Ex-situ and In-situ Conservation Approach for the Malayan sun bear (*Helarctos malayanus*)

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SYNOPSIS

Habitat loss and fragmentation, the illegal wildlife trade and exploitation for Traditional Chinese Medicine (TCM) threaten the sun bear \((Helarctos malayanus)\) with extinction. The confiscation or rescue of bears affected by each of these threats results in an ethical dilemma for conservation practitioners as they must decide over the fate of the confiscated bear and which decision is in the interest of long-term species survival.

To understand the conservation status of any species, it is essential to have access to as much long-term information as possible. This requires a standardized approach to communicate research results and approaches in order to inform scientists without the risk of data being mis-interpreted. Using the standardized PRISMA-p model (commonly applied in medical professions) in the systematic review of white literature on sun bears (Chapter 2) resulted in the Helarctos-database that will be made publicly available. This method allowed for a standardized reporting on the current literature and revealed that the sun bear has been neglected by the scientific community. Furthermore, it identified genetic (and genomic) research on the sun bear as one of the main research gaps.

Genetics and genomics find many applications in ex-situ and in-situ conservation. To protect species, it is essential to understand their evolutionary history and adaptation potential to a changing environment. In Chapter 3 of this thesis, the mitochondrial genomes of museum-derived, as well as contemporary, sun bear samples covering most of the distribution range of the sun bear were sequenced using next-generation sequencing methods. While phylogeography helps to resolve taxonomic uncertainties, identify species and sub-species that may be warranted conservation priority, it also allows the identification of geographical barriers that may have led to speciation events. This first phylogeographic study of the sun bear revealed that there are two lineages: the ‘mainland’ lineage and the ‘sunda’ lineage. These lineages were disconnected at the Isthmus of Kra (Thailand), due to seawater level rises during the Pleistocene. This study demonstrated that waterbodies, if large enough, can create a geographical barrier for sun bears. Understanding barriers to gene-flow is essential for the effective protection of a species.
As the human landscape alteration is causing the fragmentation of habitats, understanding the population structure, gene-flow between populations and their genetic variability is becoming essential for conservation management. Chapter 4 of this thesis demonstrates for sun bears in Cambodia how microsatellite (nuclear) markers can identify the number of populations and how these populations are connected. Microsatellite markers are a useful tool to identify hybridization and inbreeding in populations and are therefore capable of identifying populations of conservation concern. This research revealed that there are two sun bear populations, East and West of the Mekong river in Cambodia, and that these two populations are exchanging genes (as is an admixed population). In the population in West Cambodia, inbreeding was detected, indicating that the current exchange of genes between the two populations is not sufficient to maintain genetic diversity in the West population. Currently it is not known what may have caused the loss of genetic diversity of the Western population but illegal wildlife trade may be implicated. Microsatellite markers could be used to develop forensic markers to combat the illegal wildlife trade and to identify endangered animals in TCM products.

Although the sun bear is used in TCM products, it is not understood to what level the TCM industry is threatening the sun bear. The fifth Chapter of the thesis tested forensic DNA extraction protocols, extraction kits and Gas-Chromatography Mass Spectrometry (GC-MS) on different Traditional Chinese Medicine products, to determine whether these samples contain sun bear derivative. Currently there are many forensic methods with reference databases for the Asiatic black bear but none regarding the sun bear. The forensic genetic methods employed here were not successful in heavily processed TCM products as they likely did not appear to contain any intact DNA. Therefore, the further development of genetic and non-genetic forensic method to detect sun bear is required.

The final Chapter discusses how the findings of the previous Chapters can inform sun bear conservation practitioners who must decide whether they release a sun bear or whether they maintain it in captivity and advises on direct conservation action. Releasing a bear requires detailed knowledge and access to this knowledge about the species. The Helarctos-database would provide a one-stop for sun bear research. The phylogeography analysis identified that two genetically distinct lineages of sun bears. Although the study did not obtain enough samples to conclude on species and subspecies classification, understanding that the sun bears on the mainland are different to
the sun bears on the Sunda islands is important for ex-situ breeding and release efforts. The microsatellite study demonstrated that nuclear markers can identify population hybridization, geographical barrier and inbreeding. In the West Cambodian sun bear population inbreeding was detected. This informs conservation actions such as potential augmentation, establishment of connectivity and further research. This study also successfully assigned sun bears of unknown origin to a potential source population, which is critical information for release projects. Furthermore, the forensic analysis of TCM products of sun bears highlighted that forensic methods currently available are ineffective on heavily processed TCM samples and need to be further developed. Being able to test whether sun bear derivate is present in TCM products is necessary to judge the level of threat imposed by the TCM industry on the Malayan sun bear.
STATEMENT OF ORIGINALITY

This work has not previously been submitted for a degree or diploma in any university. To the best of my knowledge and belief, the thesis contains no material previously published or written by another person except where due reference is made in the thesis itself.

Miriam Nora Kunde
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PERMITS AND CONTRACTS

Here is a summary of the essential permits, certificates and contracts that were required to execute the research presented in this Ph.D. research. Further documentation and original copies of the issued permits and contracts are available on request.

Ethical Statement

The samples obtained for the research carried out were collected following the guidelines of the Animal Ethics Committee from Griffith University and approved by the committee (GU Ref No: ENV/04/13/AEC issued on 22 April 2013, and GU Ref No: ENV/02/13/AEC issued on 07 June 2013).

As the Malayan Sun bear is CITES Appendix I listed species, we required and obtained all CITES export and import permits required. We obtained import permits of the samples into Australia and obtained quarantine and custom clearance.

Quarantine Permit

The import of the samples was permitted by the Department of Agriculture, Fisheries and Forestry branch of the Australian Government. Permit to Import Quarantine Material (IP15007699, issued on 22/05/2015) IP13008469, issued 22/05/2013). Quarantine Awareness and the Quarantine Approved Premises for Accredited Persons (Class 2-8) were certified (Attainment number 31324, issued on 16/10/2014).

Multiple Use Import Permit

To import sun bear samples from any potential donating country, a ‘multiple use import permit’ has been obtained. This permit was issued by the Department of Sustainability, Environment, Water, Population and Communities branch of the Australian government (permit number PWS2014-AU-001113) and the import was reported on the Specimen Import Record 22/10/2014.

CITES Import Permit

As the sun bear is listed on the CITES Appendix I, I obtained CITES Import Permits for the samples imported from Cambodia. The permits were issued by the Wildlife trade
and Biosecurity Branch of the Australian government (permit PWS2014-AU-001113 was issued on 22/06/2014, permit PWS2014-AU-002163 was issued on 16/12/2014, and permit PWS2015-AU-000922 was issued on 02/06/2015).

**CITES Export Permits**

For the phylogeographic as well as the microsatellite study in this PhD thesis, sun bear samples from the Kingdom of Cambodia were obtained. The CITES export permit for the export of the samples from Cambodia to Australia was obtained from the CITES Management Authority of Cambodia (No KH0936, issued 10/09/2014). The movement of the Cambodian sun bear samples from Australia to Germany were permitted by the Kingdom of Cambodia and Free the Bears on 26/01/2016. The samples remain under property of the Kingdom of Cambodia and under my custodianship.

**CITES Transfer-Agreement**

The Traditional Chinese Medicine (TCM) samples analyzed in this Ph.D. research were obtained via a CITES Transfer-Agreement between the Wildlife Trade Compliancy Section and the Wildlife Trade and Biosecurity Branch of the Australian Government and myself. Through this agreement, I agreed to take possession of the 60 TCM samples provided to me. The Transfer-agreement was signed on 29/05/2015.

**Registration of Scientific Institutions for the exchange of Scientific specimen**

Griffith had been a CITES registered institution since 2003 but only for Australian native specimen. For the exchange of CITES Appendix I listed sun bear samples between Griffith University and the Institute for Zoo and Wildlife Research in Berlin, I obtained an extension to the existing approval. Griffith University has been approved for species listed on the Appendices to the Convention on International Trade in Endangered Species of Wild Fauna and Flora (AU026, extension approved by the Wildlife Trade Assessment branch of the Australian Government on 06/11/2015).

**Research Contracts**

As this PhD research was a collaborative project between Griffith University, Australia, Free the Bears, Kingdom of Cambodia and the Institute for Zoo and Wildlife Research
(IZW) in Germany, research and working contracts were negotiated and agreed upon. These contracts will be made available for revision on request.

**Transfer of Museum Specimen**

For the Phylogeographic study on the sun bear, I obtained museum sample from the Museum für Naturkunde in Berlin, Germany (9/2016, issued on 12/09/2016). These samples were obtained while I was under research contract with the IZW. The other museum samples used in this research were provided by the IZW as agreed in the collaboration contract.

**Nagoya Protocol Agreement**

The Nagoya agreement permit has not been required as all samples were collected before the 12th of October 2014 and therefore pre-date the enforcement of the Nagoya agreement.
SUPPLEMENTARY MATERIAL

Documents that are too big to be included in the body of the thesis (e.g. excel spreadsheets) are provided for review as supplementary material. Supplementary materials will be submitted as a separate document online.

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that field of conservation is best led by example. I am grateful to the conservation practitioners who have communicated and shared their unpublished data with me.

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Thank you to my fellow Ph.D. colleagues at Griffith University who cheered me up when things got tough, who had way too many coffees with me and who always appeared to be happy to listen to my endless rambling about sun bears. I thank you for your friendship and company through these years. Thank you, Michelle Baker for drawing the sun bear for my thesis cover page. Brett Parker, thank you so much for helping me with my maps, R–related struggles and your helpful comments on my written works and Joe McMahon, thank you for being my technical support. Kyle Barton, thank you for the brainstorming sessions and Aaron Sandhu for his contagious enthusiasm and motivation. Paula Marie Lewis, I cannot thank you enough! Over the course of the Ph.D. you have become my close friend, my sound of reason and an ethical role model. I thank you for all your help throughout the years, for your advice (which I would repeatedly ignore and then regret that I did so) and for being my number one contact whenever I found myself in a crisis.

This Ph.D. would not have been possible if I did not have parents, Ingeborg and Michael Kunde, who love me unconditionally and always support me, my decisions and ideas, regardless how stupid they may seem. I would also thank my siblings Jan and Judith Kunde who have always had my back and cheered for me. I am also grateful for my best friend and biggest fan Ryan Fritsch and his family for looking after me, hosting me and loving me as if I was a family member.
Contextualizing my interest in the conservation of sun bears

Having always had a very keen interest in animals, I decided to study Animal Conservation Science in the UK with the ultimate goal of one day being able to release an animal back into the wild. When I graduated in 2011, I applied for a position as a “Sun Bear Release Fellow” with a wildlife NGO in Central Kalimantan, Indonesia. This job seemed to be my childhood dream come true: I was being hired to ‘walk-release’ a sun bear cub until it was established enough to fend for itself in the wild. Excited over this once-in-a-lifetime opportunity, I started researching sun bears and the release method I was being asked to apply. Despite considerable effort, I was not able to gather much information on sun bears or the release method from the scientific literature; instead, most of the knowledge I obtained came from personal communications and grey literature. Three days before I was scheduled to arrive in Kalimantan, I received a message from the NGO that the release site for the sun bear was burnt by local farmers and that the sun bear release attempts would be called off. Although this was obviously disappointing, because of my background in animal husbandry with a focus on primates, the NGO still wanted me to work at their facility to assist with the orangutan release training and to help with the husbandry of other species.

I arrived as scheduled and was soon confronted with the far less romantic reality of conservation practices in Indonesia. Despite being a novice in reintroduction biology and mainly learning “on the job”, it was still obvious to me that even if the release site had not burnt down, the sun bears at the facility were not suitable candidates for release. The male sun bear I had initially been asked to release was already too old for the walk release method, and hand-raising had resulted in a virtually domesticated animal that reminded me of a loyal family dog. The other two bears were an old female that had been crippled by machete wounds and a young female that had deformations due to malnourishment. Given these conditions, none of these bears should have been seriously considered for release.

During the orangutan release work, I learned that many care facilities that take in baby orangutans do not really have the veterinary capacity or husbandry understanding to rear and release animals in a responsible manner (the care facility did not even have an x-ray machine and simply did not have enough funding, facilities or qualified staff to thoroughly conduct the necessary pre-release health screenings). This was even more frustrating once I realized that I could not change the circumstances or
situation for these animals. The work I did had no tangible or practical impact on any of the animals I cared for. As a result, I decided that it was essential for me to learn more about sun bears and the release methods and approaches from someone with more experience than me.

Siew T. Wong, co-founder and manager of the Bornean Sun Bear Conservation Center (BSBCC), allowed me to stay and work at his facility in Sabah, Malaysia. Here, husbandry and management of the sun bears were more advanced than at the Indonesian facility. Siew T. Wong taught me a lot about sun bears, invited me on release walks with his bear Mary and allowed me to accompany him when setting up traps to translocate ‘problem’ bears. Nonetheless, the lack of baseline research and knowledge about sun bears remained a significant concern even here. For example, not knowing how wild sun bears behave in the wild provided a challenge when trying to monitor behaviour and welfare of the captive animals and to prepare enrichment to stimulate natural behaviour. Many times I observed sun bears where I was unable to determine whether their behaviour was within the natural range, or was abnormal and a possible cause for concern.

Not only did I learn about the neglect of sun bears in the scientific community during my time with these bears, I also witnessed a variety of threats to sun bears. The sanctuaries were filled with bear cubs that had fallen victim to the illegal pet trade or had been confiscated from restaurants. The rescue staff were repeatedly called out to remove ‘problem’ sun bears. On one occasion, we drove three hours through oil palm plantations and 200 000 hectares of desert-like environment to trap a female sun bear with cub that had been accused of attacking the workers at the logging camp. The time in Kalimantan and Sabah enabled me to experience the threats to sun bears first hand and reminded me that scientific research is indeed required by conservation managers and practitioners. During a talk with Siew T Wong, I learnt that genetic research on sun bears was much needed and relevant for the work he does. Inspired by my time in the field, I decided that the best I could do to help sun bear conservation at this stage was to go back to university and try to fill in some of the knowledge gaps for conservation practitioners.

During my PhD research, I was fortunate to visit other sun bear scientists and facilities in Sumatra, Kalimantan and Cambodia (including Free the bears in Cambodia who became key collaborators in this research). Observing sun bears across their distribution range showed me that these animals indeed looked and behaved differently; for example, sun bears at the Bornean facilities had the tendency to tap their enrichment items with their claws whereas Cambodian sun bears did not express that investigative
behavior towards their enrichment. Talking to different sun bear conservation practitioners, it became clear that each sun bear conservation researcher and practitioner had their own research priorities for sun bear conservation and ranked the threats differently in severity. There did not seem to be a consensus for where future research efforts should be directed. With the research undertaken during this PhD, I attempt to contribute to the scientific body on sun bears through my findings and conclusions and I hope that my results will find application through conservation practitioners in the field.
CHAPTER 1

General Introduction
DEFINITIONS

Translocation: The IUCN defines Translocation as: “the human-mediated movement of living organisms from one area, with release in another.” Here we will focus on the translocation processes associated with conservation techniques. The IUCN defines these processes as ‘Conservation Translocation’ is the intentional movement and release of a living organism where the primary objective is a conservation benefit: this will usually comprise improving the conservation status of the focal species locally or globally, and/or restoring natural ecosystem functions or processes.” (IUCN 2012)

Reintroduction: The IUCN defines Reintroduction as “the intentional movement and release of an organism inside its indigenous range from which it has disappeared. Reintroduction aims to re-establish a viable population of the focal species within its indigenous range.” (2012)

Reinforcement: The IUCN defines Reinforcement as ‘the intentional movement and release of an organism into an existing population of conspecifics. Reinforcement aims to enhance population viability, for instance by increasing population size, by increasing genetic diversity, or by increasing the representation of specific demographic groups or stages’. (Synonyms: Augmentation; Supplementation; Re–stocking; Enhancement (plants only) (2012)

Rehabilitation: is the managed process whereby a sick, injured, misplaced or orphaned animal regains the health and skills it requires to function normally and live self–sufficiently’ (Seddon et al. 2012, p.4).

Captive management: Captive management is the ex–situ conservation breeding, husbandry of animals kept in captivity lifelong or temporarily including facilitating and training release candidates.
1.0 GENERAL INTRODUCTION

This thesis is concerned with Malayan sun bear (*Helarctos malayanus*) conservation. The first Chapter aims to introduce the reader to sun bears, their biology, behavior, taxonomy, the threats they are facing, and what conservation actions are in place. After providing basic understanding of the species and the conservation challenges, the Chapter concludes with a description of the thesis structure and a short discussion of how the research carried out during this Ph.D. is contributing to sun bear conservation.

1.1 Destruction of habitat and loss of biodiversity in Southeast Asia: the background

Southeast Asia has been recognized for its unique biodiversity and likewise for the unprecedented rate of its loss of habitat (Normua et al. 2004; Goossens and Ambu 2012; Vijay et al. 2016). One of the driving factors of this habitat loss is the global increase in demand for oil palm (*Elaeis guineensis*) products which has resulted in large-scale deforestation in order to accommodate plantations of this economically important tree crop (Vijay et al. 2016).

The associated clear cutting of forests and the subsequent fragmentation of forested landscapes has had a detrimental impact on the biodiversity of the region (Azhar et al. 2014), with species such as orangutan (*Pongo* spp.) (Swarna Nantha & Tisdell 2009), Sumatran elephant (*Elephas maximus sumatranus*) (Sitompul et al. 2013), Sumatran tiger (*Panthera tigris sumatrae*) (Linkie et al. 2003; Bhagabati et al. 2014) and Sumatran rhinoceros (*Dicerorhinus sumatrensis*) (Goossens et al. 2013; Kretzschmar et al. 2016) all being well-known casualties of the deforestation of SE Asia.

Besides the catastrophic impact of large-scale deforestation on species conservation, Southeast Asia is also strongly influenced by periodic-climatic events such as El Niño and La Niña which often lead to severe droughts or destructive flooding, depending on the region affected by these climate phenomena (Fredriksson & Wich 2006).

In drought periods, burning practices associated with palm oil production have particularly severe impacts. Oil palm trees are typically productive for approximately 25-30 years (Verheye 2010). After this time, the mature plant is then cut down and the plantation typically burned before re-planting a new crop of oil palm trees (Simorangkir
When plantation burning is executed in drought periods, the resulting fire can quickly burn out of control creating large scale bushfires (Field et al. 2009; Fredriksson 2012a). For instance, in 1997 in the province of East Kalimantan in Indonesian Borneo, 5 million hectares of forest (including 2.6 million hectares of forest) was lost due to such fires. These fires also destroyed national parks and protected areas resulting in the destruction and further endangerment of many local plant and animal populations (Fredriksson 2012b; Chisholm et al. 2016; Gaveau et al. 2016).

To make matters worse, areas in Southeast Asia such as Sumatra and Borneo, often have peat soils (Hooijer et al. 2010; Gaveau et al. 2014). These peat soils make the land vulnerable to burning (Fredriksson 2003; Fredriksson 2012a) and may smolder and burn underground for long periods, destroying the roots of the vegetation and making fire control very challenging (Sastry 2002; Chisholm et al. 2016). This prolonged burning above and below ground results in the loss of vital habitat for many forest-dependent species.

The loss of habitat due to forest fire, large-scale logging and fragmentation has endangered many iconic and unique wildlife species in Southeast Asia (Meijaard 1999; Linkie et al. 2003; Fredriksson et al. 2006; Swarna Nantha & Tisdell 2009; Gaveau et al. 2016). Tragically, as the animals becoming rarer, they become even more prized as possessions and attractive goods for the illegal wildlife trade which further impacts the ability of these species to survive (Angulo et al. 2009; Graham-Rowe 2011).

An additional cause for the loss of biodiversity in SE Asia can be accounted to the consumption and commercial exploitation of wildlife. The use of animals and animal parts has been culturally embedded in many civilizations over time (Mills 1994; Still 2003; da Nóbrega Alves et al. 2008; Zhang et al. 2008; Feng et al. 2009). In Southeast Asia, many animal species are also popular game species (Servheen 1999), used for fur and meat consumption (Mills 1994; Phillips 2004; Shepherd & Nijman 2007; Ling et al. 2015) and for the production of traditional medicine (INTERPOL 2014; Krishnasamy & Shepherd 2014; Lee et al. 2015; Ling et al. 2015). A common belief in SE Asia is that if an animal is consumed, then the attractive properties and characteristics are transferred to the person consuming the animal (Sethy and Chauhan 2011; Ling et al. 2015). For example, in Sarawak it is believed that sun bears carry a stick with them at all times and that if one manages to kill the bear and retrieve the stick, one will be as strong and enduring as the animal (Mathai, J. 2016 pers. comm.).

If an animal is considered dangerous, however attractive or rare, it can commonly be found for sale in local wildlife markets (Zimmerman 2003), on websites (Sun Wyler &
Sheikh 2013) as well as on social media platforms such as Facebook where they are sold as pets (Krishnasamy & Stoner 2016). The important economic concept of supply and demand determines that the value of a commodity increases as supply decreases (McEachern 2006). Unfortunately, this concept also directly translates to wildlife (Damania & Bulte 2007; McNamara et al. 2016).

In some cultures having access to rare animals (even if that means possessing or consuming one illegally) may also increase social status (Zhang et al. 2008; Wyatt et al. 2009). The increase of wealth in many Asian countries has resulted in an increase in consumption of endangered animals for food and medicine, thereby displaying the ‘wealth’ of the consuming party (Mills 1994; Warchol 2004; Courchamp et al. 2006; Fabiny 2011).

These combined threats of loss of habitat, floods, drought, fire and illegal trade are all impacting much of Southeast Asia’s wildlife. This thesis, however, concentrates on a particular animal species: the sun bear. The sun bear population trend is declining due to large-scale habitat loss and fragmentation (Fredriksson 2005; Fredriksson et al. 2008; Steinmetz & Garshelis 2008). The sun bear is hunted and consumed on a commercial scale (Vu & Cowell 2010) and subjected to an uncontrolled and illegal global trade (INTERPOL 2014; K Krishnasamy & Shepherd 2014; Krishnasamy & Stoner 2016).

As sun bears (like all bears) reproduce slowly and for this reason are a designated k-selected species1 (Jule et al. 2008; Tsangaras 2014), it is unlikely that the wild sun bear population can reproduce fast enough to sustain its depletion from the wild (Johns 1985). The sun bear may be especially vulnerable to extinction in the future as the conservation plan in place currently only states that this bear species appears to be neglected by the research and conservation community, and no overarching conservation plan is available (Servheen 1999).

### 1.2 THE SUN BEAR (*HELARCTOS MALAYANUS*)

This chapter seeks to introduce the target species of this research: the Malayan sun bear. Understanding the morphology, taxonomy, distribution, habitat requirements, life history, behavior, diet and threats are crucial to addressing and solving the conservation issues facing this bear species.

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1 k-selected species often have a long gestation period, and they reproduce in low numbers of offspring. The offspring matures slowly, have a long lifespan, and require parental care.
1.2.1 Morphology

The sun bear (Figure 1.1a) is the smallest of the eight extant bear species (Family Ursidae). The adult sun bear measures between 1m – 1.4m in length (head and body) with an average shoulder height of 70cm (Fitzgerald & Krausman 2002). Their adult weight varies between 25 kg and 65 kg (Servheen et al. 1999). Males are heavier and bigger but there are great size and mass variations within sun bears. Sun bears on Borneo are significantly smaller in body mass than sun bears on Sumatra or the mainland of Southeast Asia (McNab 2010). Their fur (pelt) is short, thick and jet-black with an orange, cream colored or white ‘U shaped’ collar on the chest (individual collar shape varies greatly among individuals) (Wong et al. 2002). Their muzzles are short and show grey, silver or orange coloration, with most bears displaying brownish patches above their eyes.

![Figure 1.1: (a) An adult sun bear (Helarctos malayanus) (Stuart Robertson Reynolds, 2011). (b) The 20–25 cm long tongue of the sun bear (Wayne Lawler / Auscape International) (www.arkive.org)](image)

Sun bears have small, round ears (4.0-6.0 cm) and a short tail (Fitzgerald & Krausman 2002). Their body shape is stocky and they have large paws with naked soles and long,
curled claws. The claws consist of bones and are strong enough to be used as spikes, allowing the bears to climb trees (Sasaki et al. 2005). Further adaptations for an arboreal lifestyle are displayed in their forelimbs which are bent inwards, providing a better grip around tree trunks (Valkenburgh 1987; Sasaki et al. 2005). The sun bear’s tongue is exceptionally long (20-25 cm) (Figure 1.1 b), allowing it to reach far into the nests of invertebrates such as termites and ants, while also allowing it to access honey (Meijaard 1997).

1.2.2 Taxonomic position and genetics

Sun bears, also known as Honey Bears, Dog Bears, Malay Bears, Collared Bears or Malaysian Bears (Fitzgerald and Krausman 2002), are the least studied of the large bear species (Meijaard & Nooteboom 1999; Servheen 1999; Wong, Servheen & Ambu 2004). In Malaysia, the species is known as Basindo nan tenggil, which means “He who likes to sit high” (San Diego Zoo, http://animals.sandiegozoo.org/animals/sun-bear), referring to its arboreal lifestyle. Horsfield (1825) first used the current scientific name of the sun bear, Helarctos malayanus, in 1825 based on the conspicuous markings on the animal’s chest (Fitzgerald & Krausman 2002). This name has its origin in the Greek language with bela meaning ‘sun’ and arcto meaning ‘bear’.

The taxonomic status of this carnivore remains uncertain as they have been interchangeably assigned to the most basal Ursus as well as to its own genus Helarctos (Kitchener 2010).

The extant sun bear is suspected to be a descendent of the Late Pleistocene bear Ursus minimus (Fitzgerald & Krausman 2002; Meijaard 2004). Historically, sun bears have been classified using morphological characteristics. Significant differences have been noted in the physical appearance of the sun bears on Borneo compared to those found on Sumatra and in mainland Asia. Morphological differences (such as differences in craniometrics, dentation and body size) (Meijaard 2004), lead to a proposal that the Bornean population(s) warrants re-classification at a sub-species level (Fitzgerald & Krausman 2002; Meijaard 2004). This resulted in the listing of the Bornean Sun Bear (Helarctos malayanus eurispylus) as a subspecies of the Malayan Sun Bear (Helarctos malayanus) (Chasen 1940; Meijaard 2004).

However, morphological differences are not necessarily indicative of genetic isolation over a prolonged time (Wayne et al. 1992). Phylogenetic analysis of sun bears can
inform our understanding of the evolutionary history of species and identify populations that may be genetically distinct (Onuma et al. 2006). Conservation strategies that include evolutionary distinct units are important in maintaining biodiversity and enhancing the potential for species to adapt to a rapidly changing environment (Goossens et al. 2013). Consequently, the International Union of the Conservation of Nature (IUCN) has declared the protection of genetic diversity one of its main goals for the preservation of diversity (Schwass 2002; Tracy et al. 2011). Genetic analysis should therefore be a key stepping stone in the process of ex-situ and in-situ conservation of this species (Frankham 1995; Frankham et al. 2014).

1.2.3 Distribution and Habitat

Currently, the sun bear is found in China, Cambodia, Vietnam, Myanmar, Lao People’s Democratic Republic, Thailand, and Malaysia, Indonesia, Bangladesh, India and Brunei Darussalam (Fredriksson et al. 2008) (Figure 1.2). In the mainland of Southeast Asia, their range expands to Bangladesh and northeast India and west as far as Yunnan Province in China, although the current distribution in China is poorly understood (Fredriksson et al. 2008). Their southern distribution extends to the islands of Sumatra and Borneo. Sun bear fossils from the Pleistocene period have also been found on Java (Punung) (Tougard 2001) although the species has not been present there in recent historical times (Meijaard & Nooteboom 1999).

Due to extensive habitat loss throughout their range, sun bear populations are now highly fragmented with local extinctions in some of their former distribution range (Fredriksson et al. 2008). Laos, where the terrain is rugged with large areas of intact forest due to sparse human settlement, may be the last remaining stronghold for sun bear populations (Mills & Servheen 1991). The key habitat for sun bears is tropical primary lowland rainforest (below 500 m, although sometime extending to 800 m altitude) (Fredriksson et al. 2008). Tropical rainforest encompasses a variety of forest types such as lower montane forest, lowland and hill dipterocarp, peat and freshwater swamp, and limestone hills. Sun bears on Borneo, Sumatra and the Malaysian peninsula inhabit evergreen rainforest with evenly distributed rainfall throughout the year, whereas sun bears on mainland Southeast Asia occupy ecosystems with long dry seasons (Fredriksson 2012a).
Figure 1.2 Distribution of the Malayan sun bear (*Helarctos malayanus*), modified from the IUCN Red List map (Fredriksson et al. 2008). The colors symbolize the confirmed present (green) and probable present (blue) distribution ranges, as well as areas where the sun bear is likely extinct (red).

### 1.2.4 Life history

Sun bears can live up to 30 years in captivity (Scotson & Hunt 2008) and produce only one or two offspring in their lifetime (Frederick et al. 2012). Unlike most other bear species, sun bears are non-seasonal, polyestrous breeders with a gestation period of 95 - 107 days. In the event of the loss/death of a cub, the sow goes into estrus again (Schwarzenberger et al. 2004). Under these circumstances, the inter-birth interval is approximately 126 ±9 days, suggesting that there is no delayed implantation in sun bears (Fitzgerald & Krausman 2002; Schwarzenberger et al. 2004). They become sexually mature between two to three years of age (Feng & Wang 1991). Their follicular and luteral phases are two weeks and three months in length (Schwarzenberger et al. 2004). In captivity, cubs can be born all year round (Feng & Wang 1991) with an average litter size of one or two cubs (although twins are rare) (Schwarzenberger et al. 2004).
wild, most bear cubs will stay with their mother until they are fully grown at approximately two years of age (Schwarzenberger et al. 2004; Huber 2010).

1.2.5 Behaviour

Sun bears do not hibernate (Fredriksson et al. 2008). They are the most arboreal of bears (Fitzgerald & Krausman 2002) building nests in trees when their habitat is disturbed by human presence (Meijaard & Nooteboom 1999; Wong et al. 2004). Little is known about many aspects of wild sun bears and this is especially the case with the species’ behaviour (Onuma et al. 2001).

Some behavioral aspects are known only from investigations of captive sun bears (Hall & Swaisgood 2009). For example, research on a captive sun bear female in the US found pronounced maternal care; the mother was observed to carry and cradle the cub, keeping it off the ground (Figure 1.3) and sheltering it from environmental exposure (Hall & Swaisgood 2009).

Sun bear cubs have also been observed to vocalize (‘mutter’) when begging for food from the mother (Peters et al. 2007). Although most bear cubs tend to be independent at seven to eight months of age, it is assumed that sun bear cubs remain with their mother for two years, allowing her to protect the cubs from other bears in their range until they have attained a larger size (Dijk 2005).

Bears of most species have been known to kill other bears and cubs of their own species (Swenson et al. 1997; Taylor et al. 2017). There is no written record of sun bears engaging in this behavior, however, sun bears held in captivity do fight and sometimes kill each other (Wong, S.T. 2017 pers. comm.).
Whether sun bears are naturally nocturnal or diurnal is unknown, but their activity patterns appear to have been influenced by human activities (Griffiths & Van Schaik 1993; Wong, et al. 2004). Sun bears living near human-dominated area tend to be more active at night, presumably in an attempt to minimize detection (Fitzgerald & Krausman 2002; Wong et al. 2004). Unfortunately, poaching of sun bears is mostly committed during the night-time with the animals often being shot from vehicles (Wong et al. 2004). Although sun bears seem to avoid confrontation, they also have a reputation for being aggressive and willing to attack humans without provocation (Lekagul & McNeely 1977; Fitzgerald & Krausman 2002; Wong et al. 2004). Attacks on humans may be a result of poor eyesight, with the bears reacting with aggressive defensive behaviour when surprised and with no immediate escape route in sight (Fitzgerald & Krausman 2002). If they feel threatened, they will stand upright, suggesting that they are trying to sniff or look at the potential threat (Lekagul & McNeely 1977). Sun bears can be very vocal and will bark, grunt and occasionally roar if they feel threatened (Fitzgerald & Krausman 2002).
1.2.6 Diet

Sun bears are opportunistic feeders and eat a large number of fruit types (Fredriksson & Wich 2006). Their main diet consists of figs, invertebrates such as termites, ants, beetle larvae, as well as honeycomb and honey (Fredriksson et al. 2008). Sun bears will also consume human crops such as fruits, palm oil and coconuts and are often described as a nuisance by local farmers (Scotson et al. 2014; Wong et al. 2015).

Their mostly frugivorous dietary behaviour plays a vital role in seed dispersal, especially for plants with larger seeds (McConkey & Galetti 1999). Additionally, their active foraging behaviour when searching for food benefits natural ecosystems through nutrient mixing - turning soil over, thereby helping to maintain trophic relationships, community structure and dynamics (Augeri 2005). Because of these ecological interactions, sun bears are considered an umbrella species (Reza et al. 2013).

1.2.7 Threats

The main threats to sun bears are habitat loss and degradation, and commercial hunting (Mills 1994; Fredriksson 2005; Fredriksson et al. 2008; Sethy & Chauhan 2016). Large areas of the forests in Southeast Asia, in which sun bears are found, continue to be logged for timber and cleared for monoculture tree crops such as oil palm and rubber plantations (Whitehouse & Mulyana 2004; Vijay et al. 2016). Illegal and uncontrolled logging of forests diminishes protected areas, and is fragmenting and degrading sun bear habitat. As a result, sun bears have lost between 30 and 60% of their habitat over the last 30 years (Meijaard 1999).

Additionally, natural and human-caused forest fires have resulted in the large-scale destruction of tropical rainforest in the region, with fires being particularly severe during the droughts accompanying El Niño weather events (Fredriksson 2012b; Wooster et al. 2012). This irregular climatic phenomenon exacerbates the impact of habitat loss caused by fires and logging, disrupting the mast\(^2\) fruiting pattern of Dipterocarp trees, a predominant component of forest composition in Southeast Asia (Moore et al. 2016). These masting events are of critical importance as a foraging resource for sun bears and the combination of habitat loss and El Nino effects significantly affect their persistence in an area (Wich & Freiksson 2006).

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\(^2\) A mast is a reproductive strategy of trees where synchronized and supra-annual fruiting of large fruit crop oversaturates predator demand.
Although sun bears have been legally protected throughout their entire range since 1979 (Servheen 1999; Wong et al. 2004; Augeri 2005), they remain a popular game species in Malaysia (Servheen 1999; Fitzgerald & Krausman 2002; Murphy 2007; Reyes-García et al. 2016). Killing sun bears is strictly prohibited, nevertheless, wildlife law enforcement is typically ineffectual in many countries (Shepherd & Nijman 2007; INTERPOL 2014; Livingstone & Shepherd 2014). Sun bear products are used to make soup, cold meat cuttings and as ingredients of Traditional Chinese Medicine (TCM) (Scotson & Hunt 2008; Scotson & Downie 2009). Some bear parts have been used for centuries as ceremonial clothing (Figure 1.4), food, jewelry, lucky charms (Figure 1.5), hunting trophies (Meijaard 1999) and even furniture: in Sarawak, for example, sun bear hides were traditionally used for decorative seating pads (Krishnasamy & Shepherd 2014).

Figure 1.4 Traditional clothing of the Indonesia Dayak tribe. The traditional clothing displays derivate of birds and mammals. Photo credit: @ infis
Figure 1.5: A sun bear claw as a lucky charm. Despite the protection status of the sun bear and all trade of live bears and bear derivate being illegal, this sun bear claw is freely advertised and sold online (including free shipping). The description under the product reads: “The indigenous Dayak tribes living in the dense forest of Kalimantan believe that the Malayan sun bear possesses mystical powers of protection. It is known as a dangerous animal that will seriously harm any person or animal which offends the bear by stepping foot on its territory. The bears claws are therefore a natural weapon of defense, highly effective to fend off attacks, and to strike in a counter-attack. Indonesians generally ascribe great powers to the claw of this fear instilling animal. Bear claws are especially sought after by practitioners of traditional Indonesian martial arts (pencak silat), who wear the claw as an amulet for evasion of danger, and to practice invulnerability (hururugan) and invincibility (kudjuddayan) against attacks of sharp objects. Hence, this genuine Malayan sun bear claw is a rare and exclusive item of both mystical and practical value.”
invulnerability (‘kanuragan’) and invincibility (‘kadigdayaan’) against attacks of sharp objects.

Also, the ferocious claws of the Malayan sun bear are a powerful talisman for the kind of person who demands respect and authority, i.e. a social or political leader, a military official etc. Hence, this genuine Malayan sun bear claw is a rare and exclusive item of both mystical and practical power.”

Sun bear cubs are also in high demand for the illegal pet trade (Krishnasamy & Shepherd 2014; Krishnasamy & Stoner 2016). Servheen (1997) reported that about 1000 sun bears are taken from the wild each year. According to an INTERPOL report, the illegal bear trade almost tripled between 2000 and 2011 (INTERPOL 2014). To obtain a cub, hunters often kill the mother and then sell the cub as a pet. As the cub grows, they naturally become less attractive, more aggressive and challenging to maintain in captivity. At this point, the animals are often sold for food or TCM products (Meijaard 2001). The use of sun bears for TCM products will be discussed in greater detail in Chapter 5.

An increase of human encroachment on sun bear habitats has accompanied the loss of habitat and hunting, and resulted in a surge of human-bear conflicts (Fredriksson 2005). As opportunistic omnivores, sun bears increasingly feed on agricultural crops and are considered a pest in many countries which frequently results in retaliation killings of sun bears (Scotson & Vannachomchan 2014). Sun bears are feared by local people for their aggressive nature, and also killed out of superstitious beliefs, for example the sun bear claw is seen as a lucky charm or killing of a bear can transform its powers to the person who killed it (Figure 1.5) (Meijaard 1999; Sethy & Chauhan 2012). These forms of uncontrolled (and largely unreported) hunting can have devastating impacts on local sun bear populations. Local hunters in Thailand, for example, estimated that hunting of sun bears reduced their abundance in some areas by 50% within 20 years (Fredriksson et al. 2008).

1.2.8 Conservation status and management actions

Although reliable population estimates for the sun bear are currently lacking, large scale habitat loss and uncontrolled hunting are estimated to have resulted in an overall decline of approximately 30% over the last 30 years throughout the species’ distribution
Fredriksson et al. (2008). The level of detailed information on populations varies greatly between countries. For example, the Malayan sun bear (*Helarctos malayanus*) is currently classified as ‘vulnerable to extinction’ on the IUCN Red List of Threatened Species (Fredriksson et al. 2008) and has been listed in the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES) Appendix I since 1979 (Convention on International Trade in Endangered Species (CITES) 2013). Although national wildlife laws throughout the distribution range of sun bears strictly prohibit killing or trading them, the killing occurs frequently and wildlife laws are not implemented (Shepherd & Nijman 2007). Furthermore, the illegal trade of live bears and bear parts results in the confiscation and/or translocation of bears by wildlife officials faster than wildlife sanctuaries can be created (Fredriksson 2005a). I will discuss the trade of bears and bear parts in greater detail in Chapter 5 of this thesis.

As a result of the illegal trade in bears and bear derivate, taking care of confiscated bears and orphan bear cubs has become a serious conservation concern (Fredriksson 1998) and wildlife officials must decide the fate of each rescued animal. In these situations, the IUCN recommends: a) euthanasia, b) lifetime maintenance in captivity, or c) return to the wild (IUCN SSC 2000, 2016).

1.2.8 a) Euthanasia

The dominant religions in Southeast Asia are predominantly Islam and Buddhism. Religion has a strong influence on the local government (Crouch 2012). In Buddhism, the intent of killing or euthanizing an animal has to be pure and can only be done, if an animal is suffering (Bristol 2016). Euthanasia of healthy animals is illegal in many SE Asian countries including Indonesia and Malaysia (Scotson & Hunt 2008; Fredriksson 2005). For these reasons the humane destruction of confiscated bears are not an option. This reduces the conservation management decision to either returning the sun bears to the wild or to keeping them in captivity for the rest of their lives (Fredriksson 2005a).

1.2.8 b) Lifetime maintenance in captivity

Captive bears that are kept under good animal welfare conditions and modern husbandry standards hold great conservation value as ambassadors of wild bear populations. Ambassador animals assist in promoting conservation education and attract donations. People are more inclined to donate to an animal with a tragic history
or are willing to ‘adopt’ a bear than to give money to protect a forest. These donations can then support research ex-situ as well as in-situ. Ambassador bears hold great financial value to the conservation community and furthermore, captive animals allow essential research projects to be carried out without further endangering wild bear populations. For example, captive animals allow veterinary research, the development of more accurate animal husbandry protocols as well as testing and the development of new telemetry technology.

Taking care of a large carnivore as long-lived as a sun bear (average life expectancy is 30 years in captivity) is expensive (Scotson & Hunt 2008). Limited space or housing opportunities often invokes the development of abnormal and stereotype behavior, resulting in significant animal welfare issues (Vickery & Mason 2003; Vickery and Mason 2005). Many NGO’s opt to release their bears as they may not be able to afford an animal-friendly captive environment or may feel pressured by the public eye and donators to engage in pro-active and attractive conservation methods (Dodd & Seigel 1991a).

1.2.8 c) Return to the wild

Rehabilitation, augmentation and releases have been controversial as conservation tools used for bear species (Scotson & Hunt 2008). Although their success rates are unclear, as follow-up research is rarely funded and failures probably unreported (Fischer & Lindenmayer 2000; Jule et al. 2008), augmentations and rehabilitations have established themselves as popular conservation methods (Seddon et al. 2012). When bear populations become small and isolated, augmentation is considered an acceptable approach to supplement the existing breeding populations (Taberlet & Bouvet 1994; Kocijan et al. 2011).

A successful gradual/soft release method applied for the American black bear (*Ursus americanus*) is the ‘winter-den’ method (Clark et al. 2002). In the ‘winter-den’ method, pregnant black bear sows or sows with cubs are moved from their home den into artificial dens in the release site. The presence of cubs reduces the dispersal of released females, insuring that the female is likely to remain in the area of release (Clark et al. 2002). Furthermore, bear cubs appear to have an innate fear of humans once they emerge from hibernation, limiting the threat of human-caused mortality (Smeeton et al. 2005).
Unfortunately, as sun bears do not hibernate, the ‘winter-den’ release methods cannot be applied to this bear species and other release methods need to be considered.

Fredriksson has developed a release method referred to as the ‘walk release’ method (Fredriksson 1998) during which the sun bear cub is walked by its sole caregiver on a daily basis in the habitat chosen as its future territory. By walking the bear cub all day every day till it reaches two years of age (the time when the natural weaning process would commence), the caregiver has provided the cub with enough natural stimulation to express and develop natural patterns of behavior as well as establishing its territory. This release method is a gradual release into a known area and is therefore considered a ‘delayed’ release method (Parker et al. 2012).

The most recent sun bear releases were carried out through the Bornean Sun Bear Conservation Centre (BSBCC) where the sun bears were held in the center with access to a forest enclosure where the bears could express natural behaviors (www.bsbcc.org.my). The release candidate was then airlifted by helicopter into a release site in the Tabin wildlife reserve (BSBCC 2015). Although the sun bear has had access to high standards of animal husbandry and a forest enclosure big enough to express natural behavior (pers. obs. 2015), the airlifting of animals into an unknown territory would have to be considered a ‘hard’ release method (Parker et al. 2012).

As sun bears are aggressive in nature (Fitzgerald & Krausman 2002) the provision of release sites with no present sun bear population, is a prerequisite for their reintroduction to the wild (Fredriksson 1998) and it is not uncommon for bears to be killed by conspecifics when entering their territory (Scotson & Hunt 2008). Unfortunately, finding a suitable release sites large enough to provide appropriate habitat without impacting on wild populations seems unlikely (Fredriksson 1998).

It is also essential to remember that sun bears are illegally traded across Southeast Asia, and whether populations are genetically different across the region has not been established (Onuma et al. 2006). When deciding to opt for the release of a confiscated bear, eliminating the threat of genetically impacting the wild population is critical (more detail in Chapter 4). Prior to release, testing whether the release candidate is genetically compatible with the sun bear population it is released into is of paramount importance. Releasing animals back into the wild before such genetic and genomic research is carried out is not recommended.

Besides the first tentative rehabilitation projects of confiscated sun bears back into the wild of Borneo’s National parks, no clear national conservation measures exist for sun
bears in any of their range countries. The first (and thus far only) “Sun bear Conservation Action Plan” (Servheen 1999) stated that the lack of scientific research has created a challenging situation for conservation practitioners in the field. Although there is to date no population consensus in place, the IUCN bear specialist group agrees that the nature of the threats facing sun bears can only lead to a decrease in the sun bear numbers (Fredriksson et al. 2008).

For a k-selected species such as the sun bear, the fast pace at which habitat is disappearing and the steady demand for sun bears in the illegal animal trade is very likely to be detrimental. For conservation practitioners, it is therefore of paramount importance to understand and eliminate these threats, making basic research on sun bears its highest research priority. Currently, still too little is known about sun bears to develop a sun bear conservation management plan and the lack of research on sun bears has made them a research priority for the IUCN bear specialist group (Servheen 1999; Wong et al. 2015).

### 1.3 THE STRUCTURE OF THE THESIS

The research conducted during the course of this PhD candidature is intended to provide much-needed information necessary for the design of a species management plan for the sun bear. To achieve this, the six chapters of this thesis introduce the species and its conservation threats, identify research gaps relevant to the conservation biology of the sun bear, provide the first phylogeographic research conducted on sun bears and introduce the first microsatellite study to assess the population genetics of the Cambodian sun bear population. Furthermore, this thesis will test Traditional Asian Medicine (TAM) for the content of sun bear DNA and discuss current forensic methods in detecting illegal products on the wildlife market. The final chapter then compiles the research results of the previous chapters and provides its relevance for the conservation management of sun bears.

This thesis is structured as follows:

**Chapter 2.** Here, the available peer-reviewed literature on sun bears is assessed through systematic quantitative literature review on the Malayan sun bear (*Helarctos malayanus*). I ask what research focus has been dedicated to the sun bear and aim to identify research gaps. The findings are then collected in an accessible data file (Appendix X) which will be made available to the IUCN bear species specialist group. This resource will assist in
the identification and design of future research projects on sun bears and is made available to conservation practitioners and scientists alike.

Proposed paper:

*Systematic quantitative literature review on the current knowledge of the Malayan sun bear (Helarctos malayanus).*

**Chapter 3** Once I have identified the current knowledge and research gaps on sun bears, I ask what knowledge might be required before sun bears held in captivity can be released back to the wild in a responsible manner. I focus on the importance of genetics for wildlife management and reintroduction biology.

In Chapter 3, I present my study on the phylogeography of the Malayan sun bear (*Helarctos malayanus*) and explain how the knowledge of phylogeography finds application in the conservation management of this species. I aim to investigate the evolutionary history of the sun bear across its distribution range to identify taxonomic barriers to genetic flow between populations and to gain an insight into the adaptability of sun bears to a changing environment.

Proposed paper:

*Phylogeography of the Malayan sun bear (Helarctos malayanus).*

**Chapter 4.** In this Chapter I focus upon the population structure within one of the sun bear populations using DNA microsatellite markers. Microsatellites are useful to detect inbreeding depression or hybridization and allow us to make assumptions of the genetic health of the studied sun bear population.

Proposed paper:

*Microsatellite analysis of the population structure in Cambodian sun bears (Helarctos malayanus).*

**Chapter 5.** Once the genetic assessment of the sun bear has been conducted, I apply this information to address an additional threat to sun bears: the illegal trade and use of sun bears in Traditional Chinese Medicine (TCM). I analyze TCM products donated by CITES Enforcement Authority Australia for their content with sun bear DNA as my target trace. The results of this research will then be reported back to the CITES Enforcement Authority Australia and the TCM and bear bile industry discussed in this thesis.

Proposed Report:
The forensic analysis of Traditional Chinese Medicine samples (TCM) for traces of sun bear (Helarctos malayanus) DNA.

Chapter 6. In my final Chapter I compile my research results and provide information how this newly gained knowledge could inform a sun bear action plan for conservation practitioners in the field. This research is intended to aid sun bear conservation in both in- and ex-situ settings to assist scientists approaching sun bears as their species of interest. By making my research results available to fellow researchers (including the IUCN bear specialist group) and for future generations, my hope is that the Malayan sun bear will no longer referred to as ‘the forgotten bear’.

As this thesis contains chapters from different research disciplines, the writing style may reflect that.
CHAPTER 2

The Forgotten Bear

A systematic literature review of Malayan sun bear

(Helarctos malayanus) research
2.1 INTRODUCTION

The Malayan sun bear (*Helarctos malayanus*) has been described as the ‘neglected’ or ‘forgotten’ bear (Meijaard & Nooteboom 1999), a claim based on an apparent lack of scientific research that has been carried out on this species when compared with other Ursids. Listed as data deficient in 1995, the sun bear is now classified as vulnerable to extinction on the IUCN red list and protected under CITES Appendix I (Fredriksson et al. 2008).

The Malayan sun bear’s dire state of existence is threatened by habitat loss and fragmentation, the illegal pet trade and commercial hunting (Fredriksson 2005; Foley et al. 2011; Krishnasamy & Stoner 2016). Although the species is strictly protected by law, nevertheless the illegal trade of bears and bear parts continues to increase (INTERPOL 2014). Bears recovered from this trade are confiscated and placed into the care of non-governmental organizations (NGOs) which then must decide the future of these animals.

Taking care of a large carnivore as long-lived as a sun bear is expensive (Scotson & Hunt 2008). Consequently, most NGOs decisions to release animals are born out of the desperation that arises when an animal-friendly environment cannot be found and when organizations benefit financially by promoting release projects (Dodd & Seigel 1991b).

Recent sun bear conservation efforts seem to have focused on translocations and rehabilitation of confiscated bears (Kunde 2012, *pers. obs.*, Guharajan 2018, *per. comm*). Although translocations and rehabilitations are an increasingly popular conservation tool for a wide range of species (Maguire 2013; Stolwijk 2013), such release projects have largely remained underreported (Fischer & Lindenmayer 2000), poorly documented (Parker et al. 2012) or were unsuccessful (Fischer & Lindenmayer 2000; Huber 2005; Richardson et al. 2015).

To prevent further population-decline of the sun bear, it is vital to gather all existing knowledge of the biology, behavioral ecology, physiology, genetics and threats to this species. To maximize the chance of survival it is critical to engage across multiple and often disparate disciplines, for example: wildlife management, logistical expertise, captive husbandry, veterinary science and technical field approaches. In addition to this broad research base, it is also necessary that high level research skills, innovativeness and political acumen be employed to provide the best chance for sun bear survival.

Each of these elements are necessary to ensure that conservation management plans are based upon a sound foundation of reliable and current information (Parker et al. 2012).
Understanding the importance and interplay between these features is essential for conservation practitioners in the field. In this chapter, the scientific peer-reviewed knowledge relating to sun bears is collated and analyzed with the following questions in mind:

1) How many peer-reviewed publications have been published on the Malayan sun bear (*Helarctos malayanus*)?

2) How many of these publications have the sun bear as its sole research focus?

3) Which disciplines were represented in these publications?

4) How much of this research has been carried out on wild compared to captive sun bears?

To answer these questions a systematic and quantitative review of the Malayan sun bear research literature was performed. Analyses of the work published in peer-reviewed literature was captured and presented in the form of a freely accessible database (Helarctos-database) which will allow conservation scientists to identify the most urgent research questions (research gaps). Importantly, the database is extensible and scientists studying sun bears are invited to include their research findings to the Helarctos-database (Supplementary_Chapter 2 Table TS1). By providing one access point for scientists with an interest in sun bears, it is hoped that future researchers may be able to identify their research target more efficiently, identify fellow researchers, avoid double-research and potentially lead to further collaboration amongst scientists.
2.2 METHOD

DEFINITIONS

The research method applied here contains different search methods and criterion which I define as follows:

Open search: The 'open search' does not restrict the time in which publications were released. The open search aims to gather as many published papers as possible.

Restricted search: The restricted search restricts the search to publications released between the years 1999 and 2016. This time restriction is looking at the time frame between the release of the sun bear action plan from 1999 to current time.

Full search: In the 'full search', all publications that have the key words in the title AND abstract are included in the search. The result is the full dataset.

Concise search: In the 'concise search', all publications with the key words in the title only are included in the search. The result is the concise dataset.
The aim of this work described in this chapter was to undertake a quantitative review of the Malayan sun bear research as it appears in peer-reviewed literature (as described in (Ansong & Pickering 2013). The Preferred Reporting Items for Systematic review and Meta-Analysis protocols (PRISMA-P) (Moher et al. 2009) guidelines were followed to ensure objectivity and replicability of the research findings and to ensure that results are reported in a standardized format.

To test whether the sun bear is indeed the ‘forgotten’ bear species two databases, Google Scholar and Web of Science, were examined for all extant bear species within two time frames: an unrestricted period and a restricted period limited to publications appearing between 1999-2016 (Table 2.1). As the ‘Sun Bear Conservation Action Plan’, released in 1999, established the sun bear as the highest research priority of all Ursidae species, we question here whether this call for more research on sun bears has been answered compared to the amount of research conducted on other bear species. Therefore, the search was restricted to the period between 1999 and 2016. The key words used for the searches followed the same structure:

“common name” OR “binominal nomenclature”

e.g. “brown bear” OR “Ursus arctos”.

As the aim was to understand the general trend of research focus across the extant bear species, the key words were searched for and restricted within titles only.

Table 2.1: A summary of the search methods applied to compile the published literature on the family Ursidae in the timeframe from 1999–2016. (The search process employed is detailed on page 26).

<table>
<thead>
<tr>
<th></th>
<th>open search</th>
<th>restricted search</th>
</tr>
</thead>
<tbody>
<tr>
<td>full search</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>concise search</td>
<td>X</td>
<td>✓</td>
</tr>
</tbody>
</table>

Number of databases searched: 2
Once the frequency of publications for all eight extant bear species was assessed, a replicable search approach to detect peer-reviewed literature that had the Malayan sun bear as the primary and only target was defined.

In addition to the published data that focuses on sun bears as the primary research target, a secondary literature review was conducted (Table 2.2). For this review, eight electronic databases for peer-reviewed journal articles as of the 4th of September 2016 were searched (Web of Science, Scopus, Science Direct (expert search), ProQuest, JSTOR, EBSCO host (greenFile-smart search), Griffith (expanded search) and Google Scholar3). These databases were searched with the keywords ("sun bear" OR "Helarctos malayanus" OR "Ursus malayanus") ("sun bear*" OR "Helarctos malayanus" OR "Ursus malayanus") where the “*” is used to search databases that allow wildcard searches.

Table 2.2: A summary of the search methods applied to compile the published literature on the sun bear. (Please refer to the definitions on page 25 and the search process employed is detailed on page 27 and 28).

<table>
<thead>
<tr>
<th></th>
<th>open search</th>
<th>restricted search</th>
</tr>
</thead>
<tbody>
<tr>
<td>full search</td>
<td>✔</td>
<td>✔</td>
</tr>
<tr>
<td>concise search</td>
<td>✔</td>
<td>✔</td>
</tr>
</tbody>
</table>

Number of databases searched: 8

---

3 Google Scholar is a database that provides search results of white and grey literature. To filter out grey literature and to test if the results were “peer-reviewed or refereed” Ulrichsweb (wwwUlrichsweb.com) was used. The initial search with the keywords produced 3900 search results. To test all of these in Ulrichsweb would have been prohibitively time-consuming. Hence, it was decided to search using the Google Scholar advanced setting and separately search the key-words in title and abstract in peer reviewed literature only. This way the exclusion and inclusion criteria reduced the initial search hits to 97 papers. These 97 papers were then individually tested in Ulrichsweb for credibility.
The retrieved literature was then filtered using the following inclusion criteria:

1. The title or abstract must contain the key words "sun bear" OR "Helarctos malayanus" OR "Ursus malayanus";

2. For databases that collect grey and white literature (e.g. Google Scholar), these publications must be peer-review tested through Ulrichsweb, a website that assesses whether a publication has been peer reviewed or not (Accessible under www.ulrichsweb.com).

3. The publications (not just abstracts) had to have been written in English. Books and book chapters were excluded as it is assumed that relevant research would have progressed into publication in a scientific peer-reviewed journal. Furthermore, critiques of literature or literature reviews that did not conduct original research and therefore did not contribute to scientific understanding of sun bears were excluded from this research. As the aim was to capture all available knowledge on sun bears, no specific cut-off point for the year of publication was set.

To ensure that the search results were genuine and replicable, two independent reviewers tested the keywords and databases (APPENDIX I). The acquired literature was then assessed and analyzed to create a unique database record for each publication.

The information gathered included:

- **Administrative/citation information** (e.g. year of publication, authors, journals published in, title of the paper, key words used by authors, research objectives of the paper, funding body behind the research).

- **Geographic information** (e.g. country and province the research had been conducted in, GPS coordinates of the research location if provided, vegetation type of the research area, the country the publishing author came from, where the research facility was located, and which country had financially contributed to the research).

- **Study type** (e.g. fundamental or applied, qualitative or quantitative as was the research approach utilized and the general type of method employed (e.g. was the research experimental or observational? Were there surveys or interviews conducted? Have animals been trapped or camera trapped? Did the researchers
collect physical samples? Did the researcher collect new data? Did they use photography for identification and detection, GIS or telemetry?).

- **Setting** (was the animal in captivity, in the wild or in protected areas or unknown).
- **Research disciplines and topic** (e.g. did the research aim at ecology or veterinary science? Each discipline was then sub-categorized into special research interest topics e.g. feeding ecology or anatomy).
- **Target species and management recommendations** (e.g. was the sun bear the only target species of the research and did the research provide conservation management recommendations?).

As this research is interested in sun bears as a primary research target, the literature has been reviewed as two different datasets: a ‘full dataset’ (keywords in abstract and title) and a ‘concise’ dataset (keywords ONLY in the title). It is assumed that the full dataset may contain research where sun bears have not been the primary research target. For the concise dataset, it is assumed that scientists who have sun bears as their primary research target will mention the species name or common name in the title of the publication.

As the aim was to evaluate the research undertaken on sun bears, only publications meeting this selection criterion were considered. Every match of a criterion is marked with a ‘1’ and every miss-match with a ‘0’ (as illustrated in the snap-shot in Figure 2.1 below). To allow replicability and to minimize irregularities between assessments of different reviewers, I provide a glossary of terms used in the assessment spreadsheet (APPENDIX II).
Figure 2.1: Screen-shot of the Helarctos-database developed during this PhD. The literature was divided into categories and disciplines (grey field). Each record was assigned “1” for a match and “0” for no match. The colors underlined under each data match allows for a quick visualization of research gaps. The column with the letters A, H, and X symbolize a biome. The legend for these biomes as well as the Helarctos-database can be reviewed under Supplementary Chapter 2 Table TS1.
2.3 RESULTS

2.3.1 Relative frequency of publications for each bear species

Brown bears were the most studied bear species of the extant Ursidae (Table 2.3). Using Google Scholar, 990 publications featuring brown bears as a key word in the title were retrieved between 1999 and 2016 (restricted search), comprising 30% of the published literature on all Ursidae. This figure increased to 1380 articles for all published literature (open search) amounting for 31% of published literature on Ursidae. The giant panda (24% of restricted, 24% of open) and the polar bear (23% restricted and 24% of open) were the next most published bear species.

A search of the Web of Science database showed that brown bears dominated the literature on bear species with 476 articles (28%) in a restricted search and accounted for 686 articles (28%) in an open search. In contrast to the Google Scholar results, polar bears were the second-most researched species (25% restricted and 27% open) and the giant panda was third (25% restricted and 23% open).

The search in Google Scholar identified the sun bear as the least studied bear species with only 24 publications constituting 1% of the published literature between 1999 and 2016, (93 publications and 2% using and open search). The second least researched bear was the sloth bear (2% of restricted, 2% of open) and the spectacled bear (3% of restricted, 3% of open) (Figure 2.2).

In contrast, Web of Science found the spectacled bear to be the least studied bear using a restricted search with 20 publications (1% published literature) followed by the sloth bear with 25 publications (1% of published literature). Using the open search parameters, only 32 publications on sloth bears (1%) and 34 papers on sun bears (1%) were identified in the Web of Science database indicating that these two are the least-studied bear species. Identified brown bears, polar bears and panda bears as the most-studied bear species, accounting for 78% of the published Ursidae literature.
Figure 2.2 Geographic distribution of the extant bears and their research coverage in peer reviewed literature. The percentage coverage is derived from the search in Google Scholar (% of publications all times). This map was created using Arc GIS and spatial data provided by the IUCN.
Table 2.3 Open and restricted search results for publications on all extant bear research in two databases. All searches were performed to search for the key words occurring in the publication title.

<table>
<thead>
<tr>
<th>Google Scholar</th>
<th># of publications between 1999–2016 (restricted)</th>
<th>% of publications in 1999–2016 (restricted)</th>
<th># of publications all times (open)</th>
<th>% of publications all times (open)</th>
</tr>
</thead>
<tbody>
<tr>
<td>“brown bear” OR “Ursus arctos”</td>
<td>990</td>
<td>30</td>
<td>1380</td>
<td>31</td>
</tr>
<tr>
<td>“giant panda” OR “Ailuropoda melanoleuca”</td>
<td>787</td>
<td>24</td>
<td>1060</td>
<td>23</td>
</tr>
<tr>
<td>“polar bear” OR “Ursus maritimus”</td>
<td>738</td>
<td>23</td>
<td>1090</td>
<td>24</td>
</tr>
<tr>
<td>“American black bear” OR “Ursus americanus”</td>
<td>336</td>
<td>10</td>
<td>446</td>
<td>10</td>
</tr>
<tr>
<td>“Asiatic black bear” OR “Ursus thibetanus”</td>
<td>217</td>
<td>7</td>
<td>241</td>
<td>5</td>
</tr>
<tr>
<td>“spectacled bear” OR “Tremarctos ornatus”</td>
<td>96</td>
<td>3</td>
<td>128</td>
<td>3</td>
</tr>
<tr>
<td>“sloth bear” OR “Melursus ursinus”</td>
<td>62</td>
<td>2</td>
<td>75</td>
<td>2</td>
</tr>
<tr>
<td>“sun bear” OR “Helarctos malayanus”</td>
<td>24</td>
<td>1</td>
<td>93</td>
<td>2</td>
</tr>
</tbody>
</table>
Table 2.3 continued

<table>
<thead>
<tr>
<th>Web of Science</th>
<th># of publications between 1999–2016 (restricted)</th>
<th>% of publications in 1999–2016 (restricted)</th>
<th># of publications all times (open)</th>
<th>% of publications all times (open)</th>
</tr>
</thead>
<tbody>
<tr>
<td>“brown bear” OR “Ursus arctos”</td>
<td>476</td>
<td>28</td>
<td>686</td>
<td>28</td>
</tr>
<tr>
<td>“polar bear” OR “Ursus maritimus”</td>
<td>429</td>
<td>25</td>
<td>656</td>
<td>27</td>
</tr>
<tr>
<td>“giant panda” OR “Ailuropoda melanoleuca”</td>
<td>429</td>
<td>25</td>
<td>556</td>
<td>23</td>
</tr>
<tr>
<td>“American black bear” OR “Ursus americanus”</td>
<td>202</td>
<td>12</td>
<td>327</td>
<td>13</td>
</tr>
<tr>
<td>“Asiatic black bear” OR “Ursus thibetanus”</td>
<td>107</td>
<td>6</td>
<td>120</td>
<td>5</td>
</tr>
<tr>
<td>“sun bear” OR “Helarctos malayanus”</td>
<td>30</td>
<td>2</td>
<td>34</td>
<td>1</td>
</tr>
<tr>
<td>“sloth bear” OR “Melursus ursinus”</td>
<td>25</td>
<td>1</td>
<td>32</td>
<td>1</td>
</tr>
<tr>
<td>“spectacled bear” OR “Tremarctos ornatus”</td>
<td>20</td>
<td>1</td>
<td>47</td>
<td>2</td>
</tr>
</tbody>
</table>

01/01/1999–16/08/2016, keywords only in title
2.3.2 Publications focused on sun bears

To determine the frequency of publications focused on sun bear research, the search of eight databases initially returned a total of 1269 journal articles (APPENDIX I). Once all duplicates were removed from the databases and tested through the exclusion and inclusion criteria, 84 unique research papers were identified.

2.3.3 Frequency of publications for sun bears (full dataset)

Between 1923 and 2016 a total of 84 publications on sun bears were published containing the relevant key words ("sun bear" OR “Helarctos malayanus” OR "Ursus malayanus") ("sun bear*" OR “Helarctos malayanus” OR "Ursus malayanus") in the title or abstract, and passing the exclusion filter (given in Supplementary Chapter_2_Table_TS1). The earliest publication detected during the inclusion criteria was published in 1923. Over the 93 years spanning the publication range, low rates of publication were observed (mode=1, mean=0.9 publications/year). An increase in publications in the years 2004 and 2005 was seen (7 publications per year) with an overall peak of 11 papers in 2013 (Figure 2.3).

Figure 2.3: Number of sun bear publications per year for the full dataset (1923–2016).
Between the first publication in 1923 and the second publication in 1972 was a 49-yearlong research gap with no publications on sun bears. After 1999, there has been at least one publication each year.

2.3.4 Journals and disciplines covered (full dataset)

The research articles detected in the search of eight databases with the key words ("sun bear" OR "Helarctos malayanus" OR "Ursus malayanus") ("sun bear*" OR "Helarctos malayanus" OR "Ursus malayanus") in the title or abstract were published in 45 different journals and covered 15 different research disciplines (Table 2.4). The average number of publications per journal was low with a mean=1.87 publications and a mode=1. The journal Ursus, a journal dedicated to publishing on research carried out on Ursidae, published most of the literature (11 publications), followed by Zoo Biology (six publications). The research discipline associated with the most publications was veterinary science with 21 publications across 12 different journals (Table 2.5).
Table 2.4: The number of publications per journal that had published sun bear research, collated in the full dataset. The search process employed is detailed on page 27–29.

<table>
<thead>
<tr>
<th>Journal</th>
<th>N publications</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ursus</td>
<td>11</td>
</tr>
<tr>
<td>Zoo Biology</td>
<td>6</td>
</tr>
<tr>
<td>Molecular Phylogenetics and Evolution</td>
<td>5</td>
</tr>
<tr>
<td>Journal of Mammalogy</td>
<td>4</td>
</tr>
<tr>
<td>Journal of Zoo and Wildlife Medicine</td>
<td>4</td>
</tr>
<tr>
<td>Theriogenology</td>
<td>4</td>
</tr>
<tr>
<td>Plos One</td>
<td>3</td>
</tr>
<tr>
<td>Biological Conservation</td>
<td>3</td>
</tr>
<tr>
<td>Annals of Anatomy</td>
<td>2</td>
</tr>
<tr>
<td>Biodiversity Conservation</td>
<td>2</td>
</tr>
<tr>
<td>Journal of Tropical Ecology</td>
<td>2</td>
</tr>
<tr>
<td>Journal of Veterinary Diagnostic Investigation</td>
<td>2</td>
</tr>
<tr>
<td>Journal Veterinarian</td>
<td>2</td>
</tr>
<tr>
<td>The Raffles Bulletin of Zoology</td>
<td>2</td>
</tr>
<tr>
<td>Traffic Bulletin (short communication)</td>
<td>2</td>
</tr>
<tr>
<td>Animal Welfare</td>
<td>1</td>
</tr>
<tr>
<td>American Society of Mammalogists</td>
<td>1</td>
</tr>
<tr>
<td>Animal Conservation</td>
<td>1</td>
</tr>
<tr>
<td>Applied Animal Behaviour Science</td>
<td>1</td>
</tr>
<tr>
<td>Applied Ecology and Environmental Research</td>
<td>1</td>
</tr>
<tr>
<td>Biological Journal of the Linnean Society</td>
<td>1</td>
</tr>
<tr>
<td>BMC Evolutionary Biology</td>
<td>1</td>
</tr>
<tr>
<td>Comparative Biochemistry and Physiology</td>
<td>1</td>
</tr>
<tr>
<td>Conservation Genetic Resources</td>
<td>1</td>
</tr>
<tr>
<td>Diversity and Distributions</td>
<td>1</td>
</tr>
<tr>
<td>Ecological Indications</td>
<td>1</td>
</tr>
<tr>
<td>Environmental Management</td>
<td>1</td>
</tr>
<tr>
<td>Evolution</td>
<td>1</td>
</tr>
<tr>
<td>General and Comparative Endocrinology</td>
<td>1</td>
</tr>
<tr>
<td>Human Ecology</td>
<td>1</td>
</tr>
<tr>
<td>International Journal of Conservation Science</td>
<td>1</td>
</tr>
<tr>
<td>Japanese Journal of Veterinary Research</td>
<td>1</td>
</tr>
<tr>
<td>JAVMA</td>
<td>1</td>
</tr>
<tr>
<td>Journal of Applied Ecology</td>
<td>1</td>
</tr>
<tr>
<td>Journal of Comparative Pathology</td>
<td>1</td>
</tr>
<tr>
<td>Journal of Conservation Biology</td>
<td>1</td>
</tr>
<tr>
<td>Journal of Morphology</td>
<td>1</td>
</tr>
</tbody>
</table>
Table 2.5: The number of journals and the number of publications on sun bear research that have been published in the different research disciplines (full dataset). (The search process employed is detailed on page 27–29).

<table>
<thead>
<tr>
<th>Research Discipline</th>
<th>Number of journals</th>
<th>Number of publications</th>
</tr>
</thead>
<tbody>
<tr>
<td>Veterinary science</td>
<td>12</td>
<td>21</td>
</tr>
<tr>
<td>Conservation</td>
<td>6</td>
<td>9</td>
</tr>
<tr>
<td>Ecology</td>
<td>5</td>
<td>6</td>
</tr>
<tr>
<td>Management</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>Evolution</td>
<td>4</td>
<td>8</td>
</tr>
<tr>
<td>Zoology</td>
<td>4</td>
<td>10</td>
</tr>
<tr>
<td>Mammalogy</td>
<td>2</td>
<td>5</td>
</tr>
<tr>
<td>Animal welfare</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Behaviour</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Biology</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Genetics</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Diversity</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>General</td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td>Trade</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>Ursidae</td>
<td>1</td>
<td>11</td>
</tr>
<tr>
<td><strong>TOTAL</strong></td>
<td><strong>45</strong></td>
<td><strong>84</strong></td>
</tr>
</tbody>
</table>
2.3.5 Countries in which research was carried out (full dataset)

The research described in the 84 publications given in Supplementary Chapter_2_Table_TS1 were carried out in 17 different countries, of which eight were without wild sun bear populations. The largest percentage of publications came from the USA on captive sun bears in zoological facilities (25 studies constituting 28% of all research carried out to date). Malaysia facilitated 17 of these studies (19% of published studies) and was the highest-ranking location for sun bear research undertaken within the distribution range of this bear species. Cambodia and Germany both conducted three studies (equating to 3% of published literature), even though Germany does not have natural populations of sun bears. Vietnam and Myanmar have their own sun bear populations but were associated with only single studies; Venezuela, Canada, New Zealand and Australia each had single studies of captive animals (Table 2.6).
Table 2.6: Countries in which sun bear research was carried out (full dataset). The **bold** values highlight the countries that are within the distribution of the Malayan sun bear. Some studies had multiple research sites across different countries, therefore the total of the numbers of studies in this table does not respond to the total number of studies carried out. (The search process employed is detailed on page 27–29).

<table>
<thead>
<tr>
<th>Country</th>
<th>Number of studies</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>USA</td>
<td>25</td>
<td>28%</td>
</tr>
<tr>
<td><strong>Malaysia</strong></td>
<td>17</td>
<td>19%</td>
</tr>
<tr>
<td><strong>Indonesia</strong></td>
<td>12</td>
<td>13%</td>
</tr>
<tr>
<td><strong>Thailand</strong></td>
<td>9</td>
<td>10%</td>
</tr>
<tr>
<td><strong>India</strong></td>
<td>6</td>
<td>7%</td>
</tr>
<tr>
<td><strong>Cambodia</strong></td>
<td>3</td>
<td>3%</td>
</tr>
<tr>
<td>Germany</td>
<td>3</td>
<td>3%</td>
</tr>
<tr>
<td><strong>China</strong></td>
<td>2</td>
<td>2%</td>
</tr>
<tr>
<td>Denmark</td>
<td>2</td>
<td>2%</td>
</tr>
<tr>
<td>Japan</td>
<td>2</td>
<td>2%</td>
</tr>
<tr>
<td><strong>Lao</strong></td>
<td>2</td>
<td>2%</td>
</tr>
<tr>
<td>Australia</td>
<td>1</td>
<td>1%</td>
</tr>
<tr>
<td><strong>Myanmar</strong></td>
<td>1</td>
<td>1%</td>
</tr>
<tr>
<td>NZ</td>
<td>1</td>
<td>1%</td>
</tr>
<tr>
<td>Canada</td>
<td>1</td>
<td>1%</td>
</tr>
<tr>
<td>Venezuela</td>
<td>1</td>
<td>1%</td>
</tr>
<tr>
<td><strong>Vietnam</strong></td>
<td>1</td>
<td>1%</td>
</tr>
<tr>
<td><strong>TOTAL</strong></td>
<td>89</td>
<td>100%</td>
</tr>
</tbody>
</table>
2.3.6 Sun bear publications 1999–2016 (concise dataset)

The ‘full dataset’ encompasses 84 research papers. Of these, 46 papers included the keywords in the title and comprise the ‘concise dataset’. The following results analyze the ‘concise dataset’. As this research tries to identify how much research has been carried out since the Sun Bear Conservation Action Plan from 1999, the time frame was chosen from 1999 to 2016. Further, this study tries to identify how much research had the sun bear as the primary research focus. Therefore, the search criteria was restricted to the key words in the title only as it is expected that scientists name the scientific or common name of their research target in the title of their publication. When analyzing the concise dataset (as well as the full dataset), it reveals that only 34 papers had the sun bear as their sole research target species. This means that sun bear data is often collected as a by-product of research carried out on other species (such as tigers or Asiatic black bears).

2.3.7 Number of sun bear publications per year

During the 17 years of research covered by the concise dataset, the mean number of annual publications was 2.7 with mode=1. The largest number for one year was seven (2013) though the years 2004-6 yielded 14 in total.

2.3.8 Journals and disciplines covered (concise dataset)

During the 17-year period encompassed by the concise dataset, 25 journals (Table 2.7) in 15 different disciplines (Table 2.8) published on sun bears with Ursus being the leading journal publishing sun bear research (six publications in 17 years equating to a publication mean of 0.35 papers a year with the key words in the title). Most of the research on sun bears was published in the veterinary research discipline with 13 publications in eight different veterinary research journals. Genetic research could not be identified when searching for publications.
Table 2.7: The number of publications per journal that published sun bear research (concise dataset). (The search process employed is detailed on page 27–29).

<table>
<thead>
<tr>
<th>Journal</th>
<th>N publications</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ursus</td>
<td>6</td>
</tr>
<tr>
<td>Zoo Biology</td>
<td>5</td>
</tr>
<tr>
<td>Theriogenology</td>
<td>4</td>
</tr>
<tr>
<td>Biodiversity Conservation</td>
<td>3</td>
</tr>
<tr>
<td>Journal of Mammalogy</td>
<td>2</td>
</tr>
<tr>
<td>Journal of Tropical Ecology</td>
<td>2</td>
</tr>
<tr>
<td>Journal of Zoo and Wildlife Medicine</td>
<td>2</td>
</tr>
<tr>
<td>Jurnal Veterinar</td>
<td>2</td>
</tr>
<tr>
<td>Plos One</td>
<td>2</td>
</tr>
<tr>
<td>The Raffles Bulletin of Zoology</td>
<td>2</td>
</tr>
<tr>
<td>Traffic Bulletin (short communication)</td>
<td>2</td>
</tr>
<tr>
<td>Animal Welfare</td>
<td>1</td>
</tr>
<tr>
<td>Animal Conservation</td>
<td>1</td>
</tr>
<tr>
<td>Annals of Anatomy</td>
<td>1</td>
</tr>
<tr>
<td>Applied Ecology and Environmental Research</td>
<td>1</td>
</tr>
<tr>
<td>Biological Journal of the Linnean Society</td>
<td>1</td>
</tr>
<tr>
<td>Diversity and Distributions</td>
<td>1</td>
</tr>
<tr>
<td>Human Ecology</td>
<td>1</td>
</tr>
<tr>
<td>International Journal of Conservation Science</td>
<td>1</td>
</tr>
<tr>
<td>Japanese Journal of Veterinary Research,</td>
<td>1</td>
</tr>
<tr>
<td>JAVMA</td>
<td>1</td>
</tr>
<tr>
<td>Journal of Conservation Biology</td>
<td>1</td>
</tr>
<tr>
<td>Journal of Veterinary Diagnostic Investigation</td>
<td>1</td>
</tr>
<tr>
<td>Research in Veterinary Science</td>
<td>1</td>
</tr>
<tr>
<td>The Journal of Wildlife Management</td>
<td>1</td>
</tr>
<tr>
<td><strong>TOTAL</strong></td>
<td><strong>46</strong></td>
</tr>
</tbody>
</table>
Table 2.8: The number of publications per Research Discipline that published sun bear research (concise dataset). (The search process employed is detailed on page 27–29).

<table>
<thead>
<tr>
<th>Research Discipline</th>
<th>N journals</th>
<th>N publications</th>
</tr>
</thead>
<tbody>
<tr>
<td>Veterinary science</td>
<td>8</td>
<td>13</td>
</tr>
<tr>
<td>Conservation</td>
<td>4</td>
<td>6</td>
</tr>
<tr>
<td>Ecology</td>
<td>3</td>
<td>4</td>
</tr>
<tr>
<td>Zoology</td>
<td>2</td>
<td>7</td>
</tr>
<tr>
<td>Ursidae</td>
<td>1</td>
<td>6</td>
</tr>
<tr>
<td>Mammalogy</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>General</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>Trade</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>Diversity</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Management</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Evolution</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Animal welfare</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Behaviour</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Biology</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Genetics</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td><strong>TOTAL</strong></td>
<td><strong>25</strong></td>
<td><strong>46</strong></td>
</tr>
</tbody>
</table>
2.3.9 Countries in which research was performed (concise dataset)

The search to find publications where the key words occurred only in the title yielded public studies performed in ten different countries, with 60% of the studies carried out in countries within the distribution range of the sun bear. Malaysia hosted 15 studies and accounted for 29% of sun bear research and is therefore with the leading country for research on this species (Table 2.9). Malaysia and Indonesia account for 50% of all sun bear research carried out, with a focus being on sun bears on Borneo, leaving other countries within the sun bear distribution being markedly understudied. For example, China participated in one study, Cambodia in two and Laos, Myanmar and Vietnam had no studies on sun bears.
**Table 2.9:** Countries that facilitated sun bear research (concise dataset). The **bold** values highlight the countries that are within the distribution of the Malayan sun bear. Some studies had multiple research sites across different countries, therefore the total of the numbers of studies in this table does not respond to the total number of studies carried out. (The search process employed is detailed on page 27–29).

<table>
<thead>
<tr>
<th>Country</th>
<th>N studies</th>
<th>N studies (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Malaysia</td>
<td>15</td>
<td>29%</td>
</tr>
<tr>
<td>Indonesia</td>
<td>11</td>
<td>22%</td>
</tr>
<tr>
<td>USA</td>
<td>10</td>
<td>20%</td>
</tr>
<tr>
<td>Thailand</td>
<td>4</td>
<td>8%</td>
</tr>
<tr>
<td>India</td>
<td>4</td>
<td>8%</td>
</tr>
<tr>
<td>Cambodia</td>
<td>2</td>
<td>4%</td>
</tr>
<tr>
<td>Germany</td>
<td>2</td>
<td>4%</td>
</tr>
<tr>
<td>China</td>
<td>1</td>
<td>2%</td>
</tr>
<tr>
<td>Japan</td>
<td>1</td>
<td>2%</td>
</tr>
<tr>
<td>New Zealand</td>
<td>1</td>
<td>2%</td>
</tr>
<tr>
<td>Denmark</td>
<td>0</td>
<td>0%</td>
</tr>
<tr>
<td>Lao</td>
<td>0</td>
<td>0%</td>
</tr>
<tr>
<td>Australia</td>
<td>0</td>
<td>0%</td>
</tr>
<tr>
<td>Myanmar</td>
<td>0</td>
<td>0%</td>
</tr>
<tr>
<td>Canada</td>
<td>0</td>
<td>0%</td>
</tr>
<tr>
<td>Venezuela</td>
<td>0</td>
<td>0%</td>
</tr>
<tr>
<td>Vietnam</td>
<td>0</td>
<td>0%</td>
</tr>
<tr>
<td><strong>TOTAL</strong></td>
<td><strong>51</strong></td>
<td><strong>100%</strong></td>
</tr>
</tbody>
</table>
2.4 DISCUSSION

The informal status of the sun bear as the ‘forgotten’ or ‘neglected’ bear (Meijaard & Nooteboom 1999; Wong et al. 2002) seems justified in the light of the relative amount of research directed toward it and the topics covered. Most current conservation measures seem to concentrate on release efforts rather than expanding the scientific understanding of this species or the threats it faces.

In this study, I successfully applied the systematic quantitative literature review to investigate whether the sun bear is indeed a ‘forgotten’ species and whether the conservation community has access to the level of relevant information needed to plan and execute sun bear release in a responsible manner.

2.4.1 Dataset limitations

The method employed here has important limitations that need to be addressed. The systematic quantitative literature review is a very useful tool for analyzing “white” literature available through peer-reviewed scientific journals but largely excluded theses and “grey” literature (Mahood et al. 2014; Adams et al. 2016). Such literature, however, can play an important role in conservation science, especially in reintroduction biology (Guisan et al. 2013; Haddaway & Bayliss 2015). This situation arises as many conservation practitioners may not have an academic background, do not have the time or support to write up their release efforts, do not attempt to publish their results due to the small sample size of their work or may have organizations opposing publication (Parker et al. 2012). Often, release projects involve individual animals and these will not accumulate statistically significant data (Armstrong & Mccarthy 2007). Another reason why so many release projects may not result in publication is that few studies manage to secure the funding and qualified staff required to apply long-term monitoring of the released animals, which in turn hampers any attempts to gather valuable information and inform future management of the species (Griffith 1989).

2.4.2 Ursidae research

“Why do we spend more on conservation of grizzly bears, timber wolves, and bald eagles living in the continental United States than we do on the sun bear, maned wolf, and
Madagascar fish eagle even though the former species are reasonable secure in Canada and Alaska, and the latter are threatened with global extinction?” (Hunter and Hutchinson 1994).

Hunter and Hutchinson (1994) have discussed why conservation funding and research attention is not distributed in accordance to conservation priority. Animals that are threatened do not necessarily obtain more funding and attention than animals that are of least concern to go extinct in the wild. This might be due to parochialism and the fact that in most instances conservation is reliant on local politics and governmental funding agencies that place the emphasis on local projects. Consequently, wealthier countries are more likely to fund conservation research projects, with governmental agencies providing funding for the local flora and fauna. Funding allocation from a political model might not concentrate on the animal species most valuable ecologically, focusing rather on species that are more charismatic, attractive, ‘interesting’ or familiar and which captivate people’s attention. To compete for limited conservation research funds, many scientists opt for research projects that they feel are certain to obtain permission to study and/or those guaranteed to deliver data for publication.
2.4.2 a) Conservation status and permits

DEFINITIONS

CITES: CITES stands for the ‘Convention on International Trade in Endangered Species of Wild Fauna and Flora’. CITES is regulating the trade of endangered species by listing endangered species into three different Appendices.

CITES Appendix I: Appendix I is listing species that are threatened with extinction. The international trade (export and import) of these species is strictly prohibited under CITES. The only exception is made if the import is not for commercial purpose (e.g. for scientific research). In these cases, trade requires authorized import and export permits.

CITES Appendix II: Appendix II is listing species that are not immediately threatened with extinction but that may become so without a strict controlled trade. The international trade of these species may be authorized under the premises of acquiring export permits or re-export certificates. Generally, no import permit is necessary for those species under CITES Appendix II.

CITES Appendix III: Appendix II includes species that can be traded internationally if appropriate certificates and permits can be presented.

(www.CITES.org)

When comparing the publication frequencies of all bear species, brown and polar bear, as well as the giant panda, were the most studied species, with sloth and sun bears receiving the least research attention. Brown bears have a large distribution range (See Figure 2.2) and are listed as ‘least concern’ in the IUCN red list, and are protected under CITES Appendix II (McLellan et al. 2017). Consequently, this species is relatively accessible to study and research permits can be easier to obtain than those for the more threatened and protected species. The brown bear being designated of ‘least concern’ also encourages more attractive and riskier research projects. For example, the extensive research conducted across its distribution range has led to the identification of several subspecies of brown bears (McLellan et al. 2017) of which some are more threatened (Pérez et al. 2009; Gippoliti 2016) than others continuously fueling further research into this species.
Table 2.10: The protection and conservation status of the extant bear species. The IUCN categories state the conservation status (LC=Least Concern, VU=Vulnerable) and the CITES Appendix informs on the level of protection. References in this table are listed numerically as followed: 1=(McLellan et al. 2017), 2=(Wiig et al. 2015), 3=(Swaisgood et al. 2016), 4=(Garshelis et al. 2016), 5=(Garshelis & Steinmetz 2016), 6=(Velez-Liendo & Garcia-Rangel 2017), 7=(Dharaiya et al. 2016), 8=(Scotson et al. 2017)

<table>
<thead>
<tr>
<th>Common name</th>
<th>Bio–nomenclature</th>
<th>IUCN category</th>
<th>CITES Appendix</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Brown bears</td>
<td>Ursus arctos</td>
<td>LC</td>
<td>II</td>
<td>1</td>
</tr>
<tr>
<td>Polar bear</td>
<td>Ursus maritimus</td>
<td>VU</td>
<td>II</td>
<td>2</td>
</tr>
<tr>
<td>Giant panda</td>
<td>Ailuropoda melanoleuca</td>
<td>VU</td>
<td>I</td>
<td>3</td>
</tr>
<tr>
<td>American black bear</td>
<td>Ursus americanus</td>
<td>LC</td>
<td>II</td>
<td>4</td>
</tr>
<tr>
<td>Asiatic black bear</td>
<td>Ursus thibetanus</td>
<td>VU</td>
<td>I</td>
<td>5</td>
</tr>
<tr>
<td>Spectacled bear</td>
<td>Tremarctos ornatus</td>
<td>VU</td>
<td>I</td>
<td>6</td>
</tr>
<tr>
<td>Sloth bear</td>
<td>Melursus ursinus</td>
<td>VU</td>
<td>I</td>
<td>7</td>
</tr>
<tr>
<td>Sun bear</td>
<td>Helarctos malayanus</td>
<td>VU</td>
<td>I</td>
<td>8</td>
</tr>
</tbody>
</table>

The polar bear is listed as ‘vulnerable’ in the IUCN red list and is protected under CITES Appendix II (Wiig et al. 2015). Although the polar bear is more threatened than the brown bear, it too has a large distribution range and a lower protection status than other bear species and has become an indicator species and mascot in the wake of climate change.

The giant panda has become the globally recognized mascot for conservation organizations, portrayed variously as charismatic, clumsy, lethargic, cute and cuddly. Although its range is small and the protection status is high (Swaisgood et al. 2016), the conservation efforts and research on the giant panda are some of the most substantial conservation efforts made for any threatened species to date. The giant panda is the only animal that had its habitat declared a UNESCO World Heritage site (UNESCO World Heritage Centre 2017).
The American black bear has not garnered as much attention as the brown or polar bears. Although the identification of subspecies appears to be a popular research topic (currently 16 subspecies are identified but these results are still debated in science (Garshelis et al. 2016), the American black bear is only the 4th popular bear species in the Ursidae research field. This is most likely because its distribution does not expand beyond North America (Garshelis et al. 2016), it does not have the same global appeal and is not a symbol of climate change as like the polar bear.

Although the Asiatic black bear is a protected species, it is nonetheless commercially farmed across Asia to produce Traditional Chinese Medicine (Peppin et al. 2008). The commercial popularity of this the Asiatic black bear has likely resulted in increased research attention.

The spectacled bear has (in comparison to the other bear species) a small distribution range (please refer to the range map Figure 2.2) and is under strict protection (Velez-Liendo & Garcia-Rangel 2017). Access to the spectacled bear to conduct research is challenging and it is not surprising, therefore, that so little research has been conducted on this species. In general, small distribution ranges limit the amount of research that is carried out and often limits the research to the local academics.

About 90% of all sloth bears are distributed in India where traditionally they have been used as dancing bears. Their distribution range is limited, however, with most sloth bears confined to remote parts of India (Dharaiya et al. 2016). It is not surprising, therefore, that research studies are not forthcoming as it is known that international collaborations with India are challenging (Iqbal et al. 2015).

The sun bear is listed as ‘vulnerable’ on the IUCN red list and strictly protected under CITES Appendix I (Fredriksson et al. 2008). The sun bear has an arboreal lifestyle that makes research more challenging despite its wide distributed across Southeast Asia. The lack of research carried out on this bear is hard to explain (but will be further expanded on in sections below).

It is worth noting though that the comparison of research carried out on the Ursidae family is limited to searches in two databases and has not been filtered in accordance to the PRISMA-p model.
2.4.3 Publication frequencies for the concise dataset

The analysis of the full dataset with an open search showed that in 93 years only 84 publications mentioned the key-words in title or abstract (a publication mode=1, publication mean=0.9 annually). Using a more concise dataset in which the key-words are mentioned only in the title, between 1999 and 2016 (restricted) a total of 47 papers are found. Of these 47 papers, 33 papers focus solely on the sun bear as the main research target.

The low mean and mode numbers provide statistical support for the notion that the sun bear does not attract significant amounts of research. Although Sloth and Spectacled bears are also not receiving much research attention (as this review showed), the sun bear has a large distribution range (Scotson et al. 2017) and is also essential for the ecosystem it occurs in (Augeri 2005; McConkey & Galetti 1999; Scotson et al. 2017). The amount of research carried out does not reflect the fact that the sun bear occurs in many SE Asian countries is of ecological importance. The peaks around the years 2004 and 2005 (see Figure 2.3) can be attributed to three single authors who published work relating to their PhD research concerning sun bears over this period. The peak in 2013 shows a brief interest in captive behavior of sun bears and veterinary research, although this interest has since dropped off.

2.4.4 Research countries

Examination of the full dataset showed the USA was top of the list of countries that conducted research on sun bears. The USA is not part of the natural distribution range of the sun bear, therefore the research focused on sun bears in captivity or on museum specimen. If the key word is in title alone, then the main countries that carry out research are Malaysia and Indonesia, suggesting that thus far only the sun bears of Borneo have been studied. Sun bear research on bears from mainland Asia have thus far been largely neglected with no research on bears in Vietnam, Laos and Myanmar, and only one study of Chinese sun bears.

2.4.5 Journals and Disciplines

More detailed examination of the sun bear research literature shows that the majority of research was carried out in the veterinary profession, followed by disciplines concerned
with conservation and ecology. As sun bears are elusive animals, research on wild sun bears clearly remains a challenge. Veterinary scientists typically investigate animals housed in captivity, with the relative abundance of sun bears in zoos allowing easy access. Disciplines focusing on conservation and ecology have started integrating modelling and field sign (marking and droppings) surveys into their research focus, facilitating publications on otherwise cryptic species (Steinmetz & Garshelis 2008).

Sun bears are very hard to trap and study in the wild (Fredriksson 2012b; Guharajan 2016). The lack of trapping success and the general shy nature of the sun bear and limited research funding might explain the lack of research carried out on this species. This is further supported by the fact that sun bear research has often occurred as a by-product of more comprehensive interspecies research and have not been the sole interest of the studies, for example, the lack of acquired data may not have allowed publication. When the literature obtained for the key words in the title is considered, it shows that the disciplines of animal behavior, biology and genetics have no associated publications. This demonstrates quite clearly that the main research areas required for successful conservation planning for this species are being completely neglected.

IUCN reintroduction guidelines strongly support the importance of understanding and considering genetic knowledge of a species and indicate that the preservation of genetic diversity to be a priority (IUCN /SSC 2013). Thus far, no comprehensive studies on the genetics of sun bears have been published, nor have any studies examining molecular-level differences between geographically separated populations. Morphology suggests that geographical differences occur, however, no genetic research has been carried out thus far to test if subspecies classification is warranted.

2.4.6 Limitations

The limitations of systematic literature reviews (SLRs) are that the databases themselves are often not standardized with respect to search-ability. For example, Web of Science has different search options and fields to Scopus and each database will also accept or reject typing combinations of the keywords differently to one another. This inevitably leads to slight variations in how keywords are entered into each database search, which in turn impacts the search results. In addition, most databases are ‘active’, meaning that publications are taken from, and added to, the database. It is essential, therefore, to record the date (timeframe) in which the research has been carried out. While there is no setting in the databases that allows to replicate a search conducted at a given date, quality control
through a second and/or third reviewer can be used to confirm replicability of the search when matched to publicly available changes made to the database.

Additionally, Universities and institutions will often have different subscriptions and access to the journals within these databases which, therefore, challenges the replicability of this approach. This can even apply to open access databases such as Google as Google has access to the individual’s metadata and search results may therefore vary between individuals.

Any search of a database is affected by the scope and structure of the electronic database used and the search criteria supplied. The use of more than one electronic database is recommended, as is carefully considering the search strategy to ensure that it is as effective as possible. Ultimately the goal of the database searching is to obtain all relevant papers in a timely fashion without evaluating papers that do not fit the search criteria. Google Scholar is very broad, partly based on what it covers, but also on the algorithm process it uses. In contrast, other databases, such as Web of Knowledge, are limited to a relatively small number of journals and so will not cover as many articles.

Lastly, the systematic review might not filter out papers that do not contain appropriate key words for their search criteria. I therefore suggest a standardized search method (“common name” OR “binominal nomenclature”) and I strongly encourage scientific authors to mention the species name and or binominal nomenclature in the title of their publication so that their paper is detected through the search method.
2.5 CONCLUSION

This systematic quantitative literature review looked at the current scientific knowledge of the sun bear and asked if the moniker the ‘forgotten bear’ is justified. The results of this study show a clear publication bias towards brown bears, giant panda and polar bears. With only 34 studies published between 1999 and 2016 focusing on the sun bear as the main research target, the sun bear is indeed the ‘forgotten’ species of all bears. The ‘Sun Bear Conservation Action Plan’ (Servheen 1999) has stated that the lack of research combined with the multitude of threats facing these bears, has created an ‘ominous situation’ for the conservation of this species. The IUCN bear specialist group has, therefore, called the basic research on the sun bear as the highest research priority for Ursidae species (Servheen 1999). This call for research action on the sun bear has been left unanswered and it appears that the only conservation attempts currently in place are public awareness efforts and the release of individual sun bears that have been confiscated by authorities (Kunde 2012, pers. obs., Guharajan 2018, per. comm.).
CHAPTER 3

Phylogeography of the Malayan Sun Bear

(*Helarctos malayanus*) through Next Generation sequencing

of fresh and museum mitogenomes
3.0 ABSTRACT

Southeast Asia is undergoing the highest rate of deforestation on the planet, posing an immediate and serious threat to its unique biodiversity. Habitat fragmentation and loss, the illegal pet trade, and commercial hunting are threatening the sun bear (*Helarctos malayanus*) throughout its southeast Asian distribution. Current conservation measures include translocation and release of confiscated bears. Genetic assessment prior to a bear’s release would enhance potential reintegration into its source population. However, the genetic data making this possible is currently lacking. Here we present the first comprehensive genetic study of sun bears throughout much of their distribution range. Based on 31 mitochondrial genomes from both historical and contemporary sun bears, we were able to survey the mitochondrial genetic diversity across the species’ range. Our results indicated that sun bears diverged into two distinct mitochondrial lineages in the Mid Pleistocene: a ‘Mainland clade’ and a ‘Sunda clade’, with samples from Thailand belonging to both lineages. Although our dataset is limited, our results suggest that conservation practitioners should consider managing sun bear populations according to their matrilineal lineages and to avoid releases of ‘Sunda clade’ bears into populations of the ‘Mainland clade’ and vice versa. We also encourage extending the research to include data of nuclear loci and to extend sampling to regions of the sun bear distribution range we could not include.
3.1 INTRODUCTION

The Malayan sun bear (*Helarctos malayanus*) is the least studied bear species (Servheen 1999; Wong et al. 2004; Linkie et al. 2007; Sethy & Chauhan 2016) which has earned it the monikers: the ‘forgotten’ or ‘neglected’ bear (Meijaard & Nooteboom 1999). These small bears had been widely distributed across southeast (SE) Asia, occurring in the eastern part of India, Myanmar, Laos, Cambodia, Vietnam, Thailand, Malaysia, Sumatra, and Borneo (Figure 3.1), but their distribution has diminished dramatically to small patches of primary low land (mostly under 500 m) and rainforest remnants (Fredriksson 2001). Sun bears are important seed dispersers and help to maintain trophic relationships (Augeri 2005). Yet despite threats to their survival in the wild, they remain understudied, particularly with respect to their genetic diversity.

Sun bears are threatened by multiple factors such as excessive hunting, habitat loss and habitat fragmentation, as well as inconsistent wildlife law enforcement (Meijaard & Nooteboom 1999; Fredriksson 2005; INTERPOL 2014; Krishnasamy & Shepherd 2014). Despite being globally strictly protected since 1979 (Servheen 1999; Wong et al. 2004; Augeri 2005), they are, for example, still a popular game species in Malaysia (Servheen, 1999). In addition, cubs are hunted for the illegal pet trade (Foley et al. 2011; Krishnasamy & Shepherd 2014; Ling et al. 2015), and body parts are used for making wine, soup, lucky charms and traditional Chinese medicine (Scotson & Hunt 2008; Scotson & Downie 2009; Shepherd & Shepherd 2010). Sun bears are opportunistic omnivores (Fitzgerald and Krausman 2002). On Borneo during mast fruiting events, the sun bear diet is frugivory (feeding almost to 100% on fruits, predominantly on figs) (Fredriksson et al 2006). During inter-mast periods, sun bears are mostly insectivorous but will also feed on fruits, palm oil and coconuts and are thus sometimes considered a nuisance or pest by local farmers (Fredriksson 1998; Fredriksson 2005).
Figure 3.1: Sun bear (*Helarctos malayanus*) distribution map, altered from the IUCN Red List map (Fredriksson et al. 2008). The colours represent the confirmed present (green) and probable present (blue) distribution ranges, as well as the areas where the sun bear is hypothesized to have gone extinct (red). Black circles indicate sampling localities for this study (details in Appendix III). The map has been produced using ArcGIS (10.2) with spatial distribution information provided by the IUCN 2017–1(www.iucnredlist.org).

Sun bears have lost 30-60% of their habitat over the past 30 years (Meijaard & Nooteboom 1999; Fredriksson et al. 2008), leading to a severe decline in population size. As a result, the species has been listed as *Vulnerable* on the IUCN Red List of threatened species (Fredriksson et al. 2008) and in Appendix 1 of CITES (CITES, 2013).

Alarming decline of population size, deficiency in life history data, lack of knowledge about its distribution, high mortality rates, and diminishing population health strongly suggest
that conservation strategies for sun bears are not working, emphasizing why the sun bear has been assigned the highest research priority within the bear family Ursidae (Servheen 1999; Linkie et al. 2007; Linkie et al. 2013). For breeding and release programs it is of paramount importance to genetically assess the sun bear’s overall population structure and connectivity, and to identify taxonomically or management relevant units to re-evaluate conservation priorities and species management (Onuma et al. 2006).

Here, we present the first comprehensive phylogeographic study of the Malayan sun bear Helarctos malayanus. To assess the distribution of intraspecific genetic variation in this threatened species, we sequenced complete mitochondrial genomes (mitogenomes) of sun bears across their current and historical range. This study can provide a first overview of the distribution of genetic variation of this species. Nonetheless, mtDNA can only provide insight into the matrilineal history of the species and although this is a vital starting point into understanding the genetics of this species, nuclear DNA data is still necessary to obtain enough information for establishing conservation genetic management plans for the sun bear.
3.2 MATERIAL AND METHOD

3.2.1 Samples

Dried skull tissue or tissue from the nasal cavity of 29 archival sun bear specimens were collected from several natural history museums in Europe (Supplementary Chapter 3 Table S1). In collaboration with, and under the supervision of, “Free the Bears” Cambodia, we also collected scat and saliva samples from 21 animals from 12 provinces in Cambodia where we had precise information regarding their origin in the wild. We attempted to include two samples per Cambodian province in order to obtain a balanced geographic coverage of Cambodia (Figure 3.1).

3.2.2 Archival and modern DNA

DNA from archival samples was extracted following Wilting et al. (2016) in a laboratory designed for and limited to the use of archival material. DNA extractions were conducted in batches consisting of three samples and one negative control, to minimize cross contamination. DNA extractions were verified to be of sun bear origin by amplification of a 147 bp long portion of the mitochondrial ATP6 gene (Supplementary Chapter 3 Table S1). DNA extractions from modern samples (saliva and scat) were carried out using the GEN-IAL First DNA All-tissue DNA extraction Kit (GEN-IAL GmbH, Troisdorf, Germany) following manufacturer’s instructions. This work was carried out in a separate facility, designed for molecular work with non-invasively collected samples.

3.2.3 Library building and hybridization capture

Illumina sequencing libraries were constructed following (Fortes & Paijmans 2015), using double-indexing with 8-bp barcodes. Prior to library building, DNA of the 21 modern samples was sheared to a target size of 300 bp using the Covaris M220 (Woburn, MA, USA). We conducted qPCRs to determine the optimal PCR cycle number for each sample prior to indexing (and post-capture, below).

As archival DNA is highly degraded and likely to contain external contamination from preparation, handling, storage and exposure of the archival specimen, we enriched the 29 libraries of the archival samples for mitochondrial DNA (mtDNA) using hybridization capture. To avoid methodological bias, we also applied hybridization capture to the 21 fresh samples.
Baits for hybridization capture were generated using long range (LR) PCR products that spanned three large overlapping regions of the mitogenome (using a fresh *Helarctos malayanus* sample from the IZW tissue sample archive). The LR PCR primers were designed using the reference sequence NC_009968: HMA1-F (5’-ACGACCTCGATTTGGATCAGG-3’) and HMA1-R (5’-AGGGCTACAGCGAACTCGAGA-3’), yielding a 6152 bp long product, HMA2-F (5’-GCCACACTCATTCAACCTACCA-3’) and HMA2-R (5’-AGTCCTTTCTGGTTGGAGACTGTG-3’), yielding a 5299 bp long product, and HMA3-F (5’-ACCAACGCCTGAGGCCCTACT-3’) and HMA3-R (5’-GCGCTTTAGTGAGGGAGGCC-3’), yielding a 6416 bp long product. Amplifications were carried out using the following protocol: 18 µl dH₂O, 25 µl Bioline Myfi mix (buffer plus enzyme) (Bioline GmbH, Germany), 2 µl of 10 µM primer F, 2 µl of 10 µM primer R, 3 µl of 100ng/µl DNA template. PCR conditions: initial denaturation at 94°C for 3 min, followed by 40 cycles of denaturation at 94°C for 30 s; annealing at 60°C for 30 s; extension at 68°C for 7 min with the final extension at 68°C for 10 min and final storage at 8°C.

LR-PCR products were sheared, pooled equimolarly and then converted into biotinylated baits following Maricic et al. (2010). In-solution capture was carried out using hybridization temperatures appropriate for sample type (Paijmans et al. 2016). Paired-end sequencing was carried out on the Illumina MiSeq platform (Illumina, San Diego, CA, USA) using v3 150-cycle kits.

### 3.2.4 Bioinformatic workflow

We de-multiplexed the paired-end reads using BCL2FASTQ v2.17.1.14 (Illumina, Inc.) and removed the adapter sequences using C U T A D A P T v1.3 (Martin 2011). Using TRIMMOMATIC (Bolger, Lohse, and Usadel 2014), we applied a sliding window approach for quality trimming with the phred quality threshold set at Q=20. Next, we merged the adapter-clipped and quality-trimmed sequences using FLASH v1.2.8 (Magoc and Salzberg 2011). These sequences were then mapped to the reference sun bear mitogenome sequence (GenBank accession no. NC_009968) with BWA v0.7.10 (H. Li and Durbin 2009). The on-target sequences were then de-duplicated using MARKDUPicates from PICARD-TOOLS v1.106 (https://github.com/broadinstitute/picard), followed by variant calling using SAMTOOLS v1.1 (Li et al. 2009) and BCFTOOLS v1.2.
(http://github.com/samtools/bcftools). For maximum data recovery, we applied iterative mapping, involving a second round of mapping to the sample consensus again using BWA v0.7.10. Positions with a sequence coverage <10×, or variants with depth of ≤80% representation were excluded and ‘N’-masked using GATK (genome analysis toolkit, Broad Institute, Cambridge, MA, USA).

The final data set of complete or near-complete mitogenome consensus sequences consisted of 13 archival and 17 fresh samples (plus one sequence from GenBank NC_009968), which were aligned using MAFFT v.7.245 (Kato and Standley 2013).

3.2.5 Phylogeographic analyses

To reconstruct the phylogeny among our sun bear samples we used a Maximum Likelihood (ML) approach implemented in RAXML (Stamatakis 2014). The best fitting substitution model for our data was obtained by applying the AIC criterion in jMODELTEST (Posada 2008), and was GTR+G+I.

We estimated the molecular clock rate using the Multispecies Coalescent model in BEAST2 (BEAST v2.4.5 Bouckaert et al. 2014) and applied it to four sun bear mitogenomes (three mitogenomes produced in this study and one from Genbank [NC_009968]) along with mitogenomes from ten other bear species (Supplementary Chapter 3 Table S1). The best fitting substitution model for this data was GTR+G+I. For the Bayesian inference (BI) tree we used a Log normal uncorrelated relaxed clock with species tree priors taken from the published, mitogenome-based phylogeny of bears (Wu et al. 2015). Two independent runs were performed for 100,000,000 generations, with sampling of model parameters and trees every 3000th generation. The first 10% of generations were discarded as burn-in before the results of the two runs were combined in LOGCOMBINER (BEAST v2.4.3). Convergence and effective sample size (ESS > 200) were assessed in TRACER v1.6 (Rambaut, Drummond, and Suchard 2013). We then applied the estimated clock-rate as prior in order to infer the divergence date from intraspecies sun bear data. We used the strict clock (mean=0.009, SD=1E-5) and a Yule tree model with 100,000,000 generations, sampling tree and model parameters at every 3000th generation. Again, the ESS value (> 200) and convergence were assessed in TRACER v1.6. TREEANNOTATOR (implemented in BEAST) was used to compute a maximum clade-credibility tree with mean height. The dated tree was then visualized in FigTree v1.4.1 (http://tree.bio.ed.ac.uk/software/figtree/). To
visualize the relationship among haplotypes, we generated a median joining (MJ) network using POPART v.1.7. (Bandelt et al.1999).

As only one Bornean sample was included for analysis after quality filtering, we could not test for the genetic differentiation of this population as reported in Onuma et al. (2006b). Therefore, we extracted the d-loop region from 30 mitogenomes generated in this study and created a dataset which additionally included 14 d-loop sequences of Bornean individuals obtained from (Manabu Onuma, Suzuki, and Ohtaishi 2006) and the extracted d-loop region from a Bornean sample available in Genbank (see details in Supplementary Chapter 3 Table S1) Sequences were aligned using MAFFT with the auto setting and the phylogeographical pattern was estimated in Popart vXX using the TCS model.

3.2.6 Summary statistics

We examined several diversity measures, both for the entire dataset, as well for subdivisions of the dataset based on lineages apparent from the phylogeographic analyses (above). DNASP v.5.10 (Librado & Rozas 2009) was used to estimate haplotype and nucleotide diversity, Tajima’s D and the number of segregating sites and average differences. ARLEQUIN v.3.5.1.2 (Excoffier et al. 2005) was used to calculate $F_{ST}$ between clades (significance was tested using 10100 permutations).
3.3 RESULTS

In total, we obtained DNA in sufficient amounts and quality from 50 samples (29 archival and 21 fresh samples) with known geographical origin. For 30 of these (13 archival and 17 fresh samples; Figure 3.2; Appendix 3.1), we were able to generate full mitogenome sequences of 16789 bp length, which we analyzed together with the published mitogenome from a Chinese sun bear (Yu et al. 2004); acc.no.: NC_009968).

Figure 3.2: Result–map of the pylogeographic sequencing of museum and contemporary sun bear (*Helarctos malayanus*) samples. The black circles symbolise the samples that have been sequenced successfully, the grey circles indicate the samples that did not amplify successfully. The circle size is representative for the number of samples (n). The smallest circles represent n=1, the second largest circles represent n=2 and the largest circles represent n=3. The white star in some of the circles are indicating that the exact sample location is not known for this sample (the source country would have been identified but not the exact location within the country). The dotted line symbolises the Isthmus of Kra (IoK).
3.3.1 Phylogeography and divergence dating

The topologies of both the ML and the BI mitogenome tree were identical and all deep nodes had high support. The depicted BI tree (Figure 3.3) and MJ network (Figure 3.4) show two major lineages: a ‘Mainland lineage’, comprised of samples from China, Cambodia and Thailand, and a ‘Sunda lineage’ comprised of samples from the Sundaic islands (Borneo and Sumatra), from the Malay Peninsula, as well as three samples from Thailand. Whether the latter were from North or South of the Isthmus of Kra (IoK) was unfortunately not known. The divergence time between the two major lineages was estimated at around 676 ky (CI95%: 518-848 ky; (Figure 3.3)), during the upper Mid Pleistocene. Both major lineages were further subdivided into two clades. The split within the Mainland lineage was estimated to be 388 ky old (CI95%: 288-500 ky), while the one within the Sunda lineage was around 297 ky old (CI95%: 221-378 ky). All (sub-) lineages apparent in the phylogenetic analyses are also evident in the MJ network; the two major lineages are separated by a minimum of 131 mutations (Figure 3.4).

The TCS network of the d-loop region remained unresolved (possibly attributed to the short fragment size of only 300 bp of the d-loop region) but also indicate a split into the two clades of ‘mainland’ and “Sunda” bears and revealed a lack of genetic differentiation as samples from Borneo shared haplotypes with Sumatra, Malaysia and Thailand individuals. The TCS network supports that Thailand is clustering with both clades (Figure 3.5). However, it did reveal large genetic diversity on Borneo. Furthermore, when adding the dloop region of samples of unknown origin, the dloop network showed that they fall into an own clade. This indicates that the phylogeography is still not fully resolved and provides the opportunity for further research, expanding the dataset and including more locations from the sun bear’s distribution range.
Figure 3.3: Phylogenetic reconstruction with BEAST of 31 mitogenomes (13 archival and 17 fresh and one NCBI sequence) with 16789 bp in length. Numbers below the branches represent BPP values and bootstrap values as obtained from RaxML analysis. Here we only display values greater than 80% (RaXML) and greater than 0.9 (posterior probability values for Bayesian trees). The error bars are showing at height 95% HPD. The numbers above the trees and arrows represent the age of the node.
Figure 3.4: Median Joining (MJ) network showing the relationship among 31 sun bear mitogenomes. Each circle represents a haplotype, and a larger circle indicates several samples with the same haplotype. The circle colour represents the samples' origin (see legend). The numbers in brackets indicate the number of mutations between haplotypes.
**Figure 3.5**: TCS haplotype network of the extracted d–loop region of 31 sun bear mitogenomes and 14 d–loop regions (300 bp) from sequences submitted to NCBI (Accession number listed in table A). The dashes on the branches indicate the amount of mutations between the nodes. The circles are color-coded in accordance with region of origin. The size of the node indicates that a few samples share the same haplotype. The colored, larger nodes with different colors indicate that samples from different origin share the same haplotype.
3.3.2 Summary statistics

Among the 31 complete mitogenomes (incl. NCBI, put number here) we detected 24 unique haplotypes. Diversity measures for the whole dataset, as well as subsets thereof are given in Table 3.1. As samples from Thailand carried sequences from both major lineages (Sunda and Mainland), diversity measures are presented both with and without these samples. Out of the 31 samples, 20 belong to the Mainland lineage (14 haplotypes). The remaining 11 samples belong to the Sunda lineage (10 haplotypes). Exclusion of the samples from Thailand changes the measures of diversity (Table 3.1).

Table 3.1: Summary statistics for the number of samples utilised in this study (N), haplotypes (h) identified, haplotype diversity (Hd), segregating sites (S), nucleotide diversity (π), average differences (k), and number of sites excluding sites with gaps/missing data (n) for the for the sun bear mitogenome.

<table>
<thead>
<tr>
<th></th>
<th>N</th>
<th>h</th>
<th>Hd</th>
<th>π</th>
<th>k</th>
<th>S</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>All</td>
<td>31</td>
<td>24</td>
<td>0.981</td>
<td>0.00578</td>
<td>76.815</td>
<td>337</td>
<td>13287</td>
</tr>
<tr>
<td>Mainland with Thailand</td>
<td>20</td>
<td>14</td>
<td>0.958</td>
<td>0.00121</td>
<td>16.268</td>
<td>125</td>
<td>13287</td>
</tr>
<tr>
<td>Mainland excluding Thailand</td>
<td>18</td>
<td>13</td>
<td>0.961</td>
<td>0.00185</td>
<td>25.69</td>
<td>157</td>
<td>13912</td>
</tr>
<tr>
<td>Sunda with Thailand</td>
<td>11</td>
<td>10</td>
<td>0.982</td>
<td>0.00389</td>
<td>63.055</td>
<td>203</td>
<td>16228</td>
</tr>
<tr>
<td>Sunda without Thailand</td>
<td>8</td>
<td>8</td>
<td>1</td>
<td>0.004</td>
<td>66.393</td>
<td>172</td>
<td>16552</td>
</tr>
</tbody>
</table>

Differentiation between the two major lineages (measured as Fst) was high and significant, both when samples from Thailand were included (Fst = 0.7678, P < 0.001) or excluded (Fst = 0.7650, P < 0.001). Differentiation between geographic localities (Table 3.2) was significant in all cases, even in pairwise comparisons between localities within the same lineage.
Table 3.2 The pairwise population differentiation between the geographic localities

*** $P < 0.001$, **$P < 0.01$, *$P > 0.01$

<table>
<thead>
<tr>
<th></th>
<th>Malaysia</th>
<th>Sumatra</th>
<th>Borneo</th>
<th>Thailand</th>
<th>China</th>
<th>Cambodia</th>
</tr>
</thead>
<tbody>
<tr>
<td>Malaysia</td>
<td>0</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sumatra</td>
<td>0.07313*</td>
<td>0</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Borneo</td>
<td>-0.14706*</td>
<td>-0.05185*</td>
<td>0</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Thailand</td>
<td>-0.02356*</td>
<td>0.13572*</td>
<td>-0.26013*</td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>China</td>
<td>0.55043*</td>
<td>0.68409*</td>
<td>1.00000*</td>
<td>-0.02604*</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Cambodia</td>
<td>0.80788***</td>
<td>0.80253***</td>
<td>0.8426*</td>
<td>0.49085***</td>
<td>0.15243*</td>
<td>0</td>
</tr>
</tbody>
</table>

The pairwise population differentiation between the geographic localities showed that the $F_{ST}$ value between the samples representing the Sunda lineage are not significant. The only significance appears to be between samples from Cambodia and the samples of the Sunda lineage. However, as there are more Cambodian sun bears represented in this study than from other localities, it is likely that the results from these $F_{ST}$ values are biased and not truly representative of the geographic population differentiation between the two lineages.
3.4 DISCUSSION

This study represents the most comprehensive survey of matrilineal genetic diversity of the sun bear. Using hybridization capture coupled with high throughput sequencing we successfully retrieved data for whole mitochondrial genomes of sun bears from a large portion of their geographic distribution by incorporating archival material in our sampling. In this manner, we were able to cover areas of the distribution range that would have been inaccessible using contemporary samples of this elusive carnivore.

3.4.1 Phylogeography of the sun bear

Sun bears originated in Southeast Asia, from where they followed the expansion of rainforest southwards to Sumatra, Java and Borneo (Meijaard 2004). However, populations in Java have not persisted (Long et al. 1996; Fredriksson et al. 2008). Our mitogenome data indicates the presence of two matrilines within the current distribution of the species we sampled: one matriline restricted to portions of mainland SE Asia (encompassing China, Cambodia and Thailand), and a second inhabiting the Sundaic Islands of Sumatra and Borneo, as well as portions of the mainland (Peninsular Malaysia and Thailand). Despite our efforts, the failure of some archival samples to yield reliable results means that our sampling of mainland SE Asia is somewhat limited and biased; we are missing data for Vietnam, Laos, Myanmar, and India.

The estimated time of the divergence of these matrilines suggests that sun bear populations experienced some form of isolation during the Middle Pleistocene. This appears to have occurred on the mainland, rather than representing a vicariance between mainland and Sundaic sun bears, as it pre-dates the presence of *H. malayanus* on the Sundaic islands (Medway 1964; Long, et al 996; Tougard 2001). While molecular estimates of divergence times need to be considered with caution, our mitogenome data suggests the arrival of sun bears on the Sundaic islands in the late Middle Pleistocene / early Late Pleistocene, consistent with the fossil evidence (Tougard 2001; Meijaard 2004).

Fossil evidence for the presence of sun bears on the mainland in the Middle Pleistocene is poor (Tougard 2001; Meijaard 2004). Thus, it is not possible to ascertain the distribution of sun bears during this period or deduce the cause for their divergence ~676kya. However, based on the current distribution of the matrilines, a split could have occurred in a north-south fashion (or, perhaps northeast-southwest fashion). Following this isolation, gene flow between populations
appears to have been reestablished, as samples from Thailand are members of both matrilines. Further sampling is needed to gain a better insight into this, including the geographic extent of the secondary contact zone between the two lineages. Additionally, nuclear data would be required to verify that the members belonging to the matrilines interbreed.

We do not have enough samples from Sundaic sun bears to determine if *H. malayanus* moved between the islands following colonization. Such movement appears possible due to the exposed shelf that connected the major islands of the Sundaland during the low sea levels of the Pleistocene (Bird et al. 2005), and has been inferred for other large mammals, such as leopard *Panthera pardus* (Wilting et al. 2016), leopard cat *Prionailurus bengalensis* (Patel et al. 2017), and muntjak *Muntiacus muntjak* (Martins et al. 2017). These corridors were, however, composed of savannah-like vegetation which probably acted as a barrier to dispersal of forest-dependent species (Bird et al. 2005) such as the sun bear. Other authors have described a patchier distribution of habitats throughout the newly available landmass with occurrence of closed and open forest (Meijaard & Nijman 2003) or even that rainforest had periods of expansion in the past (Cannon et al. 2009). Thus, further sampling of sun bears from Borneo and Sumatra is needed to understand whether this rainforest-dependent species moved between the Sunda Islands during periods when the shelf was exposed.

### 3.4.2 Taxonomic status of the Bornean sun bear

There are clear differences in the physical appearance of the sun bears on Borneo (*Helarctos malayanus euryspilus*) (Horsfield 1825) in comparison to those on Sumatra and mainland Asia (*Helarctos malayanus malayanus*) (Raffles 1821), and a split into sub-species *H. m. malayanus* and *H. m. euryspilus* was thus suggested (Chasen 1940; Fitzgerald & Krausman 2002; Meijaard 2004). These differences indicate phenotypic responses to food availability and foraging behavior and potentially mislead taxonomy (a well-nourished bear may simply grow bigger than a malnourished bear) (Kitchener 2010). The rainforest on Borneo is less fruit-bearing than rainforest on Sumatra or on the mainland of Asia, and this reduced food availability may have favored smaller body size and modified dentition and skull morphology on Borneo (Meijaard 2004; Wich et al. 2011). This phenotypic divergence would be indicative of genetic differentiation reflected in the nuclear genome, which we did not investigate here. Our genetic analyses based on mitochondrial DNA were not consistent with the suggestion that the Bornean sun bear represents a separate sub-species. However, to determine sub-species classification and find a taxonomic consensus, is it required to combine morphological data, mtDNA and nuclear
markers, applied to a comprehensive dataset. Our study has clearly indicated that phylogeographical relationships between the islands is more complex and the analysis of nuclear genetic markers, in a broad geographic context, is required to make a conclusive suggestion regarding the classification of the Bornean population.

3.4.3 Conservation recommendations

A phylogeographic approach can reveal management and evolutionary units in species (Avise 1992; Li et al. 2005; Onuma et al. 2006), help to identify conservation priorities (Goossens et al. 2013), identify cases of hybridization/secondary contact (Edwards et al. 2011), as well as assist in pinpointing suitable reintroduction locations (Apollonio et al. 2014). Thus far, the classification of sun bear populations has been based on geography and morphological traits, the latter having been only investigated using a small dataset. It is important to corroborate/supplement such efforts using molecular studies (Kitchener 2010). Our research reveals a clear (and deep) split into two matrilines that divide sun bears from the Sundaic region (Borneo, Sumatra, Peninsular Malaysia, part of Thailand) from others on the Indochinese mainland (China, Cambodia, parts of Thailand).

Considering our results, we encourage ex-situ conservation breeding to take the existence of two highly differentiated lineages (Sunda and mainland) into account; a distinction that lacks the ‘resolution’ to differentiate the morphologically distinct Bornean sun bears (see above).

A further complicating factor stems from the illegal bear trade. We cannot exclude the possibility that the sun bear from Borneo that shares the same haplotype with the sun bear from Thailand may have been traded. We also obtained museum samples of sun bears that supposedly originated from Jakarta on Java; but we know that sun bears do not occur on Java (Meijaard & Nooteboom 1999; Meijaard 2004; Fredriksson et al. 2008) and that Jakarta is a hotspot for the wildlife trade (Nijman et al. 2015; Shepherd et al. 2016). Therefore, recent and historical trade of sun bears can potentially confound results of molecular studies, particularly those employing non-recombining genetic markers such as mtDNA. The hypothesis that the Bornean sun bear sample included in this study may have been subject to trade is supported by the d-loop network.

Should a confiscated bear cub undergo genetic screening for the purpose of identifying a suitable release site for said cub, we caution here that our research results are limited in its practicality for conservation practitioners. Therefore, the bear cub should undergo further genetic screening.
with nuclear markers such as microsatellites. Microsatellites allow to identify and define populations, provide a useful genetic tool for the forensic genetics and would be a useful tool to assign bear cubs of unknown origin to their most suitable population to be released back into.

We hope that our work may encourage further research on sun bears so that they may no longer be referred to as the ‘forgotten bear’. Large mammals, such as sun bears, are often keystone species for their habitat (Treves & Karanth 2003; Karanth 2009). Protecting the sun bear will come at a cost, but the benefits are immense (Augeri 2005; Lorraine Scotson and Hunt 2008; Lorraine Scotson and Downie 2009).
3.5 AUTHORSHIP CONTRIBUTIONS

DWF and AW designed the study. AW secured international collaboration opportunities between the Leibniz Institute for Zoo and Wildlife Research (IZW) and Griffith University, and arranged the collection of museum samples. MK secured CITES export and import permits as well as ethical clearances and biosecurity import permits. MK sampled the contemporary samples from Cambodia, RM and RP collected the museum samples. MK performed experiments. RP and MK analyzed the data. MK, RM, RP, DF wrote the early drafts and all authors contributed to writing the final manuscript.

3.6 DATA AVAILABILITY

The sun bear mitogenome sequences from this study will be deposited in Genbank to a later date.
CHAPTER 4

Forgotten and fragmented: population genetics of Cambodian sun bears (*Helarctos malayanus*) using microsatellite loci
4.0 ABSTRACT

The sun bear (*Helarctos malayanus*) is threatened by habitat loss and fragmentation of its habitat, and the illegal trade of both live bears and bear parts. Sharply declining population numbers and a lack of natural dispersal between populations potentially threaten the genetic status of this species through loss of genetic variation.

Microsatellite markers are a useful tool to investigate population genetics, providing information about levels of genetic diversity and gene flow between populations (i.e. connectivity). In this Chapter, we analyzed 78 sun bears from Cambodia using a set of 14 microsatellites. The aims of the research were to 1) assess genetic structure of the Cambodian population(s), 2) estimate the level of genetic diversity within this population(s), and 3) assign bears of unknown origin to the area they most likely come from. This research showed that there are two genetically distinct populations East and West of the Mekong River and that there is gene-flow between these populations. However, the gene-flow is not frequent enough to alleviate inbreeding in the West populations. Therefore, the sun bear conservation action plans should consider promoting connectivity between the populations.
4.1 INTRODUCTION

Southeast (SE) Asia is a global biodiversity hotspot providing vital habitat to a range of endemic flora and fauna (Myers et al. 2000). SE Asia is also one of the areas of the world that has experienced the most severe anthropogenic disturbance (Hughes 2017), making it a region of global conservation concern (Sodhi et al. 2010). Located on the SE Asian mainland, Cambodia holds an outstanding amount of protected wildlife reserves, covering approximately 20% of the land-cover (Hsu et al. 2016). However, Cambodia’s wildlife is threatened by poaching, (Gray et al. 2012; Gray et al. 2017) and has one of the highest rates of deforestation worldwide (Kapos et al. 2010; Avtar et al. 2013; Hughes 2017). Large scale clearing for agriculture and construction projects extends into wildlife sanctuaries and national parks; consequently creating a highly fragmented landscape of vital wildlife habitat (Le Billon 2002). Habitat fragmentation directly reduces suitable habitat, and also creates patches of hostile and uninhabitable habitat between surrounding fragments (Bender et al. 1998; Franklin et al. 2002). Furthermore, habitat fragmentation leads to a reduced resource availability that lowers the carrying capacity to maintain large wildlife populations (Bender et al. 1998).

When fragmentation impacts the ability of individuals to traverse to other populations, this can potentially result in the isolation of breeding populations (Bender et al. 1998; Fahrig 2003; Lancaster et al. 2016). This is of concern because isolated populations are subjected to an overall loss of genetic diversity (Paetkau et al. 1995), and potentially face problems relating to inbreeding (Frankham 2005). This is particularly an issue when population sizes are low, and can ultimately lead to the local extinction of the population. Inbreeding can have significant impacts on fitness, for example reducing sperm quality and quantity (Fitzpatrick & Evans 2009), reducing competitive abilities (Haag et al. 2002), compromising mating ability (Frankham 2005), reducing the number of offspring produced and offspring survival rates (Liberg et al. 2005), increasing developmental time (Fox & Reed 2010) and reducing disease resistance and immune competence (Ilmonen et al. 2008). These impacts endanger populations, particularly in conjunction with reduced and disturbed habitat. Furthermore, a reduction in genetic diversity can be detrimental to the adaptability of a species to a changing environment (Lande 1988). Therefore, the International Union for Conservation of Nature (IUCN) acknowledges the importance of the understanding the genetic profile of a target species to optimize its conservation management plan (Reed & Frankham 2003).

The sun bear (*Helarctos malayanus*) is a small Ursid, distributed across SE Asia (see Chapter 1, Figure 1.1). Although very little research has been conducted to evaluate the status of this bear
species (Meijaard & Nooteboom 1999; Wong & Linkie 2013), there is a consensus in the scientific community that the sun bear is under threat (Fredriksson et al. 2008). The sun bear is therefore listed as ‘vulnerable’ on the IUCN red list (Fredriksson et al. 2008) and is CITES Appendix I protected (Convention on International Trade in Endangered Species (CITES) 2013). Habitat fragmentation coupled with the uncontrolled illegal trade of live bears and bear parts has resulted in a population decline and fragmentation (Augeri 2005; Fredriksson et al. 2008; Wong et al. 2015).

Poaching and the lack of viable habitat are two of the main causes that increase the demand for wildlife officials to translocate sun bears that come into conflict with humans (Crudge et al. 2016). However, translocation\(^4\), augmentation\(^5\) or rehabilitation\(^6\) of bears into an existing bear population can endanger the genetic and physiological health of the remaining wild sun bear populations (Dijk 2005; Fredriksson 2001; Huber 2010). The aims of this study were (1) to assess the genetic structure in the Cambodian sun bear population(s), (2) to estimate the level of genetic diversity in the population(s), and (3) to assign bears of unknown origin to the area they most likely come from. The results of this study will be translated into action points for the sun bear conservation action plan.

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\(^4\) Translocation: “the human-mediated movement of living organisms from one area, with release in another.”, IUCN 2012

\(^5\) Augmentation: “the intentional movement and release of an organism into an existing population of conspecifics.”, IUCN 2012

\(^6\) Rehabilitation: “the process where a sick, injured, orphan or displaced animal regains the health and skills to function normally and live self-sufficiently” IWRC 2009
4.2 METHOD & MATERIAL

4.2.1 Study area and sample collection

The Kingdom of Cambodia is a country in Southeast Asia, bordering Thailand, Laos, and Vietnam. For this research, we sampled DNA from bears from 17 different provinces in Cambodia (Supplementary Chapter 4 Table TS1): Kampot (n=1), Kandal (n=7), Koh Kong (n=5), Kompong Cham (n=2), Kompong Som (n=1), Kompong Speu (n=1), Kompong Thom (n=3), Kratie (n=4), Oddar Meanchey (n=3), Phnom Penh (n=18), Phnom Penh (Mondulkiri market) (n=2), Preah Vihear (n=3), Prey Vihear (n=1), Pursat (n=1), Ratanakiri (n=13), Siem Reap (n=7), Stung Treng (n=12) and Takeo (n=3) (Figure 4.1).

Figure 4.1: Locations from which the sun bears from the ‘Free the Bears’ sanctuary in Phnom Tamao Zoo and Wildlife Rescue Centre (Cambodia) originated from.
Collection permits (ethics, collection, export and import permits) for the samples were obtained as required. Samples were taken from sun bears housed at the ‘Free the Bears’ sanctuary in Phnom Tamao Zoo and Wildlife Rescue Centre, Cambodia. Samples were collected non-invasively, primarily through the collection of the mucus layer of scat samples. Additionally, saliva, tissue and blood samples were collected as opportunity allowed. Over the course of ten days in August 2013, 99 saliva, 81 scat, two claws, one blood, one phlegm, and one tissue sample (a total of 185 samples) from 94 individual bears were collected (Supplementary Chapter 4 Table TS1). From these 185 samples, 91 samples were used for further analysis (we attempted to get one sample of each of the 94 individual animals but not all samples passed the quality filter). Additionally, blood samples from two deceased zoo animals (IZW ID# 2449 and IZW ID# 2462) were used to test and optimize microsatellite primers (below).

4.2.2 Laboratory Procedures

All DNA extractions were carried out using the “GEN-IAL First DNA All-tissue DNA extraction Kit” (GEN-IAL GmbH, Troisdorf, Germany), following manufacturer’s protocol. Genotyping was conducted using 17 microsatellite loci: Umar01, Umar02, Umar03, Umar04, Umar05, Umar05, Umar06, Umar07, Umar08, Umar09, Umar10, (Poissant and Davis 2011), G10X (Straka et al. 2012), Ma05, G1D (Andreassen et al. 2012), UarD3139, UarT739, UarD1585 (Kleven et al. 2012), MU50 (Bellemain and Taberlet 2004) (Table 4.1).

Polymerase chain reactions (PCR) were carried out using 5µL 2x Qiagen multiplex Master Mix (Qiagen), 1 µL Q-Solution (Qiagen), 1 µL primer mix (0.5 µL primer F + 0.5 µL primer R), 1 µL template DNA and 2 µL RNase-free water. Amplifications were carried out under the following conditions: initial denaturing at 95°C for 5 min, followed by 35 cycles of denaturation at 94°C for 30 secs, annealing at 63°C, 61°C, 59°C, 57°C and 55°C for 1 min and 30 secs, and extension at 72°C for 30 secs. The final extension was at 60°C for 30 min.

The amplified fragments were separated on an A3130xl DNA sequencer (ABI Applied Biosystems, Foster, CA, USA). Allele sizes were determined on an ABI3130xl Genetic Analyser using GeneScan™ 500 ROX (both Thermo Fischer Scientific Darmstadt, Germany) as internal size standard. The allele sizes were scored using the GeneMapper v 4.0 application (ABI).
Table 4.1: Cambodian sun bear population(s) structure was analysed using 17 Ursidae microsatellite markers. Umar 04, Umar 06, and Umar 08 were excluded from further analysis as they lacked variation.

<table>
<thead>
<tr>
<th>Locus</th>
<th>Repeat motif</th>
<th>Size range*</th>
<th>Author</th>
</tr>
</thead>
<tbody>
<tr>
<td>UarD1585</td>
<td>(GT)16</td>
<td>121–131</td>
<td>Kleven et al., 2012</td>
</tr>
<tr>
<td>UarD3139</td>
<td>(GT)22</td>
<td>107–121</td>
<td>Kleven et al., 2012</td>
</tr>
<tr>
<td>UarT739</td>
<td>(TATC)10</td>
<td>108–132</td>
<td>Kleven et al., 2012</td>
</tr>
<tr>
<td>Mu50</td>
<td>(CA)17</td>
<td>106–136</td>
<td>Bellemain &amp; Taberlet 2004</td>
</tr>
<tr>
<td>Umar 01</td>
<td>(CA)17</td>
<td>225–233</td>
<td>Poissant &amp; Davis, 2011</td>
</tr>
<tr>
<td>Umar 02</td>
<td>(GT)14</td>
<td>200–212</td>
<td>Poissant &amp; Davis, 2011</td>
</tr>
<tr>
<td>Umar 03</td>
<td>(TG)14</td>
<td>232–240</td>
<td>Poissant &amp; Davis, 2011</td>
</tr>
<tr>
<td>Umar 04</td>
<td>(TG)19TTTGA(GT)4</td>
<td>117–127</td>
<td>Poissant &amp; Davis, 2011</td>
</tr>
<tr>
<td>Umar 05</td>
<td>(GT)3C(TG)13</td>
<td>202–218</td>
<td>Poissant &amp; Davis, 2011</td>
</tr>
<tr>
<td>Umar 06</td>
<td>(TG)2CG(TG)2CT(TG)3C(GT)11</td>
<td>247–249</td>
<td>Poissant &amp; Davis, 2011</td>
</tr>
<tr>
<td>Umar 07</td>
<td>(CA)14</td>
<td>184–192</td>
<td>Poissant &amp; Davis, 2011</td>
</tr>
<tr>
<td>Umar 08</td>
<td>(TA)3(TG)6CGTGA(TG)8</td>
<td>202–204</td>
<td>Poissant &amp; Davis, 2011</td>
</tr>
<tr>
<td>Umar 09</td>
<td>(CA)19</td>
<td>229–243</td>
<td>Poissant &amp; Davis, 2011</td>
</tr>
<tr>
<td>Umar 10</td>
<td>(CA)16</td>
<td>226–234</td>
<td>Poissant &amp; Davis, 2011</td>
</tr>
<tr>
<td>G10X</td>
<td>132–156</td>
<td></td>
<td>Straka et al., 2012</td>
</tr>
<tr>
<td>G1D</td>
<td>123–139</td>
<td></td>
<td>Andreassen et al., 2012</td>
</tr>
<tr>
<td>Mu05</td>
<td>109–133</td>
<td></td>
<td>Andreassen et al., 2012</td>
</tr>
</tbody>
</table>

* as given in publication
4.2.3 Analytical approach

Three loci (Umar04, Umar06 and Umar08) we excluded from further analysis due to a lack of variation. All samples that had data missing for more than one locus were removed. This resulted in a final dataset of 78 samples (from the original 91 samples) with data for at least 13 of the remaining 14 microsatellite loci. The final dataset was comprised of n= 44 samples of a ‘good origin accuracy’ (animals with good record keep and the origin of the individual sample could be traced back to the location of capture), n= 7 with a ‘poor origin accuracy’ (animals where the exact origin is not certain but allowed an estimate of the province of origin), n= 26 with a ‘bad origin accuracy’ (animals that may have been subjected to wildlife trafficking and the origin is therefore uncertain) and n=1 of a captive born bear. Samples of ‘poor’ or ‘bad’ origin accuracy as well as the captive born bear were considered as samples of unknown origin that required population assignment.

MICRO-CHECKER 2.2 (van Oosterhout et al. 2004) was used to check the microsatellite loci for null alleles (when one or more alleles did not amplify in the PCR). Null alleles can create erroneous homozygotes and may cause false levels of genetic differentiation.

To determine the population structure of the Cambodian sun bear, samples of good origin accuracy were analyzed using STRUCTURE applying the Markov Chain Monte Carlo (MCMC) estimation (excluding the samples of poor or bad origin accuracy). After the microsatellite dataset was tested on the good origin accuracy samples, genotype frequencies were assessed for consistency with the Hardy-Weinberg Equilibrium (HWE, calculates the expected allele frequency and tests for non-random association of alleles within diploid individuals). The data was then further checked for linkage disequilibrium (LD) and null alleles applying the Bonferroni corrections for pairwise comparison using ARLEQUIN 3.5.x (Excoffier et al. 2005). GENETIX was used to calculate the expected heterozygosity (H_e) and the observed heterozygosity (H_o) for each locus, as well as the effective population size (N_e). The F-statistics (measure of total inbreeding within a population [F_{is}], and the differentiation among sub-populations [F_{st}]) was computed using GENETIX. To determine the likely number of genetic clusters (K) among the sun bear samples (i.e. the number of populations), a range of K values from K=1 to K=5 were tested, with 10 repeats each (i.e. 50 runs in total), with a burn-in period of 200,000, followed by an additional 500,000 MCMC steps, using the Bayesian clustering program STRUCTURE v2.3.4 (Pritchard et al. 2000). The most likely K in this dataset was calculated applying the ΔK analysis (Evanno et al.
as implemented in STRUCTUREHARVESTER (Earl & von Holdt 2012): http://taylor0.biology.ucla.edu/structureHarvester/. Furthermore, the data was tested with a factual correspondence analysis (FCA) using GENETIX (Belkhir et al., 1996-2004) to identify (and confirm) the number of population clusters.

Once population structure was identified using the ‘good origin accuracy’ samples, samples of ‘poor’ and ‘bad origin accuracy’ were assigned to the populations (with an assignment of > 90%) using STRUCTURE. The assigned samples were distributed among the populations and each population (now the entire dataset) was tested individually for HWE and LD. The expected heterozygosity ($H_e$), the observed heterozygosity ($H_o$) and the effective population size ($N_e$) as well as the $F$ statistics were obtained using GENETIX. A Principal Component Analysis (PCA) plot was generated in R using the ADEGENET package.
4.3 RESULTS

4.3.1 General summary

A total of 17 microsatellite loci were analyzed in 91 samples. Among these 17 microsatellites, three were invariable and were excluded from further analysis. We excluded 13 samples due to missing data, with the result that 78 samples remained with data for at least 13 of the 14 microsatellite loci; these comprised 44 samples with ‘good origin accuracy’, and 34 of ‘poor’, ‘bad’ (this includes the captive born bear) origin accuracy. The remaining (variable) 14 microsatellite loci showed no signs of linkage disequilibrium (LD) or null alleles.

4.3.2 Determination of population structure

To determine the population structure within the Cambodian sun bears, we analyzed 44 samples with ‘good origin accuracy’ using STRUCTURE. The ΔK statistics indicated K=2 as the best supported population structure, meaning that there are two distinct sun bear populations in Cambodia. Both the STRUCTURE analysis and the PCA (Figure 4.2) suggested that there is gene-flow between these two populations, and that there are admixed individuals with a genetic background from both populations (‘admixed’). From the good origin accuracy samples, 12 belonged to the population 1, 22 to the population 2, and 10 were admixed (Table 4.2). The F<sub>ST</sub> value of 0.14498 between the two populations was highly significant (P<0.0001), indicating significant population differentiation.

4.3.3 Assignment of ‘bad origin’ samples

Once the population structure in Cambodia was identified using the samples with good origin accuracy, the ‘poor’ and ‘bad’ origin samples were assigned to the two populations using STRUCTURE. The tested samples had to have a high proportion of assignment to a population (>90%) before they were assigned to a population. Any assignment below 90% was marked as a ‘admixed’. From the poor origin accuracy, six animals assigned to the population 1, and one to population 2. From the bad sample origin nine animals assigned to population 2, 10 animals to population 1 and seven animals to the ‘admixed’ population (Table 4.1).
Figure 4.2: The PCA plot based on 78 sun bear samples and 14 microsatellites (full dataset). Red circles represent individuals from population 1, blue circles represent individuals from population 2, and the white circles symbolise ‘admixed’ individuals between population 1 and 2.

4.3.4 Summary statistics

From the 78 individual animals, 28 were assigned to population 1, 32 were identified as belonging to population 2, and 18 were admixed (Table 4.2). Population 1 (red circles) was comprised of all samples from West Cambodia and population 2 (blue circles) from the East, with both populations separated by the Mekong River. From the complete dataset (n=78), 18 individuals were admixed. The admixed individuals appear to be geographically located between these two populations (Figure 4.3).

Table 4.2 Assignment of samples according to their population

<table>
<thead>
<tr>
<th></th>
<th>Pop.1</th>
<th>Pop.2</th>
<th>admixed</th>
<th>N</th>
</tr>
</thead>
<tbody>
<tr>
<td>good</td>
<td>12</td>
<td>22</td>
<td>10</td>
<td>44</td>
</tr>
<tr>
<td>poor</td>
<td>6</td>
<td>1</td>
<td>0</td>
<td>7</td>
</tr>
<tr>
<td>bad</td>
<td>10</td>
<td>9</td>
<td>7</td>
<td>26</td>
</tr>
<tr>
<td>captive</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Total</td>
<td>32</td>
<td>28</td>
<td>18</td>
<td>78</td>
</tr>
</tbody>
</table>
Figure 4.3: Population distribution of the Cambodian sun bear. The circles represent the location of the individual sun bear samples, the number inside the circles represent the individual sample number. The colors represent the population according to the microsatellite assignment: red = population 1 (West–Cambodia population), blue = population 2 (East–Cambodia population) and white = ‘admixed’ population. The Mekong River appears to separate the two populations; ‘admixed’ samples are mostly located between the two populations.
Table 4.3: Summary statistics of the genetic diversity for the East and West populations, for all samples that fall under good origin accuracy (n=44) and the full dataset (n=78). N=number of samples, $H_e$= expected heterozygosity, $H_o$=observed heterozygosity, $N_A$=Number of Alleles, $F_{IS}$=inbreeding coefficient.

<table>
<thead>
<tr>
<th>Samples</th>
<th>$N$</th>
<th>$H_e$</th>
<th>$H_o$</th>
<th>$N_A$</th>
<th>$F_{IS}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>East-population (good accuracy)</td>
<td>12</td>
<td>0.513</td>
<td>0.503</td>
<td>4.4</td>
<td>0.064</td>
</tr>
<tr>
<td>West population (good accuracy)</td>
<td>22</td>
<td>0.553</td>
<td>0.390</td>
<td>4.4</td>
<td>0.316*</td>
</tr>
<tr>
<td>Good Accuracy (all)</td>
<td>44</td>
<td>0.599</td>
<td>0.431</td>
<td>5.9</td>
<td>0.291*</td>
</tr>
<tr>
<td>Full dataset</td>
<td>78</td>
<td>0.597</td>
<td>0.463</td>
<td>6.8</td>
<td>0.232*</td>
</tr>
</tbody>
</table>

Measures of diversity were calculated for the West and East populations of ‘good origin accuracy’ (Table 4.3). The West population displayed significant inbreeding ($F_{IS} = 0.316$) and reduced observed heterozygosity ($H_o = 0.390$) compared to expected heterozygosity ($H_e = 0.553$); three loci (Umar01, Umar07 and Umar10) in this population were not in HWE. The observed heterozygosity of the East population ($H_o=0.503$) was similar to the expected heterozygosity ($H_e=0.513$), and no loci significantly deviated from HWE. The expected and observed heterozygosities for the full dataset (which comprises ‘good’, ‘poor’ and ‘bad’ accuracy samples) had a similar range to the values for samples with ‘good’ origin accuracy only (Table 4.3).
Monitoring biodiversity and species population health is becoming increasingly more important in the Anthropocene (McGill et al. 2015). Humans are all-pervading, changing the environment and climate rapidly and therewith forcing species to either adapt or go extinct (Palumbi et al. 2001). Conservation scientists rely on methods enabling them to monitor species without further endangering them. Genetic monitoring can be a non-invasive, cost-effective and useful approach to gather information on a species’ ecology and evolutionary potential (Schwart et al. 2007). Furthermore, genetic monitoring is an effective way to gather data on rare and elusive species, and where the physical capture of animals is too difficult or risky, for the animal or human (Tammelheht et al. 2010). Genetic markers offer a safe and affordable alternative method to gather data relevant for in-situ conservation. Furthermore, genetic markers can be used to help inform practitioners about many population parameters, such as genetic variability, abundance, geographical range, vital-rates and reproductive success, hybridization, effective population size, population structure and health (Schwart et al. 2007).

Information about the genetic status of wild populations (and the captive stock) obtained using nuclear markers such as microsatellites can provide conservation practitioners with a valuable tool to identify and define wild bear populations (Goossens & Salgado-Lynn 2013). Monitoring and understanding genetic diversity and gene flow of a species allows the identification of populations and habitat of conservation priority (Petit et al. 2008; Brown et al. 2014; Ashikaga et al. 2015). Further, microsatellites are a useful tool in reintroduction biology as it assists in identifying which release candidate is genetically most suitable for release into the area provided to them (Jakob-Hoff et al. 2015, p. 209). This is valuable information to conservation practitioners as it facilitates screening of confiscated bears to assign them to the population they most likely originated from. Microsatellite markers have been proven to be a valuable tool as forensic markers to combat the illegal trade of wildlife (Arif & Khan 2009; Mucci et al. 2014). Being able to identify populations that are particularly susceptible to illegal poaching activities might encourage a more effective implementation of the wildlife protection laws in those areas and consequently combat the illegal trade of sun bears in this area (Arif et al. 2011).

As the trade of sun bears is a major threat to their survival (Krishnasamy & Shepherd 2014), understanding population structures and being able to assign bears of unknown origin to a source population is advantageous for their conservation management. This is the first microsatellite study conducted on sun bears. As Cambodia is the country with the highest
confiscation rates of illegally traded sun bears (Foley et al. 2011), this research concentrated on identifying the population structure and genetic variability of the Cambodian sun bear.

4.4.1 Population structure of sun bears in Cambodia

This study revealed that Cambodia has two genetically distinct populations that are geographically split by the Mekong River into an East- and West population. The Mekong emerges from the Tibetan Plateau and runs through China’s Yunnan Province (China), Myanmar, Laos, Thailand, Cambodia, and Vietnam (Kite 2001). This river has been a dispersal barrier for many mammal species (e.g. Roosevelt’s muntjac Muntiacus rooseveltorum (Meijaard & Groves 2006), the Tonkin snub-nosed monkey Rhinopithecus avunculus (Blair et al. 2011), the black crested gibbon Hylobates concolor (Thin et al. 2010), and the Malayan tapir Tapirus indicus (Tracholt et al. 2016)), restricting most of these species to the East of the river (Meijaard & Groves 2006). However, the river is not a barrier for many other species such as the Asiatic black bear Ursus thibetanus (Garshelis & Steinmetz 2016) or the Javan rhinoceros Rhinoceros sondaicus (van Strien et al. 2008). Macaque species are present to either side of the river and do not show any indication of subspecies differentiation (Fooden 1996). Whether the Mekong is a geographical barrier appears to be species dependent. Meijaard & Groves (2006) identified that the sun bear belongs to the group of species that are not limited by any of the main rivers, including the Mekong.

This research suggests that the Mekong River is a barrier for dispersal and gene-flow, as there are two genetically distinct populations to the East- and West of the river. This barrier can be overcome by sun bears as there is an ‘admixed’ population which seems to encompass F1 individuals as well as back-crossed individuals. Consequently, the East and West population are connected across the river, but the gene-flow between these population is not sufficient to alleviate the loss of genetic diversity in the West population. As the admixed animals are found along the length of the Mekong River, it appears to be a true geographical pattern. Another explanation for this pattern could be a human-mediated exchange of sun bears, as the Mekong River is an important trade route (Masviriyakul 2004).
4.4.2 Conservation application

Understanding the genetic structure and health of a population allows conservation scientists to re-evaluate the protection status of a species or subspecies and to identify populations of conservation concern. Genetic monitoring of Ursidae species has allowed researchers and conservationists to identify genetically stable (diverse) and potentially endangered populations (some that may suffer from inbreeding depression). The brown bear (Ursus arctos) has a large distribution range and is one of the best studied bear species (Swenson et al. 2011; Tammeleht 2011). Genetic monitoring has allowed scientists to recognize that some brown bear populations display signs of significant inbreeding (Taberlet et al. 1997; Benazzo et al. 2017), making them more vulnerable to extinction (Pérez et al. 2009). Consequently, these populations receive more protection and a higher conservation priority (Paetkau & Strobeck 1998).

As there are no other microsatellite studies that have investigated the sun bear, the results from this study are compared with the genetic diversity reported for brown bears (Ursus arctos). Brown bear populations are well studied and their conservation status is assessed according to their genetic diversity. Conservation practitioners know which brown bear population could be considered as genetically stable and which ones are not and require conservation management. Comparing the heterozygosity values of the Cambodian sun bear population with brown bear populations may inform whether the Cambodian sun bear is of conservation concern.

To conduct appropriate comparisons across species, this Cambodian sun bear study is compared to brown bear studies that had similar sample sizes and number of loci used. This study tested 78 individuals with 14 microsatellite markers. Straka et al. (2012) used 13 loci and tested 71 samples from Northern Slovakia, 96 samples from Central Slovakia and 109 samples from Romania. Their expected heterozygosities ranged from $H_e = 0.70$-$0.81$ and the observed heterozygosities ranged from $H_o = 0.69$-$0.76$. Therefore, and the Slovakian and Romanian brown bear populations are considered as genetically diverse and stable. In comparison, the expected and observed heterozygosities for the Cambodian sun bear ($H_e = 0.59$, $H_o = 0.46$) were substantially lower. This level of genetic diversity is more similar to that found in the Spanish brown bear populations (West-Spain $H_o = 0.44$, $H_e = 0.45$; East-Spain $H_o = 0.28$, $H_e = 0.25$; 18 microsatellite loci, 39 and 71 samples, respectively; Pérez et al. 2009). The brown bear populations in Spain are considered as endangered, and has resulted in the development and implementation of a species recovery plan (Pérez et al. 2009). Since the genetic diversity of the Cambodian sun bears is comparable to the genetic diversity of an endangered brown bear population in Spain, it could be argued that the Cambodian sun bears should receive the same level of protection and conservation focus as the Spanish brown bears. For the brown bears in Spain, promoting
connectivity between populations to alleviate inbreeding of threatened populations has been recommended (Pérez et al. 2009). Enhancing connectivity and encouraging gene-flow (potentially through augmentation) between the two populations in Cambodia may be a conservation action to prevent the further inbreeding of the West-Cambodian population. However, this conservation strategy would not be successful if the initial cause for inbreeding is due to poaching pressure. Further research is required to assess the impact of poaching and trade in the genetic variability of sun bear populations.
4.5 CONCLUSION

In this first study of sun bears using nuclear microsatellites, we have identified two distinct populations in Cambodia that appear to be connected by ongoing gene flow. The Mekong River may be a barrier between these populations. While both populations display only moderate levels of genetic diversity, the West-Cambodian population is characterized by both lower diversity and increased inbreeding. Gene flow from East-Cambodia does not seem to be sufficient to counteract the genetic impoverishment of the population to the west of the river. Therefore, conservation action plans could potentially focus on an increased connectivity between populations or augmentation (reinforcement) of the wild population. Our results also indicate that the development a reference database of the sun bear populations across its distribution range is required, to accurately assess these and other sun bear populations. Therefore, further microsatellite (or SNP) research on sun bears is recommended to develop such a reference database. This reference database could also serve as a forensic tool to combat the illegal wildlife trade that threatens the sun bear.
CHAPTER 5

“Analysis of current forensic methods to detect bear derivate in
Traditional Chinese Medicine (TCM) – with focus on sun bears”
5.1 INTRODUCTION

Global wildlife trafficking is estimated to be the 4th largest organized and transnational crime, following drug, human and counterfeit goods trafficking (WWF/Dalberg 2012), and estimated to be worth between US$5 to 23 billion per year (May & Clough 2017). Combined with poaching, the global and predominantly illegal trade threatens the survival of bear species across the world (Mills 1994; Burgess et al. 2014). The number of bears traded in Asia is estimated to have tripled between 2000 and 2011, and the annual world-market in bear parts is valued at a minimum of USD 2 billion (INTERPOL 2014). Although most illegal activities are focused on rare species and rare animal materials (e.g., rhinoceros horn and ivory), the impact of Traditional Chinese Medicine (TCM) on less rare species, such as bear species, is often overlooked (Byard 2016).

Traditional Chinese Medicine originated over 3000 years ago in China (Peppin et al. 2008) and uses both herbal and animal resources for the preparation of remedies and potions. Bear bile and bear gall bladders are sought after ingredients in TCM. The first known record of TCM using bear bile is found in the materia Medica dated 659 AD (the Tang Dynasty) (Feng et al. 2009; Foley et al. 2011). Bear bile, from the bear gall bladder and obtained by killing the wild bear, led to a decline in wild bear populations in the 1980s (Foley et al. 2011; Willcox et al. 2016). To reduce the pressure on wild bear populations and to further satisfy the demand for bear bile, North Korea developed commercial bear farming that allows the extraction of bear bile without killing the animal (Li 2004). Due to the unsanitary bile extraction methods and poor animal husbandry in the bear farms, the life expectancy of farmed bears averages five years (Mills et al. 1995; Peppin et al. 2008; Foley et al. 2011). As bear farming is legal, but the killing of wild bears is not, many bear farmers admitted to restocking their farmed bears with bears from the wild (Willcox et al. 2016). Furthermore, the unsanitary conditions in the bear farms impacts on the quality of the bear bile that is sold to the consumers (Dutton et al. 2011). The consumers therefore show a clear preference for wild bear bile (which means the bear would have to be killed to obtain the bile) and are willingly paying up to 65 times the price they would for farmed bear bile (INTERPOL 2014). This has increased the hunting pressure on wild bears dramatically and even led to the collapse of wild Asiatic black bear (Ursus thibetanus) populations in Vietnam (Crudge et al. 2016). Ironically, initial attempts to protect wild bear populations through the introduction of farmed bears has not protected the wild stock but accelerated its depletion (Dutton et al. 2011; Livingstone & Shepherd 2014).

Bear bile is used to treat epilepsy, sore throats, sore eyes, asthma, burns, bruises, pains, and to clear and repair the liver after alcohol intoxication (Traffic Network 1995; Foley et al. 2011).
Although animals have always been utilized for medicinal purposes, the increasing rarity of many animal species has contributed to a soaring demand, as their consumption (for whatever purposes) has additionally become a status symbol (Zhang et al. 2008; Clifton & Rastogi 2016). With an increase of wealth in the Asian world, the trade in bears and bear parts has steadily increased (Mills & Servheen 1991; Graham-Rowe 2011; Kikuchi 2012).

One of the key sources and consumer countries of bear products is Malaysia. A recent decision to develop a joint Chinese/Malaysian TCM research center in Malaysia is likely to further promote this country as a hub of this industry (Lee et al. 2015).

Although Asiatic black bears are commonly farmed for bear bile, this species does not occur in Malaysia. It does, however, have its own bear species: the Malayan sun bear (Helarctos malayanus). Sun bears are usually not farmed for their gall bladder, but a study conducted by TRAFFIC in 2015 concluded that nearly 60% of the commercially available Malaysian bear gall had been sourced from local sun bears (Lee et al. 2015). With an increase of TCM manufacturing and trade in Malaysia, the hunting pressure on sun bears may escalate in the near future.

The TCM related illegal trade of bear bile and gall bladders endangers sun bears throughout their distribution range (See Chapter 1, Figure 1.2) (Mills & Servheen 1991; Mills 1994; Meijaard 1999; Shepherd & Shepherd 2010; Cao et al. 2014a). The IUCN highlighted that the reduction of commercial trade and poaching of sun bears would be essential to the conservation of this species, and CITES calls for ‘immediate actions to demonstrably reduce the illegal trade in bear parts and derivate’ (Foley et al. 2011). The Malayan sun bear is listed as ‘vulnerable’ on the International Union for Conservation of Nature and Natural Resources (IUCN) Red List of endangered species (Fredriksson et al. 2008) and is at risk of extinction in the near future (Zhang et al. 2008). Consequently, the commercial trade of sun bears or sun bear parts is prohibited as this species is listed under Appendix I of the Convention on International Trade in Endangered Species (CITES 2013).

To fully assess the potential threat of TCM practices for the wild sun bear population, forensic testing of TCM samples for the content of sun bear derivatives is an important step. Various TCM products that claim to contain bear may in fact consist either of constituents from other species (such as domestic animals such as pigs or goats) (Peppin et al. 2008) or from bear species listed under CITES Appendix II (INTERPOL 2014). These products cannot be easily differentiated from products containing authentic bear components, and this creates an obstacle to effective regulatory oversight and law enforcement within the industry. Forensic techniques to analyze the content of TCM for bear DNA have been developed (Peppin et al. 2008; Lee et al. 2015) but these remain costly, time consuming and rely on access to an appropriately equipped
laboratory facility. The overwhelming quantities of suspicious confiscated materials that remain untested underscores the need for rapid, affordable, and efficient forensic genetic assays, particularly those that can identify Asian bears (Asiatic black bears as well as sun bears) – the bears most likely to be in the vicinities of the TCM industry (Peppin et al. 2008; Foley et al. 2011; Lee et al. 2015).

Aims & Objectives
The original objective of this study was to conduct analyses on confiscated TCM samples for the potential content of sun bear derivatives. We aim to test currently available forensic DNA extraction methods and kits for their efficiency to successfully extract DNA from these samples. Successfully extracted DNA samples will further be sequenced and their genetic profiles will be assigned to a reference database. The ability to detect the presence of sun bear DNA in TCM or illegally trafficked products will help to assess and to characterize the scale of the threat to wild populations from TCM.
5.2 METHODS

5.2.1 Samples used
The Malayan sun bear reference samples used to test and develop the cytochrome b (cyt b) and control region (CR) PCR primers to identify sun bear DNA in the samples (tested at Griffith University; Supplementary Chapter 5 Table TS1) were collected non-invasively (mucus layer from scat samples) over the course of ten days in August 2013 at the ‘Free the Bears’ facility in Cambodia. All required permits were obtained. Dromedary (Camelus dromedarius) and stone marten (Martes foina) DNA was used as positive controls for mammal primers. The positive controls were successfully extracted with the Gen-IAL kit, standard protocol.

The samples analyzed in this study were confiscated under Part 13A of the Environmental Protection and Biodiversity Conservation Act (1999) by the Department of the Environment, Australia (the CITES implementing governmental agency). The confiscated products were suspected to contain bear derivatives and were donated by The Wildlife Trade and Biosecurity Branch/ CITES Enforcement Authority of Australia to the study (Table 5.1). Most of the donated samples were classified as TCM samples, with many products containing the Chinese symbol for bear bile on the package (Figure 5.1). The samples were suspected to have originated from China, Japan and Vietnam and were divided into nine different groups (groups i-ix, Table 5.2). The samples were cataloged and logged with all details available.
5.2.2 Sample nature

CITES donated 60 individual samples (see Table 5.1 and Table 5.2) grouped into 22 different CITES IDs. Upon closer inspection of the samples, it was decided that each CITES ID should be representative of a sample, rather than each of the 60 individual items for the following reasons: CITES ID 339 contained 15 capsules and one vial containing crystals, resulting in the listing of 16 samples in total that are registered under the same ID. However, these 15 capsules were found to be empty and were intended to be filled by the end consumer with the crystals in the vial. Consequently, from the initial 16 items registered under ID 339, 15 of these items did not contain any material for forensic analysis and as their inclusion in the analyses would skew the success-rate of the tested extraction protocols these were treated as a single sample.

Some items came in bundles, with all items having been produced by the same manufacturer. For example, CITES ID 262 was a packet containing three identical sachets with each being produced in the same way by the same manufacturer claiming to contain the same ingredients. Thus, it was not necessary to examine all three sachets as they were representing just one product. Another issue for deciding on the possible inclusion of samples was that of likely contamination. For example, CITES ID 326 consisted of six visually similar balls (each counting as a separate sample) bundled into the same zip-lock bag. Because they were confiscated as one suspicious sample they had all been given the same CITES ID. The storage of the balls in the
same zip-lock bag would have caused cross contamination (even if the balls had been made from different ingredients), thus they were considered as one sample.

These considerations reduced the initial number of samples from 60 to 22. Furthermore, CITES IDs 2 and 4 were assigned to hair samples (assumed to have originated from polar bear (Ursus maritimus) and grizzly bear (Ursus arctos ssp.), which are not classified as TCM products. Thus, although they had been included in the tests, these samples were later excluded from the summary statistics, resulting in a final TCM sample size of n=20.
Table 5.1: List of TCM samples confiscated by the Department of the Environment, Australia and made available for this study.

<table>
<thead>
<tr>
<th>CITES Ref #</th>
<th># and form of sample</th>
<th>Seized as (species)</th>
<th>Sample source</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>&lt;1gr hairs</td>
<td>Ursus maritimus</td>
<td>Tanned whole skin</td>
</tr>
<tr>
<td>42</td>
<td>&lt;1gr hairs</td>
<td>Ursus arctos</td>
<td>Tanned whole skin</td>
</tr>
<tr>
<td>133</td>
<td>1 x bottle liquid-27 gr gross</td>
<td>Ursidae spp.</td>
<td>TCM</td>
</tr>
<tr>
<td>138</td>
<td>1 x vial liquid-66 gr gross</td>
<td>Ursidae spp.</td>
<td>TCM</td>
</tr>
<tr>
<td>139</td>
<td>1 x bottle balls 15 gr gross</td>
<td>Ursidae spp.</td>
<td>TCM</td>
</tr>
<tr>
<td>167</td>
<td>1 x vial crystals-&lt;1gr gross</td>
<td>Ursidae spp.</td>
<td>TCM</td>
</tr>
<tr>
<td>252</td>
<td>1 x bottle powder 8 gr gross &amp; 3 x capsules</td>
<td>Ursidae spp.</td>
<td>TCM</td>
</tr>
<tr>
<td>255</td>
<td>2 x balls</td>
<td>Ursidae spp.</td>
<td>TCM</td>
</tr>
<tr>
<td>256</td>
<td>4 x balls</td>
<td>Ursidae spp.</td>
<td>TCM</td>
</tr>
<tr>
<td>262</td>
<td>3 x sachets</td>
<td>Ursidae spp.</td>
<td>TCM</td>
</tr>
<tr>
<td>263</td>
<td>2 x balls</td>
<td>Ursidae spp.</td>
<td>TCM</td>
</tr>
<tr>
<td>264</td>
<td>2 x balls</td>
<td>Ursidae spp.</td>
<td>TCM</td>
</tr>
<tr>
<td>300</td>
<td>2 x balls</td>
<td>Ursidae spp.</td>
<td>TCM</td>
</tr>
<tr>
<td>301</td>
<td>2 x balls</td>
<td>Ursidae spp.</td>
<td>TCM</td>
</tr>
<tr>
<td>303</td>
<td>1 tablet</td>
<td>Ursidae spp.</td>
<td>TCM</td>
</tr>
<tr>
<td>307</td>
<td>2 x balls</td>
<td>Ursidae spp.</td>
<td>TCM</td>
</tr>
<tr>
<td>308</td>
<td>2 x balls</td>
<td>Ursidae spp.</td>
<td>TCM</td>
</tr>
<tr>
<td>309</td>
<td>2 x balls</td>
<td>Ursidae spp.</td>
<td>TCM</td>
</tr>
<tr>
<td>316</td>
<td>2 x balls</td>
<td>Ursidae spp.</td>
<td>TCM</td>
</tr>
<tr>
<td>324</td>
<td>2 x balls</td>
<td>Ursidae spp.</td>
<td>TCM</td>
</tr>
<tr>
<td>326</td>
<td>6 x balls</td>
<td>Ursidae spp.</td>
<td>TCM</td>
</tr>
<tr>
<td>339</td>
<td>1 x vial crystals-15 gr gross &amp; 15 capsules</td>
<td>Ursidae spp.</td>
<td>TCM</td>
</tr>
</tbody>
</table>

Table 5.2: Categorization (sample code i–ix) of the samples into nine different sample types.

<table>
<thead>
<tr>
<th>sample code</th>
<th>sample type</th>
<th>Samples matching this type</th>
</tr>
</thead>
<tbody>
<tr>
<td>i</td>
<td>Tablet, black in colour, rectangled</td>
<td>303</td>
</tr>
<tr>
<td>ii</td>
<td>small, brown ball</td>
<td>316, 308, 324, 264, 256, 309, 305, 300, 307, 301, 263, 326, 139</td>
</tr>
<tr>
<td>iii</td>
<td>small golden ball</td>
<td></td>
</tr>
<tr>
<td>iv</td>
<td>dark-brown crystal</td>
<td>167, 339</td>
</tr>
<tr>
<td>v</td>
<td>hair</td>
<td>2, 42</td>
</tr>
<tr>
<td>vi</td>
<td>powder</td>
<td>252</td>
</tr>
<tr>
<td>vii</td>
<td>sachet</td>
<td>262</td>
</tr>
<tr>
<td>viii</td>
<td>liquid, yellow bear-alcohol</td>
<td>133</td>
</tr>
<tr>
<td>ix</td>
<td>liquid, brown non-transparent</td>
<td>138</td>
</tr>
</tbody>
</table>
5.2.3 DNA extractions and inhibitor removal

The reference DNA was extracted using the GEN-IAL First DNA All-tissue DNA extraction Kit (GEN-IAL GmbH, Troisdorf, Germany) following manufacturer's instructions of the standard extraction protocol. As the composition and inner structure of the TCM samples was unknown, we tested several DNA extraction kits and protocols for their suitability (Supplementary Chapter 5 Table TS1). As we suspected the presence of PCR inhibitors in the TCM samples, we also tested several measures to remove potential PCR inhibitors from the samples, including the OneStep PCR Inhibitor Removal Kit (from Zymo Research), the QIAGEN clean-up kit (from Qiagen), Ethanol precipitation (several steps) or Agencourt AMPure magnetic beads (Beckman Coulter).

5.2.4 PCR Primers

The control region (CR) primer pairs CR2 F/R and CR5 F/R as well as the cytochrome b (cytb) cytb5 F/R (APPENDIX III) were designed and tested with the Primer-Blast tool available on the NCBI website, using the sun bear sequence with the accession number AB098551.1. and FM177765.1 as a template. The primer pair for the cytochrome c oxidase subunit I (COI) region (COIUTR) was developed by Shahzadi et al. (2014). The primer pairs CB770R/ 613 F, rR 4c/4a and cyBb253 R/133 I were developed by Burger et al. (2007). Primer CB1L und CB2H were primers designed by Kocher et al. (1989). The primers were re-suspended to a 10pmol/µL working solution.

5.2.5 Target specific Amplification

The reaction mix (final volume 15µl) contained 0.025 U/µL GoTaq (Promega), 3.0 µL 5x reaction Buffer (Promega), 2 mM MgCl₂ (Promega), 0.4 mM dNTPs (Bioline), 0.4 mM Primer L (Biolegio), 0.4 mM primer H (Biolegio), 7.4 µL H₂O, and 1.5 µL template DNA. Amplifications were carried out with an initial denaturation at 95°C for 5 min, followed by 40 cycles of: denaturation at 95°C for 30 secs, annealing at 50°C for 30 sec, and extension at 72°C for 45 sec. The final extension was at 72°C for 7 min. Amplifications were conducted adding positive
controls (DNA extracts from contemporary sun bear samples, stone marten and dromedary samples) and negative controls (using dH₂O instead of DNA). The PCR products (1µl) were then separated on a 1.5% agarose gel (1x TAE buffer, pH 8.0, 100mA) and visualized under UV light using Gel Red Nucleic Acid Gel Stain (Biotium Inc). To test for the presence of inhibitors in samples that did not amplify, we diluted the remains of the PCR reaction with the PCR reaction mix (without target DNA) to ratios of 1:10 and 1:100, respectively, then spiked the samples with mammalian DNA and controlled if the added DNA would amplify. Failure of amplification would indicate the presence of inhibitors in the sample analyzed.

5.2.6 Sequencing

The PCR products were purified from primers and nucleotides using the ExoCiap enzyme mix (Fermentas). This mix contains exonuclease I (to digest unfinished PCR products and primer oligonucleotides) and calf intestine alkaline phosphate (to digest excess dNTPs). Each sample (1µl) was incubated with 0.1µl exonuclease I, 0.25µl phosphatase, and 4.65µl H₂O for 15 min at 37°C, followed by a 15 mins heat inactivation of the enzymes at 85°C.

Sequencing was performed using the ABI BigDye™ v.1.1 (Applied Biosystems, Foster City, CA) reaction mix. Per sample (6µl) we added 2µl sequencing buffer, 1µl BigDye™ (both ABI), and 1µl primer (10pmol/µl). The sequencing PCR was conducted as follows (14 cycles): 10s/95°C, 5s/50°C, 1.5min/60°C. The sequences were then resolved on the capillary electrophoresis system of the ABI PRISM 3130xl Genetic Analyzer using the software RUN 3130XL DATA COLLECTION v.3.0 and SEQUENCING ANALYSIS v.5.2 (both ABI).

5.2.7 Gas Chromatography/Mass Spectrometry (GC–MS)

Gas Chromatography/Mass Spectrometry analyses were conducted on sample 326 (small golden pill) and sample 300 (brown pill) (representing the samples where DNA extractions had previously proven difficult), utilizing the Agilent 7890A Gas Chromatograph (Agilent Technologies, Böblingen, Germany) interfaced with the Agilent 5975C mass selective detector. The analysis was carried out by Dr. M. Dehnhard (Department for Reproduction
Biology) at the Leibniz-Institute for Zoo and Wildlife Research (IZW) in Berlin, Germany following a published protocol (Pribbenow et al. 2014).
5.3 RESULTS

5.3.1 DNA extraction protocols and inhibition removal

DNA extraction was executed using seven different extraction protocols (Supplementary Chapter 5 Table TS1). Here we tested phenol chloroform extractions as well as three different DNA extraction kits: GEN-IAL First DNA All-tissue DNA extraction Kit (GEN-IAL GmbH, Troisdorf, Germany), Invitrogen™ Purelink™ Genomic DNA Mini Kit (Invitrogen™ K182001) and QIAamp DNA stool Mini Kit (QIAGEN Inc., 28159 Avenue Stanford, Valencia, CA 91355). The samples caused the filter from the Genomic DNA Mini kit (Invitrogen) to clog up and prevented the samples to spin through the membrane. The DNA eluates showed discoloration and maintained a strong smell. As TCM samples quite commonly contain plant material and essential oils, we suspected that the samples may contain additional chemical compounds, potentially inhibitors (Figure 5.2).

![Figure 5.2: Discolouration of DNA eluates after having used the PureLink Genomic DNA Mini Kit (Mammalian tissue and mouse/rat tail lysis, Invitrogen™). Samples (CITES ID) from left to right: 309, 255, 300, 307, 301, 263, 167, negative control.](image-url)
5.3.2 DNA extractions and PCR amplification

We successfully extracted DNA from two samples (IDs 262, 339) using the GEN-IAL First DNA All-tissue DNA extraction Kit. Applying the Phenol Chloroform extraction protocol also successfully extracted from another sample (ID 138). From these samples we were also able to amplify a fragment of the cytochrome b gene region using the generic mammalian cyt*b primer CB 1L and CB 2H (Kocher et al. 1989) (Figure 5.3), tallying the successful DNA extractions and amplifications to a success rate of 3 out of 20 (15%). The other extraction kits and primers failed to produce any results.
A= 262–1 (1:10 dilution),
C= 262–2 (1:10 dilution),
E=262–3 (1:10 dilution),
G= 309  (1:10 dilution),
I= 139  (1:10 dilution),

B= 262–1 (1:100 dilution),
D= 262–2 (1:100 dilution),
F= 262–3 (1:100 dilution),
H= 309  (1:100 dilution),
J= 139  (1:100 dilution).

K= 339 (1:10 dilution),
M= extraction negative control,
O=2nd PCR negative control (dH₂O),
Q= 1st positive control (stone marten),

L= 339 (1:100 dilution),
N=1st PCR negative control (dH₂O),
P= 3rd PCR negative control (dH₂O),
R= 2nd positive control (dromedary).

The ladder (outer left and outer right) used was a 100bp gene ruler DNA Ladder Mix.

**Figure 5.3:** PCR results visualized on a 1.5 % agarose gel (1x TAE buffer, pH 8.0, 100mA) and visualized under UV light using Gel Red Nucleic Acid Gel Stain (Biotium Inc). The samples were extracted with the standard protocol from the GEN-IAL First DNA All-tissue DNA extraction Kit and amplified using the cytochrome b CB 1L and CB 2H primer (Kocher et al. 1989)
5.3.3 *Sanger sequencing and BLAST similarity search*

The *cytb* amplicons were sequenced using the Sanger sequencing (Sanger et al. 1977) method. The successfully sequenced samples were then submitted to a similarity search using the online ‘basic local alignment search tool’ BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi).

The *cytb* sequence of sample 339 had 99% similarity with the *cytb* sequence from the Asiatic black bear. Examination of the registration number Z19991102, revealed that sample ID 339 had been produced by a farm that is ‘bear bile drugs approved by State F&D Administration’ (Animals Asia 2010) (Shenyang Fangxi Farm, for Huanren Qunshan Bears Pharmaceuticals Co. Ltd.). However, seizure of the samples from customs at an airport suggests that this sample has been traded illegally as the Asiatic Black bear is a CITES Appendix I listed species and trading products containing material from this species is strictly prohibited (even if produced in a legally operated bear farm).

The *cytb* sequence of sample 262 had a 98% similarity with the *cytb* sequence from *Homo sapiens* (human). Unfortunately, sequencing of the (supposed) *cytb* fragment from sample ID 138 failed.

5.3.4 *Gas Chromatography Mass Spectrometry (GC–MS)*

5.3.4 a) *Volatile components*

The volatile components of sample ID 326 (small golden pill) contained high amounts of borneol, which was identified based on the comparison of its mass spectrum with the National Institute of Standards and Technology (NIST) spectral library. Borneol is a compound of the bear root (*Ligusticum porteri*), a plant commonly used in TCM products.

The volatile components in sample 300 did not contain any borneol, but a distinct amount of different phenols. Due to the lack of reference substances (standards) the chemical composition of these phenolic mixtures could not be determined down to compound 7.

5.3.4 b) *Non-volatiles*

The analyses of non-volatiles revealed large amounts of cholesterol in sample 326. The GC-MS could not confirm cholesterol in sample 300.

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7 Even though we had the equipment for a GC-MS analysis, we were unable to identify individual phenols and their individual concentrations. Identification can only be achieved if the chemical compounds in varying concentration levels are defined as standards in the headspace analysis. Unfortunately, there were no funds to further develop this method at the time.
5.4 DISCUSSION

In this study, we tested three DNA extraction kits with seven different extraction protocols, four different primer pairs and two inhibitor removal kits to test TCM samples for the content of sun bear DNA (without prior knowledge if any of these contained any DNA). Our results demonstrated that the commercially available standard DNA extraction kits and protocols tested in this study are not suitable for a general application in the forensic analysis of TCM products. In none of the samples analyzed we were able to detect sun bear DNA. The possible explanations for our negative results are that the TCM samples a) failed to amplify as due to inhibition, b) did not contain mammal DNA (and we therefore used the wrong primers), c) did not contain DNA at all to begin with or that the DNA had been degraded beyond detection.

**Figure 5.4:** Flow-chart illustrating under what circumstances the extraction and sequencing of the TCM samples tested would have been successful. To get a successful result, the flow of the green boxes must be followed.
5.4.1 Inhibition in composite samples

TCM products are often not labelled truthfully and either claim to contain animals but are entirely plant based (so they can be sold to a higher price) or they may claim to contain bear but are containing dog (Canis lupus familiaris) or pig (Sus scrofa domesticus) (Willcox et al. 2016). In other instances, the TCM producer may list only plants as ingredients but the sample may actually contain derivate of endangered animals (Coghlan et al. 2012). Therefore, TCM samples always require forensic analysis to exclude the possibility of containing bear derivate. As we expected to have samples that were composite samples of plant and animal derivate, we tested all sample types with mammal primers. A potential explanation for the negative results may be that most samples (55%) composed of all of mainly plant-based materials such as in’ Seirogan pills (Lee man Shan Pills (Lee Man Shan Medicine MFG. Ltd. and Trumpet Brand Seirogan (Taiko Pharmaceutical Co. LTD). Seirogan is a traditional Japanese medicine which may differ in composition depending on the manufacturer but is usually entirely plant based.

Plant secondary metabolites as well as lipids are known to be PCR inhibiting and do not allow for successful sequencing if the source of the inhibition is not removed (Cao et al. 2014b). The tested inhibition removing protocols were successful in cleaning the samples but seem to have also removed any residue target DNA. However, this issue seems currently insurmountable (“Sometimes you just can’t get past the inhibition.” (M Burnham Curtis 2016, pers. comm.)). A negative PCR result may therefore mean that the inhibition has removed all remaining mammal DNA or that the sample never contained mammal in the first place.

5.4.2 Primers

The primers used in this study were target specific, developed to amplify sun bear cytochrome b (cytb) and control region (CR) DNA. We further used broad range mammal primers, to test if any other mammalian DNA was detectable. Using these primers we successfully amplified Asiatic black bear DNA and human DNA (likely a contamination during either the production process or on the way to the IZW) from two TCM samples. However, if most of the TCM samples we analyzed were entirely plant-based Sairogan pills, the primers would not have been able to hybridize to any target DNA and thus could not have resulted in successful (mammal) DNA amplifications.
5.4.3 Sample Production

During the production of TCM products, ingredients are commonly cooked, bleached or come from different sources (Lv et al. 2009; Coghlan et al. 2012). If plant material or oils are among the ingredients, their PCR-polymerase inhibiting substances may cause problems for a PCR based forensic genetic analysis (Cao et al. 2014a). In addition, the prolonged exposure to heat and DNA denaturing substances such as bleach will cause the destruction of intact DNA (Herrmann and Wink 2014). Furthermore, bile acid, the main compound of bear bile (Urusol) is now commonly produced synthetically. Synthetically produced bile does not contain any DNA and therefore cannot be detected via forensic genetics (M. Burnham Curtis 2016, pers. comm.). The National Fish & Wildlife Forensic Laboratory in the United States (Ashland, Oregon) achieve a success rate of less than 50% using the Qiagen Stool kit for bile samples, probably because most of their samples not contain DNA. Even when working with freshly extracted pure bear bile, their DNA extraction success rate is around 75% (J. LeMay 2016, pers. comm.).

5.4.4 Forensic GC-MS analysis

Due to the high cholesterol readings, it appears that the golden pills were derived from bile (M. Dehnhard 2017, pers. comm.). However, we were unable to differentiate the chemical composition between bear bile or bile derived from other animals (such as pigs), or whether the product had been produced synthetically. To differentiate between pig and bear bile, we propose to further develop a method to confirm tauroursodeoxycholic acid and ursodeoxycholic acid by comparing retention time and mass spectra in GC-MS and a reference database for different bile, including synthetically produced.

Based on our results from the GC-MS, there is no conclusive evidence that the black pill is derived from bile. As there is currently no volatiles or non-volatiles GC-MS reference database for sun bear derivate, we would not be able to clearly identify whether sun bear has been used in TCM.
5.5 IMPLEMENTATION RECOMMENDATION

Although many publications report to have successfully extracted and sequenced DNA from TCM products (Peppin et al. 2008; Lv et al. 2009; Coghlan et al. 2012), our study tested these protocols and failed to extract DNA from the samples seized by Australian authorities. The analysis carried out on the samples did not allow us to clearly determine the presence or absence of bear bile in the samples. However, the ‘failure to detect presence’ does not mean that bear bile is not present in these samples.

The currently available PCR based forensic procedures to detect bear derivate in TCM is time consuming, easily manipulated and has a poor success rate. Peppin et al. (2008) recognized that the forensic analysis of heavily processed and complex (containing many ingredients) TCM products requires development as the DNA extractions are often inconclusive or failed. If the forensic analysis of TCM products generates high execution costs at only poor success rates, the application of wildlife forensics in the combat of the illegal trade will not be of great impact. This study highlights the need for further development of forensic methods targeting the analysis of TCM products, potentially considering forensic approaches that are not DNA analysis based.

Furthermore, it would be recommended to establish a reference GC-MS database that includes the volatile and non-volatile components for sun bears and bile derived from sun bears. Additionally, this study has indicated the need for the exchange of scientific analysis protocols and accessible communication to fellow forensic scientists. Wildlife forensic analysis would benefit greatly from a central database for TCM products, and state laboratories for forensic analysis that provide reference materials and protocols. The Society for Wildlife Forensic Science has been a valuable contact during this study and further development of these services would assist in the combat of the illegal wildlife trade.

Having reliable forensic methods is essential to implement and enforce existing wildlife protection laws (Chen et al. 2015). Currently, the open and international trade demonstrates that the enforcement of wildlife protection laws and CITES has been neglected or inadequate in some of the CITES member countries (Shaw 2003; Shepherd & Nijman 2007; INTERPOL 2014). Furthermore, as the cross-border trade is endangering bears across the globe we recommend reviewing current existing wildlife protection laws to close all potential loop holes and to have unified legislation. Legislation need to clearly regulate the use of TCM products and
bear harvest across borders with equal force to protect bears effectively. Legal loopholes need to be sealed (for example in the republic of Korea, bear bile extraction is illegal but keeping bears is not. These bears can be killed for their parts once they are 10 years old and older (Willcox et al. 2016)), current wildlife laws need to be enforced and corruption must be tackled as a serious threat to remaining wildlife (Foley et al. 2011).

To enforce the current wildlife laws, the collection of intelligence is of paramount importance. In 2017, INTERPOL successfully carried out operation ‘Thunderbird’ (Fig 5.5). For this operation police officers, custom and border agencies, and environment and wildlife officials of 49 countries collaborated to confiscate 1,300 illicit products (estimated worth of USD 5.1 million) and identified nearly 900 suspects within three weeks. Thus far, 88 people have been jailed and further 390 investigations are ongoing. The operation aimed at the entire consumer-supply chain and was a clear win against the wildlife trade. However, operations like this can be jeopardized quickly if there is information is leaked by any of the 190 member countries that collaborate with INTERPOL (INTERPOL 2017). In conjunction with tough enforcement of wildlife laws, conservation needs to address the cause of the issue and not only treat the symptoms. Conservation scientists interested in combatting the illegal trade of wildlife need to understand why there is a demand for wildlife and wildlife products (for example, the beliefs that promote the usage of TCM (Vu & Cowell 2010) or lucky charms (Varner 2008) and the use of wildlife to demonstrate status (Clifton & Rastogi 2016) and how to alter this behaviour (Schultz 2011).
The illegal trade is driven by a variety of different economic and social factors (Ling et al. 2015). The complexity of this issue requires a multi-disciplinary approach to reduce the demand, a variety of forensic methods, the collection of intelligence, as well as appropriate law enforcement and prosecution of offenders (INTERPOL 2014; Challender et al. 2015).
CHAPTER 6

Lessons learned for sun bear management
6.1 INTRODUCTION

Animal conservation is a complex matter as the threats that species face can vary geographically, politically, and socio-economically. The sun bears of Borneo are facing habitat loss that is driven by the conversion of land into oil and rubber plantations, as well as large scale bush fires (Fredriksson 2012a; Scotson et al. 2017). On the mainland of Southeast Asia, sun bears are primarily threatened by the increasing rates of illegal wildlife trade and poaching (Guharajan et al. 2017). Habitat loss through land conversion and fire management require a different approach than trying to combat the illegal trade in wildlife. Therefore, it is important that the sun bear action plan considers the differences in threats that sun bears face and responds with a multi-disciplinary conservation approach. As the conservation priorities are different for sun bears in Borneo than to those on the mainland of Southeast Asia, it may be worth developing local sun bear action plans that then feeds into a more general action plan for the sun bear across the entire distribution range. Further, it is imperative that the biological professions (such as ecology, genetics and behavior, ex-situ facility managers) collaborate with veterinarians, as well as social disciplines (such as anthropology and psychology) so that all aspects of sun bear conservation (ranging from baseline research on the species to public awareness and conservation marketing) can be addressed. If we are to succeed with sun bear conservation we must work with the governmental agencies, stakeholders (such as plantation-owners and hunters) and teachers towards a common and clearly defined goal. As I cannot cover all (research) disciplines to propose a complete action plan, I will elaborate how my personal experiences (please refer to the Preface) and the findings of my thesis may inform a sun bear conservation plan.

My personal experiences introduced me to the problems surrounding the commercial hunting and trade of sun bears that often results in the orphanage of bear cubs (Beecham et al. 2015). Due to the increasing nature of this issue, taking care of cubs is becoming a serious conservation concern (Fredriksson 1998). Most wildlife organizations or NGOs have a limited supply of funding and facility available (Fredriksson 1998; Scotson & Hunt 2008), which makes taking care of a large and long-lived carnivore challenging (Soorae 2005). The IUCN (Species Survival Commission 2000) provides the practitioners with three options: 1) release, 2) lifelong maintenance in captivity and 3) euthanasia. However, as mentioned in chapter 1, euthanasia is illegal in many countries and usually finds no donor and public support. Euthanasia should be more widely discussed and considered under animal welfare aspects, but it will not be further debated here. In this section, the focus will be given to captive management and reintroduction.
biology as a conservation tool for sun bears, and how the research findings from this Ph.D. inform these options. Furthermore, this chapter explains why the development of a PRISMA-p report model would be highly beneficial for re-introduction and conservation biology. The chapter then concludes with which of the management options are the most ex-situ and in-situ conservation sensitive with the current state of knowledge on the sun bear.

6.2 IUCN OPTION: RELEASE

The term ‘release’ is an umbrella term that encompasses different reintroduction biology methods. These include:

- the re-introduction of an animal into its former distribution range,
- the re-enforcement of animal population by releasing individuals, and
- the translocation (wild-to wild), where wild animals are moved from one location into another.

Sun bear conservation currently employs these management strategies. Here, re-introduction, re-enforcement and translocations will be discussed.

6.2.1 Translocation

With an ever-increasing encroachment of humans into the last remaining wild spaces, the conflict between sun bears and the local human settlements is forcing wildlife officials to catch and translocate sun bears that have ventured into villages or were feeding of plantation crop (Scotson & Vannachomchan 2014). Translocation of sun bears is seen as a humane way to handle these conflict situations as opposed to killing the animals (Seddon et al. 2012; Parker et al. 2012). However, when a sun bear is moved from one area to another, it is likely that the translocated bear is released into an area where there is a resident wild sun bear population present (Fredriksson 1998). This is problematic for the released bear and the receiving population, as this could mean that the translocated animal is potentially introducing parasites and disease (Ewan et al. 2015) or is compromising the genetic structure of the wild population (Soorae 2005; Benjamin-Fink 2017). For translocation purposes, conservation practitioners require information on population structure and genetic health (Moritz 1999; Storfer 1999; Weeks et al. 2011). In chapter 4, microsatellites loci were used to identify the population structure of the Cambodian sun bear populations. This research identified two distinct populations located East and West of the Mekong River. Furthermore, the research revealed that
the West-Cambodian population is inbreeding and, although the populations are hybridizing, the
gene exchange is not sufficient. The conservation practitioner can choose to release the
translocated bear on the same side of the Mekong River as its capture location as this would
maintain the purity of each population or the practitioner could opt for a deliberate conservation
augmentation. Microsatellite research can identify gene-flow and geographical barriers.
Expanding our knowledge on the population structure across the distribution range of the sun
bear would advise conservation practitioners when making translocation or reinforcement or re-
introduction decisions.

6.2.2 Re-introduction

Human alterations to the landscape have resulted in habitat fragmentation, that is detrimental for
sun bear conservation (Fredriksson 2001; Fredriksson et al. 2008). Fragmentation can result in
the isolation of populations (Lancaster et al. 2016), leaving them vulnerable to stochastic events
(Tracy et al. 2011), or inbreeding depression (Charlesworth & Willis 2009). The re-introduction
of animals into areas where they have recently disappeared from can be a mean to re-connect
isolated populations (Majolo et al. 2013). Although reintroductions appear to be a valuable
conservation technique for bear conservation in general (Clark & Westrum 1989; J. Clark et al.
2002; Preatoni et al. 2005), reintroducing an animal into its former distribution range may not
always be a good idea (Clack 2009). If bear has not been present in an area of its historical range
for a longer period, evoloutional processes could have changed that area so that it may no longer
be a suitable habitat for the reintroduced species (Osborne & Seddon 2012). Understanding the
adaptive and evolutionary potential of a species over time can be essential to re-introduction
biology. Understanding the evolutionary history and how and why speciation may have occurred
is relevant for conservation as it allows to identify species or sub-species of conservation priority
(Wilting et al. 2016) as well as identify the influence of geographical features that impact the
dispersal of the sun bear. The phylogeography study presented in this thesis (chapter 3) is the
first phylogeography study on sun bears, covering most of its distribution range. The results of
this research concluded that there are two different lineages of sun bears that have been divided
by geographical barrier known as Isthmus of Kra (IOK) in Thailand. This means that large
waterbodies such as the IOK have separated sun bears during the Pleistocene. Furthermore, the
research also showed that sun bears have adaptive potential as Thailand has been identified as a
secondary contact zone (sun bears re-colonized Thailand once the water levels were low enough
to expose land-bridges), allowing and increased gene-flow over the last 1000 years.
Understanding how the geography has influenced dispersal of sun bears over time provides reintroduction practitioners with guidance when they decide on a reintroduction location.

### 6.2.3 Reinforcement

Maintaining genetic diversity in wild populations is essential for the adaptability to a changing environment and the persistence of a species over an evolutionary time frame (Martinez et al. 2012). Consequently, reinforcement of a population is a powerful conservation approach for genetically depleted populations to alleviate inbreeding depression (Frankham 1995). The introduction of genetic diversity into these inbred populations is then often referred to as ‘genetic rescue’ (Frankham 2015). Commonly, reinforcement (or augmentation) projects choose wild born animals that only have been housed captive for a short period of time and are then released to an area inhabited by a depleted or locally extinct population (wild to wild restoration) (Jule et al. 2008). The sun bear is an opportunist with a large distribution range and therefore has good chances of persisting over time. However, the increased human pressure on the remaining habitat may lead to individual populations going extinct. These threatened populations may not endure further removals of individuals from the wild to augment other populations (Clark et al. 2013). The Microsatellite conducted on the Cambodian sun bear identified the West-Cambodian sun bear population as inbred and threatened. Conservation practitioners may want to consider increasing connectivity between the populations or to establish augmentation projects. As a result, conservation scientists may have to consider releasing captive bread and reared bears for augmentation purposes (Griffith 1989; Mallinson 1995; Vickery & Mason 2005; Witzenberger & Hochkirch 2011; Hoffmann et al. 2015). Breeding and rearing of potential release candidates for conservation purposes requires the knowledge of the correct taxonomy (chapter 3) and population genetics (as demonstrated in chapter 4) (Clark & Westrum 1989; Fischer & Lindenmayer 2000). Population genetics allows the monitoring of the impact captivity has on the genetic structure of the species (for example loss of genetic variation due to uninformed breeding) (Frankham 1995).
6.3 THE DEVELOPMENT OF A PRISMA-P MODEL FOR SUN BEAR CONSERVATION

To protect a species from extinction, it is essential to monitor its conservation status over time. As conservation trends are not likely to be evident in five-year time intervals, long-term monitoring is a necessity (Lindenmayer & Likens 2009; Magurran et al. 2010; Proença et al. 2017), however, this is not possible if reporting systems are incompatible. For long term monitoring to be achieved, researchers must record their data in a standard way that cannot be miss-interpreted by future researchers (Thessen & Patterson 2011). Currently, conservation biologists do not follow standardized reporting systems for their research outputs that facilitate generational transition (Thessen & Patterson 2011; Enke et al. 2012). Re-introduction biology is one of the biological sub-disciplines that has been identified for poor method-and result reporting (Fischer & Lindenmayer 2000). The reasons for that may be manifold: conservation practitioners carrying out the releases may not have any academic training, may not have the time to write up their experiments and results, may not report on their release as sample sizes or datasets are too small (Parker et al. 2012) or because their results may harm their career (Meek et al. 2015). However, even the studies that have been published do not report on what could be considered essential information (Fischer & Lindenmayer 2000).

A literature review carried out by Fischer & Lindenmayer (2000) concluded that most publications in re-introduction biology did not report on the initial reason for the execution of the re-introduction, did not list the costs or budget of their project and did not clearly define objectives, goals and targets for the release. Many reintroductions projects do not define what a reintroduction success constitutes to them or they have an unknown success rate due to lack of reports of failure and a lack of follow up research. Furthermore, the publications do not provide detailed explanations of staff requirements, or describe human actions and decision-making and the resulting outcome (Clark & Westrum 1989). Conservation Biology lacks information on failure (Dodd & Seigel 1991b; Fischer & Lindenmayer 2000; Meek et al. 2015), methodology, measures of success and provision of long-term follow-up studies (Fischer & Lindenmayer 2000; Seddon et al. 2007). Results and method (regardless whether is led to negative or positive outcomes) should be published and reported on in a standardized way as proposed in chapter 2. To succeed in conservation of a species, one must first know the cause of decline, biological constraints, habitat constraints, population genetics and structure, and disease transmission (Dodd & Seigel 1991b; Ewan et al. 2015). To identify research gaps and to communicate
research data and findings to fellow researchers, the implementation of a PRISMA-p model (Moher et al. 2009) as described in chapter 2 is recommended. Once these research gaps are filled in and reported in a way that the results cannot be misinterpreted, re-introduction biology will develop into a stronger conservation management.

Although Translocations, reintroductions and reinforcements are popular in conservation biology, their success rate is poor (Linnell et al. 1997; Teixeira et al. 2007; Seddon et al. 2012). Therefore, translocation, re-introductions and reinforcements should be considered as a last resort management approach (Griffith 1989) and lifetime maintenance in captivity should be evaluated as a conservation option.

6.4 IUCN OPTION: LIFELONG MAINTENANCE IN CAPTIVITY

Although captive bears may have to be considered for re-introduction and reinforcement purposes, captive reared bears have a twice as high mortality rate post-release when compared to wild bears used in augmentations (Jule et al. 2008; Parker et al. 2012). Although bears are robust and survive bad husbandry for a long time (Law & Reid 2010), captivity has a strong negative effect on the mental wellbeing of animals with large home ranges and complex life styles, such as bears (Vickery & Mason 2003). Therefore, bears are especially vulnerable to develop stereotypical or abnormal behavior if deprived of natural stimuli (Vickery & Mason 2005). Stereotypical captive behavior can indicate aspects that the surrounding does not meet the natural environment (e.g.: pacing is linked to home range). Even animals that do not show stereotype behaviour cannot be assumed unaffected as individual differences in the animals and their determination and persistence variability could also have an impact on which animals display abnormal behavior in captivity. Animals with developed stereotypical behavior lack adaptability to novel environments (Vickery & Mason 2003) and display certain persistence in their behavior which could result in fitness limitations (even death) if they were released into the wild (Jule et al. 2008). Therefore, it is of paramount important to preserve species typical behavior and it is recommended that bears are offered large natural enclosures (semi-wild) and use the offspring for reintroduction (Vickery & Mason 2003).

Even though captivity may appear as the least favourable management option, it is worth reminding that bears in captivity allow research that is essential for the success of reintroduction biology (IUCN/SSC 2014). Samples for this thesis research into the phylogeography and
population genetics were obtained from bears housed in a sanctuary. These bears and other zoo bears from the Tierpark in Berlin were used to develop and test genetic primers which are essential for all genetic research. Though not only genetic research benefits from captive bears.

As mentioned in earlier chapters, the usage of TCM products is threatening most bear species across their distribution range (Lee et al. 2015). The Asiatic black bear is the most targeted bear species to produce TCM products (Livingstone & Shepherd 2014) and has therefore the primary target species for the development of forensic methods to detect bear derivate in these products (Peppin et al 2008). Currently, it is still not entirely clear how far the TCM industry is threatening the sun bear. However, Malaysia has been declared a future hotspot for TCM practitioners (Shepherd & Shepherd 2010) and there are no indications thus far that TCM demand is decreasing anytime soon (INTERPOL 2014). As the Asiatic black bears does not occur in Malaysia (Garshelis & Steinmetz 2016), and Malaysia also has no bear bile farms (Shepherd & Shepherd 2010), one must consider an increasing pressure on wild sun bear populations-the only present bear species in Malaysia. Current forensic methods to detect sun bear derivate in heavily processed TCM products have been tested in chapter 5. These methods are likely to be effective when working on bear bile but have not been successful on products where the production process is aggressive enough to have destroyed the DNA (Peppin et al 2008). Therefore, it would be beneficial to utilize captive sun bears to develop and establish reference databases that allow alternative forensic species identification that is not reliant on DNA or PCR based methods, such as Gas chromatography–mass spectrometry (GC-MS). Captive animals allow the development of reference databases, enable veterinary research, are useful for the development of radio telemetry, and provided an essential breeding stock for future re-introduction and augmentation efforts (Mallinson 1995).
6.5 CONCLUDING REMARKS

Although the IUCN guidelines urges that initial threats to a species be eradicated before the animal can be released into the wild (Iucn 2012), it is unrealistic that issues as significant as habitat loss, fragmentation, and commercial hunting will be addressed and controlled before reintroductions and augmentations may become a conservation necessity. However, in the case of the sun bear, release, reintroductions and augmentations are not an immediate conservation necessity and are likely conducted due to public pressure. Most organizations rely on donation money from the public, and therefore animal welfare concerns in the husbandry of the animals in their care is counterproductive (Seddon et al. 2012). With an increase in animal welfare awareness, animals are now being released under the dogma of release being the only animal welfare responsible option to spare them from the monotone life in captivity. In fact, most organizations now use the bear cubs to attract funding by show-casing release projects of bear cubs back into ‘the wild’ (Dodd & Seigel 1991b). For the conservation of bear species, this could have been a win-win situation if the survival rates of the released bears had a high success rate. What often is not considered, however, is that most of the animals being released into the wild are not able to sustain themselves and die of starvation (Kolter & Dijk 2005; Seddon et al. 2012). Most release projects do not conduct post release monitoring (Seddon et al. 2007; Jule et al. 2008; Scotson & Hunt 2008), or if they do, not over a long enough period to conclude release projects as a successful conservation tool (Fischer & Lindenmayer 2000). Other projects have conducted surveys and have found high post release mortality (Fischer & Lindenmayer, 2000; Jule et al., 2008). One can presume that projects that have engaged in post release monitoring may have concluded a high mortality rate and did not release their findings as this could cause bad publicity for their organization or for their own career (Dodd & Seigel 1991b). This ‘fear of failure’ does not allow for a scientific learning process and is partly responsible why conservation cannot account for more successes (Meek et al. 2015). To not further endanger animals or species, it is important to allow a safe-fail environment where scientists and practitioners can report on their experiences without jeopardizing their career or conservation funding.

Conservation practitioners carry a responsibility to not further endanger wild bear populations through the inappropriate and unnecessarily release of individual bears. From a conservation biology perspective, the health of the wild population should be prioritized over animal welfare concerns of individual animals. If a confiscated bear is released into suitable bear habitat, it is likely that wild bears are occupying the release site (Fredriksson 1998). This means that an additional bear could increase the food competition above carrying capacity. Furthermore, the
released bear may introduce disease and parasites to the wild population (Ewan et al. 2015), or genetically and behaviorally compromise the wild bear stock (Benjamin-Fink 2017). Until we have a better understanding of the ‘behaviour, ecology, biology, viro-and parasitology as well as the genetics of the forgotten bear’, releasing confiscated bears has neither a conservation nor animal welfare benefit. Therefore, the maintenance of sun bears in ex-situ facilities with high animal welfare standards might be the favorable option. The sun bear deserves more research attention and a multi-disciplinary conservation management plan that is consulted by practitioners to make informed and responsible decisions. By providing recommendations on how to report research findings, identify research gaps and delivering genetic research results that are applicable for ex-situ and in-situ conservation, I hope to have contributed to the conservation of the ‘forgotten bear’.
AFTERWORD

The original and essential aim of this Ph.D. was to provide more information on sun bears so that confiscated bears could be successfully released into the wild. To me, being able to return extracted animals back to where they belong was the ultimate conservation goal. To reach this goal, more information (or any information) on the genetics of sun bears was necessary. Although my work can inform ex-situ and in-situ management alike, my understanding of release efforts as a conservation tool has changed. I now realise that the current practice of releasing individual confiscated sun bears has little to no conservation purpose. This does not mean that being able to release, re-introduce and augment wild populations of sun bears is not a potential conservation necessity in the future and therefore needs to be developed now. However, to date, we have no understanding how the release of individual bears into an area with wild sun bears present impacts on the receiving bear population or on the released individual. I am also sceptical whether the release of sun bears can even be considered as an animal welfare argument. Granted that providing suitable animal husbandry to captive sun bears is costly and challenging, the better quality ex-situ facilities can cater to many of the basic needs of sun bears by providing forest enclosures, enrichment programs, veterinary care, a balanced diet and shelter. Captive bears fulfil an important role for further research and can help to generate funding for in-situ conservation work. Most importantly, captive bears are very powerful ambassadors to educate people about the plight of the species as well as creating an important emotional connection to an otherwise illusive and cryptic animal.

The importance of conservation outreach work must not be underestimated. Knowing and understanding your species and how it may adapt or respond to human-imposed pressure is important and requires research. But such research is treating a symptom and is not addressing the cause. Conservation is dictated by human behaviour. For example, understanding the medicinal power of animal derivate in TCM seems irrelevant. It is well known that rhinoceros horn is as effective as chewing your hair or finger nails, but the trade in horn is still booming. Being informed is not enough to change behaviour. The wildlife trade and TCM usage is driven by the human ego (showing off status and wealth), and deep-rooted traditional beliefs. The only way to stop the wildlife trade is by stopping the demand. This can only be achieved if the usage of wildlife products becomes culturally (and spiritually, see quote from Gus Speth) unacceptable. Without addressing these causes, it is unlikely that co-existence between humans and wildlife is
achievable in the Anthropocene. Therefore, conservation biology is reliant on developing a multi-disciplinary and collaborative approach to species protection.

“I used to think the top environmental problems were biodiversity loss, ecosystem collapse and climate change.

I thought that with 30 years of good science we could address those problems.

But I was wrong.

The top environmental problems are selfishness, greed and apathy…

…and to deal with those we need a spiritual and cultural transformation

-and we scientists don’t know how to do that.”

Gus Speth

Although it seems evident that the future of the conservation profession will rely on a multidisciplinary research community that collaborates and shares experiences on success and failure, it is also clear that the commercialization of the academic sector does not support the free exchange of data or communication. Universities do not assign credit to scientists in accordance to their conservation impact in the field but rather to scientists who publish as many papers as possible. Consequently, scientists are pushed into a race for publications in which data and intellectual property (IP) theft, as well as authorship fights, are not uncommon. Even if universities support collaborative projects, these projects often have to deal with conflicts within the research groups and colleagues.

“Cooperation among scientists is not always a simple matter. Science is a human endeavour and, as such, is subject to those unsavoury traits which characterize human beings. Scientists, too, must content with unrestrained ambition, belligerence, arrogance, insecurity, sloth, egomania, paranoia, envy, jealousy, pettiness, anger, and vengefulness.”

(Mares 1991, pp. 59-60)

Working in a group where different egos and personas collide remains a challenge that must be met to drive scientific advancement and innovative solutions to old problems. However, social dynamics can become even more complex if one research group includes practitioners, natural and social scientists, as there is often a lack of respect for other disciplines. Species loss is usually
caused by a variety of different threats and factors that requires broad-ranging skills and knowledge from different disciplines (such as ecology, biology, genetics, veterinary science, psychology, anthropology, marketing and media). Furthermore, if we want to understand the species status of an animal over time, we need to remind ourselves that we must pass on our data to fellow research generations and think beyond our own academic and professional careers. To be able to do that, we need to start agreeing on how to report our research, so data is not misinterpreted by other scientists and to decide collectively which research is most urgent and impactful for our target species.

It was, therefore, encouraging that, while I was finishing writing this thesis, I was invited to the first sun bear symposium. This symposium was organized by Free the bears and held in Kuala Lumpur, Malaysia in September 2017. This event provided an opportunity for sun bear scientists, conservation practitioners and ex-situ managers to meet, share their research results, report on ongoing research projects, engage in discourse and debate on research targets, and to draft a conservation action plan for the species. While it was pleasing to see 94 people coming together talking about sun bear conservation, there were no psychologists, anthropologists, marketing and media experts, oil palm industry representatives, bear bile farmers or hunters present. The symposium also revealed that very few scientists focus on sun bears as their primary research target. Many of the presentations during the conference were held by scientists who work on other species (e.g., Asiatic black bears or tigers) and presented sun bear data collected as a side product. However, since this was the first sun bear symposium, I am confident that this is about to change. From now on, the sun bear and sun bear conservation can be discussed and re-evaluated on a regular basis. The next symposium will be held in Sabah, Malaysia in three years’ time. After the symposium, I left cautiously confident that from now on the ‘forgotten bear’ will get the attention it has long deserved.
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APPENDIX I

SYSTEMATIC QUANTITATIVE LITERATURE REVIEW AFTER THE PRISMA–P MODEL.

Search phrases have been searched in eight different databases by two independent reviewers (to test for replicability of the method).

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APPENDIX II

(Chapter 2)

THE PRISMA-P GUIDED MANUAL DEVELOPED FOR A
STANDARDIZED REPORTING ON PUBLISHED LITERATURE

How to fill in the excel spreadsheet:

Please review the literature for the criteria listed below. If your literature provides any information for the following sections, please score it with ‘1’ and fill the box with a background color matching the section color (by underlying the boxes with a score with a color and leaving the boxes without a score blank, it will provide a visual aid to locate the areas that lack research). If your literature fails to provide information for some of the following sections, please score it with ‘0’.

To adapt the excel spreadsheet to your target species, please add individual columns for the countries of its distribution range. By adding a column per country of its distribution range, you will be able to identify which countries (and animal populations) are understudied or overrepresented in the scientific literature.

Administrative Section

Year of publication: The year in which the article was published.
Authors: List of publication authors (Harvard Citation style).
Journal: The name of the journal the article is published in.
Title: Title of the research article.
Keywords used by authors: The key words listed in the publication (if no key word is listed, fill in “N/A”).
Objective: The objective listed in the publication
Clarity of Objectives: The objective was stated clearly in the publication (score with ‘1’ if the objectives were clearly stated and ‘0’ if the objectives were not clear).
Funding Body: List the Funding bodies acknowledged in the publication.

Geographic Section
**Country of Research:** the country in which research is carried out.

**Province of Research:** the province/state/county/etc within the country of research.

**Coordinates:** GPS coordinates of the study area (if available, “N/A” if not available).

**Country of Authors:** Country where the authors are from.

**Country of Research facility:** Country where the research facility is based (where the University is located).

**Country of Research Funding:** Country that supplied financial support for the research.

("Type of study" Section)

**Fundamental research:** ~also known as ‘basic research’ or ‘pure research’ is driven by curiosity with the intention to expand knowledge in a specific research field. It does not necessarily generate results that find immediate practical applications. Fundamental researches mainly asks why? what? or how?

**Applied Research:** ~ is solution-orientated research that is trying to apply its research findings to solve problems.

**Qualitative Research:** ~ tries to understand underlying reasons and is largely exploratory research. It tries to develop hypothesis for quantitative research, and applies qualitative research to disclose trends in opinions and thoughts. Qualitative methods may differ using semi-structured or unstructured techniques (interviews, focus groups (group discussions), and observations. Sample size tends to be small. The data analysis is non-statistical and the outcome investigative or explorative and cannot be used to generalize.

**Quantitative Research:** ~tries to quantify and generalize results using a large sample size and randomly selected respondents. The Data collection is executed in a structured manor and can
include questionnaires and street/telephone interviews. The data is analyzed in a statistical method, resulting in conclusive and descriptive findings that are then used to make recommendations of action.

“Tool” Section

**Experimental:** An experimental research design has been applied.

**Modelling:** Computational means were applied (modelling of scenarios).

**Telemetry/collaring:** The animal(s) was/were radio-collared or tracked through telemetry.

**Observational:** The research reports on observed behavior (or bear signs).

**Trapping:** The animal was trapped (including camera trapping).

**Data collection:** The researcher acquired datasets from other studies or collected data.

**Sample Collection:** Samples were collected (eg scat, hair, and tissue).

**Photography:** Photo ID was used in this research (e.g. for animal biometrics).

**Genetic:** Samples were collected with the intention of genetic research.

**Survey:** The research conducted surveys.

**GIS:** Geographic Information Systems (GIS) was applied to gather data for this research.

**Interviews:** Interviews were conducted in the research.
“Setting” Section

Unknown: The origin of the specimen might not be known in more detail (e.g., Museum specimen, author did not specify).

Captive: The animal(s) is/has been studied in a captive setting (e.g., sanctuary or zoo).

Wild: The animal(s) has/have been studied in a non-captive but in a non-protected setting (e.g., in plantations).

Protected Area: The animal(s) has/have been studied in a protected area (e.g., National Park).

Major Biome: Score the major biome in which the research has been carried out (please refer to Biome legend in the excel spread sheet)

“Research Disciplines and topics” Section

Behavioral

Foraging: The study focused on foraging behavioral research.

Social: The study focused on research into social behavior of the animal(s).

Reproductive: The study focused on reproductive behavior of the animal(s).

Maternal: The study focused on maternal behavior of the animal(s).

Stereotype: The study focused on researching stereotypical behavior of the animal(s).

Enrichment: The study focused on the impact of enrichment on the behavior of the animal(s).
**Behavioral Network:** The study focused on intra-species and inter-species networking, collaboration or competition.

**Habitat use:** The study focused on behavioral studies that allow insight into the habitat use of the animal(s).

**Adaptation:** The study focused on behavioral adaptation to changing environments.

**Activity pattern:** The study focused on behavioral studies that allow insight into the activity pattern of the animal(s).

**Veterinary**

**Parasitological:** The study focused on parasitological research.

**Disease:** The study focused on veterinary research investigating disease detection and/or cure.

**Haematology:** The study focused on research into the properties or treatment of blood.

**Serum Biochemistry:** The study focused on the chemical analysis of blood serum.

**Anatomy:** The study focused on anatomical research of the animal.

**Pain Management:** The study focused on veterinary (and animal welfare) research to detect and manage pain in the animal.

**Breeding/Reproductive Technology:** The study focused on veterinary research into animal breeding and/or reproductive technologies applied on animals.

**Nutrition:** The study focused on research into the nutrition of animals.

**Hormonal:** The study focused on research into hormones of the animals.
Restraint: The study focused on veterinary methods to restrain and immobilize (anaesthetize) the animal.

Social Science

Conflict: The study focused on conflict between humans and animals.

Threats: The study focused on threats caused by humans to animals (or vice versa).

Economics: The study focused on the economic value/gain/cost of an animal to humans.

Illegal Trade: The study focused on the illegal trade of animals.

Perception: The study focused on how animals are perceived by people.

Molecular Biology

Genetics: The study focused on genetic research of the animal(s).

Genomics: The study focused on genomic research of the animal.

Mitochondrial: The study focused on genetic or genomic research using mitochondrial DNA of the animal(s).

Phylogenetics: The study focused on researching the evolutionary relationship among species and/or individual animals.

Microsatellites: The study focused on researching into genetic diversity of an animal population using microsatellites.

Nuclear: The study focused on genetic or genomic research using nuclear DNA of an animal.
**Phylogeographic:** The study focused on researching into historical processes that may be responsible for the contemporary geographic distribution of the animal (often linked with the genetic or genomic research into the animal).

**Other**

**Stable Isotopes:** The study focused on research into an animal using stable isotopes.

**Archaeology:** The study focused on archeological research of an animal.

**Animal Welfare:** The study focused on animal welfare related issues.

**Biogeography:** The study focused on the geographical distribution of an animal.

**Morphology:** The study focused on researching into the morphological features of an animal/species.

**Reintroduction Biology**

**Release method**

**Soft:** The study focused on release methods applying ‘soft’ release (gradual release processes providing support and training for the animal released).

**Hard:** The study focused on release methods applying ‘hard’ release (sudden release of animals without prior training or post-release support).

**Re-introduction:** The study focused on research that is concerned with re-establishing an animal/species/population into its former distribution range.
Rehabilitation: The study focused on the research into releasing a recovered wild animal back into the wild.

Augmentations: The study focused on research into supplementing wild populations by releasing animals into this population.

Survival Rates: The study focused on research concerned with the survival rates of released animals (individuals).

Population Survival: The study focused on research concerned with the population survival of a threatened animal population/species.

Demographics: The study focused on the demographics of a population.

Management: The study focused on how to manage release projects or how to manage populations using reintroduction biology as a tool.

Ecology

Foraging Ecology: The study focused on researching into the foraging ecology of an animal/species.

Population Estimates: The study focused on estimating population sizes and population trends of animal populations.

Ecological Role: The study focused on the ecological role/function of an animal in its ecosystem.

Distribution: The study focused on the distribution range of the animal (through ecological drivers).

Adaptation: The study focused on how a changing environment is impacting on the ecology of an animal (and how it adapts to the emerging changes).
**Habitat Requirement:** The study focused on the ecological habitat requirement for an animal/species.

**Threats:** The study focused on threats to an animal/species caused by ecological changes.

**Ecological Network:** The study focused on how animals/species network within and among ecological niches.

**Climate Change:** The study focused on how climate change is impacting on the ecology of an animal/species.

**Model**

**Mathematical:** The study focused on the development of a mathematical model to make predictions of an animal's population survival, habitat distribution, connectivity, evolutionary significant units, etc.

**Simulation:** The study focused on simulating population survival, habitat distribution, connectivity, evolutionary significant units, etc. by changing parameters.

**Target species as research focus:** The objective of the study targeted the species of interest (here sun bears).

**Management/application recommendation:** The study provided management or application recommendations.

**Notes:** Use this section to make notes regarding the paper in that row.
### APPENDIX III

(Chapter 5)

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