly now restricted to small pockets of protected areas across their range (Walston et al., 2010b), and their numbers continue to decline in important areas despite significant conservation efforts by international agencies, local conservation groups and governments (Dinerstein et al., 2007; Seidensticker et al., 1999).

Poaching of tiger and prey has been identified as one of the major threats to tiger populations where they still persist (Aziz et al., 2013; Damania et al., 2003; Dinerstein et al., 2007; Goodrich et al., 2008; Jhala et al., 2008; Karanth and Stith, 1999; Wikramanayake et al., 2011). Tiger poaching is thought to be mainly driven by the international demand for tiger parts in traditional Asian medicine (Ellis, 2005; Jackson, 1990), while prey poaching may be driven by more localised demand (Damania et al. 2003; Mohsanin et al. 2013) and trade (Knapp et al., 2010; Madhusudan and Karanth, 2002).

However, due to the difficulty and risk involved in studying these covert and illegal activities (Karanth and Stith, 1999; Madhusudan and Karanth, 2002) it has been difficult to collect the information needed to address this problem across the 76 tiger conservation landscapes (Sanderson et al., 2006). Critical to assessing the level of tiger and prey poaching across each landscape, is the monitoring of the spatial scale and intensity of these threats to enable conservationists to design effective interventions, and to be able to monitor the impact of their activities (Duangchantrasiri et al., 2016; Hotte et al., 2016; Johnson et al., 2016; Stokes, 2010). To date, few studies have assessed the scale and spatial intensity of tiger and prey poaching to design improved law enforcement strategy at a specific site of Sumatra (Linkie et al., 2015; Rifaie et al., 2015).

To refine patrolling strategies and enhance evidence gathering efforts, it is also necessary to catalogue the specific methods that poachers employ (Karanth and Stith, 1999; Watson et al., 2013; Linkie et al., 2015). Previous studies have identified some site-specific poaching methods for tigers such as iron spring traps in India (Wright, 2010), traditional common wire cable, traps and gun in Sumatra (Linkie et al., 2015;

Shepherd and Magnus, 2004; Treep, 1973), direct shooting in the Russian Far East (Goodrich et al., 2008) , and poisoning by pesticides in Sumatra and India (Tilson et al., 2010; Treep, 1973; Wright, 2010) and explosive traps and snares in Laos and Cambodia (Johnson et al., 2016; O’Kelly et al., 2012). Likewise, the methods for prey poaching documented so far include guns and snares in India (Madhusudan and Karanth, 2002), snares in the Sundarbans (Jagrata Juba Shangha, 2003; Khan, 2004), and traps in Sumatra (Linkie et al., 2015).

The Sundarbans Reserve Forest (SRF) of Bangladesh currently has incomplete information on the scale, intensity, and methods of tiger and prey poaching. The SRF is part of the wider Sundarbans landscape, which is classified as a tiger ‘source site’ (Walston et al., 2010a) and a Class III Tiger Conservation Landscape of global priority (Sanderson et al., 2006). Tiger and prey poaching have been highlighted as key threats in this landscape for several decades (Ahmad et al., 2009; Aziz et al., 2013; Salter, 1984), and the nature and scale of local use or consumption of tiger and prey parts as well as people involved in tiger killing has recently been documented (Mohsanin et al., 2013; Saif et al., 2016, 2015). Over the last few years, law enforcement agencies have confiscated piles of tiger skins, bones, and live tiger cubs in the country (Table S1; Fig. 1). A pilot study also managed to gain insight into the scale of general illegal activities in the SRF (Hossain et al., 2016), but data on tiger and prey poaching inside the forest are still lacking.

The objectives of our study in the SRF were, therefore, to (1) identify tiger and prey poaching methods, (2) assess the spatial intensity of poaching activities, and (3) identify the factors influencing the spatial distribution of poaching. To this end, we collected and analysed field data on tiger and tiger prey poaching incidents sampled from four representative areas of the SRF. We believe that our findings will be useful in developing focused patrolling and effective law enforcement strategies to secure the survival of tigers in the SRF, and present an approach that could be replicated across all landscapes where large carnivore and ungulate poaching are a threat.

Methods

Study site

The SRF is 6,017 km², of which 1 750 km² is water (Iftekhar and Islam, 2004) consisting of a maze of rivers and creeks that make most of the forest areas accessible by water-based vessels. The SRF is bordered on the south by the Bay of Bengal and on the west by the international boundary with India, demarcated by the Raimangal and Hariabhangra rivers. The north and east sides are bounded by districts of densely populated human settlements (Hussain and Acharya, 1994) (Fig. 2).

The SRF has a high diversity of floral communities comprising 330 plant species dominated by *gewa* (*Excoecaria agallocha*) and *Sundri* (*Heritiera fomes*), and a diverse assemblage of vertebrate fauna including eight species of amphibians, 35 species of reptiles, over 300 species of birds, and 42 species of mammals (Islam and Wahab, 2005; IUCN–Bangladesh, 2001). The major ungulates which make up the tiger's prey are the Spotted deer (*Axis axis*), Wild boar (*Sus scrofa*), Rhesus monkey (*Macaca mulatta*) and Barking deer (*Muntiacus muntjak*) (Khan, 2008).

The SRF is managed as a Reserve Forest and three areas within the forest are designated as wildlife sanctuaries: Sundarbans West (715 km²), Sundarbans South (370 km²), and Sundarbans East (312 km²). These sanctuaries have been collectively declared a UNESCO World Heritage Site (Iftekhar and Islam, 2004). Administration of the SRF is overseen by three Divisional Forest Officers (DFO East, DFO West and DFO Wildlife) working under a Conservator of Forests based in Khulna. For management purposes, the SRF is delineated into 55 compartments under four ranges, with over 90 guard posts distributed across the forest (Ahmad et al., 2009) (Fig. 2).

The SRF provides a wide range of forest and aquatic resources which are fundamental to the wellbeing of local communities (Islam and Wahab, 2005; Tamang, 1993). Several million people earn their livelihood from the SRF by collecting fish, *golpata* (*Nypa fruticans*) and honey (Ahmad et al., 2009; Tamang, 1993). Fishing activities continue throughout the year but the collection of *golpata* and honey usually starts between February and April, and lasts for a few months. The Bangladesh Forest Department issues permits for limited collection of these resources across the SRF, apart from the wildlife sanctuaries. The fishermen, however tend to move towards the

wildlife sanctuaries to benefit from the perceived better fish stock in these areas (Russ and Alcalá, 2011).

No permanent human habitations exist within the forest, except forest department, navy, and coast guard camps. However, there are some temporary fishing villages on several islands (e.g., *Dublar char*) located on the south edge of forest where *ca.* 8,000 fishermen make their temporary home for fishing activities in the winter months (October to March) (Huda and Haque, 2001).

Sampling approach

We selected four areas (totalling 1,994 km²) to sample within the SRF; East Wildlife Sanctuary with additional areas (ES, 383 km²), West Wildlife Sanctuary (WS, 715 km²), Satkhira Block (SB, 342 km²), and Chandpai Block (CB, 554 km²) (Fig. 2). We selected these areas as they differed in location, protection status, and human use. 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 (Fig. 2).

Following approaches used in other studies (Kimanzi et al., 2015; Wato et al., 2006; Watson et al., 2013), to select sampling points, we first divided each of our areas into 2×2 km grid cells, creating a total of 373 grid cells for potential sampling across the four areas. We then aimed to sample all grid squares with three separate transects (using one transect each time), walked by teams of four observers. Starting points for each transect were selected by where the grid cell could be easily accessed by boat. From the start point the observers walked a transect roughly in the direction of the opposite side of the grid square. Each transect was continued for a length of 1 km, or until the observers could not continue further because of particularly dense habitat or a large water body. The observers walked in parallel along the transect line, with the distance between the first and last observer being kept to 15 m (5 m between each observer).

Five teams of four observers were used to simultaneously survey a sample area over a short (13-22 days) period of time, to reduce the possibility that poachers in the area would be able to remove signs of poaching activity due to the presence of the survey teams. Teams collected data on the number, location (using a *Garmin GPSMAP 64*), and method of tiger and prey poaching evidence encountered. We also noted any

indirect evidence of poaching such as sites where poached animals had been stored or processed. If a suspected poisoned bait carcass was encountered, we collected a sample of the poison. We then analysed the poison in the laboratory of the School of Biosciences, University of Kent, using liquid chromatography-mass spectrometry (Ameno et al., 2001; Reljić et al., 2012), to identify what type of poison it was.

We chose winter months for sampling to avoid extreme weather conditions, with sampling of SB area from 20 November to 11 December 2014 and WS area from 17 to 30 December in 2014, and with sampling of ES and CB areas from 4 to 26 February 2015. We 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. 27 grid cells were not surveyed at all due to inaccessibility and security issues.

Covariate selection and analysis

We considered a set of four covariates that might have influenced poachers on selecting sites for poaching activities: protection status (wildlife sanctuary versus reserve forest), distance to the nearest forest guard post, distance to the nearest river, and distance to the nearest human habitation. The protection status was included to investigate if poaching was distributed due to the perceived differences in protection levels (Watson et al., 2013) or abundance of tiger and prey (Kimanzi et al., 2015). Distance to forest guard posts was included to investigate if the actual intensity of protection influenced the distribution of poaching (Kimanzi et al., 2015). The distance to the nearest river was used as a covariate to investigate if poachers selected sites close to rivers because of the ease of access to those areas (Fitzgibbon and Mogaka, 1995). Likewise, the distance to forest boundary was used to investigate if areas closer to human habitation also had higher poaching levels due to ease of access (Hoffer et al. 2000; Wato et al. 2006). The covariates were analysed with respect to the density of all types of poaching evidence within a sampled grid square. Grid squares where poaching activities were not detected were not used in the analysis because of issues relating to imperfect detection (Lahoz-Monfort et al., 2014). Preliminary data analysis using a generalized linear model indicated that the dataset was over-dispersed (Crawley, 2007; Zuur et al., 2010), so we used a negative binomial regression model with Poisson distribution commonly applied for over-dispersed data (Kimanzi et al., 2015; Zuur et al., 2010).

We performed an initial analysis on our explanatory variables according to Zuur et al., (2010) to confirm that none were collinear. We also performed the global Moran's I test for each sampling area independently to check for potential spatial autocorrelation, which would be a potential constraint for regression analysis (Koenig, 1999).

Following approaches in other studies (Bavaghar, 2015; Rivera et al., 2013), we prepared a risk map with different levels of probability relating to poaching activities in the SRF. Using parameter estimates of the negative binomial regression model, the probability of poaching activity (P) was determined by,

$$Y = \beta_0 + \sum \beta_i X_i$$

where β_0 is the constant coefficient, β_i represents the significant independent variable coefficients, and X_i represents their associated independent variables. Through incorporating the natural exponential (e) into the previous equation the probability of poaching activity was constructed by the following equation,

$$P = e^Y / 1 + e^Y$$

One-way analysis of variance (ANOVA) was used to investigate the differences in poaching activity between sampled areas.

We used R (R Core Development Team 2016) and ArcGIS 10.3 for our statistical and spatial analyses.

Results

Poaching methods

The only tiger poaching method detected was poisoned baits. Spotted deer, the principal prey of Sundarbans tiger, was used as bait in all cases. Poisoned bait was typically attached to a tree trimmed to the approximate height of a tiger, and placed next to tiger trails (indicated by tiger tracks). A single spotted deer was used to create 2 bait stations, with body parts being prepared by removing the intestines, dismembering, skinning, and coating in poison (Fig. 3A). The liquid chromatography-mass spectrometry analysis identified the poison as a carbamate pesticide (Carbofuran).

The prey poaching methods detected were snares and shooting. The snares were set up to target either catch the deer's neck or leg. The neck snares were either set up individually (locally known as *fush*) or in lines of multiple snares all tied to a single rope (locally known as *daon*). The *fush* snare is held by small sticks or tree branches (for holding and acting as a trigger) with an open noose placed vertically above the ground. Each individual snare was positioned and fenced by sticks and twigs to direct the ungulate prey towards the snare set. The *daon* snare is placed in a line by clearing the forest undergrowth and suspending hundreds of nooses suspended vertically from a common rope that is tied on both ends to trees. (Fig. 3B).

The leg snare (locally called *chhitka*) contains a loop placed on animal trails and attached to a spring pole (usually adjacent to a small trimmed tree) by a fine trigger thread, with a fence of twigs and sticks to guide animals into the snare (Fig. 3D). The *chhitka* snare is the technically complex snare type, and was often found set up in conjunction with *fush* neck snares (Fig. 3D). We noted that poachers used twigs and leaves of Keora (*Sonneratia apetala*) and epiphytes as bait on either side of a snare set up in order to lure ungulates (Fig. 3C). Both neck and leg snares were made from a nylon rope (80-100 mm diameter) that is commonly used for fishing nets, which is locally available, and inexpensive. All snares appeared to be set with higher intensity around ungulate trails.

The method of shooting deer was indicated from one case of a deer carcass with bullet wounds (Fig. 3E). We also observed small-sized snares (n=9) targeted for the red jungle fowl (*Gallus gallus*) in the SB.

Poaching intensity

Of the six tiger poison baits, four were recorded in the WS and two in the ES. No bait stations were found in the CB and SB. We recorded 1,427 ungulate snare loops in 56 snare sets across the SRF. Of these, 1,141 snare loops were found in 12 *daon*, 237 snares in 15 *fush* and 29 *chhitka*. Overall, 83% were neck and 17% were leg snare sets. The number of snare loops in each set ranged from a single neck snare (usually *chhitka*) to a maximum of 296 neck snares in a single *daon*. The estimated mean density of snare set was 6 snare sets/100 km² of forest, which is equivalent to 273 snare sets (95% CI: 204-341) for the whole SRF landscape. With an average of 23 (range = 1-296, SDEV = ±54) snare loops/set, this is equivalent to approximately

6,268 (95% CI = 4,692-7,843) snare loops set out in the SRF at any one time, or 147 snare loops/100 km².

Dead animals found in snares were one Spotted deer, one Wild boar, and one red jungle fowl, all of which were found in the SB. In addition, we observed ungulate slaughter locations in the ES with evidence of Spotted deer skin (n=5), and in the South wildlife sanctuaries with skin, guts, and head of Spotted deer (n=15) and Wild boar (n=2). In the SB we released a live Spotted deer and a Rhesus monkey from snares.

Drivers influencing poaching intensity

Overall higher poaching activities were recorded in WS (37%) following ES (25%), SB and CB (19%) areas. One-way ANOVA analysis ($\alpha=0.05$) showed that the difference in poaching activities between sample areas was not significant, $F(3,59) = 2.169$, $p = 0.101$. The negative binomial regression model identified two significant drivers that likely influenced poachers to select sites for poaching activity in the SRF: distance to forest guard posts and distance to the nearest river (Table 1). The occurrence of poaching activity was significantly positively correlated with the distance from forest guard posts ($\beta = 0.06$, $SE = 0.03$, $p = 0.027$), and significantly positively correlated with the distance from the nearest river ($\beta = 2.97$, $SE = 1.03$, $p = 0.004$). However, protection status ($\beta = 0.05$, $SE = 0.51$, $p = 0.925$) and distance from the forest boundary ($\beta = -0.06$, $SE = 0.51$, $p = 0.149$), did not significantly predict the number of poaching activities (Fig. 4).

Discussion

Poaching methods

This is the first field-based study to specifically identify carbofuran as a poison used to kill tigers in the Sundarbans. Although previous studies reported unknown poison in baiting carcasses (Neumann-Denzau, 2006), and arrestees with unidentified liquid intended to poison tiger's kill in the SRF (Khan, 2004). A recent study based on interview data reported range of poison including carbofuran used in tiger killing (Saif et al., 2016). The carbofuran pesticide used to poison carcasses to catch kills tigers is readily and cheaply available in local markets, and is widely used in crop production worldwide (Reljić et al., 2012). While it appears that the use of carbofuran to kill

tigers is significant, it is not well reported in peer-reviewed literature, though there are numerous reports of its use in poisoning other wild animals (Guitart et al., 2010; Hernández and Margalida, 2008; Jung et al., 2009; Satar et al., 2005; Wobeser et al., 2004). In Africa in particular, carbofuran has led to substantial reductions in populations of lions (Frank et al., 2006), vultures and large mammals (Brown, 2006, 1991) and hyenas (Hofer and Mills, 1998). Use of such poisons may kill both the target animal and any other animal that consumes the poisoned carcass. In the SRF, for example, this would include monitor lizards, Wild boar, and lesser adjutant storks (Adam Barlow, personal observation). This is supported by our study, which found four dead monitor lizards within 3 m of a poisoned tiger bait. Carbofuran is classified by the US Environmental Protection Agency (EPA) as a group I toxin, and in most cases, animals die from respiratory failure following ingestion (Tomlin, 2000).

Poaching of deer with snares has been reported from the SRF over several decades (Jagrata Juba Shangha, 2003; Salter, 1984). A previous study documented a case where poachers had been arrested with a snare intended for deer poaching in the ES (Khan, 2004), but our study is the first to document the different types of snare sets used in the SRF. Of all the snare types identified in the SRF, the *daon* snare, with its multiple snare loops, was particularly destructive, as it had the potential to capture large number of animals at a time. The observed practice of setting snares near to trails and using prey-preferred bait plants suggests that poachers have been well adapted to the SRF landscape and applied local knowledge about the species' behaviour to increase their chances of success, as poachers have done in other landscapes (Gadgil et al., 1993).

Interestingly, in one instance we found a plastic sack full of snares, which may suggest that the poachers store their snares in the forest rather than carrying with them. This practice may reduce the poaching effort and also reduce the chances of capture with incriminating evidence by authorities.

Although poaching of tigers using snare traps or cable snare has been detected in most tiger range states (Johnson et al., 2016; Shepherd and Magnus, 2004; Wright, 2010), in the SRF the snare materials, placement, and association with prey food suggest that the snares were set up to only target tiger prey. Similar to the poison baits, snares may also lead to the capture, injury, and death of non-target species (Barlow, 2009). For

example, in 2013 in the SRF a tiger was seen with the loop of a nylon snare tightly constricting its forearm. Another tigress were rescued from a village adjacent to the SRF in 2012 that had probably escaped from a prey snare and had lost its right hind leg (Reza et al., 2012).

Although only detected once in this study, poaching of tigers and prey by shooting with a gun may be more widespread in the SRF, as it is in other South-Asian landscapes (Madhusudan and Karanth, 2002). For example, in 2016 a group of deer poachers was arrested with hides and guns from the SRF.

Poaching intensity

It seems reasonable to conclude that tiger poaching, particularly by poison bait, could be one of the underlying causes of the recorded decline in relative tiger abundance in the SRF over the past 7 years (Aziz et al., 2013; Rahman et al., 2012). Likewise, the estimated 147 snare loops/100 km² in the SRF indicates a widespread and large-scale threat to the tiger's prey base in the SRF. This level of snaring intensity in the SRF is very high compared to approximately 21 trap sets/100 km² recorded in Kerinci Seblat National Park of Sumatra (Linkie et al., 2015). The estimated snare loop density in our study is also higher than the estimate of 55 snares/100 km² reported from the Tsavo National Park, Kenya where bush meat hunting is a common practice (Wato et al., 2006). The widespread and intensified prey snaring in the SRF is likely driven by the high levels of prey meat consumed by local people, that may account for 11,195 deer being killed annually (Mohsanin et al., 2013). The continued reduction of the tigers' prey base may well also be contributing to the overall recent decline in tiger abundance (Chapron et al., 2008; Karanth and Stith, 1999). However, additional modelling of the response of the tiger population to tiger and prey poaching levels is needed to better quantify these threats.

Drivers influencing poaching intensity

The relatively high concentrations of poaching activities within the sanctuaries, may be due to the relatively high density of tigers and prey in these areas (Dey et al., 2015). We found that there was higher poaching intensity in the WS compared to the ES, which is in line with a recent study assessing the frequency of illegal human activity associated with wildlife crimes detected in these areas (Hossain et al., 2016). Of note, the highest number of prey snare loops in a single *daon* (n=296) was also

recorded in the WS. The positive relationship of poaching activities with the distance to forest guard posts suggest that poachers avoid these guard posts to reduce their chances of detection from the authorities. This also explains why a high intensity of poaching activities was recorded in the south and southwest areas of WS, where two forest guard posts were either destroyed or temporarily abandoned (Fig. 4).

While seemingly reducing their chances of detection, poachers seem to be reducing their effort by carrying out their activities close to the navigable rivers and creeks. Unlike in other landscapes where poachers avoid transport networks (Kimanzi et al., 2015), the poachers use of the SRF transport network may indicate either a low level of patrolling by the authorities (Hossain et al., 2016) or an ability of the poachers to disguise their intentions while using the transport network. The reduction of effort may also have the added advantage of enabling the poachers to check the bait and snare sets more regularly to avoid animals escaping or decomposing (Wato et al., 2006). Our observations of intensive human foot-prints around baits and snares suggest that poachers checked their bait and snare regularly to ensure a timely capture of ensnared or poisoned animals, and replacement of bait.

Our finding that there was no significant effect of distance from the forest boundary on bait and snare intensity in the SRF differed from studies in other landscapes where poaching signs were shown to either increase with distance to the forest boundary (Wato et al., 2006; Kimanzi et al., 2015), or decrease with the increase of distance from the forest boundary (Fitzgibbon and Mogaka, 1995). However, our results approached significance ($P = 0.101$), suggesting that there was some effect that may have become significant with larger samples.

A camera trap study that detected high levels of activities such as fishing and wood collection in the SRF wildlife sanctuaries did not detect any poaching activities (Hossain et al., 2016), despite there being high numbers of snare and bait sets in those areas. This indicates that poaching in the SRF is being carried out as an ancillary activity by other types of resource collectors, where nylon rope in particular can be used and transported for repairing fishing gear or making snares. This would be a similar situation to other landscapes such as Tsavo West National Park, Kenya, where honey gatherers were found to be setting snares while staying in the forest (Wato et

al., 2006). Alternatively, poachers could be disguising themselves as honey collectors or fishermen, or simply traveling with these groups.

Conservation implications

This study demonstrated a simple approach to investigating the methods, intensity and distribution of poaching, that could be replicated across all tiger landscapes to better direct mitigating actions and monitor changes to tiger and deer poaching. However, the approach we used does not appear an effective means of detecting the poachers themselves. Like the SRF, many conservation situations involve dealing with poachers that are difficult to detect because they actively avoid check points and/or disguise their activities. Although reducing poaching ultimately requires tackling the demand for the wildlife products outside the forests, there is scope to improve how poachers are detected and identified while trying to catch animals in the forest. For example, the effectiveness of existing patrolling techniques could be increased by concentrating efforts away from guard posts and close to waterways, using randomised patrol times and routes, and searching boats for poaching implements and captured prey. Likewise, improving anti-poaching intelligence networks is an effective and cost-effective way to strengthen any patrolling efforts, as well as incorporating the use of new intelligence technologies and software to enhance poaching detection. While drones or remote sensing methods may be able to detect the presence of vehicles and people in a landscape, they do not necessarily facilitate the linking of those vehicles and people to an illegal activity. As an alternative, our study suggests that remote camera traps would be a useful tool to identify poachers when they return to their snare or bait sets. Camera traps are relatively low cost, are generally more robust and harder to detect, and can produce better evidence than a forest guard trying to collect the same evidence. However, camera traps may be less intrusive than ranger patrols but they should be hidden and made secure to reduce the risk of detection and theft.

Wildlife sanctuaries in the SRF are closed to all resource extraction, including fishing and honey collection. Since the only access to the sanctuaries is by boat, excluding people from these areas would relatively simple matter by increasing the surveillance and patrolling of rivers and waterways. Boats are easily detected, relative to poachers travelling on foot through dense forest as is the case throughout much of tiger range, and therefore could be easily captured by rapid response teams equipped with

appropriate speedboats. Future developments would include technology like drones and camera traps with real time GSM or satellite uplinks which could help to guide patrols to suspect boats.

In addition, this study and others suggest that there is a risk of wide spread use of carbofuran as a poison to kill tigers across their range. Tigers have been killed by an unidentified poisons in Nepal (Martin, 1992), India (Wright, 2010) and Sumatra (Tilson et al., 2010), and the use of carbofuran is commonly reported by conservation practitioners in S and SE Asia (J. Goodrich, unpublished data). Banning the use of carbofuran, or at the very least restricting its use in the SRF and other tiger range states would be an important first step in making it more difficult for poachers to use this method. In other parts of the world such as the European Union, Canada, the United States of America and parts of Africa, carbofuran use has already been prohibited or severely restricted (Ogada, 2014; Reljić et al., 2012; Ruiz-Suárez et al., 2015).

Tables

Table 1

Predictors associated with poaching activity in the SRF using negative binomial regression fit to Poisson distribution.

Response	Predictors (drivers)	β	SE	Z value	Pr(> z)
Occurrence of poaching activity	(Intercept)	4.43	0.51	8.65	2e-16
	Protection status	0.05	0.49	0.09	0.925
	Distance to guard post	0.06	0.03	2.21	0.027
	Distance to the river	2.97	1.03	2.87	0.004
	Distance to forest boundary	-0.06	0.51	-1.44	0.149

Table S1

Records of seizures of tiger parts in Bangladesh from 2011 to 2016*.

Date	Location of seizure	Tiger parts seized	Seizure notes
26-Aug-16	Koyra, Khulna	1 tiger skin	Six poachers arrested
21-Jul-16	Paikghacha, Khulna	Tiger bones (15 pieces)	Two poachers arrested
04-Mar-16	Bhatiagat, Khulna	1 tiger skin	Two traders arrested
04-Mar-16	Koyra, Khulna	1 tiger skin	Two traders arrested
26-Aug-15	Khulna city, Khulna	1 tiger skin	Two smugglers arrested
25-Aug-15	Koyra, Khulna	Tiger bones (5 pieces)	Bones recovered by Forest Department
09-Aug-15	Mandarbaria, Sundarbans	3 tiger skins	Five poachers arrested
08-Aug-15	Rupsha, Khulna	Tiger bones (69 pieces)	Two poachers arrested
09-Jun-15	DCC market, Dhaka	1 tiger skull, 15 bones	Also seized 20 vanity bags made of skins of tiger, fishing cat, monitor lizard and snake
13-May-15	Sarankhola, Bagerhat	1 skull and 157 tiger bones, deer snares	One poacher held
05-Feb-15	Tala, Satkhira	1 tiger skin	One poacher held
25-Feb-15	Assassuni, Satkhira	1 tiger skin, 4 deer skins	Three poachers arrested
20-Feb-15	Bhandaria, Pirojpur	1 tiger skin, 14 deer skins	One poacher held
19-Jan-15	Kalabagan, Dhaka	1 skin, 5 deer skins	Tiger skin had bullet holes indicating killed by gun
14-Jan-15	Morrelganj, Bagerhat	1 skin, 1 skull, 25 bones, 29 teeth	Skin was about 10 feet long; immediate destination was Bagerhat
17-Oct-14	Satkhira sadar, Satkhira	2 tiger skins	Six poachers arrested with 2 fresh skins without any bullet signs
27-Jan-14	Khulna, Sundarbans	1 injured tiger	Female tiger rescued but eventually died; tiger escaped from rope snare on her right arm
13-Apr-13	Uttara, Dhaka	1 tiger skin	Two foreigners arrested with a skin
11-Jun-12	Shaymoli, Dhaka	3 tiger cubs	Honey collectors caught them live from Satkhira range and then handed over to group of smugglers
08-Dec-11	Mothbaria, Pirojpur	1 tiger skin, 18 pieces of bones	No sign of bullet or trap on skin; destination was Benapole close to Indian border
16-Feb-11	Sarankhola, Bagerhat	3 skins; 4 skulls, 32 kg bones (138 pieces) of tigers	Tigers poisoned with baits comprising 2 males and 1 female, with another skull

* This information was collected from different secondary sources, and validated with wildlife crime database of WildTeam, which keeps records only after verification with Forest Department and law enforcing agencies.

Figures

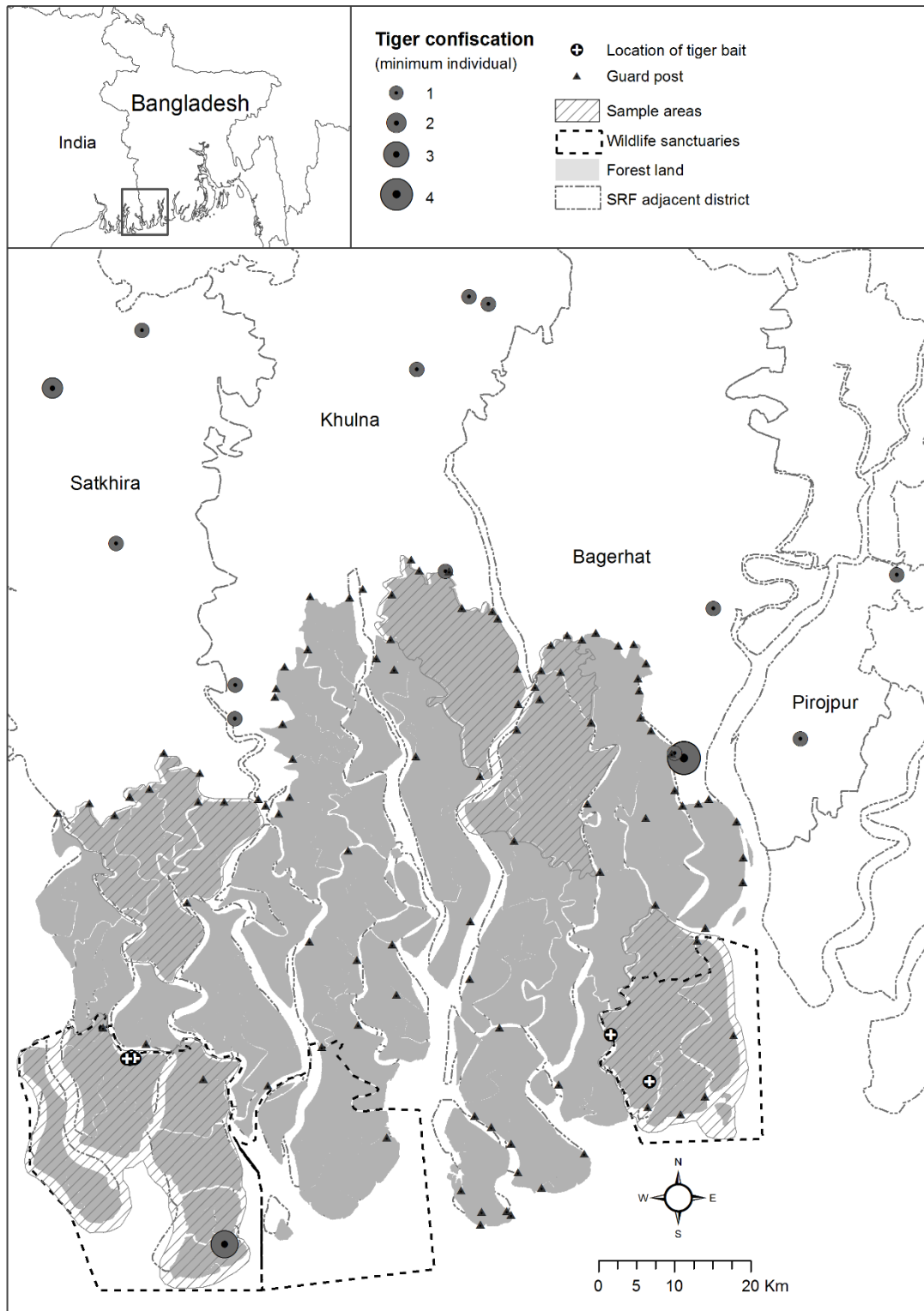


Fig. 1. Tiger confiscation locations in and around the SRF in relation to sampling areas, tiger bait stations and guard posts. Data of tiger confiscations were collected from secondary sources after validation against the wildlife crime database of WildTeam, which keeps records only after verification with Forest Department and law enforcing agencies.

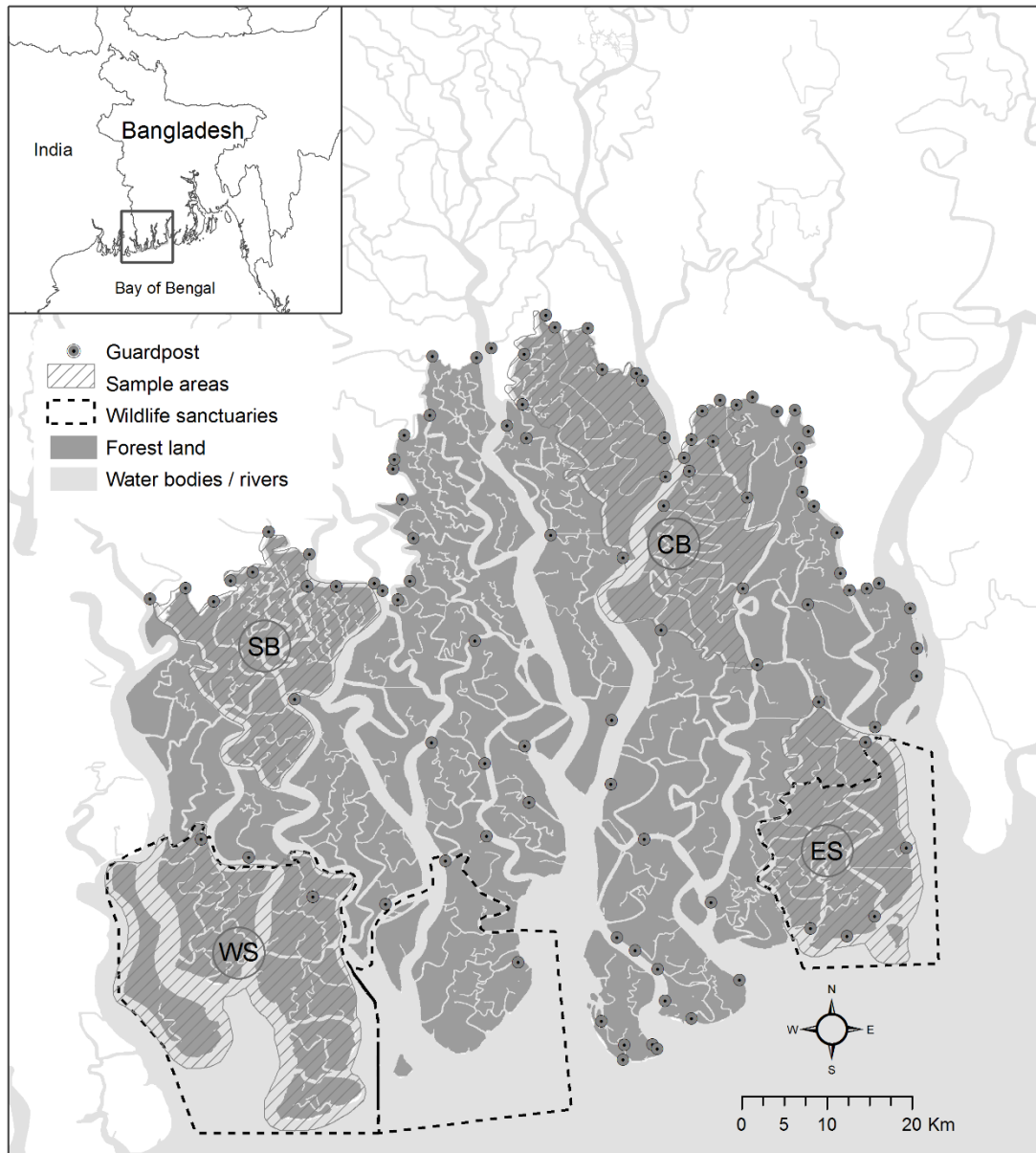


Fig. 2. Sampling area and associated features of the Bangladesh Sundarbans. SB = Satkhira Block, CB = Chandpai Block, WS = West Wildlife Sanctuary, ES = East Wildlife Sanctuary.



Fig. 3. Tiger bait, snares and killed tiger prey in the SRF: (A) Tiger bait station, (B) *Daon*, (C) *Fush* with bait, (D) *Chhitka*, and (E) Spotted deer that died from bullet injuries.

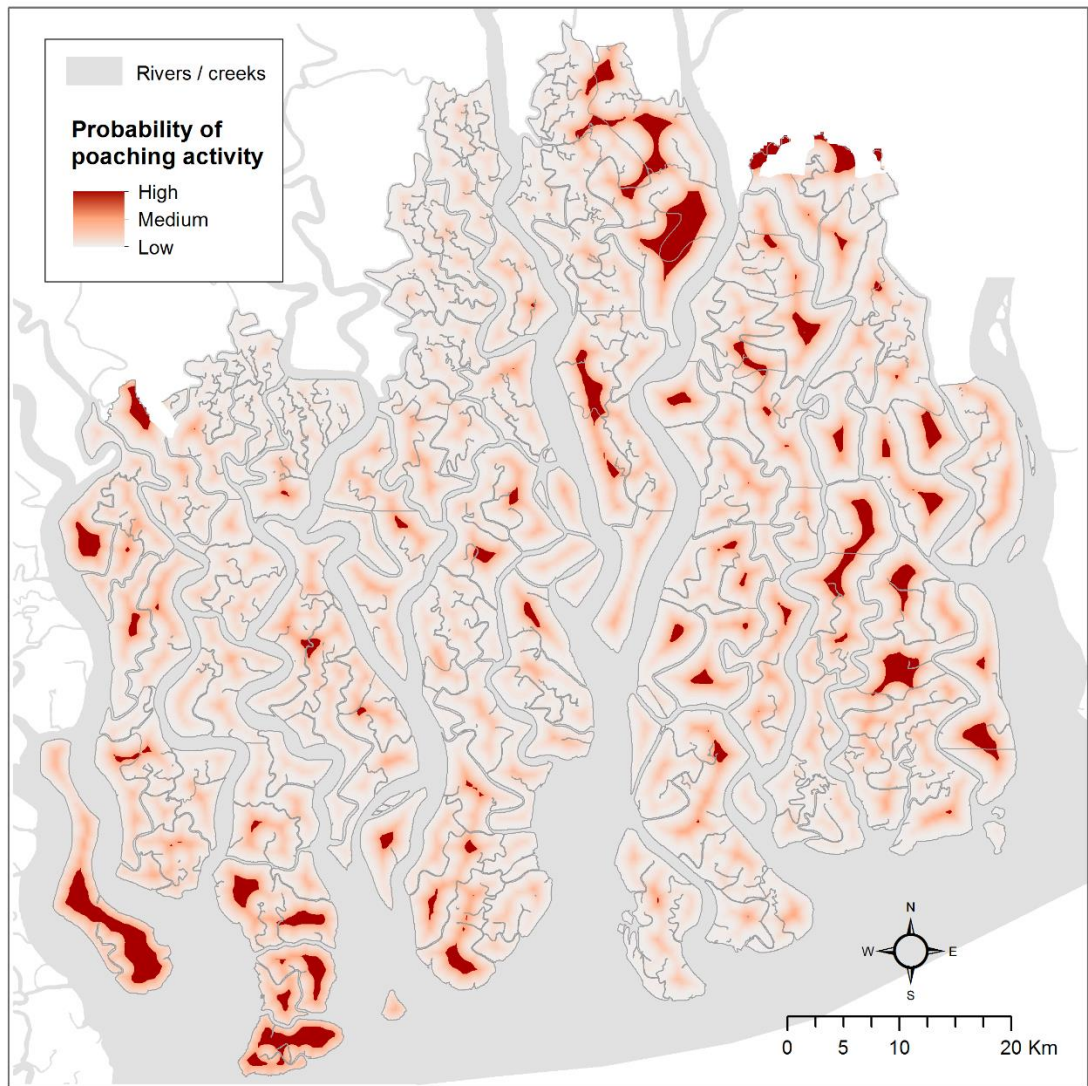


Fig. 4. Probability of poaching activity derived from negative binomial regression coefficients of the distance to guard posts and distance to water.

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Chapter 7
General discussion

The tiger is a flagship species for successful conservation of forested ecosystems where it occurs (Tilson and Seal, 1987). The Sundarbans represents a unique global priority Tiger Conservation Landscape, (Sanderson et al., 2006) and supports one of the most globally important tiger populations (Dinerstein et al., 2007; Gopal and Chauhan, 2006; Sanderson et al., 2006). The Bangladesh Sundarbans is the last stronghold for critically endangered tigers in the country, representing nearly half of the remaining forest of the country (Hussain and Acharya, 1994), and has provided a wide range of economic and ecosystem services to millions of people in local communities for centuries (Biswas et al., 2008; Islam and Peterson, 2008). Therefore, ensuring the continued existence of the forest relies on the survival of the tiger and its role as a flagship species of the ecosystem (Ahmad et al., 2009). Prior to the onset of this PhD research, information was lacking on many aspects of tigers living in the Sundarbans forest, including knowledge about their phylogenetic history and affiliations and genetic structure (Ahmad et al., 2009). This study has investigated these important areas of research and has produced a number of original research findings that fill the gap in knowledge for tigers in the Sundarbans.

Genetic sampling is a viable population monitoring method for tigers

Facing a myriad of challenges in applying conventional photographic camera-trapping methods for monitoring tigers in the Sundarbans (Karanth, 1995; Karanth and Nichols, 1998; Khan, 2012), a non-invasive genetic sampling technique was applied for the first time to investigate the population status of Sundarbans tigers. Overall this study provided exciting results (121 tigers; 95% CI: 90-164), with population estimates aligning to previous estimates (Dey et al., 2015), demonstrating that non-invasive sampling as a viable method for monitoring tigers in the Sundarbans. This sampling technique can be applied elsewhere in the tiger range where application of camera-trapping is challenging due to constraints in relation to logistics and habitat conditions.

The decline of the Sundarbans tiger population from as many as 300-500 tigers (Barlow, 2009) to only 106 tigers (Dey et al., 2015) in over five years is a catastrophe for the Sundarbans landscape. This steep decline undoubtedly jeopardizes the future of this important population because the long-term persistence depends on the size of the population. As such, a population with a large number of individuals is considered to

be able to more effectively retain its genetic diversity and maintain evolutionary potential, which increases their ability to adapt to changing environmental conditions (Vrijenhoek et al., 1985). On the other hand, reduction in population size leads to loss of genetic diversity, inbreeding and increase extinction risk (Frankham and Briscoe, 2002). The finding from this PhD study, an estimate of 121 individuals based on genetic surveillance, has added greater support to the estimated size of the Sundarbans population and therefore will place on a firmer footing future plans for conservation management of the population.

The Sundarbans tigers is polyphyletic within the Bengal tigers

The Sundarbans tigers were traditionally assigned to Bengal tigers, but more recently have been identified as morphologically smaller than other tigers (Barlow et al., 2010). Although the genetic status of this population has been previously investigated using limited samples collected from the Indian Sundarbans (Singh et al., 2015), the genetic ancestry and phylogenetic relationships of this tiger population was poorly known. By generating an extensive mtDNA database through collection and sequencing of samples from across the widespread Bangladesh Sundarbans, this study revealed that the Sundarbans tigers have retained three informative haplotypes, including one haplotype unique to that area, suggesting that this population is genetically more diverse than many of the remaining tiger populations elsewhere. The support of Maximum likelihood and Bayesian inferences placed the Sundarbans tigers as a polyphyletic group within the Bengal tigers. The combined ecological, demographic, and biogeographical factors might have played a key role in producing the unique phylogenetic position, and reflecting the morphological distinctiveness of the Sundarbans tigers (Barlow et al., 2010). Therefore, the ecological and phylogenetic uniqueness of the Sundarbans tigers comply with the ESU criteria, and the population should be managed in appropriate manner in the situation when global tigers are historically low (Walston et al., 2010), and left with only *ca.* 1000 breeding females (Karanth et al., 2010). This study also revealed a close phylogenetic relationship between tigers living in Bangladesh and those living in the Indian Sundarbans.

Rivers influence genetic structure of the Sundarbans tigers

Assessing fine-scale genetic structure of the currently isolated global tiger populations is critical for understanding the impact of landscape barriers on the dispersal of tigers between populations. The Sundarbans tigers have adapted to a unique mangrove landscape dominated by many wide rivers but no study has investigated the impact of these rivers on tiger dispersal, and consequently on genetic structure of this globally important tiger population. Therefore, a suite of microsatellite and mitochondrial markers were applied to investigate fine-scale genetic structure using non-invasively collected genetic samples from across the Bangladesh Sundarbans. The combined microsatellite and mtDNA analyses revealed a signal of fine-scale genetic structure, and detected significant genetic differentiation within the population in the presence of some of the larger (wider) rivers that bisect the Sundarbans, suggesting that these river systems may limit genetic exchange.

Previous studies have found significant influence of human and road density on genetic architecture of tigers in the Indian landscapes (Joshi et al., 2013; Sharma et al., 2012). This study found an impact of river systems on the fine-scale genetic structure of tigers, although this finding is unexpected given the extreme genetic dispersal of tigers (up to 650 km between tiger reserves) in Indian landscapes (Joshi et al., 2013). The role of large rivers in delimiting the geographical distribution of a species was first noted by Wallace (1852) for Amazonian monkeys, and the importance of rivers as barriers was then highlighted by Martin (1972). Subsequent studies have showed that large water bodies are among the most obvious barriers to animal dispersal and hence to gene flow (de Queiroz et al., 2005). Several case studies have dealt with the influence of rivers on the distribution and genetic structure of species, especially in the Amazon where rivers are particularly wide enough to be an efficient barrier for a range of species (Hayes and Sewlal, 2004; Lougheed et al., 1999; Moritz et al., 2000). These studies have suggested that the influence of rivers could be specific to taxa and specific geographical barriers to dispersal. For instance, rivers strictly limited population distribution and gene flow in primates (Gehring et al., 2012; Goodman and Ganzhorn, 2004; Pastorini et al., 2003), raccoon (Cullingham et al., 2009), and white-tailed deer (Blanchong et al., 2008). This study is the first of its kind that provided insights about the effect of rivers on fine-scale genetic structuring within a globally

important tiger population across the range, and the results should be taken into account in future habitat management of the Sundarbans tigers.

The Sundarbans tigers are under threat from poaching

Poaching of tigers and their prey species has been identified as among the major threats to tiger populations across their range (Miquelle et al., 1996; Karanth and Stith, 1999; Madhusudan and Karanth, 2002; Damania et al., 2003; Dinerstein et al., 2007; Jhala et al., 2008; Wikramanayake et al., 2011; Aziz et al., 2013). However, due to the difficulty and risk involved to collect information on these covert and illegal activities (Karanth and Stith, 1999; Madhusudan and Karanth, 2002) there were little information on this problem across the 76 tiger conservation landscapes (Sanderson et al., 2006). To mitigate these threats effectively on the ground, it is also necessary to identify specific methods of poaching of tiger and their prey (Watson et al., 2013; Karanth and Stith, 1999; Linkie et al., 2015). By applying a systematic sampling approach, this study has gathered valuable information on poaching methods, intensity and driving factors in relation to tiger and tiger prey poaching in the Bangladesh Sundarbans (Fig. 1). The results showed that tigers are being poached chiefly by poisoned baits, where carbamate pesticide (Carbofuran) was used with the principal prey animal, the Spotted deer in all bait stations. The prey poaching methods employed were mainly the snares and occasionally shooting. The occurrence of poaching activity was significantly positively correlated with the distance from forest guard posts and significantly positively correlated with the distance from the nearest river. Apart from tiger and prey poaching, and a previously identified range of threats (Aziz et al., 2013), illegal tree cutting, livestock grazing, chemical pollution by vessels and cargos all seem to have a serious impact on the terrestrial and aquatic habitats of the Sundarbans. Although the boundary of the Sundarbans forest has remained almost unchanged since 1933, the overall habitat quality has deteriorated considerably (Curtis, 1933; Iftexhar and Islam, 2004). Studies have already detected changes in the structure and composition of the forest, suggesting that the forest is declining in terms of tree regeneration and ecosystem vigour (Karim, 2004; Siddiqi, 2001). During this study, plenty of signs of large-sized tree loss were noted in the eastern parts for the SRF, and from around the *Dubla* Island fishery villages.

Livestock grazing appears to be one of the emerging threats as observed over extensive areas on northeast and northwest fringes of the Sundarbans (Fig. 1h). These grazing activities may significantly impair the habitat quality, in addition to creating a chance of bi-directional disease transmission between wild ungulates and grazing livestock. Several herds of buffaloes were recorded up to 5 km inside of the Sundarbans forest in Chandpai range along with intensive livestock grazing in Baidyamari, Katakali, Jewdhara in the northeast and Kaikhali in the northwest. Moreover, livestock grazing may easily allure tigers, with increasing stray-tiger situation, leading to fatal human-tiger conflict.

Serious water pollution due to cargo incidents was observed in the recent past. For instance, cargo vessels carrying furnaces oil (350,000 litres) (9 December 2014), chemical fertilizer containing potash (300 tonnes) (5 March 2015), and coal (1,235 tonnes) (22 March 2016) were capsized in Shela and Bhola rivers in the eastern part of Sundarbans. On 15 January 2017, another vessel sank in the Passur river channel containing 1,000 tonnes of coal. Pollution from these incidents could have serious consequences on aquatic ecosystem, including aquatic organisms and vegetation structures. In relation to pollution, another impending threat is probably the establishment of a coal-based 1,320 megawatt Rampal power plant located 14 km away from northern boundary, and only 4 km from the buffer zone of the Sundarbans (CEGIS, 2013).

Future research and management directions towards tiger conservation

Work with local people for their livelihoods and wellbeing towards tiger conservation

There is near universal agreement that tigers will survive only if tigers and people can coexist (Nyhus and Tilson, 2010). Unfortunately, conflict between tigers and local people is at an extreme level in the Sundarbans landscape (Barlow et al., 2009; Inskip et al., 2013), with death records as high as 76 humans and six tigers per year between 1881 and 2006 (Barlow, 2009). Several millions of people living next to the SRF earn their livelihood while an additional 10 million people benefit from a variety of related economic and subsistence activities (Hoq, 2007; Islam and Wahab, 2005). Again, approximately, 740,000 people are directly involved with resource extraction from the SRF, where 80% are collectors, and the remaining are traders relating to such activities (IPAC, 2010). The majority of these people live in absolute poverty, with

more than half of their annual income derived from forest resources, of which 50% is raised through illegal collection of resources from the Sundarbans (Abdullah, 2014). This situation clearly reflects the level of dependency of local people on the Sundarbans for their livelihoods, and the nature of their involvement with resources collection. Therefore, the success of tiger conservation remains at the heart of ensuring alternative livelihood opportunities for the wellbeing of these local communities. Moreover, studies have recommended recognising the problem of human-tiger conflict from a human perspective, where poverty alleviation for these poor, rural and marginalised communities is needed to be urgently addressed (Inskip et al., 2013). Exploring livelihood opportunities and understanding the complex nature of the relationship between local communities and the Sundarbans ecosystems would be fundamental to address this issue towards future tiger conservation.

Achieve political will towards tiger conservation

Recognising the need for concerted and collaborative conservation action to reverse the worldwide decline of tiger populations, heads of the state from all TRCs at the St. Petersburg Tiger Summit have pledged to double the population of wild tigers by 2022 (Wikramanayake et al., 2011). While this high level of commitment might have significant impact on regional tiger conservation efforts, implementation of such pledges assumes that there are adequate resources in place for timely management actions. Unfortunately, this study has noted ineffective management actions to tackle tiger and tiger prey poaching in the Sundarbans (Aziz et al., 2017). Large numbers of illegal resource collectors have been observed within the wildlife sanctuaries which is a clear violation of protected area guidelines stated in the Wildlife (Protection and Security) Act 2012, indicating that management authorities are either unable to implement management actions or allowing such activities under corrupt practices. Moreover, apart from the forest and security (navy, coast guard) staffs in the SRF, a number of local pirate groups (locally called “*dacoit*” or “*party*”) live within the forests, primarily earning money by collecting “fees” from resource collectors, and sometimes by kidnapping if resource collectors deny payment or avoid them during their work. These illegal miscreants usually live in the remote areas of forest by making temporary shelters (“*machan*”), and they often remain armed and frequently move at night (Fig. 1g). Observations suggest that these pirates and their fellow members (e.g., illegal resource collectors) might have been involved with tiger

poaching activities, being carried out with support and networks from local “elites” and “politicians”. Studies show that corrupt practices threaten the maintenance, monitoring and protection of the world’s biodiversity and natural resources (Laurance, 2004). The threat of corruption is especially problematic in developing countries that often have high levels of biodiversity, but lack the capacity to effectively monitor and protect such resources (Smith et al., 2003). Therefore, a concerted effort from all stakeholders including local elites and politicians, with a high level of political will, is needed to eradicate poaching activities from the Sundarbans.

Monitor intelligence-based wildlife and forest crime to crack down poaching networks

Crime and intelligence analysis has gained significant attention from conservationists in recent years due to the increasing advances of various forms of information technology. Such information can be used to inform policy implications as well as contribute to situational crime prevention (Pires and Moreto, 2011). Apart from applying camera-trap or GSM technology in detecting wildlife crime within the forest, community-led intelligence may help gather crime information about village-based poachers or traders who maintain networks with forest-based poacher groups. Future research and conservation effort may explore this important issue relating to the development of local intelligence network in order to expedite quick management responses for cracking down the poaching networks.

Develop frontline staff for effective protection measures

An effective patrolling force is extremely important for the prevention of illegal activities such as tiger and their prey poaching; however, this will require skilled frontline staffs and improved logistics to deliver effective patrolling (Ahmad et al., 2009). There are 17 revenue stations and 72 guard posts stationed across the 6,017 km² Sundarbans to monitor regular management activities, giving about one staff for every 5 km² of forests (Khan, 2011). Given the vastness of the forest and a high level of involvement of local people for collecting resources from the Sundarbans, adequate number of skilled frontline staffs are extremely important to act timely to the emergency management of wildlife and forest-related crimes. Besides, frequent cyclone and tidal surges across the coastal belt including the Sundarbans area (Islam and Peterson, 2008) often leave the forest stations and guard posts severely damaged. For instance, the cyclones Sidr in 2007 and Aila in 2010 badly damaged many of these

stations and guard posts; some of them are still deserted due to lack or delay of restoration works. We detected a significant negative relationship between location of guard posts and intensity of poaching activities, and interestingly we found higher incidence of prey snaring close to the deserted guard posts in the West wildlife sanctuary.

Nonetheless, law enforcement rangers are often the primary protectors of protected areas and wildlife, yet like other law enforcement agents, they are not immune to misconduct and corruption (Moreto, 2015). Given the unique role that law enforcement rangers play, it is imperative to better understand factors that may influence their behaviour and activities for protection of wildlife and their habitats. We recorded significant amount of prey snares and deer skins within the vicinity of several forest camps, suggesting that those poaching activities might have not been carried out by professional poachers; rather very likely by some dishonest staffs of management or security agencies residing within the forest. Studies show that corruption has a potentially harmful impact on a number of conservation related issues, particularly recreational hunting (Leader-Williams et al., 2009) and illegal logging (Miller, 2011). Therefore, identifying drivers and indicators of wrongdoing can provide a better understanding on the applicability, feasibility and likelihood of success of preventive measures on the ground (Moreto, 2015).

Develop DNA fingerprinting database to aid forensic investigation

The combination of scientific, technological, and analytical methods (e.g. forensic DNA) are useful to supplement and support conservation intelligence and prosecution (Wellsmith, 2011). The genotype profiles developed in this study may be useful for future genetic monitoring of the Sundarbans tigers, in addition to provide support to the forensic investigation of confiscated tiger parts in relation to poachers arrested in the recent past in Bangladesh. Therefore, future initiative may include developing a comprehensive DNA repository of the tigers in the Sundarbans, and investigate the opportunity of forensic application to expedite the prosecution process of those poaching cases.

Assess tiger's prey populations to model prey-predator relationship

Prey populations are the critical determinant to long-term viability of tiger populations (Karanth and Stith, 1999), therefore understanding the structure, density and

distribution of tiger's prey population of the Sundarbans is urgently needed. Thus future research effort should focus on assessing the density and distribution of tiger's major prey animals of the Sundarbans in a situation when both the tigers and their prey are at stake due to extensive poaching that had occurred in the recent past (Aziz et al., 2017; Mohsanin et al., 2013).

Reduce commercial and human activities across the major rivers

Habitat connectivity is fundamental to recovering regional tiger populations, because tigers need contiguous forest corridors for dispersal and genetic exchange (Smith, 1993). Therefore, reducing the impact of commercial and resource collection activities across the wide rivers (e.g., Passur, Sibsa) of the Sundarbans is crucial to facilitate tiger dispersal for preventing further genetic differentiation within the population. There are reports that the ongoing establishment of the Rampal power plant will use approximately five million tonnes of coal annually, and this coal will be imported from neighbouring India by transporting them predominantly through the Passur rivers, including other major channels of the Sundarbans. To transport this huge amount of coal, extensive dredging will be needed to maintain the navigability of the Passur river that bisects the Sundarbans (CEGIS, 2013). This level of commercial activity will further increase the disturbance in these rivers that have already been heavily used by cargos and commercial vessels. Therefore, it is very likely that the tiger dispersal will further be hampered by these impending activities which may have severe consequence on the genetic architecture of the Sundarbans tigers. Therefore, future management of the Sundarbans habitats should aim to regulate water-based commercial cargo movement, and human activities in relation to resources collection within the larger river channels of the Sundarbans. Given the close phylogenetic relationship of tiger populations between Bangladesh and Indian Sundarbans, reduction of human presence across international boarder of the Raimangal and Hariabhanga rivers is also critical to allow dispersal of tigers across the international border.

The results presented in the preceding four data chapters are the milestones of this PhD research outlined in the Figure 7 of the introductory chapter; these outcomes will be critically important for future monitoring and genetic management of the Sundarbans tigers. In particular, Chapter 3 provided substantial evidence that the non-

invasive genetic technique can a potential supplementary method in monitoring the Sundarbans tiger population in future. Chapters 4 and 5 provided insights on the fine-scale genetic structure and phylogenetic ancestry of the Sundarbans tigers which can be extremely useful in future population and habitat management of tigers of the entire Sundarbans. Finally, the techniques and intensity of poaching, and the factors influencing spatial occurrence of these poaching activities can be integrated into improved patrolling strategy and law enforcement to tackle poaching of tigers and their prey animals in the Sundarbans.

Figure



Fig. 1. Evidences of wildlife poaching activities and threats to the Sundarbans: (a) Carbofuran collected from tiger poison bait (left) and local market (right), (b) Dead monitor lizards next to a tiger poison bait, (c) Wild boar leg entangled and left in a *chhitka* snare, (d) Dead Spotted deer in a *fush* snare, (e) Spotted deer skin in a slaughter location within forest, (f) *Daon* snare burnt after removal from, (g) Shelter (*machan*) of ‘pirates’, and (h) domestic buffaloes bathing after grazing in the Sundarbans.

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