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"Generalists" & "Specialists": Closely Related Felidae Species exhibit Diverse Evolutionary Histories

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“It is not the strongest of the species that survives,

Nor the most intelligent that survives.

It is the one that is most adaptable to change”

-Charles Darwin (1809-1882)

Dedicated to my mother in law and my mother,

For their love and support.

This thesis is based on the following manuscripts:

1. Patel RP, Förster DW, Kitchener AC, Rayan MD, Mohamed SW, Werner L, Lenz D, Pfestorf H, Kramer-Schadt S, Radchuk V, Fickel J, Wilting A (2016). Two species of Southeast Asian cats in the genus *Catopuma* with diverging histories: an island endemic forest specialist and a wide-spread habitat generalist. Royal Society Open Science. DOI: 10.1098/rsos.160350. (Chapter 1)

2. Patel RP, Wutke S, Lenz D, Mukherjee S, Ramakrishnan U, Veron G, Fickel J, Wilting A, Förster DW (2017). Genetic structure and phylogeography of the Leopard cat (*Prionailurus bengalensis*) inferred from mitochondrial genomes. Journal of Heredity. DOI: 10.1093/jhered/esx017. (Chapter 2)

3. Patel RP, Lenz D, Kitchener AC., Fickel J, Förster DW, Wilting A (2017). Threatened but understudied – supporting conservation by understanding the genetic structure of the flat-headed cat. Conservation Genetics. DOI:10.1007/s10592-017-0990-2. (Chapter 3)

CONTENTS

Abbriviations

Mya	Million years ago
Kya	Thousand years ago
LGM	Last Glacial Maximum
IoK	Isthmus of Kra
dNTP	Nucleoside triphosphates
Mm	Milli molar
Nm	Nano molar
µl	Micro litre
bp	Base pairs
kb	Thousand base pairs
PCR	Polymerase chain reaction
rpm	Rotation per minutes

ZUSAMMENFASSUNG

Einese der Hauptziele der Evolutionsbiologie ist die Identifizierung von Faktoren, die die Evolution von Arten und ihre Verteilung beeinflussen. Das Verständnis darüber, wie ökologische, verhaltensbedingte oder physische Barrieren den Genfluss zwischen Populationen verringern oder gar unterbrechen, ist eine notwendige Voraussetzung, um die evolutionäre Geschichte von Arten aufklären zu können. Neben äußeren Faktoren, wie geologischen und klimatischen Ereignissen, spielen auch andere Faktoren, wie die Anpassungsfähigkeit von Arten, eine wichtige Rolle für die Verbreitung der Arten und somit die Verteilung der Artenvielfalt.

Ziel der vorliegenden Arbeit war es daher, die Artbildung in engverwandten Schwesterarten (oder Linien) mit überlappenden oder geographisch eng beieinanderliegenden Ausbreitungsgebieten zu untersuchen, da diese zwar von einem gemeinsamen Vorfahren abstammen, aber unterschiedlich auf Ereignisse der Vergangenheit wie klimatische Veränderungen oder Änderungen im Lebensraum reagiert haben könnten. Zu diesem Zweck wurde die evolutionäre Vergangenheit und phylogeographische Verteilung mehrerer südostasiatischer Schwesternartpaare von Kleinkatzen in der Unterfamilie Felinae mithilfe der Untersuchung vollständiger Mitogenome untersucht.

Kapitel 1 beschäftigt sich mit der Phylogeographie der beiden im Genus *Catopuma* zusammengefassten Schwesterarten: die Asiatische Goldkatze (*Catopuma temminckii*), ein Habitatgeneralist, und die Borneo-Katze (*Catopuma badia*), ein Habitatspezialist. Die Analyse der Daten ergab eine weit zurückliegende Aufspaltung der mitochondrialen Linien beider Arten. Mithilfe von Artverbreitungsmodellen für die drei Zeiträume Spätpleistozän (~120 Kya), letztes Eiszeitmaximum (~22 Kya) und Holozän (~6 Kya) konnte gezeigt werden, dass sich die unterschiedlichen evolutionären Entwicklungswege beider Arten sehr gut mit der Lebensraumverteilung und der Herausbildung entsprechender Präferenzen in beiden Katzenarten erklären lassen. Mittels molekularer Daten konnte außerdem gezeigt werden, dass sich die Goldkatze

bereits in eine Kontinental- und eine Sunda-Population differenziert hat. Durch die Kombination der Ergebnisse der Mitogenomuntersuchungen mit denen der Felluntersuchungen gelang es, den Ausbreitungsweg der Asiatischen Goldkatze vom kontinentalen Festland über die Malaysische Halbinsel bis nach Sumatra zu belegen.

Kapitel 2 und 3 stehen in engem inhaltlichen Zusammenhang, da sich beide mit Vertretern der Gattung *Prionailurus* befassen.

Kapitel 2 beschäftigt sich mit der genetischen Struktur zweier mitochondrialer Linien der Bengalkatze (*Prionailurus bengalensis*), einem Habitatgeneralisten. Auf der Ebene mitochondrialer DNA kann die Bengalkatze klar in eine Kontinental- und eine Sunda-Linie unterschieden werden. Die Kontinental-Linie setzt sich aus drei Unterlinien zusammen (eine südindisch-indochinesische, eine nordindisch-indochinesische, sowie eine fernöstliche), während die Sunda-Linie aus zwei Unterlinien besteht, von denen eine auf Sumatra, Borneo und den Philippinen und die andere auf Java und Bali vorkommt. Überraschenderweise gehören Bengalkatzen auf der Malaysischen Halbinsel zu beiden Hauptlinien (Kontinental- und Sunda-Linie). Für einen Habitatgeneralist, für den es auf der Malaysischen Halbinsel keine Barrieren geben sollte, ist dieses Ergebnis insofern überraschend, da eine solche Linien-Aufspaltung kennzeichnend für beginnende Artbildungsprozesse ist. Daher werden in diesem Kapitel auch mögliche Barrieren für den Genfluss diskutiert, deren Gegenwart die Aufspaltung eines Habitatgeneralisten in Unterlinien begünstigt haben könnte. Die Ergebnisse sind von taxonomischer Relevanz, da viele der bisher beschriebenen Unterarten im mitochondrialen Erbgut nicht eindeutig abgebildet sind. Eine Revision und zahlenmäßige Reduktion der bestehenden Unterarten wird daher empfohlen.

Kapitel 3 befasst sich mit der Flachkopfkatze (*Prionailurus planiceps*), einer Schwesterart der Bengalkatze und ein Habitatspezialist für Feuchtlandschaften. Die auf den Mitogenomen basierende phylogeographische Analyse zeigt klar, daß es bereits innerhalb Sundalands eine Aufspaltung in zwei abgegrenzte mitochondriale Linen gibt, deren Alter auf ~0,575 Millionen Jahre datiert werden konnte. Die eine Linie umfasst Individuen der Malaysischen Halbinsel und Sumatras, während die andere ausschließlich Individuen Borneos umfasst. Interessanterweise erbrachte die Mitogenomanalyse auch den Nachweis der Existenz von Unterlinien in beiden Hauptlinien, die auf eine Unterbrechung des Genflusses innerhalb der Hauptlinien hinweisen.

In der sich anschließenden allgemeinen Diskussion fasse ich die Ergebnisse der einzelnen Kapitel für die Habitatgeneralisten (Asiatische Goldkatze, Bengalkatze) und die Habitatspezialisten (Borneo-Katze, Flachkopfkatze) hinsichtlich deren genetischer Diversität und Differenzierung zusammen. Ich gehe außerdem darauf ein, wie sich Generalisten und Spezialisten in ihren Reaktionen auf die pleistozänen Klimaveränderungen und/oder Vikarianz-Ereignisse und somit in ihren Ausbreitungsmöglichkeiten unterscheiden, was zu ihren heutigen Verbreitungsgebieten führte.

SUMMARY

Studies identifying drivers and barriers of speciation and species distribution patterns are a priority subject for evolutionary biology. Understanding of ecological, behavioural and/or physical barriers that reduce gene flow and drive speciation is essential to infer the evolutionary history of species. Along with extrinsic factors, such as geo-climatic events, intrinsic factors such as species adaptability also play an important role in the distribution of species and thereby shape local biodiversity.

The aim of this study was to identify speciation mechanism(s) of closely related species that diverged from a common ancestor but responded differently to past environmental changes. This was achieved by inferring the phylogeography and evolutionary history of closely related species of small cats in the subfamily *Felinae* distributed in southeast Asia from their mitogenomes.

Chapter 1 deals with the phylogeography of the two species of the genus *Catopuma,* one being a habitat generalist – the Asian golden cat (*Catopuma temminckii*), and the other one being a habitat specialist – the bay cat (*Catopuma badia*). Analysis of their mitogenomes revealed a deep split between the mitochondrial lineages of both species. Our species distribution modelling data for the Late Pleistocene (~120 Kya), the Last Glacial Maximum (~22 Kya) and the Holocene (~6 Kya) suggests that both species have had diverse evolutionary histories, most likely because of their distinct habitat preferences. Our molecular data for the Asian golden cat further differentiates a mainland from a Sundaic population. Combining molecular data and pelage data explained the recent expansion and colonization of Sumatra by the Asian golden cat from mainland through Peninsular Malaysia.

Chapters 2 and 3 are closely linked as they both deal with members of the genus *Prionailurus*.

Chapter 2, describes the in-depth genetic structure of two mitochondrial lineages of the leopard cat (*Prionailurus bengalensis*) – a mainland and a Sundaic lineage. Based on mitogenome phylogeny we differentiated the mainland lineage into three sub-lineages (South India /Indochina, North India/Indochina and Far East) and the Sunda lineage into the sub-lineages Sumatra, Borneo and Philippines Islands and Java/Bali. Interestingly, samples from Peninsular Malaysia are present in both lineages. The chapter discusses probable barriers to gene flow which could separate populations of a habitat generalist into sub-lineages. The findings suggest a revision of the current taxonomy of leopard cat sub-species since their status is not unambgiously reflected in their mitogenome.

Chapter 3 focusses on the matrilineal population structure of the flat-headed cat (*Prionailurus planiceps*), a closely related species of the leopard cat and a wetlands habitat specialist. Results from mitogenome phylogeography revealed the presence of two clearly distinct lineages already in Sundaland. The split, which is ~0.575 million years old, separates genetically distinct populations in Borneo from populations in Sumatra and Peninsular Malaysia/Thailand. The results also revealed the presense of sub-lineages within the main lineages, indicating reduced or interrupted gene flow even among populations.

In the general discussion, I summarize the results of the single chapters for habitat generalists (Asian golden cat, leopard cat) and for habitat specialist (bay cat, flat-headed cat). In particular, I discuss how generalist and specialist species differ in their responses to Pleistocene gradual environmental changes and vicariance events and thus in their dispersal and distribution patterns.

GENERAL INTRODUCTION

The relative influences of geography and ecology on the process of speciation have been at the center of interest for evolutionary biologists. Since Charles Darwin (1859) and Alfred Russel Wallace (1876) expressed their views on the distribution of species from field observations, the question of how species arise in nature has fascinated biologists. A good understanding of this continuous process is essential for conserving biodiversity, particularly in the anthropocene, the era of increased human influence on the natural habitat of many species. Speciation is broadly understood as a process whereby gene flow between populations becomes sufficiently reduced to allow each population to become irreversibly committed to a different evolutionary path (Bush 1994, Schluter 2001). Such reduction of gene flow may happen in different ways, but it always requires the emergence of a barrier. A barrier may be behavioral (e.g., assortative mating), ecological (e.g., preference of different niches) or physical (vicariance). Behavioral and ecological barriers allow speciation despite physical contact zones between populations (sympatric speciation), and require the evolution of reproductive isolation to complete speciation whereas physical barrier separates populations (allopatric speciation) geographically. For both sympatric and allopatric speciation, studying the barriers to gene flow provides a rich source to understand the speciation mechanism in specific instances and enhances our understanding of the importance of the various mechanisms.

The basis for using genetics as a scientific discipline to study Darwin's theory of natural selection and evolution was provided by Theodosius Dobzhansky's *Genetics and the Origin of Species* (1937) and Ernst Mayr's *Systematics and the Origin of Species* (1942). Today's studies combine evolutionary genetics with ecology and species distribution modeling and thus try to find answers to several important questions: What are the geographical barriers to dispersal (i.e., gene flow) which might promote allopatric speciation? Does a change in habitat preference drive populations into reproductive isolation, as in the case of sympatric speciation? Why does one species diverge and become a (habitat) specialist and the other a (habitat) generalist when both are evolving from a common ancestor, and thus should have a similar scope of adaptability?

Comparison of genetic data collected from several taxa with similar distribution ranges offer the opportunity to infer general biogeographic barriers, as well as geological and/or climatological phenomena or events that may have generated the observed distribution of species. In addition to extrinsic and abiotic factors, life-history traits and consideration of the ecology of the taxa may also provide keys to understand the current genetic structure of populations. Genetics can also clarify to what extent current taxonomic assignments are useful and / or valid, since the original definitions of many taxa are often based on very few or just one specimen and a limited set of traits dominated by morphology. By establishing the true number of taxonomic units, evolutionary units or conservation management units, genetics may also be helpful for conservation as it allows the allocation of limited financial resources in a more effective way.

This dissertation aims to identify drivers of evolution and key events who shaped the distribution of species we observe today. To accomplish this aim, the study design required appropriate selection of study area, study species and study method. Particularly well suited areas to study speciation mechanisms are biodiversity hotspots because here speciation is often rapid, increasing the chances to detect physical and/or ecological barriers that drive speciation and generate heterogeneous conditions that provide different ecological niches. Southeast Asia harbours numerous biodiversity hotspots; the high biodiversity of this region is associated with a complex biogeography and rich history of geo-climatic events that include the emergence and disappearance of barriers. Although many species are widespread in southeast Asia and occur in different habitats, this region is also known for high level of endemic species that are specialized to small areas (Myers et al. 2000, Sodhi et al. 2004). To study speciation we need to choose species which diverged from their common ancestor relatively recently (closely related species) so that their molecular divergence can be compared with known geo-climatic events of the region to identify possible barriers to gene flow. To improve our understanding of how species differ in their response to environmental changes, the chosen species should have been exposed to similar environmental change scenarios but adapted to different niches. Their current distributions may, in the case of sympatric speciation, overlap or in the case of allopatric speciation may not overlap. Equally important is the availability of reliable and accurate information regarding the geographic locations of the samples.

When considering these criteria, members of the family *Felidae* fulfil these criteria very well and provide unique opportunity to study speciation. Felids are highly mobile and occur in many different habitats. Radiation of modern felids took place only within a quite short span of just ~11 million years and within that period, 38-41 species diverged from a common ancestor (Li et al. 2016). This recent evolutionary history allows relatively good estimates on the time and sequence of geographic or climatic events that may have caused lineages to split.

Study area

Southeast Asia contains four major global biodiversity hotspots, namely Indo-Burma, Sundaland, Wallacea, and the Philippines. The species diversity and richness of this region mirrors the complex tectonic and climatic processes that have shaped this region (Myers et al. 2000, de Bruyn et al. 2014). Present day southeast Asia is the result of 300 million years of collisions between continental plates, rift building in sea beds and up-lifting of mountains. Sundaland, comprising Sumatra, Borneo, Peninsular Malaysia and surroundings (see Fig. 1), was part of continental Asia until ~ 200 Mya (Hall 2009). Geography of Southeast Asia was strongly influenced by the climate fluctuations in Pliocene-Pleistocene and consequently the dramatic and repeated sea-level changes (Woodruff 2010).

During the last 2.4 million years, ~ 48 glacial-interglacial transitions have caused fluctuation in sea level from -16 ± 10 m to -62 ± 50 m (Woodruff 2010).

During periods of low sea level, especially during the Last Glacial Maximum (LGM) ~ 19-26 Kya, the shallow Sunda-shelf surrounding the Sundaic Islands of Southeast Asia became fully exposed and formed a single land mass which is also known as '**Sundaland'**. Sundaland connected mainland Asia, Sumatra, Borneo, Java and Bali. During each interglacial interval, the melted water from the receding glaciers flooded Sundaland, separating again the formerly connected islands of the Sunda-Shelf (Woodruff & Turner 2009, Woodruff 2010).

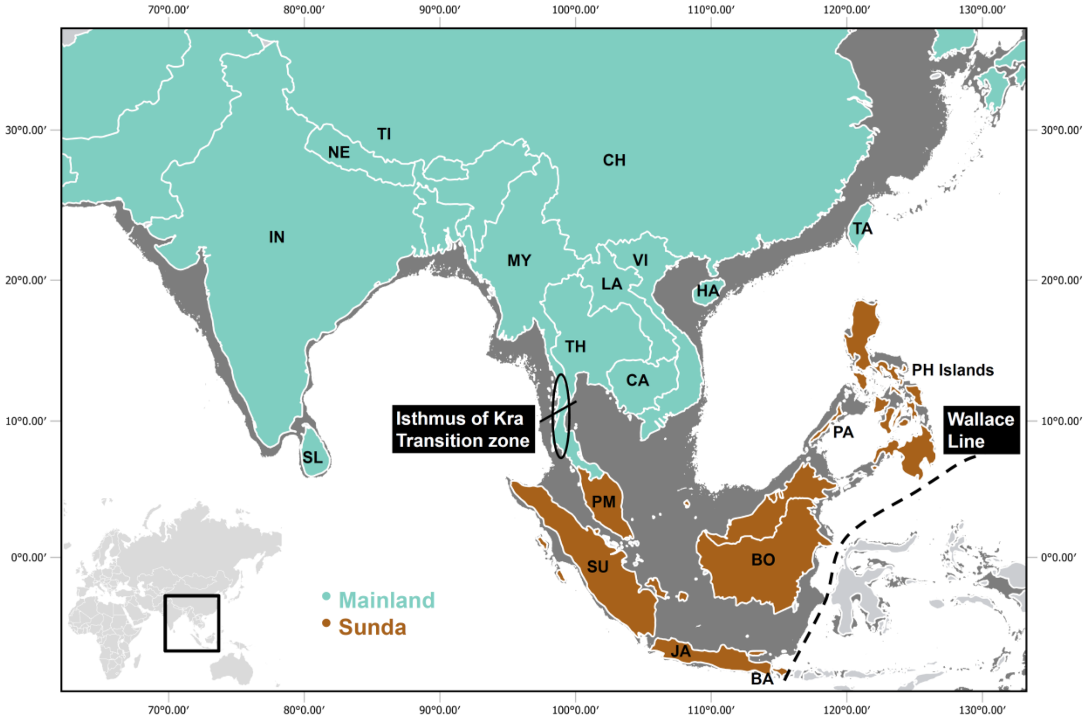

Figure 1: Map of southeast Asia. Dark gray colour (filled) area surrounding Sundaic islands and continental mainland indicates land masses emerged during low sea level (-120m lower than present day sea level). Two letters abbreviations for geographic areas are as follows: on the mainland: India (IN), Nepal (NE), Tibet (TI), China (CH), Myanmar (MY), Laos (LA), Thailand (TH), Cambodia (CA), Vietnam (VI), Hainan (HA), Taiwan (TA); in the Sundaic region: Peninsular Malaysia (PM), Sumatra (SU), Borneo (BO), Java (JA), Bali (BA), Palawan (PA) and The Philippine Islands (PH Islands). Map was created using QGIS desktop v2.16.0 and data from ETOPO1 (Amante & Eakins 2009).

The glacial and interglacial periods were also associated with major changes in the climate and vegetation of Southeast Asia (Cannon et al. 2009). During the glacial periods low sea level caused the decreased evaporation from the sea and thus vegetation might have changed to more seasonal forest and open savannah habitat, for example in south-eastern Borneo and Java. Intermidiate warm periods (interglacial periods) or abrupt episods of high sea level caused increase in humidity and rainfall thus caused increase in rainforest (Heaney 1991, Gathorne-Hardy et al. 2002, Bird et al. 2005).

Impact of geo-climatic events on Southeast Asian biota

The knowledge regarding the repeated events of isolation and connections between mainland Asia and the Sundaic islands or among islands within the Sunda-Shelf gave birth to phylogenetic and phylogeographic hypotheses regarding the evolutionary history of fauna and flora of Southeast Asia. In the east, the Sunda-Shelf extends to 'Wallace's line' (Wallace, 1860; Fig 1). This line demarks the boundary between Sunda and Wallacea, a major zoogeographic transition zone between the Asian and the Australian region (Hall & Holloway 1998, Hall et al. 2011). At the northern boundary of the Sundaic subregion, biogeographers recognized a major transition zone for forest-associated birds at 10º 30´ N, near the Isthmus of Kra (IoK) (Hughes et al. 2003, Round et al. 2003; Fig 1). Interestingly, the transition zone for plants from continental Asia and the Malaysian floral region which includes all islands of Southeast Asia is around 500 km south of the IoK (Van Steenis et al. 1950).

In previous studies, molecular data revealed a deep divergence between Indochina and Sundaland at the level of species or subspecies. Examples are amphibians and reptiles (Inger et al. 2001), freshwater crustaceans (de Bruyn et al. 2005), birds (Hughes et al. 2003, Moyle et al. 2012), bees (Smith et al. 2000) and mammals (Chiroptera: Hughes et al. 2011; Felidae: Luo et al. 2014; *Macaca* sp.: Tosi et al. 2002). One of the causes identified to explain the faunal transition around the IoK involves rapid flooding of this region, which would have dramatically decreased the landmass, thereby affecting habitat availability and composition across IoK (Woodruff et al. 2003). During several periods of the Pleistocene, the IoK was ~ 1000 km wide, which should have allowed gene flow between populations north and south of IoK (Dejtaradol et al. 2016). Due to the current complex distribution pattern of many species and unknown range limits both north and south of the IoK, the entire Thai-Malay Peninsula is referred to as a bio-geographical "crossroads" (Lohman et al. 2011).

The distances over which species could disperse during glacial and interglacial periods also depended on the adaptability of the dispersing species. During glacial periods, land connections between Sundaic islands and between the mainland and Sundaland would have allowed species to disperse across these regions. However, the land bridges were drier than inland areas of the islands and covered by open habitats such as grassland or savannah-like vegetation (Heaney 1991, Meijaard 2003, Bird et al. 2005, Wurster et al. 2010, Wurster & Bird 2016). Such open and different habitats (Cannon et al. 2009) might have not posed a problem to generalist species whereas specialist (e.g. forest dwellers) species might have been unable to disperse from their specific habitats. Therefore (if true), generalists should have had high gene flows, little genetic structure and thus low divergence among their populations. On the other hand, restricted and disconnected populations of specialist species should have had low gene-flow, causing high genetic differences, pronounced genetic structure and substantial divergence among their populations. During warm interglacial periods, expansion of evergreen forests could have allowed the dispersal of at least evergreen forest specialists although the submerged land bridges or rivers running through the Sundaic islands may have acted as physical barriers preventing admixture of some or all of these populations.

Along with the Pleistocene's gradual geo-climatic changes, vicariance events such as the Toba volcanic super-eruption on Sumatra ~73.5 Kya has also been suggested as driver of the distribution pattern of species which we observe today (Ambrose 1998) in southeast Asia. This eruption was one of the largest volcanic eruptions in the last 25 million years (Oppenheimer 2002). Although direct extinction of any species due this eruption is controversial (Louys 2012), aftermath effects of this vicariance event on species distribution in the wider area and surrounding refugia have been studied and demonstrated for many species (Nater et al. 2011, Wilting et al. 2012).

Focal species

Southeast Asia is the felid-richest region worldwide and home to at least 12 cat species (Luo et al. 2014). From several closely related cat species which are also distributed in same regions (co-distributed species) we chose species which diverged from a common ancestor but differed in their habitat preferences and can be categorized as habitat generalist or habitat specialist. To avoid species or genus specific conclusions and facilitate the recognition of general principles we included two genera of small cat species, *Catopuma* and *Prionailurus*, each represented by two closely related species.

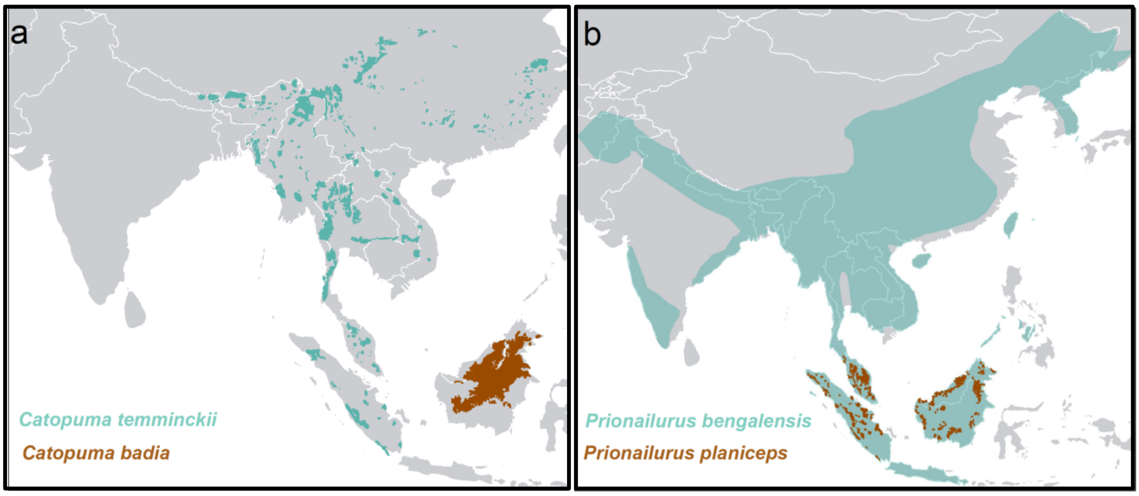

Figure 2: The current distributions of a) *Catopuma temminckii* and *C. badia b) Prionailurus bengalensis* and *P. planiceps* and of. (Source: IUCN Red List of Threatened species).

Genus *Catopuma*

Asiatic golden cat — *Catopuma temminckii* (Vigors and Horsfield 1827), habitat generalist.

The Asiatic golden cat (*Catopuma temminckii*, syn. *Pardofelis temminckii, Felis temminckii*), also known as Temminck's cat, is phenotypically similar (Sunquist & Sunquist 2002) to the African golden cat (*Profelis aurata,* syn. *Caracal aurata).* The coat coloration of the Asiatic golden cat varies between shades from red to brown and black to grey and can also have an ocelot-like spotted pattern. Its patchy but wide distribution (Fig. 2a) includes parts of northeastern India, the Himalayan foothills, Tibet, Bhutan and Nepal, Bangladesh, Myanmar, Thailand, Laos, Cambodia, Vietnam, parts of China, Peninsular Malaysia, and Sumatra (McCarthy et al. 2015). Asiatic golden cats live in a broad range of habitats: they are found in tropical and sub-tropical moist evergreen forest, mixed evergreen hill forest and dry deciduous forest, shrub land, grassland and open rocky areas (Nowell & Jackson 1996) and have been reported at elevations up to 4,033 m (Bhutan, Jigme 2011). Unfortunately, ecology and behavior of the Asiatic golden cat are little known. Its diet includes a variety of small vertebrates ranging from the size of a squirrel, rat, or muntjac up to sheep and goats (Grassman et al. 2005, Sunquist & Sunquist 2009). Three to five subspecies have been described (Sunquist & Sunquist 2009, Wilson & Mittermeier 2009), but the need for a revision of its taxonomy has been recognized (Wozencraft 2005). In the last decades, destruction of its habitats and hunting pressure are thought to be responsible for a serious population decline; the species is now classified as *Near Threatened* in the IUCN Red List of Threatened Species (McCarthy et al. 2015).

Bay cat – *Catopuma badia* (Gray, 1874), habitat specialist.

The bay cat (*Catopuma badia*, syn. *Felis badia*), also known as Bornean cat, is the sister species to *C. temminckii*. It is endemic to the island of Borneo (Fig. 2a) and one of the least known of all wild cats (together with *P. planiceps*, see below). Historical and contemporary records suggest that the bay cat was (is) distributed throughout Borneo (Azlan & Sanderson 2007, Hearn et al. 2016b). Fur colors may vary from reddish to grey. This monotypic species is classified as *Endangered* by the IUCN Red List of Threatened Species due to habitat loss and poaching (Hearn et al. 2016a). The bay cat is forest-dependent with records from hill forests, lowland forests and swamp forests. Records from selectively logged forests are scarce but do exist (Hearn et al. 2016b).

Genus *Prionailurus*

Leopard cat – *Prionailurus bengalensis* (Kerr, 1792), habitat generalist.

Leopard cats, also known as Asian leopard cats, it is the most common wild felid in Asia (apart from the domestic cat) and has the largest distribution range (Fig. 2b) of all felines, the small cats which are members of the subfamily Felinae. It occurs in Pakistan and northern and southern India (leaving a distribution gap in central India), across the Himalayan foothills and in China, in the Russian Far East and the Korean Peninsula. It is also found on the Japanese islands of Tsushima and Iriomote. The distribution range extends throughout southeast Asia, including Thailand, Myanmar, Cambodia, Laos, Peninsular Malaysia, Taiwan, Sumatra, Borneo, Java, Bali and the Philippines (Ross et al. 2015). It lives in different types of forests, including evergreen tropical rainforest, shrub forest, temperate broadleaf forest and dry coniferous forest up to an altitude of 3000 m (Sunquist & Sunquist 2002). Leopard cats can live in disturbed and human-modified habitats such as cultivated land and logged forest as well as close to settlements (Nowell & Jackson 1996). A study of the leopard cat’s diet in Sabah, Borneo, showed that it roamed in open oil palm plantations where it exploited the higher abundance and visibility of prey such as rodents (Rajaratnam et al. 2007).

Currently, 12 subspecies are recognized (Sunquist & Sunquist, 2009). A taxonomic revision using molecular data is warranted because the current classification of subspecies is often based on single (usually incomplete) specimen. The species *sensu lato* is of *Least Concern* in The IUCN Red List of Threatened Species, although some currently recognized subspecies are classified to be much more threatened (*P. b. rabori*: *Vulnerable*, *P. b. iriomotensis*: *Critically Endangered*). A recent molecular study of felids which used some leopard cat specimens but did not focus on the species already revealed two distinct clades, a Sundaic and a mainland Asia one (Luo et al. 2014).

Flat-headed cat – *Prionailurus planiceps* (Vigors & Horsfield, 1827), habitat specialist.

The flat-headed cat is one of the smallest cat species (Muul & Lim 1970), and also one of the least known species among all small cats. Its distribution is limited to Borneo, Sumatra and Peninsular Malaysia (Fig. 2b), possibly reaching the southern border of Thailand (Wilting et al. 2015b). Most records for this cat are from wetland areas such as forests close to lakes, peat–swamp forests and riverine forests. It has intriguing morphological traits such as webbed feet, which appear to fit well with available records that suggest that it inhabits freshwater and low wetland areas (Wilting et al. 2010, 2016a). There has been no previous genetic study focusing on this species.

Study method

Phylogenetic studies now frequently investigate the change in a genotype in relation to time and space. The discipline known as Phylogeography (Avise et al. 1987) describes how evolutionary biologists try to infer the 'history and formation of species' (Avise 2000) by estimating the points in time when lineages split and how their past and present distribution relates to geological events that occurred during their evolutionary history (Avise et al. 1987).

To resolve the genetic structure of species whose populations are distributed on the Indochina and on Sundaland, we used mitogenomes as molecular markers, as will be explained below. We used mostly archival samples (from natural history museum collections) with specific geographic localities.

Natural history collection

Natural history museums around the world hold irreplaceable collections of biodiversity. Specimens such as whole animals, skeletons, tissues and skins collected decades or centuries ago provide a wealth of data on geographical distribution, phenotypic variation and identity of the species/subspecies (Rowe et al. 2011). The advantage of using museum samples in phylogeographic studies of endangered and rare species is that their geographic origins are known. Also, for some species, museum collections include samples from historic distributions when species went extinct or became rare in their former distribution range

We collected material such as dried tissue fibers from post-cranial skeletons, skulls (here often from the nasal cavity), and skins (even including those of dermoplasts), from several natural history museums located in America, Europe and Asia. A few samples were donations from personal collections and wildlife park repositories. Details about specimens used in this dissertation are contained in the 'Supplementary Material' section of each chapter.

Enrichment techniques and next generation sequencing (NGS)

The caveat of using specimens from natural history museum's collections is the low amount and poor quality of the DNA extracted from them. Decades of storage, exposure to air, UV-light and chemicals degrade the DNA to small fragments, usually just a few hundred or fewer base pairs (bp) in length (Willerslev & Cooper 2005). The degradation renders it particularly challenging to amplify long stretches of DNA using traditional PCR techniques, which usually target the mitochondrial cyt*b* gene or the d-loop followed by Sanger sequencing. This hurdle can now be solved with NGS technology. One of the currently most commonly used platforms, the Illumina sequencing platform, essentially requires short fragments with sizes ranging from ~ 50-500 bp. Thus, DNA libraries prepared from historical samples are, in principle, well suited for this technique. Since DNA extracted from museum specimens is low in concentration and contaminated by exogenous sources and inhibitors, species-specific genome or partial genome sequencing is difficult and costly to perform (Hawkins et al. 2015). Targeted capture techniques, which drastically decrease the amount of non-endogenous target DNA, can circumvent some of these problems and made the use of degraded archival DNA affordable and efficient, even for phylogeographic studies comprising a large number of taxa (Bi et al. 2013). The targeted capture technique protocols which we used are described in detail in the *Methods* section of each chapter and in the Appendix.

Mitogenomes as molecular markers

Mitochondrial DNA (mtDNA) provides significant information in phylogeographic studies (Garrick et al. 2015). Mitochondrial genomes are valuable markers because of their high copy number per cell and an additional protective membrane around the mitochondria. These features increase the chances to yield mitochondrial DNA from (degraded) historical samples. Additionally, their rate of nucleotide substitution is usually higher than that of nuclear DNA, revealing a finer gradation and greater detail in the definition of phylogenetic lineages. Neverthless, mitogenome evolve as a single linkage unit. Thus, the analysis yields a single gene tree that might be resulted due to the incomplete lineage sorting during the coalescence process (Zink & Barrowclough et al. 2008). To avoid any misinterpretation, results of mitogenome phylogeny studies should be interpreted cautiously.

AIM OF THE STUDY

The aim of this study is to reveal the drivers of speciation of closely related species and to identify factors shaping their current distribution pattern. To achieve this aim I used mitogenome phylogeography approach to understand the evolutionary history of closely related species that is described in each chapter.

In the general discussion, I compared the results from each chapter for the parameters 'genetic differentiation between mainland and Sunda populations' and among Sundaic island populations and 'population structure' and for generalist and specialist species.

For the parameter '*genetic differentiation between mainland and Sunda populations'* and *'among Sundaic island populations*' I assumed that genetic differentiation among populations of generalist species should be low due to high gene flow. For the specialist species I assumed that genetic differentiation among populations should be high due to none or very low gene flow

For the parameter '*population structure*' I assumed that generalist species (Leopard cat, Asian golden cat) would either have no genetic population structure, or if there was some structure, it would be variation along a cline. For specialist species (Flat-headed cat, Bay cat) I assumed a strong genetic population structure (if suitable habitats were disconnected by unsuitable habitats).

Based on these assumptions regarding the adaptability of generalists and specialists I made predictions for these parameters under the following two hypotheses: the observed distribution patterns of fauna in southeast Asia have resulted from gradual (glacial/interglacial) environmental changes, e.g. by dispersal through land bridges or the lack of dispersal caused by savannah corridors as a barrier (hypothesis H1), or they have resulted from a vicariance event or a single catastrophic event, e.g. Toba volcanic eruption (hypothesis H2).

Table 1: Predictions for the four cat species studied depending on the habitat preferences.

		Hypothesis	
		H1: gradual environmental changes	**H2**: vicariance event
	Parameters	Dispersal and distribution pattern	
Prediction	**Generalist species (Leopard cat, Asian golden cat)**	Adapted to different habitat, species will be widely distributed and will have dispersed across Sundaland and to mainland during LGM.	Population might go extinct in affected area and populations outside that area might differentiate into distinct genetic lineages (if survival happened in more than one refuge; refugia may have differed in habitat), recolonization of affected region from refugia possible.
	Specialist species (Flat-headed cat, Bay cat)	Adapted to specific habitat, distribution will be fragmented if the habitat is fragmented, populations should have been restricted to refugia during LGM and dispersal should only have been possible during interglacial periods through suitable habitat (corridors).	Population might go extinct and current distribution will have a gap there.

CHAPTER 1

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Two species of Southeast Asian cats in the genus *Catopuma* with diverging histories: an island endemic forest specialist and a wide-spread habitat generalist.

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Author contribution

R.P.P., J.F. and A.W. designed the study; A.W. and R.P.P. collected the archival samples; R.P.P. and L.W. performed the laboratory analysis; R.P.P., D.W.F. and D.L. analysed the molecular data; H.P., S.K.-S. and V.R. performed the species distribution modelling; A.C.K., M.D.R., S.W.M. and A.W. collected the pelage data; R.P.P. analysed the pelage data; R.P.P., A.W. and J.F. led the writing and all other authors gave final approval for publication.

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Two species of Southeast Asian cats in the genus *Catopuma* with diverging histories: an island endemic forest specialist and a widespread habitat generalist

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Background. The bay cat *Catopuma badia* is endemic to Borneo, whereas its sister species the Asian golden cat *Catopuma temminckii* is distributed from the Himalayas and southern China through Indochina, Peninsular Malaysia and Sumatra. Based on morphological data, up to five subspecies of the Asian golden cat have been recognized, but a taxonomic assessment, including molecular data and morphological characters, is still lacking. *Results.* We combined molecular data (whole mitochondrial genomes), morphological data (pelage) and species distribution projections (up to the Late Pleistocene) to infer how environmental changes may have influenced the distribution of these sister species over the past 120 000 years. The molecular analysis was based on sequenced mitogenomes of 3 bay cats and 40 Asian golden cats derived mainly from archival samples. Our molecular data suggested a time of split between the two species approximately 3.16 Ma and revealed very low nucleotide diversity within the Asian golden cat population, which supports recent expansion of the population. *Discussion.* The low nucleotide diversity suggested a population bottleneck in the Asian golden cat, possibly caused by the eruption of the Toba volcano in Northern Sumatra (approx. 74 kya), followed by a continuous population expansion in the Late Pleistocene/Early Holocene. Species distribution projections, the reconstruction of the demographic history, a genetic isolation-by-distance pattern and a gradual variation of pelage pattern support the hypothesis of a post-Toba population expansion of the Asian golden cat from south China/Indochina to Peninsular Malaysia and Sumatra. Our findings reject the current classification of five subspecies for the Asian golden cat, but instead support either a monotypic species or one comprising two subspecies: (i) the Sunda golden cat, distributed south of the Isthmus of Kra: *C. t. temminckii* and (ii) Indochinese, Indian, Himalayan and Chinese golden cats, occurring north of the Isthmus: *C. t. moormensis*.

1. Background

Fluctuating geological and climatic conditions during the Pliocene and Pleistocene have shaped what is now recognized as the globally important Southeast Asian biodiversity hotspot. In particular, the Sunda Shelf which comprises Peninsular Malaysia, Sumatra, Borneo, Java, Bali and other smaller islands is of great interest to evolutionary biologists [1], as alternating glacial and interglacial periods resulted in the emergence and submergence of land bridges between the larger landmasses [2–4]. The impact of these transient land bridges on the distribution of genetic variation within and among species has received growing attention in the past years. For example, little or no genetic differentiation between island populations of a given species supports a scenario in which members of this species were free to move between islands during the Last Glacial Maximum (LGM) [5,6], while significant genetic divergence between mainland and Sundaic or among Sundaic populations of different islands supports a scenario of prolonged genetic isolation due to movement restriction. The latter has been demonstrated among others for murine rodents [7], common palm civets *Paradoxurus hermaphrodites* [8], clouded leopards *Neofelis* spp. [9] and leopards *Panthera pardus* [10].

A history of vicariant evolution is also ascribed to the two sister species in the genus *Catopuma*: the bay cat *Catopuma badia* (Gray, 1874) and the Asian golden cat *Catopuma temminckii* (Vigors & Horsfield, 1827). The monotypic bay cat is endemic to the island of Borneo. Despite continuous camera-trapping efforts, this carnivore remains one of the least known cat species [11]. A characteristic morphological trait of the bay cat is its pelage. It is dense reddish (bay) or grey, with gradual transitions between these forms being common [11]. Occasionally occurring black or almost black morphs have also been reported [12,13]. The bay cat is smaller than the Asian golden cat [14] and recent molecular studies showed that the two species probably split around 3.27 Ma [15].

In contrast with the island endemic bay cat, the Asian golden cat has a wide distribution in Southeast Asia, ranging from Northeast India and Nepal to southern China, Indochina and to Peninsular Malaysia and Sumatra in the Sunda Shelf. The species is polymorphic and has usually been divided into three subspecies [16,17]: *C. t. temminckii* (Vigors & Horsfield, 1827), distributed in Sumatra, Peninsular Malaysia, Indochina, Burma to Nepal; *C. t. dominicanorum* (Sclater, 1898), restricted to Southern China; and *C. t. tristis* (Milne-Edwards, 1872) with a distribution from Tibet, Sichuan to Upper Burma. In addition, it has been proposed that Asian golden cats from Yunnan should be separated as *C. t. bainesi* (Sowerby, 1924), and those from Nepal, southern Tibet and probably northwest Yunnan and west Sichuan in China as *C. t. moormensis* (Hodgson, 1831). Although colour variations, ranging from orange to black, including the blotched ‘ocelot’ type in northern populations, and size differences between northern and southern Sundaic populations have been observed (JH Mazák and ACK 2011, unpublished data), a detailed study assessing these morphological traits has not yet been conducted on the Asian golden cat. The first molecular study of the Asian golden cat included two mitochondrial genes, four autosomal genes, one X-linked and four Y-linked genes [18]. This study revealed low molecular diversity among the analysed samples, with a moderate distinction in mtDNA between animals from north of the Isthmus of Kra and Peninsular Malaysia. Autosomal genes and sex chromosome markers showed no differentiation. Sumatran samples were not included in this study.

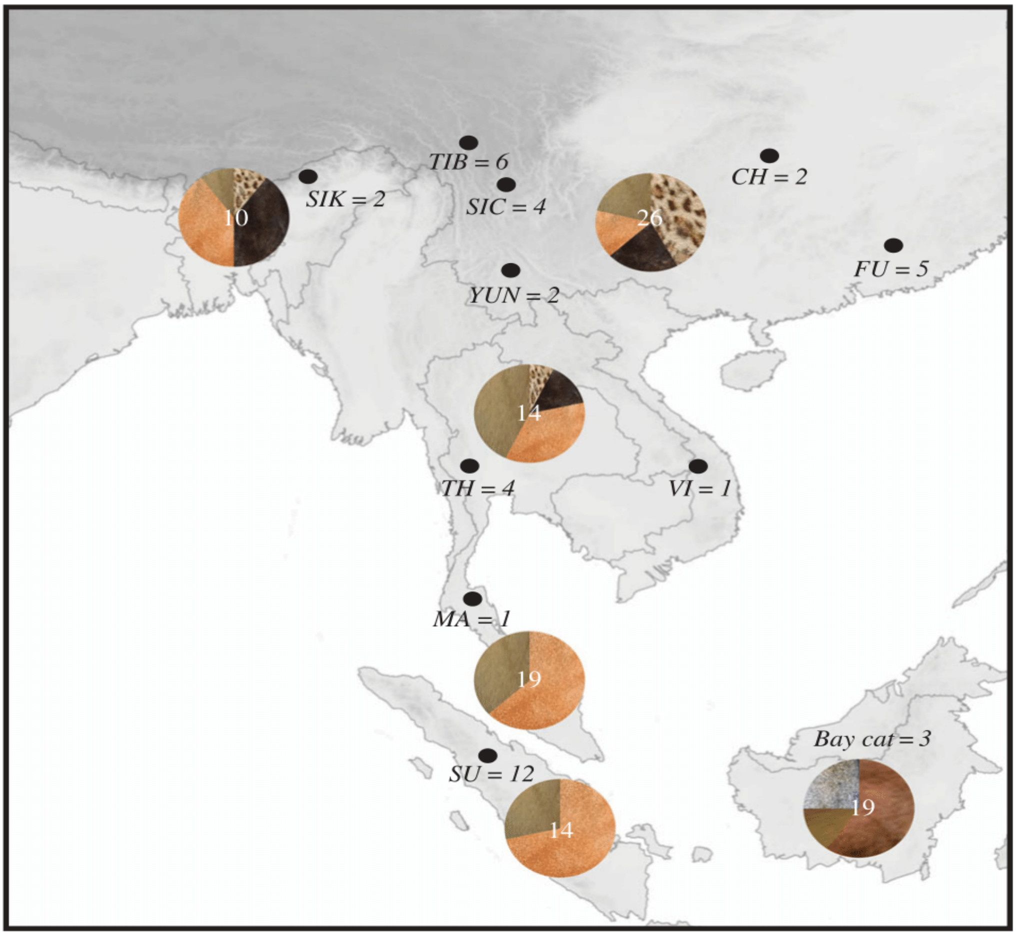

Figure 1. Geographical distribution of samples from Asian golden cats and bay cats used in the analyses of molecular data and pelage colour data. Pie charts represent coat colour proportions found in the population of that geographical area. White numbers on pie charts denote sample size used in pelage colour data analysis for that population. Black dots indicate populations used for mitogenome analysis. Initials of populations (*SIK*: Sikkim, India, *TIB:* Tibet, *SIC:* Sichuan, *YUN:* Yunnan, *CH:* China, *FU:* Fukien, *TH:* Thailand, *VI:* Vietnam, *MA:* Malaysia, *SU:* Sumatra) are given together with sample size.

In this study, we used in-solution hybridization capture of mainly archival samples to sequence whole mitochondrial genomes (mitogenomes). We combined these molecular data with morphological data from pelage pattern and Pleistocene species distribution projections to gain further insights into the evolutionary history of the bay cat and the Asian golden cat. Our sampling covered most parts of the respective distribution ranges (figure 1), allowing for a balanced perspective on how both species were impacted by environmental changes during the Late Pleistocene.

2. Material and methods

2.1. Samples

We obtained 38 archival samples (epithelial tissue from skulls or skins, or maxillo-turbinal bones) and two tissue samples (pathological reference sample collection of the IZW) for the Asian golden cat and we used two archival samples and one tissue sample for the bay cat (for samples and their origin see electronic supplementary material, S1).

2.2. DNA extraction

DNA extractions from archival samples were carried out following the Qiagen DNeasy Blood & Tissue kit protocol (Qiagen, Hilden, Germany) with an overnight lysis and a 15 min incubation period at 37°C

Table 1. Primer sequences for long-range PCR designed using *Prionailurus bengalensis* NCBI reference sequence NC 016189.

primer name	sequence 5′-3′	product size
Prion_mt_F1	AAGYATTCCRCCCCAAACATAAG	6000
Prion_mt_R1	TCCTTTTTGGGTTCATTCGTAGG	6000
Prion_mt_F2	ACTAYTACTYCCCCTCCCATGA	5500
Prion_mt_R2	ATAGTGGGGCTGTTGCTTCTTC	5500
Prion_mt_F3	CAGACCTCCTAACCCTAACATGA	6000
Prion_mt_R3	TGGTAGCACGAAGATTTTTGGAT	6000

during the elution. We included multiple extraction blanks, one per set of five samples, to control for sample cross-contamination and reagent contamination. DNA extraction and library preparation of archival samples was carried out in specially equipped laboratories dedicated to the analysis of archival samples. DNA extractions from tissue samples were carried out using the Invitek DNA extraction kit (Invitek GmbH, Berlin, Germany) in a separated laboratory dedicated to the extraction of fresh samples.

2.3. Library preparation and hybridization capture

Illumina libraries were prepared according to a modified paired-end sequencing protocol [19]. As the DNA extracted from archival samples was severely degraded, we used an enrichment technique (in-solution hybridization capture) to target complete mitogenomes. As none of our *Catopuma* samples was suited to generate baits for capture, we applied a cross-species capture approach [20]. Baits for cross-species capture were generated from the leopard cat (*Prionailurus bengalensis*) by amplifying three large (approx. 6 kb) overlapping regions of the mitogenome by long-range PCR (table 1); these were subsequently sheared to approximately 250 bp using a Covaris M220 (Covaris Inc., USA), purified using the QiaQuick kit (Qiagen GmbH, Hilden, Germany), and pooled equimolarly. The final steps in bait generation were blunt-end repair and ligation of biotinylated adapters (see [21], for details). In-solution capture was then carried out as described in [19]. After capture, enriched libraries were amplified using primers IS5 and IS6 [22] and purified, and then the enriched and re-amplified libraries underwent a second round of capture using freshly prepared baits because two consecutive rounds of capture significantly increased target yield (data not shown; see also [23]). Libraries were sequenced on the MiSeq platform (Illumina, San Diego, CA, USA) using MiSeq v. 3 150-cycle kits. Extraction blanks underwent the same laboratory steps (library construction and capture procedure) and were likewise sequenced to assure absence of contaminants.

2.4. Bioinformatic analyses of *Catopuma* mitogenomes

Adapter sequences were clipped from paired-end reads using *cutadapt* v. 1.3 [24]. Subsequent quality trimming was performed on reads more than or equal to 20 bp using a sliding window approach and a phred quality threshold of $Q = 20$. The resulting adapter-clipped and quality-trimmed reads were merged using the software FLASH v. 1.2.8 [25]. Mapping assembly was then performed using MITObim v. 1.7 [26] using a leopard cat mitogenome sequence (GenBank accession NC 016189) as a reference for the first step of the iterative mapping. For each sample, a consensus sequence was generated in Geneious v. 8.1.2 (Biomatters, Auckland, New Zealand). Each sequence was annotated for control region, CDS, tRNAs, rRNAs and ATPase in RATT [27].

2.5. Phylogeographical analyses for the Asian golden cat

The dataset of 40 Asian golden cat sequences was aligned using Geneious v. 8.1.2 (Biomatters, Auckland, New Zealand). For further analysis, we excluded the control region from the alignment. PartitionFinder v. 1.1.0 [28] was applied to search for the best fitting substitution model for the dataset, with BIC (Bayesian Information Criterion) as model selector with unlinked branch length. We used HKY + I + G as substitution rate model in MrBayes v. 3.2 [29] and GTR + I + G in RAxML as it is the only model provided [30] for phylogeny reconstruction. Median-joining (MJ) haplotype networks were constructed

Table 2. Mitogenome sequences used for estimation of TMRCA and molecular clock rate for the genus *Catopuma*.

no.	species name	common name	NCBI accession no.
1	*Panthera pardus*	leopard	EF 551002
2	*Panthera uncia*	snow leopard	EF 551004
3	*Puma concolor*	puma	JN 999997
4	*Panthera leo persica*	Asian lion	JQ 904290
5	*Felis margarita*	sand cat	KR 132580
6	*Leopardis pardalis*	ocelot	KR 132583
7	*Lynx pardinus*	Iberian lynx	KR 132583
8	*Otocolobus manul*	Pallas's cat	KR 132585
9	*Felis catus*	domestic cat	NC 001700
10	*Acinonyx jubatus*	cheetah	NC 005212
11	*Neofelis nebulosa*	clouded leopard	NC 008450
12	*Lynx rufus*	bobcat	NC 014456
13	*Prionailurus bengalensis euptilurus*	Amur leopard cat	NC 016189
14	*Panthera onca*	jaguar	NC 022842
15	*Panthera tigris*	tiger	NC 010642
16	*Lynx lynx*	Eurasian lynx	KM 982549
17	*Prionailurus viverrinus*	fishing cat	KR 135742
18	*Prionailurus rubiginosus*	rusty-spotted cat	KR 135744
19	*Prionailurus planiceps*	flat-headed cat	KR 135743
20	*Catopuma temminckii*	Asian golden cat	KR 135745
21	*Catopuma badia*	bay cat	KR 135746
22	*Pardofelis marmorata*	marbled cat	KT 288227

using Network 4.6.1.3 [31]. The MJ-network is based on 40 Asian golden cat mitogenome sequences (without d-loop; 15 460 bp).

Diversity indices for the combined Asian golden cat samples were calculated in DnaSP 5.10.01 [32], including nucleotide (π) and haplotype (h) diversity, as well as transition–transversion ratios. To check for patterns of genetic variation in geographically separated populations, we divided the Asian golden cat dataset into four geographical subsets—India, Indochina, China (including Tibet) and Sumatra. These do not correspond to the currently recognized subspecies, as these are poorly defined and partly even overlap (see above), but instead correspond to zoogeographical regions of mammals in southeast Asia [33]. Two samples were excluded from this analysis: the single sample from Peninsular Malaysia, because of the geographical separation of Peninsular Malaysia and Sumatra at the Strait of Malacca since the LGM, and zoo sample CTE 2808, because its geographical origin was unknown. Pairwise F_{ST} values among the four Asian golden cat subsets were calculated using the algorithm implemented in ARLEQUIN 3.5 [34], significance was tested by 1000 permutations. Geographical distances between the four sample subsets were calculated using a least-cost-path (LCP) analysis applied within an LGM habitat suitability model for the Asian golden cat (see below). The LCP analysis was conducted in R (library gdistance [35]). To test if the distribution of genetic variation in Asian golden cats followed an isolation-by-distance (IBD) pattern, we applied a Mantel test implemented in R (v. 3.2.0 [36] package *ade4* [37]) and plotted genetic distances as $F_{\mathrm{st}}/1\text{-}F_{\mathrm{st}}$ values [38] against geographical distances using R package *ggplot2* [39].

2.6. Estimating divergence times

The dataset used to estimate the time to the most recent common ancestor (TMRCA) of the genus *Catopuma* included all 22 Felidae mitogenomes available in NCBI (table 2). The divergence time of the

Felidae family (10.78 million years (Myr); CI: 8.38–14.45 Myr; [40] was set as calibration point. For tree reconstruction, we applied both a normal distribution prior with a Yule type speciation model and the HKY + I + G substitution rate model. Four independent analyses were conducted, using MCMC lengths of 100 million generations, logging every 3000th generation. All runs were evaluated in Tracer v. 1.6 for ESS $>$ 200. LogCombiner v. 1.8.1 was then used to combine tree logs from the independent runs. The final tree log was used to resolve the phylogenetic tree, which was visualized in FigTree v. 1.4.2. (http://tree.bio.ed.ac.uk/software/figtree/). The obtained TMRCA estimate of *Prionailurus* clade (3.71 Myr ($CI_{95\%}$: 2.36–5.04 Myr)), puma lineage (4.99 Myr ($CI_{95\%}$: 3.16–6.70 Myr)), lynx lineage (3.91 Myr ($CI_{95\%}$: 2.51–5.35 Myr)) and Pantherinae subfamily (5.54 Myr ($CI_{95\%}$: 3.59–7.57 Myr)) were similar to the ones reported in a previous study [15]. Hence, we used the TMRCA estimate of *Catopuma* (3.44 Myr (CI: 2.5–4.5 Myr)) to infer divergence times at internal nodes. As the dataset included interspecies data (Asian golden cat and bay cat sequences) we applied a multi-species coalescent tree model in *BEAST with the HKY + I + G substitution rate model. Four independent runs were conducted as described above.

2.7. Demographic analysis

To reconstruct the demographic history of the Asian golden cat, we used the TMRCA derived from the analysis conducted for the *Catopuma* genus (see above) with lognormal distribution as prior to determine root model height. Coalescent extended Bayesian skyline (EBSP) was selected as tree prior as this analysis concerned intraspecific variation. Analyses were conducted with MCMC lengths of 30 million generations, logging every 1000th generation in BEAST v. 1.8. Each run was evaluated for ESS $>$ 200 in Tracer v. 1.6. Results were plotted as skyline plots using R package *ggplots2*.

2.8. Projection of Pleistocene Asian golden cat and bay cat distributions

We used the approach described in [10] to project the Pleistocene distribution of the Asian golden cat and the bay cat. Current distribution ranges were taken from the IUCN/SSC Red List of Threatened Species. As a study area, we used the region between 87° E and 131° E longitude and between of 34° N and 12° S latitude, to ensure that the species distribution modelling is linked to the climatic conditions in South and Southeast Asia. We randomly selected 10% of the rasterized distribution ranges for the Asian golden cat (8302 occurrences) and for the bay cat (1261 occurrences) to serve as occurrences for model-fitting procedures, and then pseudo absences ($n = 8310$ for Asian golden cat, $n = 1270$ for bay cat) were sampled from the whole remaining study area. To avoid multicollinearity, only environmental predictors with $-0.7 < r < 0.7$ (Pearson's correlation) were retained for model building, resulting in three predictors for the Asian golden cat ('min. temperature of coldest month', 'temperature annual range', 'precipitation of wettest quarter') and four predictors for the bay cat ('max. temperature of warmest month', 'temperature annual range', 'precipitation of driest month', 'precipitation of wettest quarter'). We then built an ensemble model by taking the mean of the probabilities predicted by 18 models: three random presences sets fitted with the two algorithms (MAXENT and GBM) and with three cross-validation runs. All statistical analyses and shape file and raster manipulations were carried out using R packages *maptools* [41], *rgdal* [42] and *raster* [43].

2.9. Pelage coloration

In total we analysed the pelage colour variation data of 83 Asian golden cat individuals (52 museum specimens, 30 camera-trap photographs and 1 zoo sample with known origin) and 19 bay cat individuals (photographs of 8 museum specimens, 9 camera-trap photographs and 2 published accounts; [44,45]). To compare pelage variation within the Asian golden cat populations from different geographical origins, we used the geographical sample subsets created above—India, China (including Tibet), Indochina and Sumatra, and added a fifth one: Peninsular Malaysia—to estimate geographical coat colour frequencies (figure 1). We defined four coat colours—blotched, black or grey, red, and brown to divide samples into their corresponding colour or pattern (see electronic supplementary material, figure S2). The obtained frequencies were used to calculate a matrix of coat colour distances between the geographical regions. We then employed a Mantel test implemented in R to test whether the coat colour distances between the geographical regions correlated with the LCP distance (see above). Based on these distance matrices, we also calculated a cluster dendrogram using the R package *dendextend* [46].

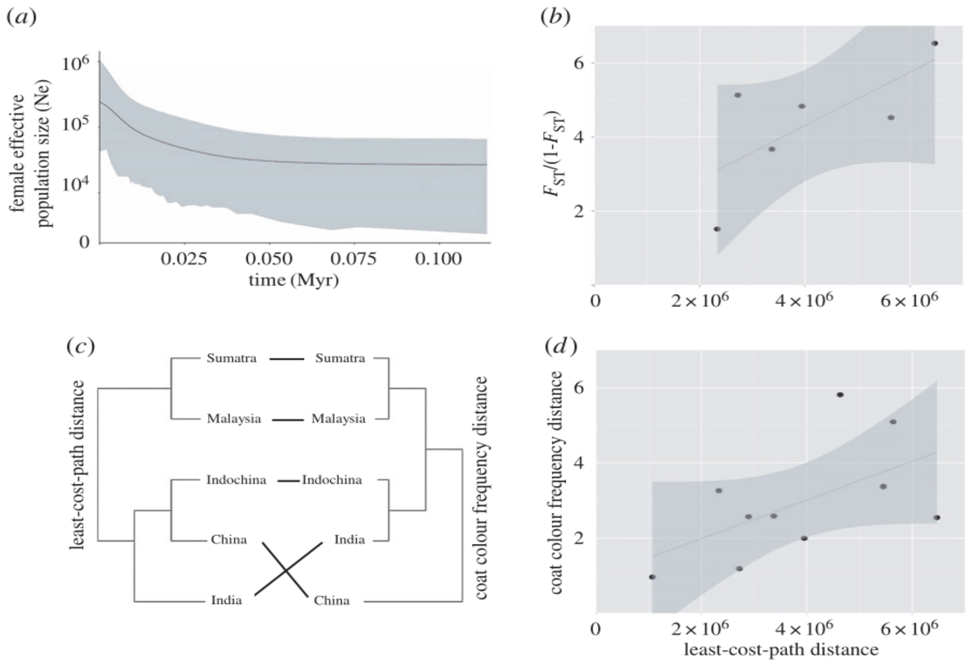

Figure 2. Population demographic analysis, isolation-by-distance analysis using genetic data, coat colour frequency and least-cost-path (geographical distance) data for the Asian golden cat population; (*a*) Extended Bayesian Skyline Plot for the Asian golden cat population; *x*-axis displays times in million years, *y*-axis displays effective population size Ne in log scale where Ne /τ ($\tau = 6.5$ years × 10^6). (*b*) Graph represents correlation between Slatkin's distance ($F_{ST}/1\text{-}F_{ST}$) and least-cost-path distance among geographical populations; (*c*) dendrogram comparison between coat colour frequency and least-cost-path distance; (*d*) graph represents correlation between coat colour and least-cost-path distance between geographical populations.

3. Results

We obtained mitogenomes (16 471 bp) for 3 bay cats and 40 Asian golden cats with a minimum coverage of 5×. Each position with coverage less than 5× was replaced with *N* (approx. 1–2% of all positions per sample) before phylogenetic and phylogeographical analyses. These mitogenome sequences were translated into protein sequences to verify the alignment based on coding frames. We also compared our sequences with the complete mitogenome of the Asian golden cat stored in the NCBI database (Accession ID KP202267) to confirm their mitochondrial origin. All mitogenomes represented individual haplotypes, indicating maternal unrelatedness of all samples. In total 787 fixed mutations separated the bay cat and the Asian golden cat mitogenomes, and among the 3 bay cat and 40 Asian golden cat mitogenomes we found 33 and 224 variable positions, respectively. The MJ-haplotype network for mitogenomes (electronic supplementary material, figure S1) showed a minimum of 44 mutations between haplotypes from Indochina (haplotypes H16–H40) and Sumatra (H3–H14), a minimum of 27 mutations between Indochina and Peninsular Malaysia (H15) and a minimum of 19 mutations between haplotypes from Peninsular Malaysia and Sumatra. It is also noteworthy that the two samples from Sikkim (H1, H2) were separated from all other haplotypes by at least 41 mutations. Nucleotide diversity among Asian golden cat mitogenomes was $\pi = 0.003$ (s.d. = 0.0023). We found a moderate, yet not significant, correlation between genetic distances among geographical subsets and their geographical distances ($r^2 = 0.70731$, $p = 0.1238$; figure 2*b*).

3.1. Phylogeography

For the Asian golden cat dataset, trees obtained by ML (maximum-likelihood) and BI (Bayesian inference) were concordant in their topologies (figure 3). Sumatran haplotypes (SU; figure 3) formed a monophyletic clade. Indian (Sikkim, SIK) haplotypes and Chinese haplotypes from Fukien (FU) also formed distinct monophyletic groups. The other samples from China (Yunnan, YUN; Tibet, TIB; Sichuan, SIC) as well as those from Thailand (TH) were paraphyletic; individuals from the same provinces were found in different clades. This indicates some recent gene flow within

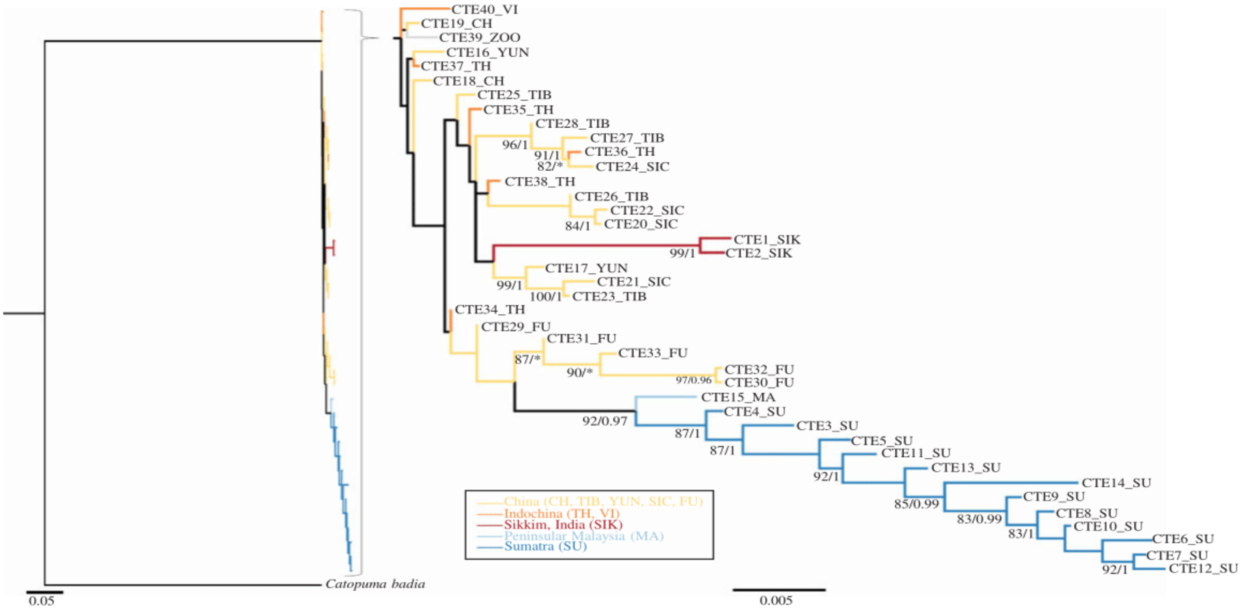

Figure 3. Maximum-likelihood phylogenetic tree derived from Asian golden cat mitogenomes using the bay cat (*Catopuma badia*) as outgroup. The Bayesian phylogenetic tree provided identical topologies. Support values for nodes were obtained from ML analysis (RaXML) and Bayesian inference (MrBayes). Only values greater than 80% (RaXML) and greater than 0.9 (posterior probability values for Bayesian trees) are shown. Smaller values are denoted with asterisk. Haplotypes and their origins are listed separately (electronic supplementary material, S1).

China but also between China and Indochina as Chinese samples were not clearly distinct from Indochinese samples.

3.2. Molecular dating

As there were no *Catopuma* fossils available for age calibration, we used the divergence time of the family Felidae [40] and calculated a molecular rate of 0.0133 substitutions per site per Myr (s.d. = 0.00288) using 22 mitogenomes of felids (table 2; electronic supplementary material, figure S3). Based on this rate and the number of differences, we estimated a divergence time between Asian golden cat and bay cat of approximately 3.16 Myr ($CI_{95\%} = 2.05$–4.54 Myr) (table 3 and figure 4). This is slightly more recent than what we had used from Felidae mitogenomes (3.44 Myr (CI: 2.5–4.5 Myr)) though it still falls in the $CI_{95\%}$ range of the splitting time reported in a previous study [15]. Based on this date, we estimated the intraspecific divergence time for the Asian golden cat clades to be approximately 112 kyr ($CI_{95\%} = 77$–151 kyr) (figure 4). The Bayesian skyline plot revealed a very recent population expansion for the Asian golden cat (figure 2*a*).

3.3. Projection of Pleistocene Asian golden cat and bay cat distributions

Habitat distribution modelling (figure 5*a–e*) indicated that large parts of Southern China contained suitable habitat for the Asian golden cat throughout the Late Pleistocene. In particular, during the LGM, most parts of the exposed Sunda Shelf were habitable, including Sumatra and Peninsular Malaysia. However, areas on Borneo, in particular in northeastern Borneo, were only marginally suitable as habitat. Although the area of suitable habitats in the Sunda Shelf receded with increasing temperatures and rising sea levels at the beginning of the Holocene, large areas in Peninsular Malaysia and Sumatra remained suitable for the Asian golden cat.

By contrast, projections for the bay cat (figure 5*f–j*) suggested that this species was probably confined to Borneo throughout the Late Pleistocene. Its range was particularly restricted during the LGM, despite the greater available Sunda land masses.

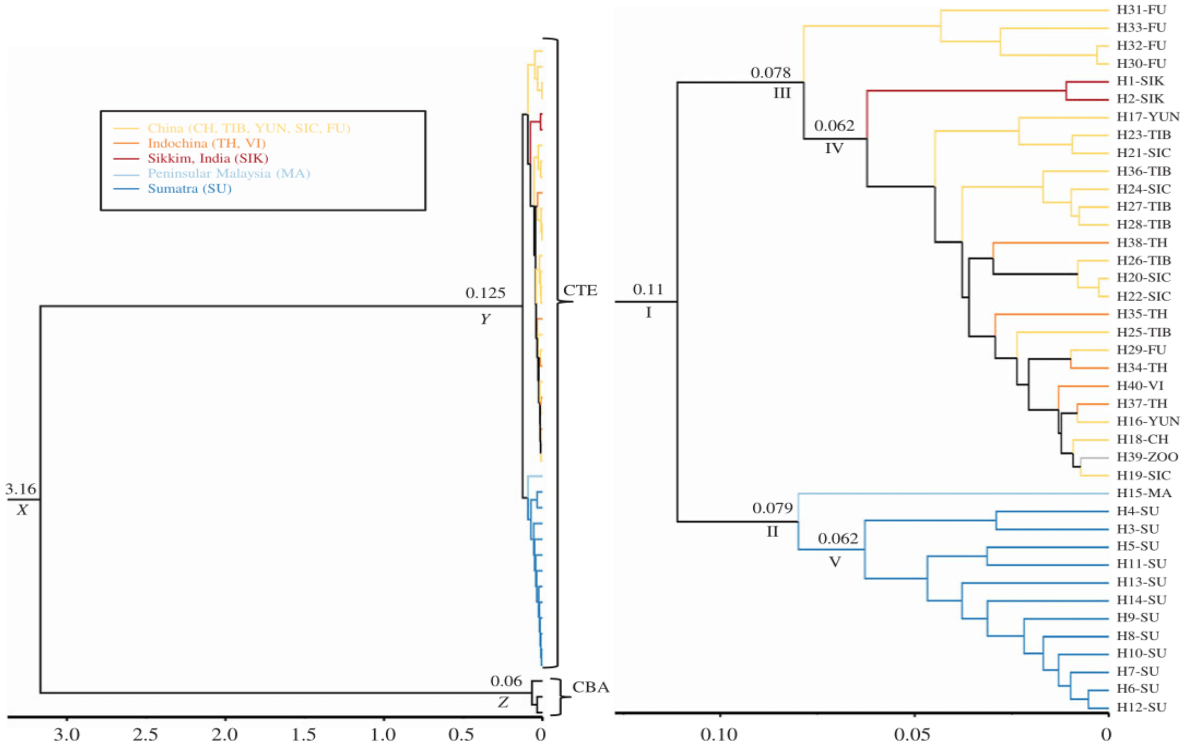

Figure 4. Divergence time estimates plotted onto a phylogenetic tree reconstructed using BEAST. Left: *Catopuma* genus-tree showing molecular dating for the Asian golden cat and the bay cat (CBA: *Catopuma badia*, CTE: *Catopuma temminckii*). Right: CTE species tree showing molecular datings within the Asian golden cat. Only nodes supported with posterior values = 1 are displayed with their age in million years (for the genus tree: nodes X, Y, Z; for the species tree nodes I–V). $CI_{95\%}$ values for nodes X, Y and Z and nodes I–V are given elsewhere (table 3).

Table 3. Estimated divergence date with 95% confidence interval ($CI_{95\%}$) and posterior values as a node support (figure 4).

node	time in Myr	$CI_{95\%}$ in Myr	posterior value
dating for *Catopuma* genus			
X	3.167	2.052–4.548	1
Y	0.125	0.076–0.188	1
Z	0.066	0.0367–0.104	1
dating for the Asian golden cat internal nodes			
I	0.111	0.077–0.151	1
II	0.079	0.054–0.112	1
III	0.078	0.052–0.11	1
IV	0.062	0.041–0.087	1
V	0.062	0.04–0.08	1

3.4. Pelage coloration

The bay cat displayed three different morphs—grey, red and brown (figure 1). Among Asian golden cats, the greatest diversity in coat coloration was observed in China, Tibet, Northeast India and Indochina, with the occurrence of all four recognized morphs: red, brown, spotted and black. By contrast, in Peninsular Malaysia and Sumatra only the red and the brown morphs were observed (figure 1). We found a moderate correlation between coat colour and our LCP distance matrix ($r^2 = 0.5604$, p-value = 0.064; figure 2*d*). This correlation is supported by the similar topologies in the dendrograms of the two distance matrices (figure 2*c*).

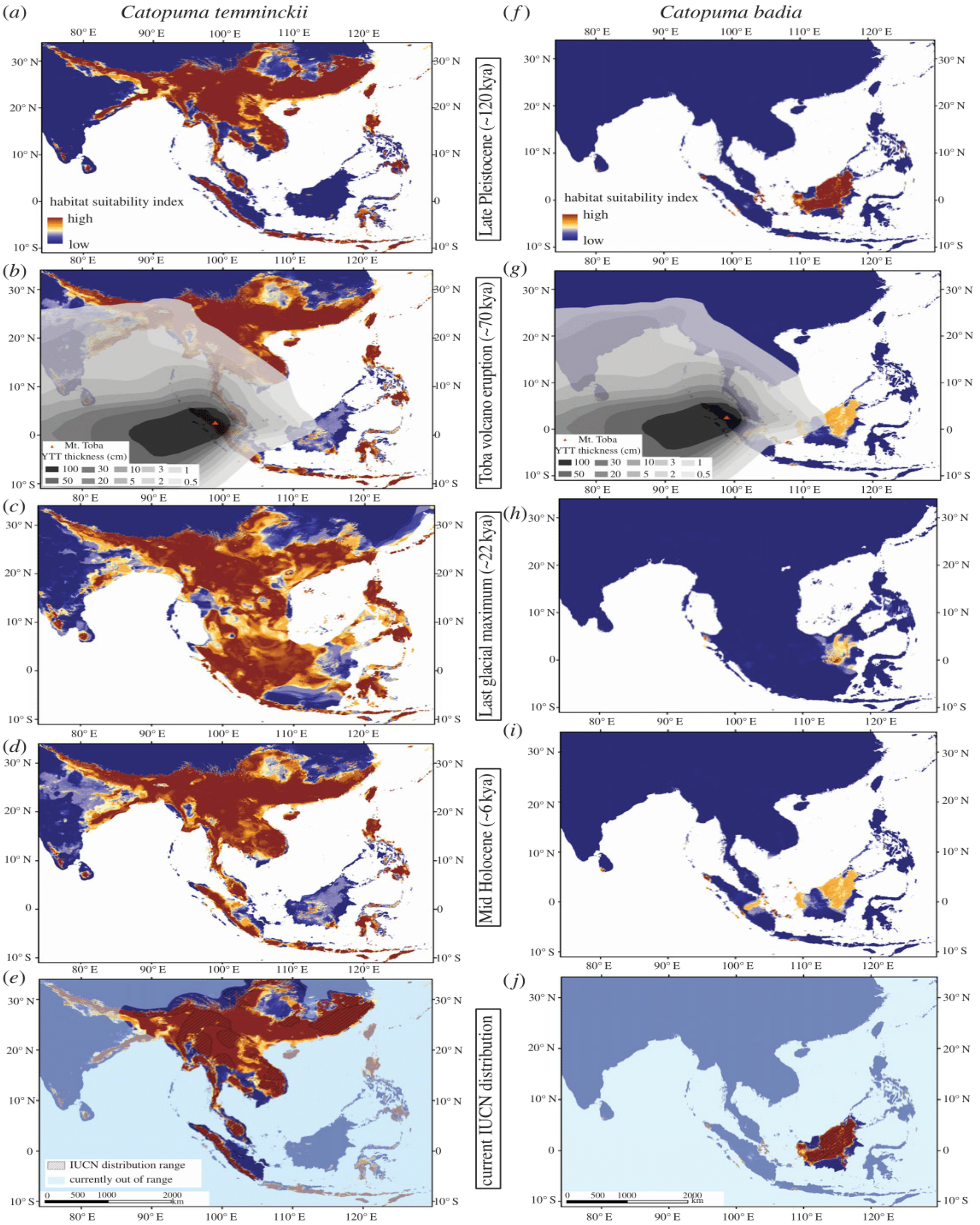

Figure 5. Projected distribution of the Asian golden cat (*a–e*) and the bay cat (*f–j*) along a time axis from 120 kya until present: (*a,f*) for the Late Pleistocene (approx. 120 kya) using the LGM projections; (*b,g*) the thickness of the Young Toba Tuffs (YTT) was superimposed on the projection to indicate the severity of impact of the Toba super volcanic eruption approximately 74 kya; (*c,h*) the Last Glacial Maximum (approx. 22 kya); (*d,i*) the mid Holocene (approx. 6 kya) and (*e,j*) the current distribution according to the IUCN Red List of Threatened Species used for the modelling (hatched pattern), while areas outside of the current distribution were shaded in light blue.

4. Discussion

4.1. Evolution history of the bay cat and the Asian golden cat

The Asian golden cat and the bay cat are allopatric species of the genus *Catopuma*. Based on our mitogenome data, we estimated that Asian golden cat and the bay cat split during the late Pliocene approximately 3.16 Ma, which is somewhat more recent than had been estimated before [15]. At this time, Borneo was still connected to other parts of the Sunda Shelf and Southeast Asia. During the Late Pliocene, however, the Isthmus of Kra was submerged by high sea levels for an extended period of time [47], geographically dividing mainland Southeast Asia and Sundaland. This Late Pliocene vicariance event is considered to be one driver of speciation in Southeast Asia, because numerous other species split during this time, e.g. rodents [7], *Macaca* spp. [48,49], masked palm civet [5] and common palm civet [8]. In the case of the two *Catopuma* species, it is conceivable that following the split into the northern Indochinese (= *temminckii*) and the southern Sundaic (= *badia*) populations, the latter specialized and became more adapted to tropical evergreen rainforests, whereas the northern population remained a more generalistic species due to the higher habitat diversity in Indochina. Subsequently, the two emerging species responded differently to the changing environmental conditions that prevailed during the Late Pliocene and Pleistocene.

During the glacials of the Pleistocene, Sunda Shelf evergreen rainforests became restricted to smaller areas due to a cooler and drier climate, particularly during the LGM [50]. Our species distribution projections indicated that the bay cat had probably been constrained to the rainforest refugia in central and northern Borneo (figure 5*h*) because the more open savannah-like habitats of western and southern Borneo [1,3,51] were unsuitable for this specialized forest dwelling species. This rainforest refugium scenario was also proposed for other Bornean species, such as colobine monkeys [52] and termites [53]. However, due to the very small sample size ($N = 3$) and the restricted distribution coverage of the bay cat samples available for molecular analyses (all were from northeastern Borneo), we could not reconstruct the population history of the species and thus we do not have the molecular means to infer potential population size fluctuations, i.e. whether the species had experienced a population bottleneck (e.g. during the LGM) and/or underwent a subsequent population size expansion. Therefore, more extensive, ideally Borneo-wide sampling is required to test the hypothesis of an LGM refugium in the rainforests of northeastern Borneo, as suggested by our species distribution model (figure 5*f*–*j*).

In contrast with its sister species, the Asian golden cat could have expanded its distribution range southward to the Sunda Shelf using land bridges that were temporarily available during glacial periods of the Pleistocene (figure 5*a*). Given the intraspecific clade split at approximately 112 kya, such a southward movement to Peninsular Malaysia and Sumatra probably took place during Late Pleistocene (126–11 kya), and presence of Asian golden cats on Sumatra at that time is supported by Late Pleistocene/Early Holocene fossils from the Lida Ayer cave in West Sumatra [54]. Our molecular data on population demography favoured a late Pleistocene (approx. 30–25 kya) population expansion of the Asian golden cat on Sumatra (figure 2*a*). We cannot exclude that Asian golden cats had reached Sumatra much earlier (shortly after the clade split), but the full colonization of Sumatra, evidenced by population expansion, took place much later. This disparity can be explained by local extinction of these 'potential early arriving' Asian golden cats on Sumatra and Peninsular Malaysia. Such extinction may have been the result of maladaption to rapidly changing environmental conditions during the succession of glacials and interglacials in the Late Pleistocene. However, such a scenario is not supported by the current distribution of Asian golden cat, which lives in habitats with diverse environmental conditions reflecting the large adaptive potential of this species. Instead, it is conceivable that the Toba super volcanic eruption on Sumatra approximately 74 kya, which has also been linked to the local extinction of other species (orangutans *Pongo* spp. [55], clouded leopards [9], tigers [6]), also impacted Asian golden cat populations (figure 5*b*). A probable post-Toba expansion of Asian golden cats from southern China and northern Indochina, areas which were less or not affected by Toba, is also supported by the sample from Peninsular Malaysia, which is the basal-most branch of the Sundaic clade and based on the network (electronic supplementary material, figure S1) and phylogenetic tree (figure 3) genetically positioned between samples from Mainland Indochina and those from Sumatra. Such a scenario—post-Toba population expansion from south China to the Sunda Shelf, accompanied by low nucleotide diversity due to the short evolutionary time frame—has also been reported for tigers [6,56].

While samples from Sumatra formed a monophyletic cluster, samples from different provinces/regions in China and Indochina did not form respective regional clusters in our phylogenetic reconstructions (figure 3), suggesting past gene flow between these populations. By contrast, samples from Sumatra, Peninsular Malaysia, Sikkim (India) and Fukien (China)—the most southern, most western and most eastern parts of the distribution range—were separated from the other Indochinese and Chinese samples indicating spatial differentiation. The tree-like pattern of the Sumatran samples in the haplotype network (electronic supplementary material, figure S1) indicated a directional north-to-south expansion of golden cats after a single colonization. However, multiple colonization events on Sumatra from Peninsular Malaysia cannot be excluded. To test these scenarios additional samples from Peninsular Malaysia and from Sumatra with precise locality information would be needed. A haplotype radiation from a few founders in Central Sumatra would have resulted in a star-like pattern [57] and is thus rather unlikely.

The southward expansion of Asian golden cats is also supported by the pelage data. Southern Chinese populations showed the greatest diversity and an almost even proportion of all four colour morphs (figure 1). Frequencies of blotched and melanistic morphs decline towards the Sundaic population, which completely lacked these morphs and was characterized by red and brown golden cat individuals only. The pronounced morphological diversity of Asian golden cats on the mainland relative to their Sundaic conspecifics may be the result of selection over a long time and can be interpreted as local adaptation to the more diverse habitats [58]: melanistic golden cats mostly occur in temperate subalpine and alpine habitats (elevation up to 5000 m.a.s.l.) in northeast India [13], while spotted golden morphs mostly occur in dry deciduous forests, tropical savannahs, grasslands and occasionally shrublands [16]. As the southward population expansion of the Asian golden cats to the Sunda Shelf was accompanied by an increased frequency of red and brown golden morphs (the subtropical habitats became more homogeneous), the probability also increased that these high frequency morphs would be the ones colonizing the Sunda Shelf.

For the bay cat, three different coat colours (red, brown and greyish black) have been recorded intermixed from different regions of Borneo. In contrast with the Asiatic golden cat's coat colour morphs, which are highly contrasting and distinctive, the bay cat's polymorphism is tonally neutral. Close examination of the pelage of the greyish black morphs show an underlying reddish coloration (e.g. FMNH 8378) and we suspect that some individuals may change coloration during their lives as has been recorded for the polymorphic African golden cat, *Caracal aurata* [59] Given that most of the bay cat's probably mammalian predators and prey have dichromatic vision, this means that these colour variants are not visually distinct from each other, especially in the low light levels of closed-canopy forests. Therefore, this observation supports the species distribution projections, which suggested that the bay cat has probably been restricted to more homogeneous evergreen rainforests during the Pliocene and Pleistocene. By contrast, the brighter Asiatic golden cat morphs could be seen as an adaptation to open deciduous forests.

4.2. Taxonomy of the Asian golden cat

Several different species and subspecies of Asian golden cat have been described based on different colour morphs [17], e.g. *Felis tristis* for the ocelot-like coat pattern. Thus, we expected the phylogeny (figure 3) to show clades consisting of particular colour morphs, reflecting their putative subspecies assignments. Although there was a clear reduction of colour morphs towards Peninsular Malaysia and Sumatra, the Asian golden cats cannot be assigned to any population based on their coloration (no colour morph was specific to any particular population). Besides rejecting the colour-morph based classification, we also found an indication that modern Asian golden cats expanded only very recently. Therefore, such a recent expansion provides poor support for recognizing any subspecies of Asian golden cat and instead suggests it should be regarded as being monotypic.

On the other hand, our molecular data showed that both the Indian and the Peninsular Malaysia/Sumatran populations were distinct from all others. We could, however, not distinguish the Indian population from Indochinese or Chinese ones based on occurrence of colour morphs, so that an Indian subspecies is likewise not supported.

As already pointed out, Asian golden cats from Sumatra and their conspecifics from the mainland formed two well-separated clusters with the sample from Malaysian peninsula in between. A split between the Asian golden cats from Peninsular Malaysia and those from Indochina had already been suggested in an earlier study [18], and also the absence of certain colour morphs in Peninsular Malaysia and Sumatra supports a distinction of these Asian golden cats from their mainland relatives. Considerable body size differences (ACK 2011, unpublished data) between Sunda and

Indochinese/Chinese/Indian Asian golden cats further support the distinction of two subspecies, one occurring north of the Isthmus of Kra and the other one south of it:

(1) North of Isthmus of Kra: *Catopuma temminckii moormensis*, distributed in Indochina (Thailand, Cambodia, Lao and Myanmar), China, Tibet, Nepal and northeast India (Sikkim). Four different colour morphs: blotched, black, red and golden brown are recorded throughout the distribution range.

(2) South of Isthmus of Kra: *Catopuma temminckii temminckii*, distributed in Peninsular Malaysia and Sumatra. Two dominant colour morphs: red and golden brown. So far, no blotched morphs have been reported from Sumatra/Peninsular Malaysia. Melanistic black individuals were not in our sample collection but have been seen occasionally [11].

5. Conclusion

Phylogenetic analyses of mitogenomes, habitat distribution models and analysis of pelage colour data of the Asian golden cat and the bay cat presented here helped to elucidate the evolutionary history of these sister species. We argue that the flooding of the Isthmus of Kra in the Pliocene and subsequent climate and vegetation variations between Sundaland and Indochina have caused a species split between the bay cat and the Asian golden cat lineages approximately 3.16 Ma. Our data suggest that the bay cat became restricted to northern Borneo during the Pleistocene and particular in the LGM, when evergreen rainforest habitats were confined to that region. As a habitat specialist for the closed evergreen rainforest, the bay cat is thus much more susceptible to extinction than its sister species, the Asian golden cat, a generalist species with a much larger distribution across different habitat types. Although its recent population expansion, its low intra-population nucleotide diversity and the cline of variation in pelage colour depict the Asian golden cat as being monotypic, we argue that a recognition of two subspecies is warranted based on the presence of two distinct mitogenomic clades, the absence of certain colour morphs in the Asian golden cats from Peninsular Malaysia and Sumatra, and the considerable size differences between Sunda and Indochinese/Chinese/Indian Asian golden cats. As a conservative approach, we recommend to collapse the 3–5 subspecies and to treat the Asian golden cat as a species with just two subspecies until further evidence (e.g. from Peninsula Malaysia) suggests otherwise.

Data accessibility. Mitogenome sequences produced in this study are deposited in GenBank under the accession numbers for the Asian golden cat KX224490–KX224529 and for the bay cat KX265094–KX265096 (see electronic supplementary material, S1). Also, mitogenome alignments are submitted at Dryad under: http://dx.doi.org/10.5061/dryad.472b9 [60].

Authors' contributions. R.P.P., J.F. and A.W. designed the study; A.W. and R.P. collected the archival samples; R.P. and L.W. performed the laboratory analysis; R.P., D.W.F. and D.L. analysed the molecular data; H.P., S.K.-S. and V.R. performed the species distribution modelling; A.C.K., M.D.R., S.W.M. and A.W. collected the pelage data; R.P. analysed the pelage data; R.P., A.W. and J.F. led the writing and all other authors gave final approval for publication.

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Supplementary material

Figure S1: Median-joining (MJ) haplotype networks for the Asian golden cat

Haplotype networks were constructed using NETWORK 4.6.1.3. Numbers above bars connecting haplotypes correspond to number of mutational steps (if > 1); (see Table S1 for haplotype details).

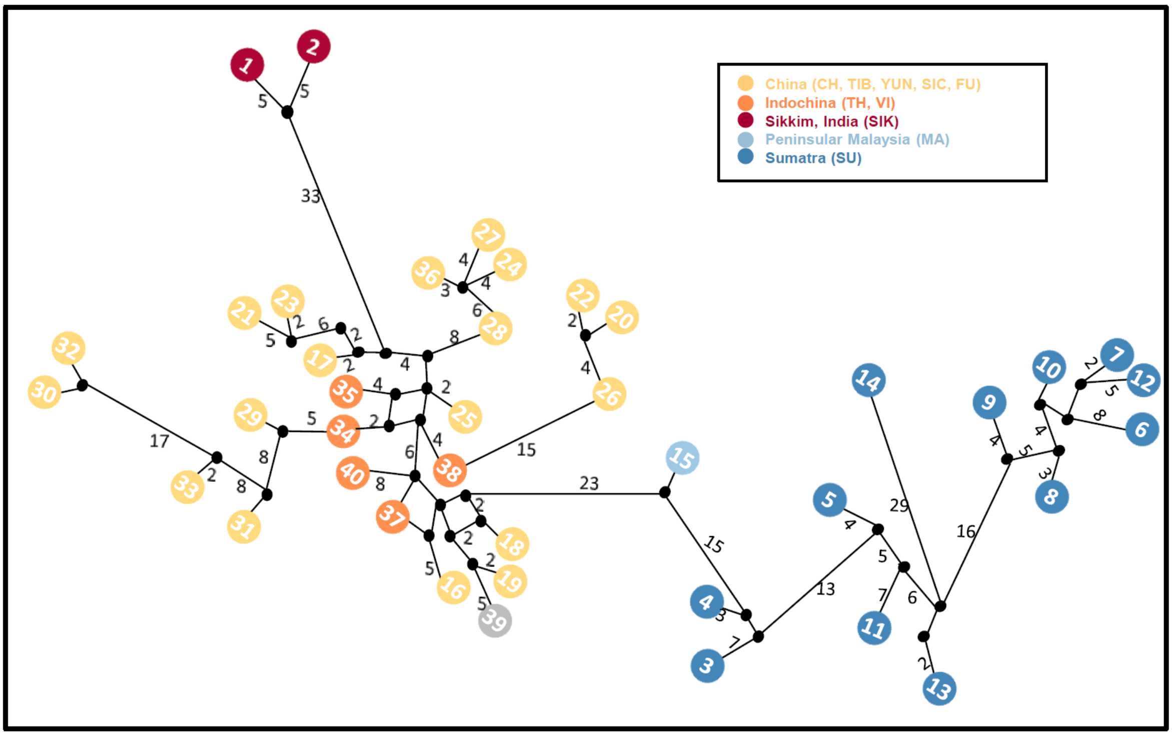


Figure S2: Different coat colour morphs of Asian golden cat

Four different coat colours from two geographic locations each are shown: **spotted morph** a) Sikkim-India, specimen ID: ZMB Mam. 91060 b) Tibet, specimen ID: ZMB Mam. 57913; **black morph** c) Yunnan- China, specimen ID: ZMB Mam. 43467 d) Sikkim- India, specimen ID: ZMB Mam. 91059; **red morph** e) Sumatra, specimen ID: NMNL Mam. 1732 f) Siam-Indochina, specimen ID: ZRC Mam. .4.1067; **brown morph** g) Tibet, specimen ID: AMNH Mam. 28250 h) Fujian-China, specimen ID: AMNH Mam. 84393

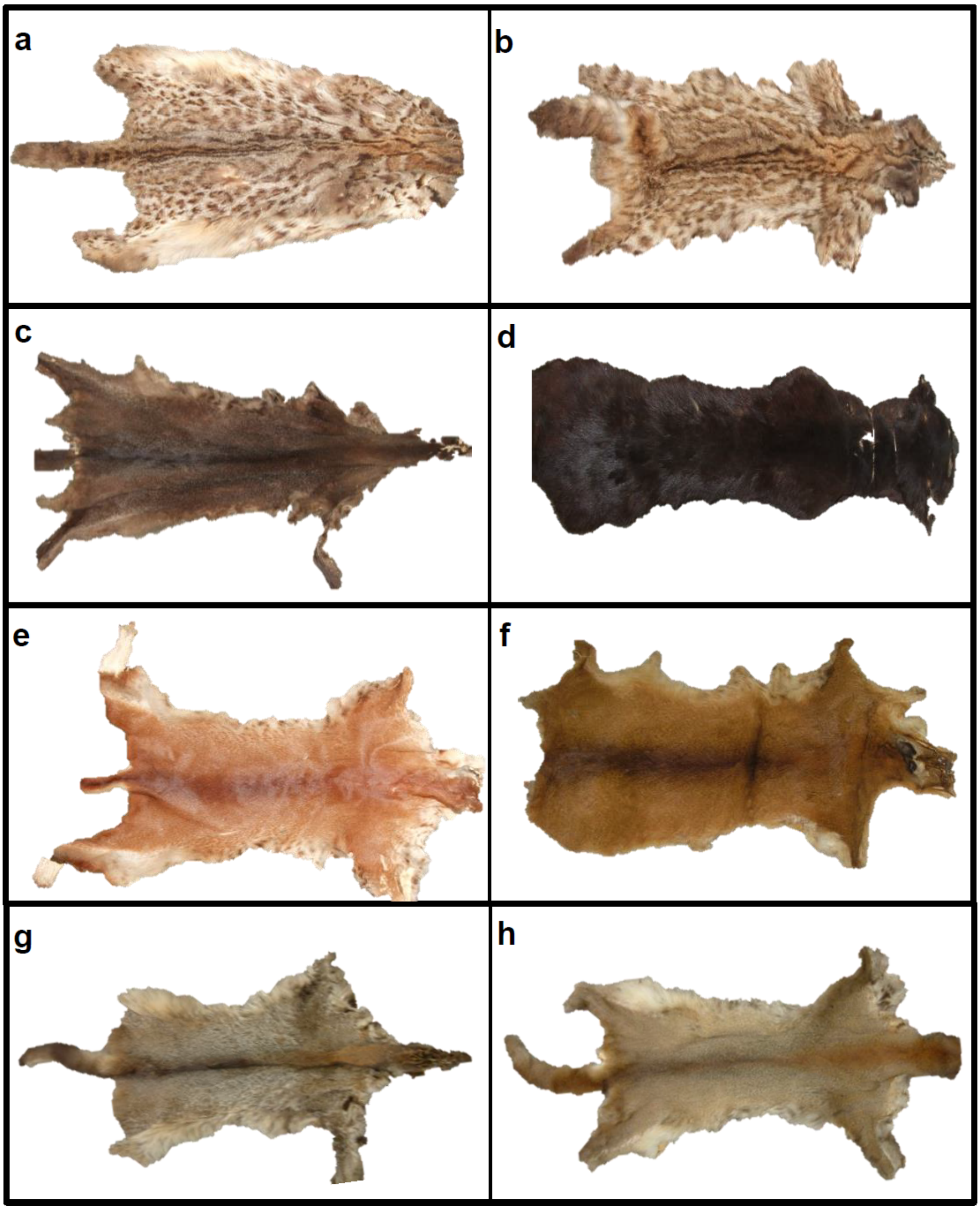
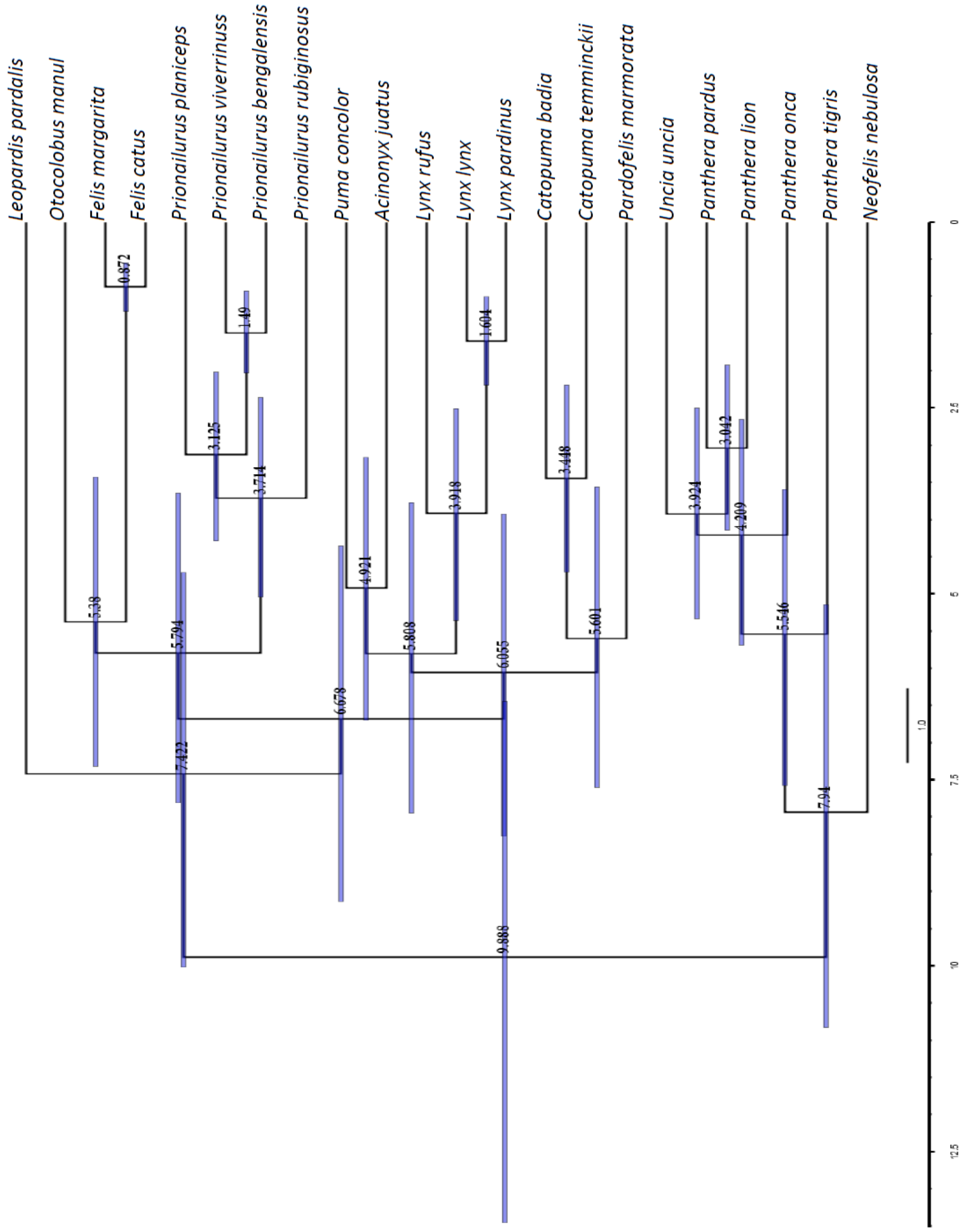


Figure S3: Molecular dating using Felidae mitogenome sequences

22 mitogenome sequences (see Table 2) were used to estimate divergence time within bay cat lineage using BEAST v1.8. Blue bars show 95% confidence intervals, numbers denote node age in million years.



Supplementary Table S1: List of specimens included in the molecular analysis

Catopuma temminckii Asian golden cat

No.	Museum or Zoo ID	Museum	Sample No	mtDNA Haplotype	GenBank accession	Sex	Source	Birth status	Year	Locale of origine	Origin contact
1	B7277	Dresden	CTE-1	H34-TH	KX224523	M	skin[a]	W	1933	South Siam, Thailand	Dr. Clara Stefen, Senckenberg natural history collection of Dresden
2	1973/334	München	CTE-2	H6-SU	KX224495	M	tissue skull[a]	W	1973	Brastagi, Province Deli, N-Sumatra	NA
3	1902	Leiden	CTE-4	H7-SU	KX224496	M	tissue skeleton[a]	W	1930	Sawah Loento, West Coast Sumatra	Dr. Pepijn Kamminga, Nationaal Natuurhistorisch Museum Leiden
4	5049	Leiden	CTE-5	H3-SU	KX224492	NA	tissue skeleton[a]	W	1941	Sumatra	Dr. Pepijn Kamminga, Nationaal Natuurhistorisch Museum Leiden
5	a.	Leiden	CTE-6	H4-SU	KX224493	M	tissue skull[a]	W	1963	Fort de Cock, Padang, Sumatra	Dr. Pepijn Kamminga, Nationaal Natuurhistorisch Museum Leiden
6	Coll. No. Schedel & Skelett b.	Leiden	CTE-7	H8-SU	KX224497	M	tissue skull[a]	W	1935	Sumatra	Dr. Pepijn Kamminga, Nationaal Natuurhistorisch Museum Leiden
7	33842	Leiden	CTE-8	H5-SU	KX224494	M	tissue skull[a]	W	1931	Peudawa, Peureula, Atjeh N-Sumatra	Dr. Pepijn Kamminga, Nationaal Natuurhistorisch Museum Leiden
8	1732	Leiden	CTE-9	H9-SU	KX224498	M	tissue skull[a]	W	1929	Djambi, Sumatra	Dr. Pepijn Kamminga, Nationaal Natuurhistorisch Museum Leiden
9	647	Leiden	CTE-11	H10-SU	KX224499	M	tissue skull[a]	W	1916	Deli, Sumatra	Dr. Pepijn Kamminga, Nationaal Natuurhistorisch Museum Leiden
10	coll. No. 315	Leiden	CTE-12	H11-SU	KX224500	NA	tissue skull[a]	W	1913	Sumatra	Dr. Pepijn Kamminga, Nationaal Natuurhistorisch Museum Leiden
11	a. für Felis tristes	Leiden	CTE-13	H29-FU_Amoy	KX224518	NA	Stuffed	W	1865	Fukien Prov, Amoy China	Dr. Pepijn Kamminga, Nationaal Natuurhistorisch Museum Leiden
12	1247	Leiden	CTE-15	H12-SU	KX224501	NA	skin[a]	W	1917	Pagar Alam, Sumatra	Dr. Pepijn Kamminga, Nationaal Natuurhistorisch Museum Leiden
13	1523	Leiden	CTE-16	H13-SU	KX224502	F	skin[a]	W	1926	Padang, Sumatra	Dr. Pepijn Kamminga, Nationaal Natuurhistorisch Museum Leiden
14	57913	Berlin	CTE-17	H23-TIB	KX224512	F	skin[a]	W	1919	Tibet	Dr. Frieder Mayer, Naturkundemuseum Berlin
15	57914	Berlin	CTE-18	H25-TIB	KX224514	F	skin[a]	W	1919	Tibet	Dr. Frieder Mayer, Naturkundemuseum Berlin

16	57915	Berlin	CTE-19	H26-TIB	KX224515	M	skin[a]	W	1919	Tibet	Dr. Frieder Mayer, Naturkundemuseum Berlin
17	91060	Berlin	CTE-20	H2-SIK	KX224491	M	skin[a]	W	1939	Gangtok, South Sikkim, Indien	Dr. Frieder Mayer, Naturkundemuseum Berlin
18	91059	Berlin	CTE-21	H1-SIK	KX224490	NA	skin[a]	W	1939	Chungtang, Mid lsikkim, Indien	Dr. Frieder Mayer, Naturkundemuseum Berlin
19	43467	Berlin	CTE-22	H16-YUN	KX224505	NA	skin[a]	W	1914	Wei Shi, Yunnan, China	Dr. Frieder Mayer, Naturkundemuseum Berlin
20	57912	Berlin	CTE-23	H18-CH	KX224507	NA	skin[a]	W	1910	Sao Kay (Tao Ray), Tonkin, China	Dr. Frieder Mayer, Naturkundemuseum Berlin
21	9193	Berlin	CTE-24	H19-SIC	KX224508	NA	skin[a]	W	NA	Batang,Sichuan, China	Dr. Frieder Mayer, Naturkundemuseum Berlin
22	38708	Berlin	CTE-25	H17-YUN	KX224506	NA	skin	W	NA	West Yunnan	Dr. Frieder Mayer, Naturkundemuseum Berlin
23	258552	Washington	CTE-27	H20-SIC	KX224509	NA	tissue skeleton[a]	W	NA	Wen Chuan, Sichuan	Kristofer M. Helgen, National Museum of Natural History, Smithsonian Institution
24	258694	Washington	CTE-28	H21-SIC	KX224510	M	tissue skeleton[a]	W	1933	Wen Chuan, Sichuan, 600-700 ft,China	Kristofer M. Helgen, National Museum of Natural History, Smithsonian Institution
25	259012	Washington	CTE-29	H22-SIC	KX224511	F	tissue skeleton[a]	W	1934	Wen Chuan, Sichuan	Kristofer M. Helgen, National Museum of Natural History, Smithsonian Institution
26	113709	New York	CTE-30	H24-SIC	KX224513	F	tissue skull[a]	W	1934	Minya Konka, Sikong, China	Dr. Nancy B. Simmons, American Museum of Natural History
27	28249-b	New York	CTE-31	H27-TIB	KX224516	NA	skin[a]	W	NA	Tibet	Dr. Nancy B. Simmons, American Museum of Natural History
28	31279	New York	CTE-32	H28-TIB	KX224517	NA	skin[a]	W	NA	Batung, Tibet	Dr. Nancy B. Simmons, American Museum of Natural History
29	55554	New York	CTE-33	H35-TH	KX224524	M	skin[a]	W	1927	Trang, Thailand	Dr. Nancy B. Simmons, American Museum of Natural History
30	84395	New York	CTE-34	H30-FU	KX224519	F	tissue skeleton[a]	W	1926	Chungan Hsien, Fukien Prov., China	Dr. Nancy B. Simmons, American Museum of Natural History
31	84393	New York	CTE-35	H31-FU	KX224520	M	tissue skull[a]	W	1926	Chungan Hsien, Fukien Prov., China	Dr. Nancy B. Simmons, American Museum of Natural History
32	84394	New York	CTE-36	H32-FU	KX224521	F	tissue skull[a]	W	1926	Chungan Hsien, Fukien Prov., China	Dr. Nancy B. Simmons, American Museum of Natural History
33	84396	New York	CTE-37	H33-FU	KX224522	M	tissue skull[a]	W	1926	Chungan Hsien, Fukien Prov., China	Dr. Nancy B. Simmons, American Museum of Natural History
34	28250	New York	CTE-38	H36-TIB	KX224525	NA	skin[a]	W	NA	Tibet	Dr. Nancy B. Simmons, American Museum of Natural History

35	ZRC. 4.1071	Singapore	CTE-39	H14-SU	KX224503	NA	Skull & skin[a]	W	1933	Indragiri, Sumatra	NA
36	ZRC. 4.1068	Singapore	CTE-40	H37-TH	KX224526	M. ad.	Skull & skin[a]	W	1916	Rapids below Chiengmai, N. Siam	NA
37	ZRC. 4.1067	Singapore	CTE-41	H38-TH	KX224527	M	skin[a]	W	1916	Nr. Chiengmai, N. Siam, Thailand	NA
38	ZRC.4.1069	Singapore	CTE-42	H15-MA	KX224504	NA	skin[a]	W	1891	Malacca, Malaysia	NA
39	NA	NA	CTE-8908	H39-ZOO	KX224528	M	Fresh tissue	captive	2008	Allwetterzoo Münster	Dr. G. Wibbelt, Leibniz Institute For Zoo and Wildlife Research, Berlin
40	NA	NA	CTE-2808	H40-VI	KX224529	M	Fresh tissue	captive	2008	Vietnam	Dr. C. Szentiks,Leibniz Institute For Zoo and Wildlife Research , Berlin

Catopuma badia **Bay cat**

No.	**Museum or Zoo ID**	**Museum**	**Sample No**	**mtDNA Haplotype**		**Sex**	**Source**	**Birth status**	**Year**	**Locale of origine**	**Origin contact**
1	25534	Leiden	CBA-2	CBA-2	KX265094	W	tissue skull[a]	W	1901	Boven, Mahakkanriver	Dr. Pepijn Kamminga, Nationaal Natuurhistorisch Museum Leiden
2	NMS.Z.2004.15.1	NA	CBA-3	CBA-3	KX265095	M	Fresh tissue	NA	2000	Soak village, Interior region, Sabah, Malaysia	Department of Natural Sciences, National Museums Scotland
3	NMS.Z.2004.15.2	NA	CBA-4	CBA-4	KX265096	F	Fresh tissue	NA	2000	Nabawan Village, Interior region, Sabah, Malaysia	Department of Natural Sciences, National Museums Scotland

[a] indicates tissue source is archival

CHAPTER 2

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Genetic Structure and Phylogeography of the Leopard Cat (*Prionailurus bengalensis*) Inferred from Mitochondrial Genomes

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Author contribution

R.P.P., J.F. and A.W. designed the study; A.W., S. M. and G. V. collected samples; R. P. P. and S.W. performed the laboratory analysis; U. R. and S. M. provided laboratory in India, R. P. P., S. W. and D.L. analysed the molecular data; R.P. P., D. W. F., A.W. and J.F. led the writing and all other authors gave final approval for publication.

CHAPTER 3

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Threatened but understudied – supporting conservation by understanding the genetic structure of the flat-headed cat

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Author contribution

R.P.P., J.F. and A.W. designed the study; A.W and A. C. K. collected samples; R. P. P. performed the laboratory analysis; R. P. P. and D.L. analyzed the molecular data; R.P. P., A.W., A. C. K. and J.F. led the writing and all other authors gave final approval for publication.

GENERAL DISCUSSION

"…microevolutionary processes operating within species can be extrapolated to explain macroevolutionary differences among species and higher taxa" – (Avise et al. 1987)

A phylogeographic assessment with many species sampled from the same geographic region (also known as comparative phylogeography) can be used to explain an evolutionary history shared among many species. Examples include continental European species showing northward range expansions following the glacial maximum (Taberlet et al. 1998, Hewitt 1999, 2000), the width of Amazonian rivers acting as a dispersal barrier to forest bird species (Hayes et al. 2004), multiple studies describing divergence within flora and fauna due to a biogeographic barrier (de Bruyn et al. 2005) or gene flow within island populations through land bridges at low sea levels in southeast Asia (Leonard et al. 2015).

Similarly, this dissertation aims to deduce the evolutionary history of two genera of closely related felid species and to identify the drivers of their evolutionary history. This work used mitogenome phylogeography in combination with the knowledge of current distribution patterns, the ecology of the species and past geological and climatic events in the Southeast Asia.

Habitat generalists and specialists

Species-specific differences in niche breadth define the dichotomy between habitat specialists and habitat generalists (Whittaker et al. 1998, Rosenzweig 1981, Janecka et al. 2016). Closely related species can diverge in the extent of their adaptibility and this may influence their abilities to persist in different habitats (in case of generalist species) or in narrow range of habitats (in case of specialist species) (Griffith et al. 2012). Specialists are known to use a narrow range of resources and are well adapted to a particular habitat (Büchi & Vuilleumier 2014), which can restrict their ability to disperse across unsuitable habitat. As a result, their distribution patterns are often fragmented and their disconnected populations increase in genetic differentiation with the time span by which they are separated from each other. In contrast, with wider niches, generalists can often cope with disturbances and changes in habitats. Hence, they may have a greater ability to disperse through less well suited habitats (Janecka et al. 2016). Thus, we would expect that gene flow among generalist populations would be higher than among specialist populations (Introduction, Tab. 1), preventing the formation of a population structure. Any occurance of population structure in generalist species is likely to require larger distances ('isolation by distance').

Genetic differentiation

The Asian golden cat (*Catopuma temminckii*) and the leopard cat (*Prionailurus bengalensis*) are both habitat generalists. They are widely distributed throughout Southeast Asia and adapted to diverse habitats. The bay cat (*Catopuma badia*) and the flat-headed cat (*Prionailurus planiceps*) are both habitat specialists. They are both restricted to the Sundaic region and are either forest-dependent (bay cat) or a wetland dweller (flat-headed cat).

Comparison between Indochina and Sundaic populations

The results obtained from the analyses of mitogenomes of the leopard cat and the Asian golden cat were in contrast to the assumption of low genetic differentiation between Indochina and Sundaic populations for generalists. The results showed for both species substantial genetic differentiation between mainland and Sundaic populations. But there were exceptions: Leopard cat samples from Peninsular Malaysia belonged to both the 'Mainland lineage' and the 'Sunda lineage' (Chapter 2, Fig. 3). The only Asian golden cat sample available from Peninsular Malaysia was positioned between the mainland and the Sundaic samples (Chapter 1, Fig. S1). This suggests recent gene flow for both species between mainland and Sundaic populations and a recolonization of southern areas from northern areas through Peninsular Malaysia.

Comparison among Sundaic populations

Gene flow between Java and other Sundaic islands was expected because Java and Bali were connected to each other and to the other Sundaic islands during glacial periods. In contrast to this expectation the leopard cat populations from Java and Bali were genetically highly differentiated from other Sundaic populations. Climate and vegetation on Java is drier and different from other parts of the Sunda-Shelf (Raes et al. 2014). But for generalist species such as the leopard cat this does not really constitute a barrier to gene flow and requires further explainations. Future studies should incorporate nuclear genes to trace bi-parental evolution of leopard cat population on Java and Bali and to evaluate the possibility of ancient admixture.

Both habitat specialist species studied here are restricted to Sundaland: the flat-headed cat up to the Isthmus of Kra, and the bay cat is limited to Borneo. Significanant genetic differentiation inferred from mitogenome analyses of the flat-headed cat (Chapter 3, result section) suggests that the two lineages, the 'Bornean lineage' and the 'Sumatra and Thai-Malay Peninsula lineage' had been separated for a long period of time. Mitogenome analysis for the later lineage did not differentiate it into further distnict clades, indicating recent gene flow between Thai-Malay Peninsula and Sumatra, but the species only spread up to the IoK. The bay cat as the southern representative of the two *Catopuma* species diverged from its common ancestor with the Asian golden cat and evolved to become a specialist of tropical evergreen rainforests. The absence of both specialists (the flat-headed cat and the bay cat) from Indochina suggests their adaptation to more homogenous habitat of Sundaland in comparison to the different habitat types in Indochina which may have posed ecological barriers that restricted their distribution to Indochina.

Population structure

As presumed, the analysis of Asian golden cat mitogenomes revealed a low genetic diversity and the lack of a genetic structure among mainland populations (Chapter 1, Figure 3). Samples from China and Indochina were present across most branches of the maximum likelihood phylogenetic tree, suggesting that Indochina was the refugium during glacial periods. Similar findings were reported for other widely distributed carnivore species such as the leopard *Panthera pardus* (Wilting et al. 2016b), the grey wolf *Canis lupus* (Pilot et al. 2006), the common palm civet *Paradoxurus hermaphroditus* (Patou et al. 2010), and the small Indian civet *Viverricula indica* (Gaubert et al. 2016). When areas such as Indochina became a refugium during glacial maxima, they provided numerous, very diverse ecological niches. Depending on how long these niches existed and how broad they had been, these niches could then have accelerated evolutionary divergence in newly evolving species. In consequence, broad niches could have enhanced the adaptive potential and resilience to changing environments in generalist species (Sacks et al. 2008), whereas narrow niches may have facilitated the evolution of specialists.

The phylogeographic analysis of leopard cat mitogenomes revealed the presence of four haplogroups among the mainland populations: Far East (Russia, Taiwan, and China), Indochina (Thailand, Vietnam, Myanmar, and Laos) and India (Northeastern India, southern India, see Chapter 2, Fig. 3). Nevertheless, leopard cat samples from Indochina were present in three out of these four haplogroups (A, B, C), indicating a very wide distribution of the leopard cat across this region in the past. Significant genetic differentiation of leopard cat populations from populaions of the Far East (haplogroup D: Russia, China and Taiwan) and from the other mainland populations in India and Indochina (Chapter 2, Table 3) may suggest geographic distance as a barrier for divergence ('isolation by distance') and/or local adaptation. Similar patterns have been also observed for other felids, e.g. for the Amur tiger *Panthera tigris* (Wilting et al. 2015a) and the Amur leopard *Panthera pardus orientalis* (Uphyrkina et al. 2001).

On the other hand, as expected for habitat specialists (Introduction, Table 1), analysis of mitogenomes from the flat-headed cat showed presence of a pronounced genetic structure among Sundaic populations (Borneo and Thai-Malay Peninsula, Sumatra; Chapter 3, Fig. 2). Moreover, the flat-headed cat populations from Thai-Malay Peninsula and Sumatra were sub-structured; each appeared to have its own sub-clade. Interestingly, samples from Thai-Malay Peninsula also appeared on the same branch with Sumatra, suggesting recent gene flow among populations from Thai-Malay Peninsula and Sumatra. A possible explaination can be that as a wet land specialist — flat-headed cats would have probably followed the shifting coastlines (due to repeatedly fluctuating sea levels) with its peat swamps and mangrove forests between Sumatra and Peninsular Malaysia during Middle and Late Pleistocene.

Unfortunately, the small sample size for the bay cat was insufficient to investigate this other habitat specialist at this level of detail.

Dispersal and distribution

Response to gradual environmental change

As predicted for generalist species (Intoduction, Table 1), the results obtained in the studies presented here showed gene-flow among the populations of the Asian golden cat, indicating continuous dispersal among populations from diverse habitats across Indochina. The noticeable separation of the Asian golden cat samples from Sikkim (North East (NE) India) and Fukien (China) — the most western and eastern parts of the species distribution range on the mainland — from other Indochinese — Chinese samples (Chapter 1, Fig. 4), may indicate spatial differentiation. Neverthless, low nucleotide diversity (see chapter 1, results section) and the estimated date of the split among Indochinese population (chapter 1, Fig. 4; node III & IV) suggest a relatively recent divergence.

The presence of leopard cat samples from Borneo, Sumatra, Peninsular Malaysia and the Philippines Islands in one clade (‘Sunda lineage’, Chapter 2, Fig. 3, clade E) would normally indicate gene flow among these populations by dispersal of the leopard cat using the land bridges connecting those islands during glacial periods. However, the presence of leopard cat populations on the Philippine islands of Negros, Panay and Cebu raises doubts on ‘colonization by natural dispersal’, because none of these Philippine islands had been connected to Sundaland (Heaney 1986, Voris 2000, Hall 2001, Reis and Garong 2001). Thus, it is much more plausible that humans introduced leopard cats to these islands in recent times.

The flat-headed cat haplotypes revealed an old matrilineal split (~ 0.57 million years old), between populations from Borneo and the other Sundaic populations (Thai-Malay Peninsular, Sumatra; Chapter 3, Fig. II). This suggests that dispersal for this wetland specialist was restricted by the drier (savanna-like) vegetation on the exposed (barrier-like) land corridors during glacial periods between Borneo and Sumatra / Peninsular

Malaysia, separating Bornean flat-headed cats from their conspecifics on Sumatra and the Thai-Malay peninsula (Heaney 1991, Louys & Meijaard 2010). On the other hand, the much shorter branch lengths of samples from Sumatra and the Thai-Malay peninsula indicated recent but limited north-to-south dispersal of flat-headed cat individuals (Chapter 3, Fig. II).

Unlike the three other felid species studied here, the bay cat is endemic to Borneo. During most of the Pleistocene and especially at glacial phases when the climate became more seasonal (see distribution modelling in Chapter 1, Fig. 5f & 5h), the central savanna corridor separating the Bornean lowland evergreen rainforest from the Sumatran lowland evergreen rainforest (Cannon et al. 2009) would have been a strong barrier to dispersing bay cats. During interglacial periods the sea itself was a barrier, preventing the bay cat from moving out of Borneo to other parts of the Sunda-Shelf. During most of the Pleistocene, northern Sarawak, Brunei, Sabah and eastern Kalimantan acted as rainforest refugia for forest-dependent species (Gathorne-Hardy et al. 2002). Hence, the distribution of the bay cat became restricted to the rainforests of Borneo.

Response to a Vicariance event

During the glacial periods of the Pleistocene the emerged land bridges of Sundaland provided the physical connection for species to disperse amond Sundaic islands and between the latter and the continental mainland. I had therefore predicted that the high ability of generalist species to cope with diverse habitats together with their capacity to disperse across less suitable habitats would result in low genetic differentiation among populations of Sundaland and the mainland (Introduction, Table 1). However, surprisingly, the mitogenome analysis revealed a pattern of significant genetic differentiation between mainland and Sundaic populations of both the leopard cat and the Asian golden cat, a result that clearly did not congruent the prediction. As Pleistocene sea level fluctuations could not explain the observed population differentiation, other causes had to be considered. One plausible cause of the isolation could have been an ancient vicariance event (e.g. high sea level and submergence of IoK) that separated the continental mainland fauna from the Sundaic fauna. But this explanation seems feeble for species whose intraspecific divergence is estimated to be < ~1-2 million years because there is no evidence for a complete submergence of IoK which is therefore discussed controversally. A hypothesis has been brought forward (Woodruff 2003a) that marine transgressions had submerged the IoK during the early/middle Miocene and the early Pliocene (24–13, 5.5–4 and 3 MYA), but this hypothesis was opposed later (Woodruff 2003b) by the notion that there is no evidence for the existence of a seaway at IoK (i.e. complete submergence of IoK) in the past 25 million years. Nevertheless, during the Pleistocene, rapid marine transgressions and regressions around IoK could have affected the area severely by narrowing the passage which in turn may have ecologically isolated mainland and Sundaic populations of species such as the leopard cat and the Asian golden cat.

Although the previous scenario cannot be fully excluded, a more convincing explanation for the observed 'Indochinese-Sundaic divergence' is provided by the Toba volcanic supereruption on Sumatra, ~74 Kya. Although its consequences, such as the wipe out of species on Sumatra and surrounding regions, are being controversially discussed (Louys 2007, Williams et al. 2009, 2010, Louys and Meijaard 2010), the deposition of tephra (pyroclastic sediment), the circulation of ash clouds, as well as the subsequent changes in vegetation and climate across IoK and most of Indochina (Song et al. 2000) might have created barriers to gene flow between mainland populations and populations of their Sundaic conspecifics. The phylogeography data for the Asian golden cat suggests a post-Toba (eruption) expansion from southern and northern China, areas which were less affected by the after-effects of the Toba eruption. For the leopard cat the data indicate an extinction of populations from Peninsular Malaysia, followed by a (recent) re-colonization of the peninsula from both the north (mainland) and the south (Sundaland).

For specialist species, we expected either absence or a fragmented distribution in response to vicariance events (i.e. Toba eruption). The presence of the flat-headed cat on Sumatra suggests a probable recolonization from northern Peninsular Malaysia and southern Thailand, areas less severly affected by the Toba eruption (Wilting et al. 2012) that could have served as a refugium. The volcanic fallout of the Toba eruption may have created an additional barrier to the already existing barrier — the open savannah corridor, hindering bay cats to disperse from Borneo to Sumatra or Peninsular Malysia. This might explain the absence of the bay cat on Sumatra and Peninsular Malaysia.

Taxonomy and implications for conservation

Conventionally, species and subspecies have been described by studying their morphological traits and phenotypic differences often using only few speciemens. Due to the low sampling size, the variation within a population could however not be assessed, and thus it remained uncertain if found differences reflected individual differences between the specimens or true differences between the populations. This led biologists to describe populations from different geographic locations or populations on different islands as different species or subspecies. The use of molecular genetic markers in combination with other traits (skull morphology, pelage, ecological traits, and other phenotypic marker) has already improved the classification process (O'Brien 1994) and will continue to do so. Additionally, molecular markers can identify and differentiate true genetic lineages. Phylogenetic studies can also guide location-based decisions for cases when confiscated individuals are being released back into the wild (Banes et al. 2016).

During the investigation of the phylogeographic history of the four felid species studied in this dissertation, distinct populations and haplogroups were recognized. These could become the subject of further studies and might be examples of evolutionarily significant units for conservation. In chapter 1, on the basis of mitogenome data and in combination with morphological data, I suggested a reduction of the number of subspecies of the Asian golden cat to just two subspecies instead of the current three to five subspecies, whose description are mostly based on coat colour morphs. Similarly, in chapter 2, the mitogenome analysis of the leopard cat suggested only four mitochondrial lineages and thus rejected the conventional classification of 14 subspecies. Moreover, the presence of samples from the Peninsular Malaysia in both the 'Mainland lineage'and the 'Sunda lineage' identified Peninsular Malaysia as a potential hybrid zone, although this needs to be verified in further studies, which need to include nuclear markers and additional samples from this region. In chapter 3, the analysis revealed separate phylogenetic clusters for the flat-headed cat populations from Borneo and the Thai-Peninsular Malaysia/Sumatran population. The surprisingly strong (and long-standing) genetic differentiation between these populations suggests that the flat-headed cat population from Boneo is genetically distinct and warrants the consideration of being declared a subspecies. Any future breeding programmes (in the wild and in zoological gardens) and as well as conservation efforts for the flat-headed cat should consider the two lineages to be separate conservation management units to maintain the genetic diversity of this rare and endangered species.

Although all four cat species in this dissertation are protected in their distribution range, illegal poaching for their pelts (leopard cat and Asian golden cat) and meat are major threats to these species. Additionally, destruction of wetland and conversion of lowland rain forests are major threats to the specialist species – the flat-headed cat and the bay cat. Currently, specialist species are declining worldwide at a higher rate than generalist species because of rapid habitat fragmentation and deforestation (Büchi and Vuilleumier 2014). Studying the evolutionary history of species and the identification of important taxonomic (evolutionarily significant) and management units will help focus conservation efforts on the particular species or subspecies.

Limitations of methods used in this study

Quality of DNA from museum samples

Specimens stored in museums have often been collected decades or even centuries ago. The storage process includes trearment with a wide range of chemicals for preservation. Consequently, extracted DNA from museum specimens is highly degraded and consists mostly of small fragments (50-200bp long) (Pääbo *et al.*, 2004). Hence, despite the use of advanced techniques and several repetitions, I failed to obtain high quality mitogenome sequence data (defined as sequences with a five-fold minimum coverage) for several samples. I could not include some of the samples with very important locations (for example in Chapter 2, the leopard cat samples from Mongolia or northern India). Also, the short reads sequenced from these degraded DNA libraries need to be mapped to very good quality references to also be able to map repeat regions (for example in the d-loop of mitogenomes, the Y chromosome and microsatellite loci).

Mitogenomes as marker

Mitogenomes have been used to measure timing and extent of the divergence between populations and to infer the phylogeography and evolutionary history of species (Desalle et al. 2017). The high mutation rate, the high copy number per cell and the fast(er) coalescence time are some of the advantages of using mitogenomes as markers to study species or populations with low intraspecies nucleotide diversity and when samples with degraded DNA are used (Zink & Barrowclough et al. 2008). Despite these advantages, all mitochondrial genes are linked and thus the analysis of mtDNA generates a single-locus tree that presents a biased view of the species' history because it excludes the selection pressure on the evolution of any species' nuclear DNA (Hung et al. 2016).

In this study, inferred evolutionary history and distribution patterns were based on mitogenome haplotypes, which only allow us to 'tell the story of the matrilineal side' of the history of the species. However, felid dispersal is male-biased, which means that felid males disperse farther than females (Sunquist and Sunquist 2002), therefore phylogeny predicted with the maternally transmitted mitogenome requires validation from Y chromosome and autosomal markers (Petit and Excoffier 2009). Studies have already reported discordance between topologies from phylogenetic trees based on mitochondrial sequences and several nuclear markers (Ting et al. 2008, Toews et al. 2012, Mendes et al. 2016; for felids see Li et al. 2016). In chapter 2, the presence of *P. bengalensis* samples from Peninsular Malaysia in both the Sunda and the mainland lineage cannot be unambiguously interpreted with only mitogenome data. Additional nuclear markers (Y chromosome and autosomal genes) would be helpful to assess the presence of potential hybrids. Population divergence patterns solely based on mitogenomes are in general reliable, but in case of mtDNA introgression and incomplete lineage sorting (=incongruent mtDNA-nuDNA phylogenies), studies require several independent markers to accurately explain the (potentially) complex biogeographic history of a species (Hung et al. 2016).

Implication for future research

The results presented here are limited to mitogenomes of four species. Future research should include nuclear markers in order to detect potential hybridization or introgression events. An integrative approach targeting independent loci across the genomes of multiple closely related species will then shed a clearer light on their speciation. The remaining sister taxa within the studied cat lineages (for genus

Prionailurus: fishing cat *P. viverrinus*, rusty-spotted cat *P. rubiginosus,* for the bay cat lineage: marbled cat *P ardofelis marmorata*), should also be studied, as this will improve our understanding of the evolution of these closely related species against the background of their shared biogeographic past. This knowledge has the potential to allow predictions about what a species-specific response to an environmental change would be. In consequence, it can be used for conservation planning to maintain the biodiversity of a region.

Concluding remarks

In this dissertation, I described how closely related species with a shared historical and biogeographic past responded differently to similar environmental challenges and diverged along their own evolutionary trajectories. When the two-sister species of *Catopuma* diverged by allopatric speciation, one became a habitat specialist endemic to the evergreen rainforests of Borneo whereas the other evolved to become a habitat generalist who expanded its range throughout Southeast Asia. On the other hand, the two *Prionailurus* species partially share their distribution range but prefer different habitats and prey species, which may even have allowed speciation in sympatry. This, however, remains speculative, since so far, no evidence exists that both species continuously lived in sympatry since they diverged from their last common ancestor ~2.4 Mya (Li et al. 2016). The matrilineal histories of the two-generalist species (*C. temminckii*, *P. bengalensis*) revealed two distinct lineages for each of them, supporting the Indochinese-Sundaic divergence. These matrilineal splits within both species was most likely caused by a vicariance event (the Toba supervolcanic eruption), because free dispersal of these generalists across Sundaland would have prevented the observed divergences to evolve. The generalist species could also have re-colonized the habitat affected by the aftermath of the Toba eruption (or disperse across it) because of their ability to cope with diverse habitats. In contrast, the two-specialist species remained either restricted to their preferred habitat – reflected by the purely Bornean distribution of the bay cat– or only dispersed if suitable (similar) habitat was available – reflected by the genetic structure of the flat-headed cat on Sundaland.

In the past, it has been common practice to postulate concordance in biogeographic barriers and extrinsic factors for the evolution of different species. The study presented here advocates a closer look at a species biogeographic history, because the species presented here revealed quite dissimilar evolutionary histories.

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APPENDIX

Lab protocol for library preparation and hybrid capture

Library Preparation

- put all componants on ice
- always prepare a control sample (water)
- after each step turn on the UV light and change gloves
- mix everything everytime prior to use

Blunt end

		1×
ddH$_2$O	(Milli Q)	3.56 µl
Tango Buffer	10x	3.5 µl
dNTPs	(25 mM)	0.14 µl
ATP	(100 mM)	0.35 µl
T4 Polynucleotide Kinase	10 U/µl	1.75 µl
T4 Polymerase	5 U/µl	0.7 µl

→ 10 µl per tube
+ 25 µl DNA template

Cycler:	25°C	20 min
	72°C	20 min
	12°C	∞

Adapter Ligation

		1×
ddH$_2$O	(Milli Q)	2.5 µl
T4 Ligase Buffer	10x	5 µl
PEG-4000		5 µl
T4 Ligase	50	1.25 µl
*Adapter-Mix	20 µM	1.25 µl (museum sample)
Template from previous step		35 µl

1. make mastermix
2. mix samples + adapter mix

3. add enzyme Ligase to mix
4. add 13.75 µl mastermix to samples

Cycler: 22°C 30 min
12°C ∞

* Enzyme ligase is very temperature sensitive → take it out only when needed and put back immediately

Clean up with Qiagen MinElute (Purification)

- colums are in the fridge under the fluidigm
- transfer PCR-products in a new 1.5 ml tube and cut the lid
- add 250 µl PB-Buffer (50µl in the PCR-Product → 5 × 50 = 250 µl)
- Tranfer to a column, spin 1 min at 13000 rpm, remove flow-through
- Add 750 ml PE-Buffer, spin 1 min at 13000 rpm, remove flow-through
- Dry centrifugation for 3 min at 13000 rpm
- Place column in a new tube (cut the lids)
- Add 10 µl EB (Elution-Buffer), wait 5 min, spin 1 min at 13000 rpm
- Repeat with 10 µl EB

➔ While waiting for elution, set up program on thermocycler and prepair mastermix for adapter fill-in

Adapter fill-in

	1×
ddH_2O	14.1 µl
Thermopol buffer	4 µl
dNTPs	0.4 µl
Bst polymerase LF	1.5 µl

→ add 20 µl to 20 µl template from previous step

Cycler: 37°C 20 min
80°C 20 min
12°C ∞

(Whatever is left can be kept as stock for library at -20°C)

Indexing amplification

- Do 3 to 4 parallel amplifications for each museum sample to avoid PCR bias towards some fragments and not others (2 -4 parallels for fresh sample)

	1x
ddH_2O	9.8 µl
AmpliTaq buffer	2 µl
dNTPs	0.2 µl
BSA	0.2 µl
$MgCl_2$	1.6 µl
IS4	1.5 µl
Index oligo	1.5 µl
AmpliTaqGold	0.2 µl

→ put first 3 µl DNA-template in new PCR-Tubes than add 17 of the mix

Cycler: **12-18 x**

94°C	10 min
Loop start	
94°C	30 sec
60°C	45 sec
72°C	45 sec
Loop ends	
72°C	5 min
12°C	∞

Clean up with Quiagen MinElute (Purification)

- add 175 µl PB-buffer to the PCR-tubes (35µl → 5 × 35 = 175 µl)
- Transfer to a column, spin 1 min at 13000 rpm, remove flow-through
- Add 750 ml PE-Buffer, spin 1 min at 13000 rpm, remove flow-through
- Dry centrifugation for 3 min at 13000 rpm
- Place column in a new tube
- Add 10 µl EB (Elution-Buffer), wait 5 min, spin 1 min at 13000 rpm
- Repead with 10 µl EB
- Quality of libraries can be determined with Tape station or Bioanalyzer instrument.

(Whatever is left can be kept as stock for library at -20°C)

Hybrid capture protocol

Library –example is for, *Prionailurus bengalensis*

Baits: *Prionailurus viverrinus*

To prepare baits: Long range PCR fragments of mitochondrial genome from fresh DNA samples are produced, which are 6-6.5 Kb in size and we shear them into 250bp fragments. Purify sheared fragments with Qiaquick and pool them equimolarly.

From the pooled and purified baits take 10µl which is around 4000ng and add 66µl of water to make 76µl of volume.

Blunt ending- NEB quick blunting kit

DNA-pooled Baits	76µl (Upto 4µg)
Blunting buffer(10x)	10µl
dNTPs(1mM)	10µl
Blunting enzyme mix	3µl
Total	100µl

Incubate the reaction at room temperature for 30min

Purify the DNA with Qiaquick and elute in 30µl single time

Adapter ligation-NEB Qiaquick ligation kit

Blunt ended DNA	30µl
Adapters Bio-T/B (50µM)	2µl
Quick ligase buffer (2x)	40µl
Quick ligase	8µl
Total	80µl

Incubate the reaction 15 min at RT

Purify the DNA with Qiaquick and elute in 30µl each.

Mmeasure concentration of purified DNA

Now add 10µl of BWT 2× buffer to the content of each tube and heat the mixture for 1min at 98°C. Put that on ice immediately after that.

Wash 5 µl of the M-270 beads with 200 µl of BWT buffer (1×)

Wash the beads with 200 µl TET buffer

Add the bait mixture that is kept on ice to the beads and resuspend the beads
Incubate for 20 minutes at RT and shake the tube every 5 min to allow the biotinylated bait to bind to the streptavidin beads. Ensure that the lids are closed.

Meanwhile-

Heat the hybridization oven to 65ºC and preheat four 1.5ml tubes with BWT 1x buffer at 50 ºC.

Prepare the hybridization mixture:

800ng of the pooled indexed libraries	1× µl	8.5× µl(× sample size)
BO 1	0.5	4.25
BO2	0.5	4.25
BO 3	0.5	4.25
BO 4	0.5	4.25
BO 5	0.5	4.25
BO 6	0.5	4.25
Agilent blocking agent 10×	5.2	44.2
Agilent hybridization buffer 2×	26	221

Distribute 34.2 µl in each tube and add 17.8µl library in each tube.
Incubate the mixture for 3 minutes at 95ºC to denature the DNA and incubate for 30 minutes at 37 ºC on PCR machine.

Bait and bead mixture can wait while you are preparing capture mixture

Collect the beads with a magnet on the tube wall and remove the supernatant which contains the bait that did not bind to the beads

Wash the beads 2 times with 200 µl of BWT buffer (1×) heated to 50ºC

Resuspend the beads in 50 µl of TET buffer, transfer the suspension to a new 0.2 µl PCR tube and store it at 4 ºC until the hybridization mixture is ready.

Remove the TET buffer from bait-coated beads by magnetically collecting them and discarding the supernatant

Add 52 µl of the mixture to the ‘dried’ beads and resuspend the beads.

Use tape to secure the lid
Use tape to attach the tubes to the rotor inside the hybridization oven and rotate slowly (12 rpm).

After 48 hours
After 48 hours elute the libraries from beads.

Wash the beads with 200µl of BWT1× buffer 3 times carefully, so that not a single bead remains stuck to lid or surface of the tubes.

Wash the beads with 200µl of HWT buffer (pre-heated at 60°C) and incubate samples at 60°C for 2 min.

Repeat this step twice.

Wash the beads with 200µl BWT 1× buffer.

Add 100µl of TET buffer and transfer into new tubes.

Elute enriched library from beads –Heat libraries on 95 °C for 3 min.

Transfer samples very quickly to new tubes and recover supernatant on magnetic rack.

Purify captured library with minielute columns in 15µl volume.

Amplify captured library to recapture the same.

Prepare fresh bait and repeat the following procedure.

Amplify recaptured samples for 10 cycles and measure them on Tape station or bio analyzer to see size distribution. Pool them equimolarly at 10 nM.

Sequencing on MiSeq platform

Required:

- Stock 1.0 N NaOH
- Lab-grade H_2O
- 10mM Tris-Cl with 0.1% Tween
- HT1 (hybridization buffer) ← needs to be on ice, delivered with MiSeq Cartridge
- MiSeq Cartridge
- 10nM Library

Protocol:

1. Prepare fresh dilution of 0.2 N NaOH from the 1 N NaOH stock.

2. Combine the following volumes to dilute the library to 4nM:
    - 10nM library (2µl)
    - 10mM Tris-Cl with 0.1% Tween (3µl)
3. Combine 4nM library and 0.2 N NaOH to give 2nM library:
    - 4nM library (5µl)
    - 0.2 N NaOH (5µl)
4. Vortex briefly
5. Centrifuge down (280 g) for ***one min***
6. Incubate for ***5 mins*** at room temperature to denature library
7. Add pre-chilled HT1 to tube containing 2nM library to result in 20pM library:
    - Denatured library (10µl)
    - Pre-chilled HT1 (990µl)
8. Dilute the denatured 20pM library to 10 to 12pM as follows:
    - 20pM denatured library (375µl)
    - Pre-chilled HT1 (225µl)

Add to MiSeq cartridge.

Curriculum Vitae

For reasons of data protection, the Curriculum Vitae is not published in the electronic version.

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