Assemblage and genetic structure of insectivorous bats in Peninsular Malaysia

Lee Sim Lim
School of Biological and Chemical Sciences
Queen Mary University of London
Mile End Road, London, E1 4NS
United Kingdom

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STATEMENT OF ORIGINALITY

I certify that this thesis, and the research to which it refers, are the product of my own work, and that any ideas or quotations from the work of other people, published or otherwise, are fully acknowledged in accordance with standard refereeing practices in biological sciences. I acknowledge the helpful guidance and support of my supervisors and other advisors in sections dedicated to this purpose. Any views expressed in the thesis without reference to others are those of the author.

Lee Sim Lim
01 February 2012

ABSTRACT

Past climate change and recent human activity have had major impacts on the distribution of habitats as well as the community and population genetic structure of the species occupying these habitats. In temperate zones, glaciation forced many taxa into southern refugia. In contrast, little is understood about the extent to which tropical taxa and habitats were affected by colder periods. In Southeast Asia, some argue that the tropical forest was replaced by savannah at the Last Glacial Maximum (LGM), whereas others suggest that the forest persisted. Studying population genetic and community structure of forest-dependent species in this region may shed light on which of these scenarios is most likely, as well as provide crucial information on the effects of recent habitat loss. To address these issues, I studied the genetic and community structure of forest-dependent insectivorous bat species in Peninsular Malaysia. Data collected at 22 sites indicated that species richness declined with latitude, consistent with post-glacial expansion of forest. To test this further, I undertook mitochondrial DNA sequencing of a widespread species, *Rhinolophus affinis,* and found high haplotype diversity, little phylogeographic structure and no demographic growth. These all suggest a long population history in the region with no post-LGM range expansion. Subsequent microsatellite analyses of *R. affinis* and the congeneric *R. lepidus* showed that genetic distance followed an isolation-by-distance model, and that allelic diversity was unexpectedly higher in the northern populations. Taken together, my results from the community and genetic analyses disagree with each other. These conflicts are perhaps best explained if observed clines in species richness pre-date the LGM. I conclude that there is little evidence of forest contraction in the LGM. The fact that the highest species diversity was detected in the south, which is experiencing the most forest loss due to human activity, has important conservation consequences.

CONTENTS

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CHAPTER ONE

General Introduction

Chapter 1: General Introduction

Climatic oscillations in shaping biodiversity on Earth

Climate change, either due to the long-term natural climatic oscillations or to human activity, can lead to local and regional changes in habitat structure and availability (Rosenzweig et al. 2008). The species occupying these habitats have been observed to show associated phenological, genetic, behavioural and distributional responses to climate change (Parmesan, 2006). Such responses have been implicated in the previous 'big five' mass extinctions, which resulted in severe global species turnover and altered global biodiversity levels (Hewitt, 2000; 2004).

In terms of the impact that natural climate change linked to past glacial cycles has had on populations, the most recent episode, known as the Last Glacial Maximum (LGM), is particularly well-understood. Recent dating studies, based on ^{14}C, ^{10}Be, and ^{3}He, have traced the maximum extent of ice-sheet coverage to 26.5-19 thousand years before present (Ky BP), and suggest de-glaciation of the northern hemisphere occurred around 19-20 Ky BP and that of the southern hemisphere around 14-15 Ky BP (Clark et al., 2009). Since the end of this glaciation and the beginning of the current interglacial period, global land cover has changed considerably with climatic warming (Adams and Faure, 1997). There has been an increase in sea level, with the present level being up to 120m higher compared with that of the LGM, when lower coastal areas were exposed and more land was available for terrestrial species (Yokoyama et al., 2000; Lambeck and Chappell, 2001).

Overall, glacial periods, including the LGM, led to the southern constriction of warmer and humid systems, whereas during warmer periods there have been polar constrictions of cold systems. The extent to which species will be impacted by environmental change will reflect their ecological flexibility (Laidre et al., 2008). While some species might remain in their original habitat, others may fail to adapt, or tolerate, new conditions and will often undergo range shifts to places with more suitable climatic conditions. In worst case scenarios, populations or species can become extinct (Davis et al., 2005). Species that were already regionally constricted, especially in colder regions

such as the poles and mountains, face especially severe stresses and appear to be the first groups to become extinct (Stirling et al., 1999; Wilson et al., 2005).

Although there is much scientific debate regarding the initial triggers and mechanisms causing climate change (Williams et al., 1998; Rind, 2002; de la Fuente Marcos and de la Fuente Marcos, 2004; Donnadieu et al., 2004), recent advancements in technology have revealed that, for at least the last 2 gigayears (Gyr), the Earth has experienced continued fluctuations of temperature, causing ice ages and interglacial periods (de la Fuente Marcos and de la Fuente Marcos, 2004). The theory behind these oscillations is still debated, however, several hypotheses have been proposed, including the eccentricity of the Earth's orbital parameters (Hays et al., 1976; Bintanja and van de Wal, 2008), variations in solar activity (Marsh and Svensmark, 2003), natural variation on the Earth's surface (Hoffman and Schrag, 2002; Donnadieu et al., 2004) and amplification of the orbit cycle that triggers the glacial cycles by positive feedback from carbon dioxide and temperature (Hogg, 2008). The interrelated nature of cycles of water and atmospheric gases, and conditions for sustaining life, mean that climatic fluctuations are of concern to researchers from various fields, ranging from astronomy and geology to biology. Indeed interdisciplinary approaches are needed to trace how past climate fluctuations affected the conditions on Earth, and in turn their impact on biota. Decades of investigation have identified five major mass extinctions in the history of the planet that have been linked to changes in climatic conditions, which occurred around 439 million years ago (Ma) (boundary of Ordovician-Silurian period), 364 Ma (Late Devonian period), 251 Ma (Permian-Triassic period, boundary of Paleozoic and Mesozoic era), 199-214 Ma (End Triassic) and 65 Ma (Cretaceous-Paleogene period, Mesozoic and Cenozoic era) (Erwin, 2001). The last three extinction events were followed by a massive turnover of global biodiversity (Alroy, 2008).

Identification of the exact causes of these mass extinctions is still in progress. However findings to date have been able to link these mass extinctions to various natural events, including direct and/or indirect impacts of volcanic activity (Erwin, 2001; Wignall et al., 2009), as well as other broad scale trends such as global cooling or changes in sea temperature (Jablonski, 1995; Erwin, 2001; Wake and Vredenburg, 2008). The first mass extinction is believed to have been triggered by great fluctuations in sea level due to a period of extensive glaciation followed by dramatic global warming. While the more recent mass extinctions have been attributed to volcanism or

meteor impacts, leading to drastic climatic change and changes in patterns of climatic oscillations (Jablonski, 1995; Erwin, 2001).

Nowadays, many scientists believe we are experiencing the sixth mass extinction, which once again is caused by changes in climate, but on this occasion, these changes are being accelerated by human activity (Martin, 2005; Jackson, 2008). Climatic oscillations normally refer to the fluctuation of temperature on Earth, which affects the humidity, the extent of ice coverage, and sea levels. In addition to experimental evidence demonstrating the impacts of temperature oscillations on individual species, there are also empirical data including shifts in the composition of fungus communities resulting from changing daily temperature cycles (Dang et al., 2009). Longer term trends can also be seen; for example, in changing copepod biodiversity in the ocean (Hooff and Peterson, 2006). Environmental heterogeneity resulting from change can encompass both spatial variation (e.g. local climates and biomes) and temporal variation (including the history of disturbance) (Pearson et al., 1996). Long time frames are typically needed to observe biotic changes; although distribution range shifts in invasive mussels along coastal areas of California were evident over the course of just one decade (Thomas et al., 2009). We can also learn about the likely ability of a species to face future extreme habitat changes by tracing how these species respond to present variation, as well as past climatic fluctuations, for example, as demonstrated for amphibians (Wake and Vredenburg, 2008).

As mentioned above, it is perhaps unsurprising that different species will vary in their sensitivity and response to climate and other habitat changes. Such differences are often reflected by contrasting present and historical broad scale distributions. Historical distribution patterns of species have been inferred from the remains of plant micro- and macro-fossils (Bos et al., 2007; Engels et al., 2008; Pini et al., 2009), from insect remains (Engels et al., 2008) and also indirectly from genetic methods (Hewitt, 1999, Schmitt and Seitz, 2001; Bigg et al., 2008; Davies et al., 2009). Increasingly, former distributions have also been reconstructed from palaeo-climate modeling (Adams and Faure, 1997; Cannon et al., 2009). Recently it was suggested that the impacts of past climatic oscillations on global geographical ranges of mammal species were mediated by selective local extinctions of small-range species during glaciations, and the re-colonization of good dispersers after glacial maxima (Davies et al., 2009). Indeed, in this study it was found that mammal species that faced more extreme temperature

fluctuations in the Quaternary are characterised by larger geographic ranges with wide habitat breadths (Davies et al., 2009). Yet today's even higher rate of climate change is likely to shape biodiversity more rapidly than during natural cycles. By applying dynamic bio-climate envelope models on current global distributions of marine fishes and invertebrates, it has been suggested that the marine biodiversity will undergo a species turnover of around 60% in the next 50 years (Cheung et al., 2009).

Climate, geology and biogeography of Southeast Asia

Southeast Asia is of great scientific interest, in part due to its complex geological and climatic history. The region is mainly encompassed by the so-called Sunda plates (Bird, 2003), which have been uniquely shaped by the active margins of seismicity and volcanism in contrast to the relative stability of the plate interior (Ben-Avraham and Emery, 1973). At different times in the past, the Sunda plates have collided with several surrounding smaller plates, such as Sino-Burma-Thailand Block, Burma Block and Indochina Block, all to the north of Sundaland, as well as other land masses in the east and west of Sundaland (Lee and Lawver, 1995; Hall, 1998; 2002). These complicated geological processes have undoubtedly played an important role in contributing to the hyper-diversity of Southeast Asia (Hall, 1998; Morley, 1999). Specifically, the collision of the tectonic plates resulted in the diversification of four biogeographical zones: Sundaland, Indo-Burma, the Philippines and Wallacea (Conservation International, 2005). Of these regions, Sundaland (the Sunda Shelf) comprises the Malay-Thai peninsula, Sumatra, Java and Borneo.

Located across the equatorial zone (about 20°N-10°S) (Goh, 2005), much of present-day Southeast Asia's land cover is composed of tropical vegetation, and is considered among the oldest rainforests in the world (Olson et al., 2001; Wikramanayake et al., 2002). Climatic conditions show greater seasonality in the continental parts of Sundaland (Myanmar, Vietnam, Laos PDR, Cambodia and Thai mainland), whereas further south in the insular part of Southeast Asia, the conditions are more balanced and aseasonal (Malay-Thai peninsula, Singapore, Borneo, Indonesia and Philippines) (Goh, 2005). In general, throughout the year the climate in insular Southeast Asia is humid (high annual rainfall up to 4000mm per year) and warm (high

mean atmosphere temperature). Insular Southeast Asia has also been defined as a "maritime continent" by meteorologists (Ramage, 1968), attributable to the complexity of its geographic structures as well as the dynamics between oceanic and atmosphere circulation systems that bring high annual rainfall (Ramage, 1968; Chang et al., 2005). In some parts of the region, differences in temperature between continental and insular parts also lead to monsoonal periods (Hastenrath, 1991).

The historical coverage of tropical rainforest in Southeast Asia, including at the time of the LGM, is a matter of debate. Studies of rock sediments also show the probable existence of rainforest tree species since Late Paleocene (60-54Ma) in Kayan, North Borneo, together with other vegetation types associated with coastal and freshwater tropical ecosystems (Morley, 1999). A long history of forest in the region is supported by a number of investigations (Sun et al., 2000; Anshari et al., 2004; Wang et al., 2009); however, at the time of the LGM, many authorities have argued for a drier climate than at present, with a more savannah-like habitat (Gathorne-Hardy et al., 2002; Wurster et al., 2008), or a mix of habitats in which tropical forest showed a limited distribution due to the lower temperatures and rainfall (Heaney, 1991; Aide and Rivera, 1998; Meijaard, 2003; Bird et al., 2005). Two recent high profile studies illustrate these conflicting claims. First, Cannon et al. (2009) combined distribution modelling of forest trees with data on past climates to argue that the Sunda Shelf was actually covered by humid forest earlier than the LGM, and reached its maximum coverage during the LGM when the sea level was at a minimum. The authors showed that the majority of present lowland Peninsular Malaysia was covered by semi-green, seasonal and transitional hill forest, whereas lowland evergreen rainforest was constricted towards lower and coastal areas in Sumatra, the southern part of Peninsular Malaysia, coastal Borneo and in the exposed shelf in the east (Cannon et al. 2009). If correct, this finding would mean that the rainforest in Sundaland can be considered to be in a refugial state at the present time. During this period, sea level was +56 m than present day (Miller et al., 2005). Indeed the latest pollen data have also supported this, suggesting that at the time of the LGM in the region, temperatures were cooler but humidity was not significantly lower, and that rainforest persisted in both lowland and montane areas (Wang et al., 2009). In the second study, authored by Wurster et al. (2010), stable isotopes from the accumulated guano of bats and birds in caves was analysed, and used to infer a completely different scenario. From caves in central Peninsular Malaysia, guano biomass from 35 Ky BP until 16 Ky BP was found to contain mostly C_4, indicative of

the presence of open savannah, as opposed to forest that has a profile of C_3. Consequently, the authors state that there was "a substantial forest contraction during the Last Glacial Period on both Peninsular Malaysia and Palawan, while rainforest was maintained in northern Borneo" (Wurster et al. 2010).

In addition to potentially influencing the ranges of populations and species via direct impacts on habitat and vegetation coverage, historical climate change will also lead to fluctuations in sea levels that, in turn, can interrupt dispersal and gene flow. Unlike temperate zones, equatorial Southeast Asia was not directly affected by ice during past glacial periods. However, in tropical zones, changing sea levels due to the sequestering of water as ice in the poles had an arguably more important role in shaping ecological and evolutionary processes (Woodruff and Turner, 2009). Sea levels fluctuated widely; at their lowest level they were approximately 120 m below the present level, and they stayed between 30 to 40 m below present sea levels for more than half of the glacial cycle (Voris, 2000; Hope, 2005). It is also known that sea levels increased very rapidly: first, 20-22m soon after the LGM (Hearty and Kaufman, 2000) and then around 16m within just 300 years at around 14.6 to 14.3 Ky BP (Hanebuth et al., 2000). During these low sea level periods, the exposed lower area of the Sunda Shelf created land bridges among the islands and mainland and caused the region to be dissected and rejoined repeatedly. These land bridges may have enabled faunal exchange across the Sunda Shelf (Hewitt, 2000; Gorog et al., 2004). Therefore, sea level changes in Southeast Asia, particularly the Sunda Shelf, are believed to have influenced the dispersal processes of terrestrial species, because during times of high sea levels, the flooded areas then served as significant geographical barriers for dispersal, thus promoting divergence (Woodruff and Turner, 2009).

In summary, the complicated tectonic structure, interactions among the maritime continent and atmosphere, and recurrent fluctuations in sea levels have all contributed to the formation of the region's current distinct and high level of biodiversity (Voris, 2000). For example, Morley (1999) pointed out three geographical events that facilitated the moulding process of floral diversity: first, the collision of different tectonic plates into the region brought along different tropical floral species into the region and allowed dispersal and intermixing extensively among these plates; second, the formation of new land masses during the tectonic plate collision with the high sea levels during interglacial cycles acted as an efficient barrier for terrestrial species which

led to an increase in the rate of formation of endemic species on islands; thirdly, the isolated setting of the region also permitted the survival of the primitive species since the early stages of rainforest formation by reducing competition (from invasion) or radical climate change (extreme continental climatic conditions).

Human activity and biodiversity loss in Southeast Asia

Apart from the effects of long-term gradual environmental change (e.g. glacial episodes and sea level fluctuations) on biodiversity, human activity has also had major impacts, leading to modification of the structure and function of ecosystems and the wildlife assemblages they support (White and Pickett, 1985). In fact such anthropogenic disturbance has taken a far shorter time to create a similar amount of change: within the past two centuries, the scale of human impacts in the region has dramatically increased, leading to alteration and fragmentation of the previously large and continuous tract of natural rainforest. Of all tropical regions, Southeast Asia is known to have the highest current rates of destruction in the world (Achard et al., 2002): it is predicted that 75% of its primary forest and 42% of its biodiversity will be lost, by the end of the century, putting the region in a critical status in terms of its unique endemic biota (Sodhi et al., 2004). Plant, bird and mammal diversity have all been negatively affected by logging, forest fragmentation and large-scale monoculture agriculture (Sodhi et al., 2004), while the impacts of these activities on amphibians and reptiles are less well understood but are subject to ongoing studies (Sodhi et al., 2010a).

Despite the impacts of biodiversity loss of the scale seen in Indo-China, this topic has generally been under-studied. In Thailand, there is good evidence for disturbance of seasonal evergreen rainforest for the past 250 years, with documented small populations of secondary long-lived tree species and irregular canopy size distributions of common tree species (Baker et al., 2005). Probably the worst scenario can be seen in Singapore, where massive development since the beginning of colonial history has resulted in large-scale forest clearance on the island, causing a total of 95% of forest loss (Lane et al., 2006). Recently, a comparison between disturbed forest in Singapore and some undisturbed forest in the southern end of Peninsular Malaysia, found significant changes in dung beetle species diversity, probably due to anthropogenic influences and, to some extent, the island effect (Lee et al., 2009).

Further studies on the regional biodiversity are needed to improve our understanding of the sustainability of the biota, and how these are affected by the joint processes of climate change and habitat modification (Sodhi et al., 2010a; Chazdon et al., 2009). Such research can also help to inform aspects of conservation management, including logging and plantation practices, which are necessary steps for achieving conservation goals for Southeast Asia's biodiversity (Sodhi et al., 2010b).

Habitat fragmentation, degradation and local microclimate change

Current habitat change is the major conservation concern for specialist tropical species, whereas anthropogenic climate change is often viewed to be the principal threat to global biodiversity (Thomas et al., 2004). Therefore, regions such as Southeast Asia are considered to be particularly vulnerable because they face both types of threat. Moreover, the potential loss of intact rainforest in Southeast Asia is even more alarming in light of evidence indicating it might already be in a refugial stage, so much reduced from the former larger distribution at the time of the previous glacial maximum (Cannon et al. 2009).

As well as the net loss of forest, habitat modification in Southeast Asia has involved extensive fragmentation of the remaining forest. Although fragmentation is sometimes a natural process arising from events such as flooding or hurricanes, it is more commonly the result of human activity (Andren et al., 1997; Frankham et al., 2002). Forest fragmentation occurs when continuous forest is converted into several smaller pieces, a process that involves a reduction in patch size and an increase in the distance and thus level of isolation among remaining habitat patches. Of particular concern is that habitat fragmentation increases exposure to habitat edges (so-called 'edge effects') that may alter several aspects of the newly fragmented ecosystem, included the local climate (Lovejoy et al., 1986; Laurance et al., 2006). Indeed the local microclimate of patches is often characterised by higher temperatures and lower relative humidity (Dreistadt et al., 1990) and these effects are further promoted by extreme weather conditions such as in times of drought (Laurence et al., 2001). The nature of the microclimate is important in determining the position of the edge-interior boundary of a habitat patch, along with its vegetation composition and other local conditions (Bender et al., 1998).

The process of fragmentation leads to the formation of new habitats among gaps (matrix), so leaving a mosaic landscape (Andren, 1994). The habitat characteristics of fragments and areas of the matrix may vary depending on their size and level of disturbance. Generally, areas of disturbed forest will retain more humid microclimates when they are surrounded by primary forest (Avendaño-Mendoza et al., 2005). Such habitat modification can affect species composition and diversity across the landscape. In fact, fragmentation can even lead to higher species diversity due to the increased numbers of habitat types in the landscape. For example, Laidlaw (2000) reported higher mammal species richness or abundance in disturbed forests than protected ones. Nonetheless, it is also clear that excessive disturbance and habitat heterogeneity can cause the loss of species that are sensitive to environmental change (Pearson et al., 1996). In fact, forests can take many years to recover from disturbance, with one study showing that tree canopies were affected by logging for over 40 years after it had been stopped (Okuda et al., 2003).

In Southeast Asia, a major contribution to fragmentation of forest has been the rapid expansion of agricultural land for commercial production of oil palm and other cash crops (Fitzherbert et al., 2008). This type of land conversion contrasts with smaller-scale forest clearance that occurs in more traditional agricultural practices such as shifting cultivation, and when unsustainable can also result in the formation of savannah and secondary forest (Bogaert et al., 2008). Oil palm monocultures are structurally less complex and support fewer species than tropical forests (Fitzherbert et al., 2008) and as such, represent an inhospitable matrix to forest-interior species. Where matrix habitats are barriers to dispersal, over time fragmentation can lead to reduced gene flow across the landscape and, this in turn is associated with the loss of genetic diversity and inbreeding depression, which may reduce the capacity of populations to adapt to future change (Frankham et al., 2002). Such consequences are important considerations when planning strategies and policies for wildlife management.

Insectivorous bat diversity in Peninsular Malaysia

Within Southeast Asia, Sundaland is a biodiversity hotspot and this high diversity is well reflected in the region's bat fauna, which comprises over 330 reported species (Simmons, 2005; Kingston et al., 2006; Kingston, 2010). Bat species in the region are currently classified into nine families, eight of which represent insectivorous bats (Francis, 2008). In Peninsular Malaysia, the insectivorous bat fauna is among the richest and highest density in the world (Kingston et al., 2003; Yoshiyuki and Lim, 2005; Kingston et al., 2006; Francis et al., 2007). Many of these species live in the highly cluttered environment of the tropical forest interior, whereas others are adapted to forest gaps, vegetation edges and the open spaces surrounding forests.

A high proportion of the insectivorous bats present in Peninsular Malaysia belong to the Old World families of Rhinolophidae and Hipposideridae, and to two Old World subfamilies of the family Vespertilionidae: the Murininae and Kerivoulinae. The bats from these groups are characterized by ecomorphological traits that result in slow flight in highly cluttered environments, and sensory signals for hunting in clutter (Kingston et al., 2003). They are thus highly adapted to life in the forest interior, as supported by distribution data from Southeast Asia (Kingston et al., 2006; Francis, 2008; SAMD, 2009). These insectivorous species play a major role as nocturnal insect predators; every night, one insectivorous bat is estimated to consume a minimum of half their body-weight in insects, while a large colony may consume up to 2,000 tonnes of insects per night (Kingston et al., 2006).

Like most of the mammal species in the region, insectivorous bats are facing habitat threats. The negative impact of deforestation on Sundaland's bats has been demonstrated by the loss of bat fauna in Singapore due to rapid development over the past 50 years. Here, massive deforestation has been linked to the loss of 38% of the island's insectivorous bat diversity (Lane et al., 2006), as well as 34-87% of butterflies and vertebrates (Brook et al., 2003). In spite of the recent rediscovery in 2009 of *Hipposideros bicolor* and a new record of *Kerivoula hardwickii* (Leong and Lim, 2009), the situation for Singapore's bats is a long-term concern. In central Sumatra comparison of bat assemblages across habitat types revealed very few species in rubber and oil palm plantations compared to forests (Danielsen and Heegard, 1995). Fukuda et al. (2009) also found that, with the exception of three Old World fruit bat species that use orchards

as a primary food source, many bat species seldom feed in agricultural lands. Other research on the bat assemblages in Pahang state in Peninsular Malaysia reported declines in insectivorous bat abundance and diversity in small forest fragments (Struebig et al. 2008).

Apart from a few notable exceptions, such as those described above, there have been very few investigations into the impacts of environmental change on bats of the Asian and African Old World tropics. This is unfortunate given that Sundaland and, in particular, Peninsular Malaysia, is both a centre of Old World bat diversity and has also experienced dramatic habitat modification due to historical climate change, and more recently due to human activity. In recent years, records of bats and other mammals from this region have been collated into a Southeast Asia Mammals Databank (SAMD), which together with field guides (Medway, 1982; Kingston et al., 2006; Francis, 2008) and taxonomic revisions (Csorba et al., 2003; Simmons, 2005) has aided research (see SAMD, 2009). However, conservation initiatives would still benefit from longer-term monitoring studies of mammals, of the sort that are commonly practiced in Europe and America. In particular, monitoring would help to determine the differential responses of Old World bat species to habitat change.

With such little work conducted in Southeast Asia, some of our understanding of the potential vulnerability of tropical forest bats to habitat loss and fragmentation has come from bat work in the better-studied Neotropical bat assemblages (Cosson et al., 1999; Law et al., 1999; Estrada and Coates-Estrada, 2002; Gorresen and Willig, 2004). However, it is important to note that there are fundamental differences in the sensory and morphological characters of Neotropical versus Palaeotropical forest bats; the former are often frugivorous and are better adapted for flying over longer distances. In contrast, the Old World bats use either flutter detection or whispering echolocation calls to find insect prey in clutter, and are poorly adapted to disperse over open spaces. Therefore, there are good reasons to suspect bats from Southeast Asian forests will be especially susceptible to the unparalleled rate of forest fragmentation.

Project aims and objectives

This project aimed to characterize the community and population genetic structure of forest-interior bat species in Peninsular Malaysia, in order to determine the impact of both past climatic fluctuations and on-going human-induced habitat change on bat populations. It was anticipated that the results would provide indirect evidence on whether the forest of Southeast Asia has a long history, or has expanded in area since the Last Glacial Maximum (LGM). In addition, I aimed to assess whether local forest conservation management will safeguard current levels of biodiversity in Peninsular Malaysia in the face of future threats. The main objectives were as follows:

i. Characterize the broad scale patterns of species diversity and assemblage structure in forest-interior bats across Peninsular Malaysia. Determine whether site-wise assemblage structure relates to geographical distance, and also whether species diversity declines with latitude, as expected if the forest has undergone a post-LGM expansion.

ii. Reconstruct the phylogeographic and demographic history of the most widespread bat species, *Rhinolophus affinis* across Peninsular Malaysia, to assess whether this species has undergone recent population growth consistent with expansion of the forest, or whether it appears to have a long and stable history in the region.

iii. Characterize the population genetic structure of two related forest-interior bat species, *Rhinolophus affinis* and *Rhinolophus lepidus* to assess the pattern and nature of gene flow. Also, to assess whether genetic diversity in these taxa declines with latitude (as would be consistent with forest expansion) and thus whether species and genetic diversity are correlated.

CHAPTER TWO

Broad scale patterns of assemblage structure in insectivorous bats in Peninsular Malaysia

Chapter 2: Broad scale patterns of assemblage structure in insectivorous bats in Peninsular Malaysia

Chapter summary

Understanding geographical patterns of assemblage structure can provide information on past ranges, the impact of past habitat change, as well as the conservation implications of current and future habitat change. Here I undertook the most detailed study to date of mammalian assemblage structure in Peninsular Malaysia, focusing on bat species that are critically dependent on the forest interior. Tropical forest in Malaysia contains the highest bat diversity of anywhere in the Palaeotropics, however, whether or not this forest has contracted or expanded since the Last Glacial Maximum is debated. Bat surveys were taken across Peninsular Malaysia and supplemented with published data. The assemblage structure was constructed both at the level of sites (α-diversity in terms of species richness, abundance) and at the level of dissimilarity of species between sites (β-diversity). Results indicated that bat assemblages were consistently dominated by six cave roosting species from the families Rhinolophidae and Hipposideridae, while another 16 captured species were classified as rare in this study. Species richness decreased with increasing latitude, consistent with hypothesised northern shift and expansion of tropical rainforest species since the Last Glacial Maximum. Analyses of β-diversity showed that differences between communities were not related to geographical distance, although there was evidence of greater differences in species numbers between the most distant sites. *Rhinolophus affinis* was the most dominant species across Peninsular Malaysia and variation in the abundance of this species among sites correlated with overall patterns of assemblage similarity. Greatest bat diversity was recorded in areas that are undergoing the most intensive forest loss, highlighting conservation priorities.

Introduction

Biogeographical factors shaping assemblage structure

Studying patterns of community or assemblage structure can help us to understand how past events have shaped existing levels of biodiversity, as well as allow us to predict the impact of environmental change on species survival in the future. Species responses to environmental change can be physiological, ecological and/or behavioural, and the capacity of species to respond may affect their adaptability and thus long-term survival (Lichatowich and Mobrand, 1995; Wiens and Donoghue, 2004; Hillerbrand et al., 2008).

Most rapid environmental change is typically due to non-sustainable human activities such as construction, agriculture, logging, mining or war (Millemium Ecosystem Assessment, 2005). These anthropogenic alterations cause changes in the landscape, often reducing the availability of natural habitats. Massive change within a short timeframe imposes stress on communities, potentially leading to a loss in species diversity due to species emigration and extinction events. Large-scale anthropogenic alterations can also result in changes in species dominance and evenness, which often occur more quickly than changes to actual species richness (Chapin et al., 2000). Indeed a recent review revealed that human-induced environmental changes commonly led to increased regional dominance and thus declines in the variation in species diversity among sites (beta diversity) (Hillebrand et al., 2008).

Broad clearance of land for agriculture, or linear clearings formed by human construction, such as highways or wide logging roads, will divide large continuous natural habitat into smaller fragments, which may affect local biodiversity either positively (Ferris, 1979) or negatively (Tija, 1988; Laurance et al., 2009; Pohlman et al., 2009). The affected ecosystem then faces scale changes in patch size, shape and vegetation structure. Island biogeography theory suggests that larger patches of habitat support higher species richness (MacArthur and Wilson, 1967). However, elements of landscape variation due to fragmentation, including shape and edge effects (patch shape index), are also important factors in determining species diversity (Barbaro et al., 2005). Indeed, low interior-to-edge ratios in fragments are of greater benefit to so-called edge species (species that primarily live near the perimeter of a landscape (*sensu* Forman,

1995)) than to interior species (species that live primarily away from the landscape perimeter in the interior of the landscape patch (*sensu* Forman, 1995)). Whether or not fragments will suffer long-term declines in interior species will be partly determined by the strength of neutralising source-sink effects, which can allow species to persist in unsuitable sites if there are sources of colonisers nearby (Hanski, 1999). In other cases, fragments might form foraging sites for assemblages of mobile species, as found in the case of Neotropical bats assemblages in Panama (Estrada-Villegas et al., 2010).

In addition to environmental change caused by human activities, assemblage structure will also be affected by natural events, particularly over longer time periods. It is proposed that climatic fluctuations have been especially important in shaping biodiversity (Erwin, 2009). Efforts to reconstruct past and present climates, habitats and species distributions have been intensively undertaken in recent years (Walther, 2000; Walther et al., 2002; Kostopoulous et al., 2007). Several hypotheses have also been formed to explain global biodiversity patterns, including the latitudinal gradient of species richness (Willig et al., 2003) and metabolic theory of ecology (Allen et al., 2002). Of these, latitudinal effects are probably the longest recognised correlates of species richness (Willig et al., 2003).

Observations of latitudinal gradients in biodiversity levels have been noted in numerous studies; in fact, even Darwin and Wallace suggested that more species were found at warm and humid equatorial zones than at the poles (Willig et al., 2003). In the 1960s, the recognition of this natural global vegetation-climatic-latitudinal pattern led to the proposal of the Holdridge life zones, which define the distribution of natural vegetation or habitat based on three climatic factors: precipitation, heat and humidity (Holdridge, 1967). This climatic latitudinal gradient, along with dispersal limits and/or niche width differences among taxa, are all expected to contribute to decay in community similarity with distance on a north-south axis (Nekola and White, 1999). Others such as Monjeau et al. (2009) have attributed increased diversity in the tropics to either an energetic gradient (i.e. amount of energy per surface unit), or to a simple artifact of the greater land area at lower latitudes compared to at temperate or polar regions (Rosenzwig, 1992). Regardless of these explanations, recorded patterns of diversity do not always follow predictions (Clarke and Lidgard, 2000; Krystufek and Griffiths, 2002; Lambshead et al., 2002; Carranza et al., 2009).

An additional important explanatory factor for greater diversity at the topics is long-term climatic stability (stability hypothesis). Unlike temperate regions, the wet lowland tropics did not experience glacial or tundra conditions during glacial maxima (Cannon et al., 2009), and have therefore had longer to accumulate species and genetic diversity (although as mentioned the extent to which forest persisted is open to question). In contrast, in temperate zones some species have never colonized or re-colonized following de-glaciation so depressing diversity at these latitudes and contributing to the global gradient (Yalden, 1982; Hewitt, 1999; Sommer and Zachos, 2009). Regardless of the exact climatic conditions in tropical areas during the Pleistocene, it is clear that sea level changes have had lasting impacts on biodiversity. For example, a study of mammal distribution data for the Malay-Thai Peninsula showed that apart from the expected latitudinal gradient in species richness, there was also a species-area relationship (Woodruff and Turner, 2009). In this study, distribution range limits indicated that more mammal species (including bats) occur at the wider parts of the peninsula where land surface area is greater: at 5°N (north Peninsular Malaysia) and 14°N (northernmost peninsula in Thailand that joins with Asia mainland). These findings suggested that current mammal distribution patterns are shaped by the historic drastic and repeated sea level fluctuations (see Chapter 1). Major sea level rises of 120m after the Last Glacial Maximum (LGM) caused a shrinking of approximately half the area of the Malay-Thai Peninsula since the LGM, and might have led to local extinctions in these narrower areas.

Species diversity and assemblage structure

Biodiversity comprises three hierarchical components: genetic diversity, species diversity and ecosystem level diversity (World Climate Monitoring Centre, 1992; Gaston and Spicer, 1998). Studies of species diversity have included analyses of local species richness (the number of species in a community (Dyke, 2008), abundance (total individuals of a species in a defined region) (Krishnamurthy, 2003) and evenness (equitability of different species existing in a defined region, relative to the species abundance of the region) (Krishnamurthy, 2003) and, finally, assemblage and community structure analysis. With extensive research on this topic, confusion has arisen regarding some of the community ecology terms such as community, assemblage and ensemble. These terms have been reviewed and redefined by Fauth et al. (1996) and

also summarised by Magurran (2006). According to these authors, a *community* refers to all the taxa that occur in the same place at the same time regardless of phylogeny or resource use. An *assemblage* is part of the community in which phylogeny is restricted, and an *ensemble* is a sub-component of an assemblage that includes species with similar resource use.

Analyses of species diversity consider both species richness and abundance in an assemblage or community. Species abundance measures determine the importance of particular species in assemblages whereas species richness treats all species equally regardless of abundance (Magurran, 2006). It has been argued that communities with greater species evenness (i.e. low dominance) are better at responding to environmental constraints (Norberg et al., 2001), and allow greater stability of richness across temporal fluctuations (Doak et al., 1998). However, the effects of species evenness in ecosystems have been overlooked compared to those of species richness (Hillerbrand et al., 2008), possibly partially reflecting the greater effort needed to estimate evenness. Also, estimating species richness is considered important for understanding the impacts of past, present and future changes in habitat availability. Obtaining repeated measures of biodiversity over time can thus be useful in tracing environmental events or factors of ecological or conservation importance (Green et al., 2009).

In addition to quantifying diversity at a site or within an area (termed 'alpha diversity'), ecologists are also interested in differences in diversity between areas (termed 'beta diversity') and the total diversity in a region ('gamma diversity') (Lande, 1996; Magurran, 2006). Beta diversity can provide information on the dynamics of assemblage turnover and structure across sites (Whittaker, 1960). Variation in assemblage structure across space and time in natural conditions can in turn improve our understanding of the consequences of habitat modification and disturbance (Kingston, 2009). Some have modified the beta diversity definition, to incorporate comparisons at different spatial scales, with proposed measures of local beta diversity, beta diversity and delta diversity (Willig et al., 2003).

Bat assemblage studies in Peninsular Malaysia

Peninsular Malaysia is recognised as the centre of a hotspot of Old World bat diversity with more than 120 species recorded (Simmons, 2005; Kingston et al., 2006; Kingston, 2010). The Krau Wildlife Reserve in Pahang, in the centre of the territory, has a reported alpha diversity of >70 species, which is greater than anywhere else in the Palaeotropics (Kingston et al. 2003), and is also hosts the best studied bat fauna of anywhere in Southeast Asia. Long-term monitoring of insectivorous bats at Krau has taken place at five permanent study grids in the reserve (Kingston *et al.*, 2006). Bat assemblages in forest fragments surrounding the reserve have also been described by Struebig et al. (2008; 2011), who showed that some large forest patches hold greater bat species richness and abundance at standardized sample sizes than the study plots in Krau. Struebig and colleagues have also focused on examining the influence of limestone karst on local assemblage structure, and reported that two species (*R. affinis* and *R. lepidus*) dominate bat assemblages up to 11 km from major cave roosts (Struebig et al., 2009). This series of studies in and around Krau make central Pahang the most well studied region of Peninsular Malaysia for bats. Comparable (albeit slightly lower) levels of bat diversity are also known from Ulu Gombak approximately 50km away, in Selangor (Heller and Volleth, 1995).

Currently there are no published data on bat assemblage structure from elsewhere in Peninsular Malaysia. Therefore, it is not known whether forests to the north and south of Krau support the same levels of bat diversity, and whether there are clines in species richness over the peninsula. Based on expectations of latitudinal gradients of species diversity in other areas, and the proposed shift of humid tropics towards lower areas of exposed Sunda Shelf during LGM (Gathorne-Hardy et al., 2002, Meijaard, 2003; Cannon et al., 2009; Wurster et al. 2010) (see Chapter 1), assemblage structure might be expected to vary across the peninsula, with higher species richness towards refugial areas towards the equator. Given the importance of the Malaysian peninsula for regional bat diversity, gaining a greater understanding of patterns of diversity will also be important for developing effective conservation priorities in this country.

Study objectives

The objectives for this chapter were as follows:

i. To undertake surveys of bats across Peninsular Malaysia in order to yield richness and abundance data as well as collect samples for genetic analyses (Chapters 3 and 4).

ii To describe patterns of insectivorous bat assemblage structure and diversity in the forests of Peninsular Malaysia, and determine whether there is any clinal pattern of species richness as expected if forests have expanded, or whether forest specialist species are found evenly across the region.

iii. To identify the main species that shape these patterns, including characterisation of rare and common species.

Methods and Materials

Characterisation and determination of study forest sites

Using maps of forest cover in 1997 supplied by the Department of Agriculture Peninsular Malaysia, I selected 22 rainforest sites distributed across Peninsular Malaysia for sampling of bats (Figure 2.1).

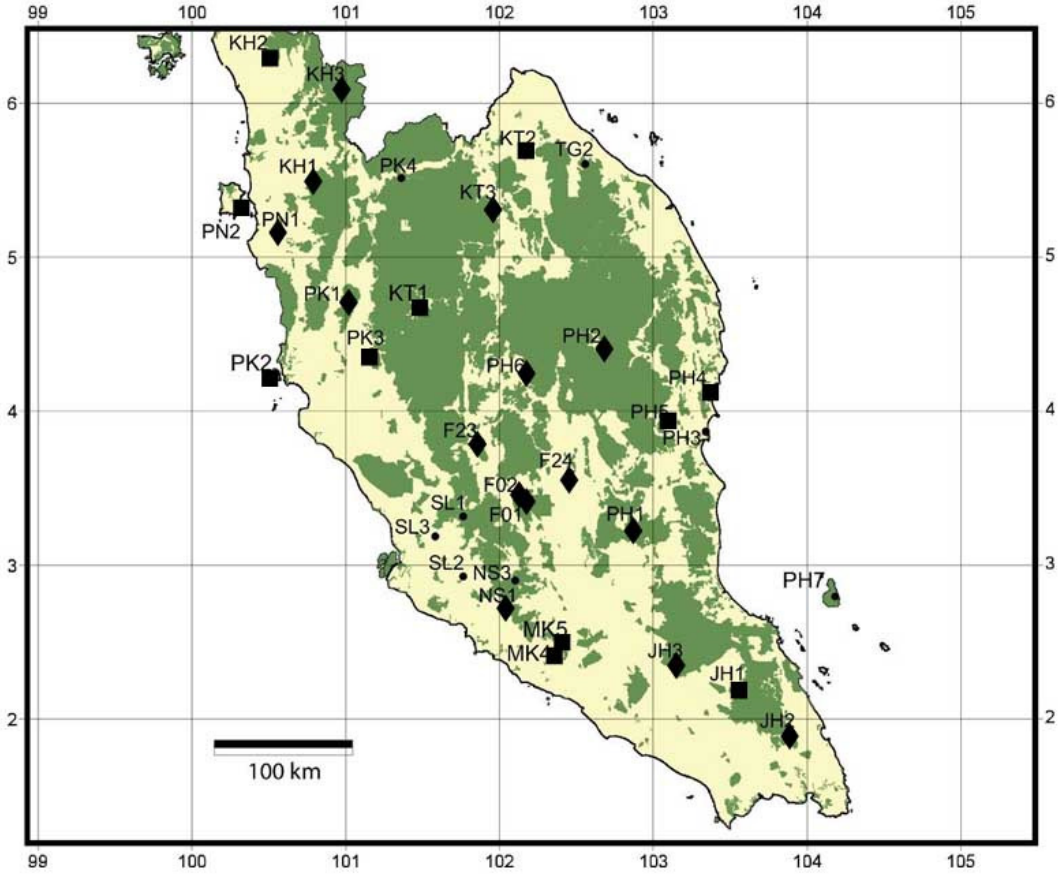


Figure 2.1

Map of Peninsular Malaysia showing sampling sites and the coverage of lowland tropical rainforest in 1997 as green areas (Forestry Department of Peninsular Malaysia, 1997). Sites for which tissue samples were obtained from third-parties but for which no capture records were available are shown as circles. Sites for which bat assemblage data were collected are shown as diamonds and sites for which bat assemblage data were not analysed due to either low capture rates or non-standard trapping methods are shown as squares.

All sites were in continuous forest or large fragments of >1000 ha and were categorised as lowland evergreen, Dipterocarp, tall forest (Adams and Faure, 1997). Sites were located at lower than 300m above sea level, which is the reported maximum elevational extent for the local lowland Dipterocarp evergreen rainforest (Vincent and Yusuf Hadi, 1993). All sites had been logged once within the last 30 years, but were now either fully protected for recreation and tourism, or managed for logging or mining (Table 2.1). The local landscape varied for each site due to geographical factors and also the type of human activities in the area, so for assemblage analyses forests were selected if they were of sufficient area and quality to be comparable with one another. A cut-off of 1000 ha was used because previous work showed that bat assemblages in forests of this size are similar in structure to those in undisturbed forest (Struebig et al., 2008). The potential influences of contemporary forest fragmentation on bat assemblage structure were therefore minimized for the final set of sites analysed, meaning that patterns in assemblage structure detected could be attributed to geographical and historical influences.

Site area was characterised using ArcView version 3.2 and the geographical distance between selected sites was calculated using GenAlEx version 6.0 (Peakall and Smouse, 2006) based on coordinates recorded by a Garmin V GPS in decimal degrees.

Bat capture and species identification

Field work was undertaken between 2007 and 2009. In order to avoid monsoonal seasons or other wet weather that can bias captures and thus affect species composition and sample sizes (see Kingston, 2003; Zortéa and Alho, 2008), I only captured bats between March and September, which is the drier season. Target species were insectivorous bats that forage or roost in cluttered forest under storey. Following Kingston (2006) and Francis (2008), bats were categorised into three main ensembles based on the level of vegetation clutter in their foraging environment: species that forage in the cluttered forest interior; those that forage around forest edges or gaps between cluttered areas; and bats that forage in open spaces. Four-bank harp traps (Figure 2.2) were used to capture bats. This trapping method has proven effective for capturing Old World forest bat species compared to mist nets which are widely applied in bat surveys in the New World (Francis, 1989). However, mist nets and hand nets

were also used on certain occasions where harp traps were not practical, mainly to obtain tissue samples for genetic analyses (Chapters 3 and 4). Individuals captured by mist nets or hand nets were not included in these analyses due to the different capturing method.

Figure 2.2 A four-bank harp trap set across a stream for this study. Parallel vertical fishing line is undetected by the bats, which fall and roost in the cloth bag beneath.

All four-bank harp traps were set across old logging skids, hunting trails, or streams, all of which are potential foraging routes for bats. The number of traps set depended on the number of available trapping positions, geographical and weather conditions and accessibility of the forest site. Between two to eight traps were set per trapping night, which were set between 1400 and 1800 and checked twice, at 2200 and at 0700. During poor weather conditions (i.e. heavy rain) or when forest access was considered dangerous, the traps were – where possible - taken down before dark, or were otherwise left up but only checked the following morning. The sampling period for each site was 3-14 days. A minimum of seven trapping days was conducted for each site.

Individual bats were identified immediately using the key by Kingston et al. (2006) and Francis (2008). External measurements (e.g. length of forearm, tibia and tail, as well as body mass) were taken and then the bats were released near to the capture points within 12 hours of processing. On occasions where identification was uncertain, detailed remarks were recorded and photographs were taken for future reference.

Table 2.1 Details of 26 sampling sites, comprising 22 visited in this study and 4 surveyed by Struebig (2008). In total 15 were used for community structure analysis. Details given are site location, land use of surrounding areas, management regime of each site, and protection status. Observed total capture species (S), total capture individuals (N) and total harp trap nights (HTN) are reported.

Site Code	Surveyed site	Surrounding land use[a]	Major human activity[b]	Protected Status[c]	S[d]	N[e]	(HTN)
JR1^	Lenggor						
	103.586°E, 2.186°N	O,F	L	M	12	99	41
JR2	Gunung Panti, Johor						
	103.914°E, 1.869°N	O,F	R,L	M	20	481	51
JR3	Labis Forest Reserve, Johor						
	103.159°E, 2.346°N	F,O,V	R	M	19	528	7
KH1	Bukit Hijau, Kedah						
	100.773°E, 5.501°N	O,R,V	R	M	15	162	6
KH2^	Wang Hill						
	100.484°E, 6.319°N	V,G	R	M	14	68	32
KH3	Ulu Muda, Kedah						
	100.963°E, 6.107°N	F,D	R	F	7	217	3
KT1^	Lojing Highland						
	101.486°E, 4.674°N	V,F,G	R,A	M	14	79	14
KT2^	Temangan Hill						
	102.168°E, 5.695°N	C,G,V	N	F	18	82	45
KT3	Gunung Stong, Kelantan						
	101.977°E, 5.340°N	O,R,F,V	R,L	M	15	123	8
MK4^	Senggeh Hill						
	102.383°E, 2.383°N	G,V,O	N	M	11	66	N/A
MK5^	Batang Melaka						
	102.417°E, 2.467°N	G,V,O	N	M	5	6	N/A
NS1	Gunung Angsi, N. Sembilan						
	102.078°E, 2.705°N	O,R,F,V	N	M	16	171	N/A
PH1	Bukit Ibam, Pahang						
	102.901°E, 3.223°N	O,C	L,M	M	19	195	24
PH2	Gunung Aais, Pahang						
	102.681°E, 4.413°N	F	L	M	21	382	21
PH3^	Beserah						
	103.357°E, 3.861°N	G	N,R	M	11	82	10
PH4^	Balok						
	103.362°E, 4.127°N	C,G	L	M	5	6	4
PH5^	Chalas						Hand
	103.033°E, 3.917°N	O	R	M	8	460	net
PH6	Forest Kenong						
	102.188°E, 4.216°N	F,O	N,R	F	17	694	15
PK1	Kledang Saiong, Perak						
	101.004°E, 4.538°N	O,G	R	F	14	131	26
PK2^	Pangkor Island						
	100.555°E, 4.220°N	G,F	R	F	13	375	N/A
PK3^	Bujang Melaka						
	101.176°E, 4.379°N	G,V	R,L	M	15	94	24
PN1	Bukit Panchor, Penang						
	100.546°E, 5.151°N	O,G,V	R	M	13	177	N/A
F01*	Kemasul 1						
	102.183°E, 3.383°N	A,O	N/A	M	15	137	28
F02*	Kemasul 2						
	102.133°E, 3.433°N	A,O	N/A	M	16	220	40
F23*	Klau Besar						
	101.890°E, 3.749°N	O,R	N/A	M	16	358	19
F24*	Jengka						
	102.455°E, 3.615°N	O,R	N/A	M	13	104	13

a. Land-use surrounding the sampling site: A, Acacia plantation; O, oil palm plantation; R, rubber plantation; C, cleared land; F, forest; G, mixed gardens with villages; V, vegetable and fruit plantation; D, dam; C, cleared land. Classes follow Struebig (2008).
b. Main human activity in the sampling site: N, low activity; R, recreational and tourism; L, logging; M, mining; A, Agriculture.
c. Protected status based on the forestry department: F, fully protected from logging or mining, and minor tourism; M, managing for logging, mining and tourism.
d. Total captured number of individuals per site.
e. Total captured species per site
* Sampling sites data obtained from Struebig (2008).
^ Surveyed sites excluded from the assemblage analysis due to low sample number or non-standard capture method

Analytical design

From the 22 sites that I surveyed, 11 sites distributed from south to north of the peninsula were used for detailed species assemblage analysis (Table 2.1). Sampling sites were excluded if they did not follow a standardised harp trapping protocol and yielded fewer than 100 captured forest-interior bats (Kingston et al., 2003). At some sites, where the number of bats captured was very high, it was necessary to release individuals of some common species immediately after identification without taking detailed measurements. This was done to prevent stress to the animals where manpower was limited. In these cases, numbers of individuals were recorded based on a 25-individuals scale (e.g. 25, 50, 75, 100, 125 individuals released). For statistical analyses, capture data were further filtered so that only forest-interior bat species were used. The 11 datasets were augmented with published data from four additional large forest fragments (Struebig, 2008) to give a total of 15 sites.

In order to identify the composition of the bat assemblages prior to analysis, a Fisher's plot was constructed. The purpose of this plot is to draw attention to the large proportion of rare species in an assemblage (Magurran, 2006). The cut off point for rare species in this study was set to describe species that represented less than 1% of total captured individuals of all species. Assemblage structure was investigated at the level of sites (α-diversity) and the dissimilarity of species between sites (β-diversity) in terms of species richness, abundance, diversity and similarity.

Site species richness, diversity and species abundance

The accuracy of species richness is very sensitive to sample size; as the number of individuals sampled increases, the higher the possibility for most of the species in a community to be detected (Nicholas and Robert, 2001). Due to time and resource constraints, sampling effort for each site was limited to a maximum of two weeks (Chapters 3 and 4). Consequently, the number of bats captured at each site varied (Table 2.1). For this reason, and to minimise potential sampling biases, species richness for a standard sample was estimated using rarefaction. Two methods were used, the first of which involved rarefying the total captured individuals of each site down to the minimum number of individuals (104 bats) using sample-based species accumulation

curves with individuals recoded as samples (Colwell et al., 2004; Mao et al., 2005). For this procedure 1000 randomisations with replacement were used to generate 95% confidence intervals for the rarefaction curves. These rarefied observed richness (S_{obs}) values were produced using EstimateS version 8.2 (Colwell, 2009). However, because information from the more thoroughly sampled sites could potentially be lost using this method, I also used a second rarefaction technique to predict species richness of sites up to the site with the maximum number of individuals (694 bats). These predicted species richness values were calculated using the Shen Multinomial Model (S_{Shen}) (Shen et al., 2003) computed in Species Prediction and Diversity Estimation (SPADE) (Chao and Shen, 2003) with 200 bootstrap replicates. The Shen predictor was chosen as it performed better than other estimators in a previous bat assemblage study based on assemblages in Malaysia (Kingston, 2009).

In addition to species richness, rarefied species evenness was estimated at each site using the reciprocal Simpson index (1/D) calculated in EstimateS version 8.2 using 1000 randomisations with replacement to generate the 95% confidence intervals. The Simpson index is a robust diversity index as it considers the variance of the species abundance distribution (Simpson, 1949; Magurran, 2006) and the reciprocal of the Simpson index has been shown to have a higher degree of discrimination. The Simpson index was designed to describe the dominance of species, and is equal to one when there is zero diversity, and decreases with greater diversity (Magurran, 1988). Thus larger values of 1/D indicate greater diversity and evenness, with the maximum equal to the total species in a sample.

Estimated metrics (S_{obs}, S_{Shen}, and 1/D) were plotted against latitude and longitude to test for clines in assemblage structure. To determine relative contributions of land area, latitude and longitude on shaping spatial patterns of bat species richness and diversity over Peninsular Malaysia, relationships between these metrics and site-level diversity were further investigated using generalised linear regression models in Systat 13 (http://www.systat.com/). Prior to running this test, I tested and confirmed that the data conformed to assumptions of normality for parametric tests (backward stepwise model: longitude: $F = 0.678$, $P = 0.426$; latitude: $F = 13.540$, $P = 0.003$; width: $F = 2.104$, $P = 0.173$; S_{Shen}: $Z = 0.885$, $P = 0.915$).

Variation in the abundance of species between sites was investigated by calculating the proportional abundance of each species at each site (i.e. ratio of total captured individuals of a species in a site to the total captured individuals of the site). Species proportional abundances were then plotted against latitude and longitude for the 15 most abundant species. Spearman rank correlations were used to test for association between latitude, longitude and population abundance.

Assemblage structure and β-diversity

To determine patterns of species turnover and assemblage similarity between sites, beta (β) diversity was calculated based on pairwise or multiple-site comparisons. Similarity indices suffer the same biases as estimates of species diversity, and so are strongly influenced by sample size. I therefore used the Morisita-Horn similarity index, calculated in SPADE, which is biased towards more common species and so less sensitive to the absence of rare species in under-sampled assemblages (Chao et al., 2008). I also repeated analyses using a recently modified version of the Sørensen similarity index (Chao et al., 2005), calculated in EstimateS with 200 bootstrap replications to generate standard errors, which accounts for potentially undetected rare species in assemblages. These distance measures consider the abundance of species in assemblages as well as their presence/absence, with higher values indicating higher similarity between the two communities (Chao and Shen, 2009).

To test for distance-decay, pairwise assemblage similarity among sites was plotted against corresponding pairwise geographic distance. To test for statistical significance of the correlation, I used a Mantel test in GenAlEx 6.2 with 999 permutations. This was also verified using the RELATE analysis in Primer version 5 (Clarke and Gorley, 2006), which is a non-parametric technique that is robust to non-linearity between the variables.

Identifying species that contributed to patterns of assemblage structure

Finally, the potential determinants of patterns of β-diversity between communities, and the species that contribute to these patterns, was also explored using non-metric

multidimensional scaling (NMDS), performed using PC-ORD version 5 (McCune and Mefford, 2006). For this analysis, the Sørensen dissimilarity index was used. NMDS is an ordination technique that attempts to reduce the difference between the rank order of dissimilarities and ordination distances. It is different from other ordination techniques in terms of design, interpretation and compatible for non-parametric assemblage data (McCune and Grace, 2002). NMDS also better preserves high dimensional analysis structure with fewer axes than principal coordinates analysis (PCoA), although both are types of ordination (Zuur et al., 2007). The number of axes for the ordination is in part determined by minimising 'stress', which is a measure of reliability of the ordination, and can be tested in PC-ORD by comparing the actual ordination with ordinations produced using Monte Carlo iterations of the original data. However, it is easier to interpret an ordination that has fewer axes.

In PC-ORD, a matrix of pairwise Sørensen dissimilarity coefficients was generated based on the abundance data of assemblages from each of the 15 sites. I then used the autopilot feature to determine the appropriate ordination solution for these data. The auto-pilot test is included in PC-ORD in order to choose the best solution for the dimensionality (i.e. the number of the axes) as well as test for the significance of the ordination solution compared with randomised data using Monte Carlo with 250 iterations. For the final ordination chosen, species that contributed most to the variation in the assemblage structure were identified by correlating the species abundance with the ordination axis score. Correlation between axis scores and both latitude and longitude was also determined.

Results

Bat capture and species identification

Between 2007 and 2009, I conducted a total survey effort of 333 harp trap nights (HTN) across 22 sites. In total, I captured 4679 individuals (excluding recaptures), consisting of 48 species from seven families. These bats included Old World fruit bat species and open space/ forest edge insectivorous species (Table S2.1, see Appendix). To obtain standardised data for assemblage analyses, I used 11 sites for which at least 100 individuals of forest-interior species were captured. These 11 sites recorded a total of 161 HTN with 3262 captured individuals of 31 species from five families. After adding in data from four sites surveyed by Struebig (2008) the final dataset from 15 sites comprised 3847 captured individuals, representing 32 species from five families. Further filtering to remove occasional captures of open space and edge species resulted in 3776 individuals of 32 species from five families. The three most commonly represented families were the Rhinolophidae (9 species), Hipposideridae (11 species) and Vespertilionidae (10 species), with the latter consisting of six species of the subfamily Kerivoulinae, three species of the subfamily Murininae, and one species of genus *Myotis* (Table 2.2). Of these 32 species, 18 were categorised as tree-roosting bats and 14 cave-roosting bats.

Table 2.2 (a) Insectivorous bat species captured at 22 sites in Peninsular Malaysia used for analyses. See Appendix S2.1 for full inventory.

FAMILY/Taxon	Species code	Red list status[a]	Distribution level[b]	Ensemble[c]	Species abundance in surveyed sites													
					JR2	JR3	KH1	KH3	KT3	NS1	PH1	PH2	PH6	PN1	PK1	F01	F02	F23	F24
MEGADERMATIDAE																			
Megaderma spasma	MSP		R	F													1		
NYCTERIDAE																			
Nycteris tragata	NTR		R	F		1	2			1									2
HIPPOSIDERIDAE																			
Hipposideros bicolor 131	HB131		W	C	118	136	11	1	4	10	11	126	2	5	31	5		1	1
Hipposideros. bicolor 142	HB142		W	C			35		27	2	3		170	6	16			21	5
Hipposideros cervinus	HCE		W	C	196	222	10			40	7	8	169	3	24	24	5	12	2
Hipposideros. larvatus	HLA		W	C	11	5	3		36	15		100	220	27	32	1	26	17	
Hipposideros armiger	HAR		R	C									2	3					
Hipposideros cineraceus	HCI		R	C		1			3										1
Hiposideros diadema	HDI		W	C	7	3	4		4		1	1	3	2		1	3		11
Hipposideros doriae	HDO	NT	R	F	1	1	2					2					1		
Hipposideros dyacorum	HDY		R	C					2				1						
Hipposideros galeritus	HGA		R	C	7	6						6	2		1				
Hipposideros ridleyi	HRI	VU	W	F	1				1		12	11				10	16		
RHINOLOPHIDAE																			
Rhinolophus affinis	RAF		W	C	68	97	25	169	14	47	64	80	94	76	8	14	23	18	21
Rhinolophus lepidus	RLE		W	C		32	55	34	1	11	2	3	10	28		2	22	2	40
Rhinolophus luctus	RLU		R	F						1		1							
Rhinolophus robinsoni	RRO		R	C	3	3				13		1	1	3	3				
Rhinolophus stheno	RST		W	C	1	2	4	8	4	1	1	1	3	4			5	4	4
Rhinolophus trifoliatus	RTR		W	F	13	3		2		1	14	3		3	4	14	22	5	4
Rhinolophus acuminatus	RAC		R	C								2							
Rhinolophus sedulus	RSE	NT	W	F	8		3				8	6			1	1	13	1	
Rhinolophus macrotis	RMA			F														1	
VESPERTILIONIDAE																			
Kerivoula hardwickii	KHA		W	F	12	5			10	7	13	1		10	2			7	
Kerivoula minuta	KMI	NT	W	F	6	3	4	1		3	8	12	7						
Kerivoula papillosa	KPA		W	F	15	1		2	3	3	20	2	5	3	4	14	14	11	6
Kerivoula pellucida	KPE		R	F	4		1		1	3	5	5	3		1	1	9	1	1
Kerivoula intermedia	KIN	NT	W	F	1	1			2		6	3	1		2	47	47		
Phoniscus atrox	PAT	NT	R	F		1					3	2				2	1		
Murina suilla	MSU		R	F	1		1		2	1	10					2	5	5	6
Murina aenea	MAE	VU	R	F	1		1				1							1	
Murina cyclotis	MCY		R	F		1					1		1		1	1		1	
Myotis ridleyi	MRI	NT	R	F	**1**					**1**									

[a] IUCN red list status: NT, near threaten; VU, vulnerable (SAMD, 2009).
[b] Distribution of species based on the total captured individuals of all species from all sites: R = rare (comprises <1% of total individuals; W = widespread (comprising >1% of total individuals). Rarity classification based on Struebig (2008).
[c]Ensemble to which species belongs: F = predominantly tree cavity and/or foliage roosting narrow-space species; C = predominantly cave roosting narrow-space species.

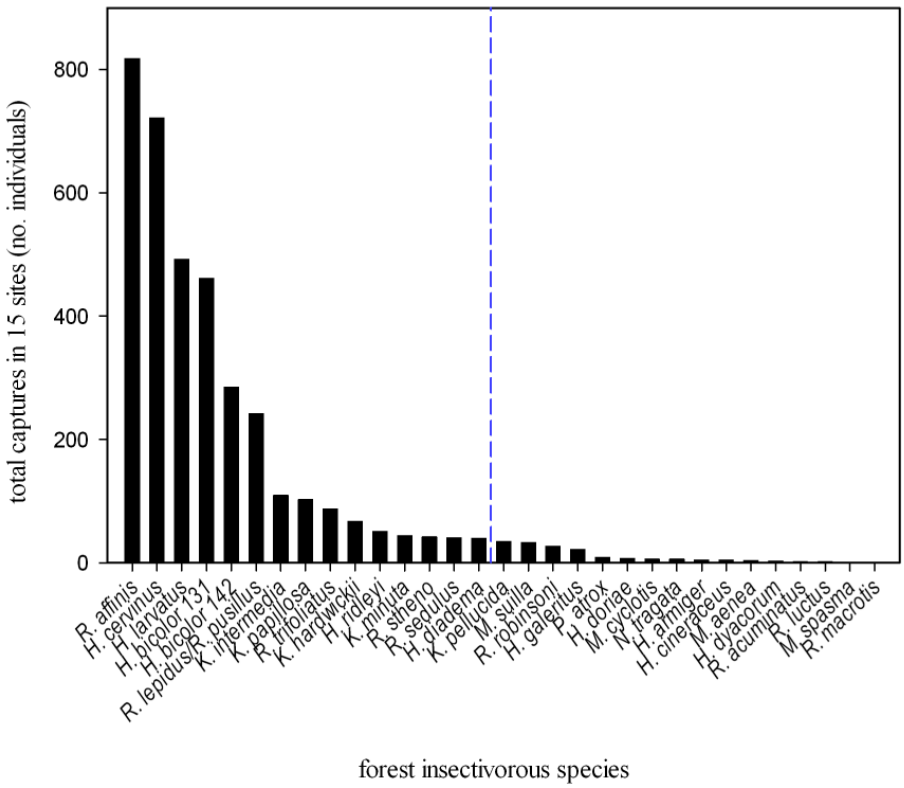
Table 2.2 (b) Insectivorous bat species captured at 22 sites in Peninsular Malaysia and used for analyses. See Appendix S2.1 for full inventory.

FAMILY/Taxon	Species code	Red list status[a]	Distribution level[b]	Ensemble[c]	Species abundance in surveyed sites										
					JR1	KH2	KT1	KT2	MK4	MK5	PH3	PH4	PH5	PK2	PK3
MEGADERMATIDAE															
Megaderma spasma	MSP		R	F										1	1
NYCTERIDAE															
Nycteris tragata	NTR		R	F				1		1		1		3	2
HIPPOSIDERIDAE															
Hipposideros bicolor 131	HB131		W	C	7		3	1	7		7				
Hipposideros. bicolor 142	HB142		W	C		15			11		4		4	57	38
Hipposideros cervinus	HCE		W	C	14				5					221	
Hipposideros. larvatus	HLA		W	C		3			7		4		1		1
Hipposideros armiger	HAR		R	C											1
Hipposideros cineraceus	HCI		R	C						1	1			2	
Hiposideros diadema	HDI		W	C		1		19	2		1				
Hipposideros doriae	HDO	NT	R	F	1										
Hipposideros dyacorum	HDY		R	C							30				
Hipposideros galeritus	HGA		R	C				1			1			2	3
Hipposideros ridleyi	HRI	VU	W	F		3		2							
RHINOLOPHIDAE															
Rhinolophus affinis	RAF		W	C	40	16	6	3	6	1	29		222	35	24
Rhinolophus lepidus	RLE		W	C	4		17	1	22		1	1	221	49	8
Rhinolophus luctus	RLU		R	F				1		1				1	1
Rhinolophus robinsoni	RRO		R	C					3	2					
Rhinolophus stheno	RST		W	C	1		27							1	5
Rhinolophus trifoliatus	RTR		W	F	4	4	4	19							
Rhinolophus acuminatus	RAC		R	C		2		2				1			
Rhinolophus sedulous	RSE	NT	W	F	1							1			
Rhinolophus macrotis	RMA			F											
VESPERTILIONIDAE															
Kerivoula hardwickii	KHA		W	F	5	4	6	2	1		3				1
Kerivoula minuta	KMI	NT	W	F		4	1								
Kerivoula papillosa	KPA		W	F	5	12	8	6							6
Kerivoula pellucida	KPE		R	F	2		1	2			1				1
Kerivoula intermedia	KIN	NT	W	F	15			2				2			
Phoniscus atrox	PAT	NT	R	F											
Murina suilla	MSU		R	F			1	1	1					1	1
Murina aenea	MAE	VU	R	F										1	
Murina cyclotis	MCY		R	F		1	2								1
Myotis ridleyi	MRI		R	F									7		

[a] IUCN red list status: NT, near threaten; VU, vulnerable (SAMD, 2009).
[b] Distribution of species based on the total captured individuals of all species from all sites: R = rare (comprises <1% of total individuals; W = widespread (comprising >1% of total individuals). Rarity classification based on Struebig (2008).
[c] Ensemble to which species belongs: F = predominantly tree cavity and/or foliage roosting narrow-space species; C = predominantly cave roosting narrow-space species.

A Fisher's species abundance plot showed clear stepwise patterns in dominance (Figure 2.3). The two most dominant species across the entire study region were *R. affinis* (21.7% of total captures) and *Hipposideros cervinus* (19.1%), although their abundance was highly variable across sites (Table 2.2). All six of the most dominant species were cave roosting species from the families Rhinolophidae and Hipposideridae, possibly reflecting the fact they live in large colony sizes (Nowak, 1994). The 15 most dominant species together represented 95.6% of total captures (Table 2.2). Based on a cut-off point of less than 1% of total captures (37 individuals), 16 species were classified as rare, which included *Kerivoula pellucida* (0.93%), *Murina suilla* (0.87%) and *Rhinolophus robinsoni* (0.72%). For *Rhinolophus luctus* and *Rhinolophus accuminatus*, a maximum of two individuals were recorded for any assemblage, whereas *Megaderma spasma* and *Rhinolophus macrotis* were represented by only 1 capture over the entire 15 sites (Table 2.2).

Figure 2.3 Fisher's plot showing the abundance of all species captured at the 15 standardised sites from 2007 to 2009. The dashed line depicts the threshold of rarity (less than 1% of total capture individuals of all species, with more common species on the left of the graph and rarer ones on the right.



Site species richness, diversity and species abundance

Raw (uncorrected) species richness at the 15 selected sites ranged from seven species (site KH3) to 21 species (PH2), with a mean of 15.73 ±1.74 (CI) species captured at a site (data not shown). After rarefaction downwards to a standardised sample size of 104 individuals, observed species richness (S_{obs}) was seen to be greatest at site PH1 (16.38 ±1.79), whereas only 5.07 ±1.04 species were found at KH3 (Table 2.3). This pattern was consistent when species richness was predicted (S_{Shen}) to the maximum sample size available (694 bats at PH6): 22.8 species at PH1 compared to 8.8 species for KH3 (Table 2.3). Compared to the actual capture record sites, F23 (358 individuals, 16 species) was predicted to host a further 8.5 species given extra survey effort. This was

relatively high compared to PH1 and KH3, also suggesting that the F23 sampling had not reached an asymptote in its species accumulation curve relative to PH1 and KH3.

The reciprocal Simpson Index, which considers both the species richness and evenness, indicated high diversity in F02 (1/D=8.86) and F23 (1/D=8.16). In contrast, KH3 exhibited low bat diversity (1/D=1.60), as only seven species were captured for 217 individuals, and the assemblage was dominated by one species (*R. affinis*) (Table 2.3).

Table 2.3

Bat species diversity at 15 selected sites based on observed rarefied species richness (S_{obs}), predicted richness using the Shen Multinomial predictor (S_{Shen}), and evenness as described by the reciprocal Simpson diversity index (1/D).

Site Code	Site name, state and location	Width of peninsular (km)	S[a]	S_{obs}[b]	S_{Shen}[c]	1/D[d]
NS1	Gunung Angsi, N. Sembilan 102.078°E, 2.705°N	237.64	17	12.60	20.60	5.49
PK1	Kledang Saiong, Perak 101.004°E, 4.538°N	315.94	14	11.96	17.10	5.84
KH1	Bukit Hijau, Kedah 100.773°E, 5.501°N	292.25	15	12.55	16.40	5.09
PH1	Bukit Ibam, Pahang 102.901°E, 3.223°N	235.93	19	16.38	22.80	6.71
PH2	Gunung Aais, Pahang 102.681°E, 4.413°N	317.96	21	13.72	23.40	4.35
KH3	Ulu Muda, Kedah 100.963°E, 6.107°N	233.19	7	5.07	8.80	1.60
PN1	Bukit Panchor, Penang 100.546°E, 5.151°N	317.23	13	11.84	13.00	4.06
JR2	Gunung Panti, Johor 103.914°E, 1.869°N	147.19	20	12.27	23.00	3.94
KT3	Gunung Stong, Kelantan 101.977°E, 5.340°N	306.07	15	12.80	16.40	5.51
JR3	Labis Forest Reserve, Johor 103.159°E, 2.346°N	209.72	19	8.86	20.90	3.56
PH6	Forest Kenong 102.188°E, 4.216°N	322.2	17	8.31	17.00	4.23
F01*	Kemasul 1 102.183°E, 3.383°N	248.47	15	12.11	21.70	5.62
F02*	Kemasul 2 102.133°E, 3.433°N	254.68	16	12.82	18.10	8.86
F23*	Klau Besar 101.890°E, 3.749°N	270.4	16	12.93	24.50	8.16
F24*	Jengka 102.455°E, 3.615°N	258.67	13	11.00	15.10	4.84

[a] Raw species richness based on harp trapping records.

[b] Rescaled sample-based to individual-based rarefied species accumulation index. This index was calculated based on the Mau Tao function estimated in EstimateS 8.2 (Colwell, 2009).

[c] Prediction diversity using the multinomial model index was calculated in SPADE (Shen et al., 2002).

[d]Reciprocal Simpson Index to show species diversity based on the sampling data in EstimateS 8.2 (Colwell, 2009).

Spatial assemblage patterns were investigated by plotting species diversity indices (S_{obs}, S_{Shen} and 1/D) against both the latitude and longitude of each site (Figure 2.4). Results showed significant negative relationship between S_{Shen} species richness and latitude (r^2 = 0.51, P = 0.001) and a positive relationship with longitude (r^2 = 0.49, P = 0.003). All of the indices examined (S_{obs}, S_{Shen} and 1/D), revealed a broad reduction in diversity with ascending latitude (south to north), although these were not always significant (Figure 2.4 a, c, e). Trends in diversity with ascending longitude (west to east) were less clear (Figure 2.4 b, d, f). Species richness indices, S_{obs} and S_{Shen}, exhibited wider 95% confident interval ranges compare to 1/D. This is partly due to the sensitivity of S_{obs} and S_{Shen} on rare species compare to 1/D, which considers both species richness and species evenness.

Variation in S_{Shen} across latitude and longitude was further examined using a general linear model. Initial models revealed that both latitude and longitude predicted S_{Shen} index independently. Therefore, latitude, longitude, quadratic latitude, quadratic longitude and interaction between latitude and longitude were also included in the models. The results indicated latitude yielded the simplest model for predicting the S_{Shen} index. In short, latitude itself is a better predictor of S_{Shen} rather than combination of latitude and longitude (linear regression model for latitude adjusted r^2=0.5031, P = 0.002, F=15.17). When the, S_{Shen} index from 15 sites was superimposed on the Peninsular Malaysia forest coverage map in Figure 2.5 a latitudinal decline in species richness was seen from south towards north.

Figure 2.4

Plots to show clinal patterns of species richness with latitude (a, c and e) and longitude (b, d and f). Plots a and b show upper 95% confidence intervals only because these are estimated from upward extrapolations of the observed values. All other plots show upper and lower 95% confidence intervals for each value.

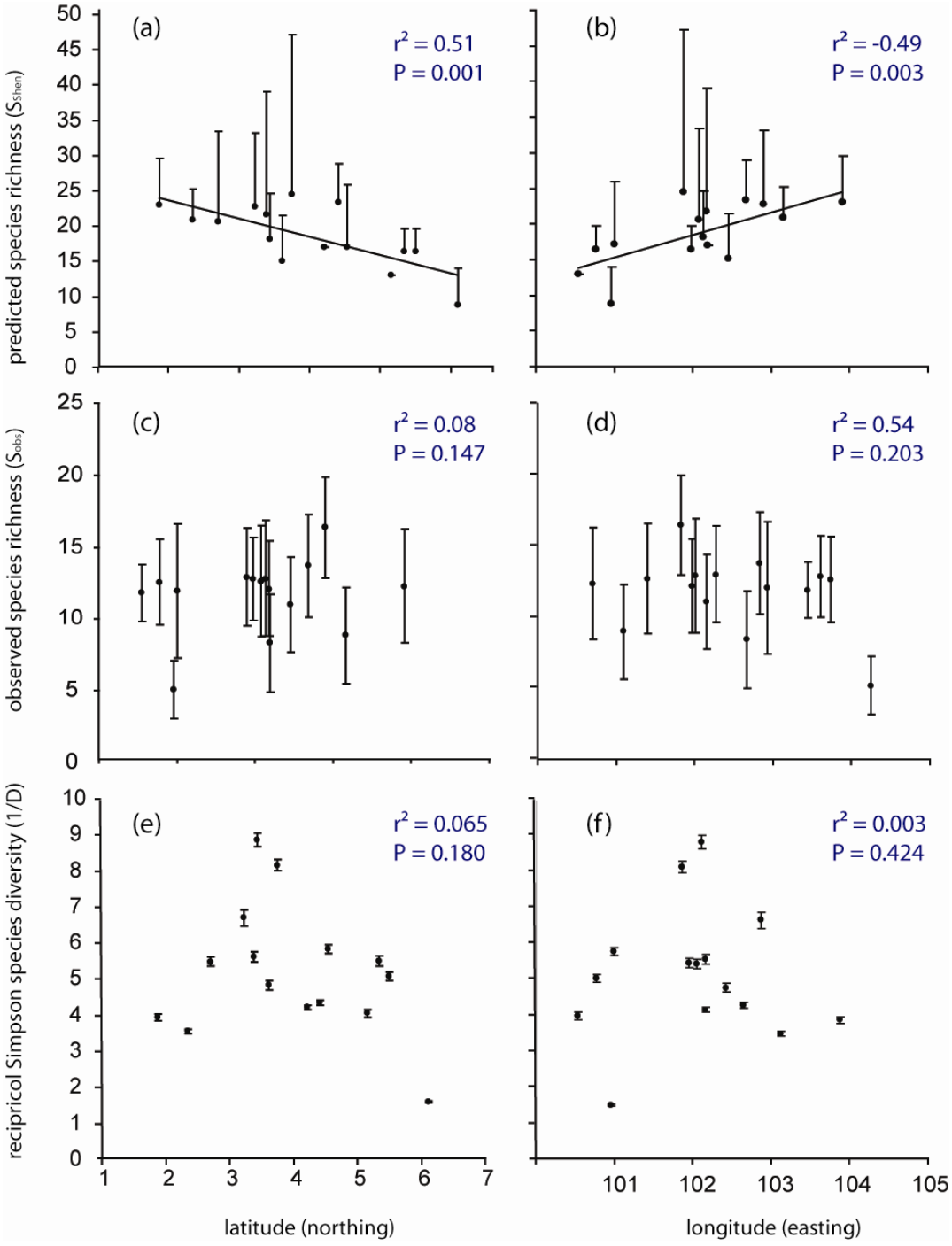
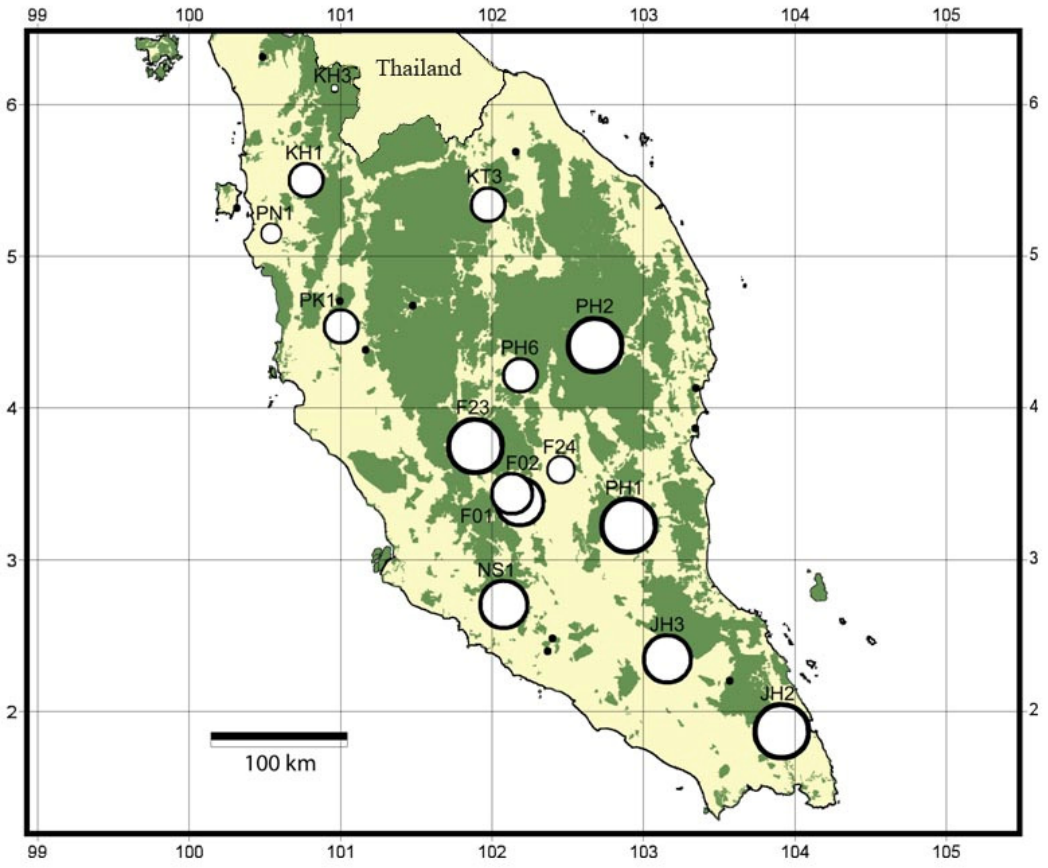


Figure 2.5

Map of Peninsular Malaysia showing the gradient of the species richness with latitude. Radii of circles are scaled by predicted species richness based on the Shen Multinomial Model (S_{Shen}) in Table 2.3. Filled small circles without labels represent those sites excluded from the assemblage analysis. Note that the circles show a broad reduction from south to north, indicating northward reduction in species richness.



Species diversity was highest in the centre of the peninsula at F02 (8.86) and F23 (8.16), which indicated a higher species evenness compared to other sites. In contrast, KH3 at the far North of the study area showed the highest dominance of *R. affinis* and *Rhinolophus lepidus* with 1/D=1.60. The proportions of abundance for two of the 15 common species were correlated with latitude (Figure 2.6). Abundance of *Hipposideros cervinus* was lower at higher latitude ($r_{Spearman}$= -0.693, P = 0.002), and the abundance of *H. bicolor* 142 was higher at higher latitude ($r_{Spearman}$= 0.456, P = 0.044). No significant correlation was detected between abundance and longitude (i.e. west-east) for any of the 15 most common species.

The proportional abundance of many species was also strongly correlated: cave-roosting species positive correlations were seen between *R. lepidus* and *R. stheno* ($r_{Spearman}$= 0.686, P = 0.002); tree/foliage-roosting species *Kerivoula intermedia* and *Hipposideros ridleyi* ($r_{Spearman}$= 0.857, P = 0.000), and tree/foliage-roosting species *Kerivoula papillosa* and *R. trifoliatus* ($r_{Spearman}$= 0.846, P = 0.000). Significant negative correlations were detected between cave-roosting *H. cervinus* and *R. stheno* ($r_{Spearman}$= -0.601, P = 0.009), cave-roosting *R. lepidus* and tree/foliage-roosting species *K. intermedia* ($r_{Spearman}$ = -0.628, P = 0.006), and between cave-roosting *H. bicolor 131* and *R. stheno* ($r_{Spearman}$ = -0.597, P = 0.009). A large number of weaker but significant correlations were also detected: between cave-roosting *R. affinis* and tree/foliage-roosting species *K. intermedia* ($r_{Spearman}$ = 0.583, P = 0.011), cave-roosting *R. stheno* and tree/foliage-roosting species *K. intermedia* ($r_{Spearman}$ = -0.561, P = 0.015), tree/foliage-roosting species *K. papillosa* and *H. ridleyi* ($r_{Spearman}$ = 0.467, P = 0.015), tree/foliage-roosting species *R. trifoliatus* and *H. ridleyi* ($r_{Spearman}$ = 0.481, P = 0.035), tree/foliage-roosting species *R. sedulus* and *K. hardwickii* ($r_{Spearman}$ = 0.472, P = 0.038), tree/foliage-roosting species *R. sedulus* and *H. ridleyi* ($r_{Spearman}$ = 0.573, P = 0.013). In general, the results suggested that abundance of the tree/foliage-roosting species *K. intermedia* decreases with increasing abundance of the common cave-roosting species *R. lepidus*, *R. affinis* and *R. stheno*.

Assemblage structure and β-diversity

Mantel tests and inspection of separate plots of geographical distance versus the three measures of assemblage similarity showed weak but non-significant relationships: Morisita-Horn similarity (r^2=0.029, P_{Mantel} =0.074), estimated Chao-Sørensen (r^2 = 0.034, P_{Mantel} = 0.084) and raw-Sorensen (r^2=0.024, P_{Mantel}=0.122) (Figure 2.7). When analyses were repeated using the RELATE procedure with non-parametric assumptions the associations remained non-significant: Chao-Morisita similarity ($r_{Spearman}$= 0.147, P = 0.101), estimated Chao-Sørensen ($r_{Spearman}$= 0.169, P = 0.089) and raw Sørensen ($r_{Spearman}$ = 0.107, P = 0.196) (Figure 2.7). Therefore, similarity in assemblage structure did not show a pattern of distance decay, and neighbouring populations were no more similar to distant ones (Figure 2.7). However, pairwise differences in species richness was positively correlated with pairwise geographical distance (r^2= 0.1932, P_{Mantel} = 0.010) (Figure 2.7).

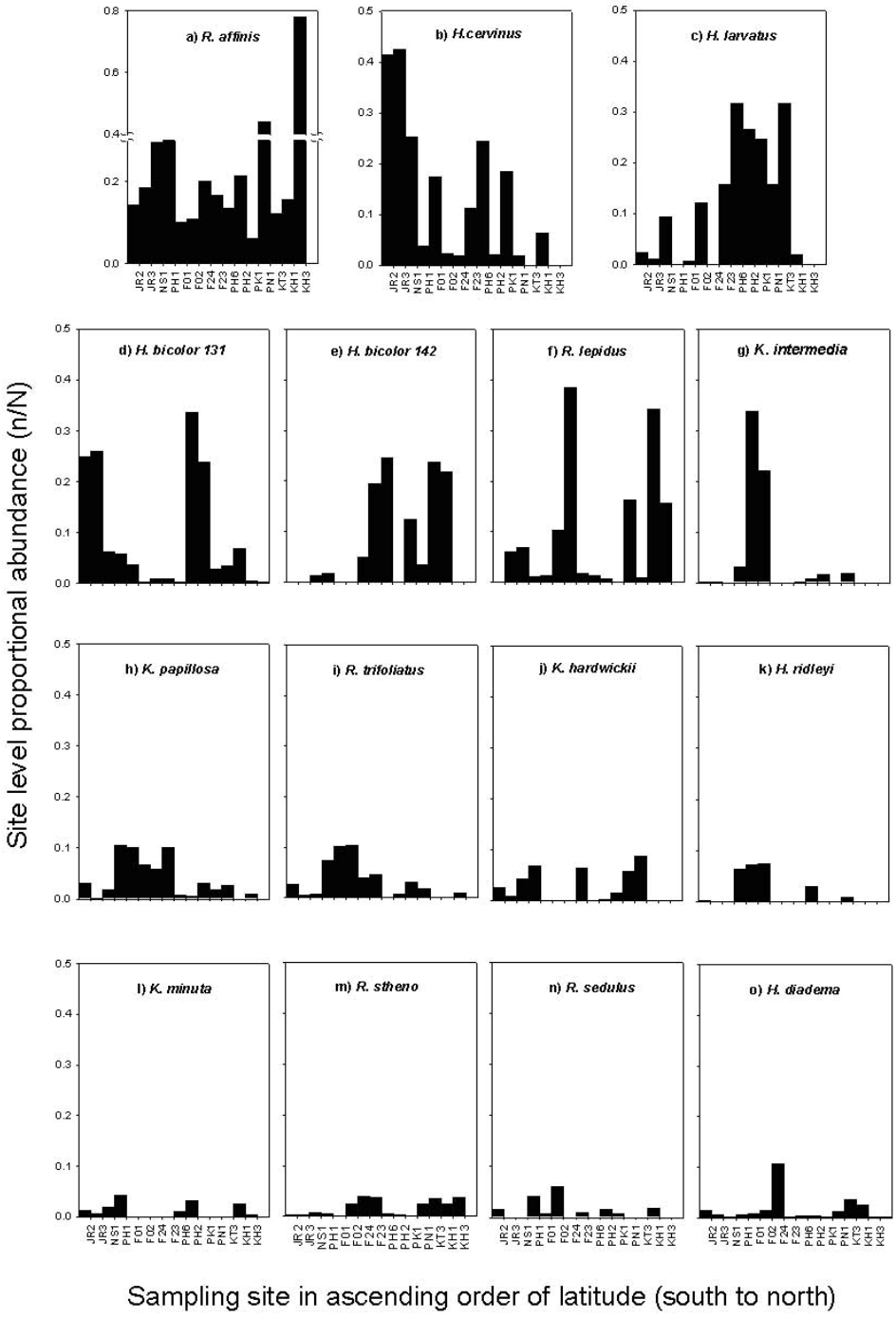
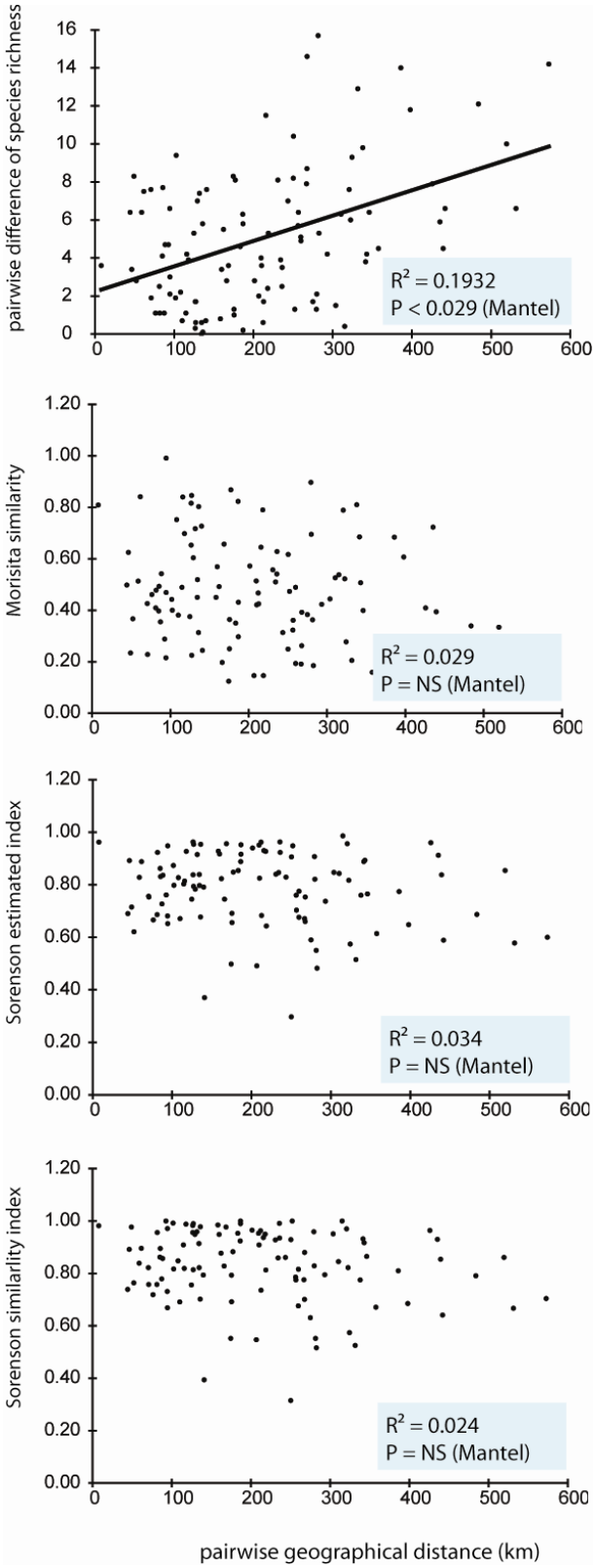


Figure 2.6 Latitudinal variation in the relative abundance (proportional to total number of captures) of the 15 most common species. Proportional abundance was less than 0.5 for all species except for *R. affinis*.

Figure 2.7 Plots showing species similarity (beta-diversity) versus geographical distance (km)



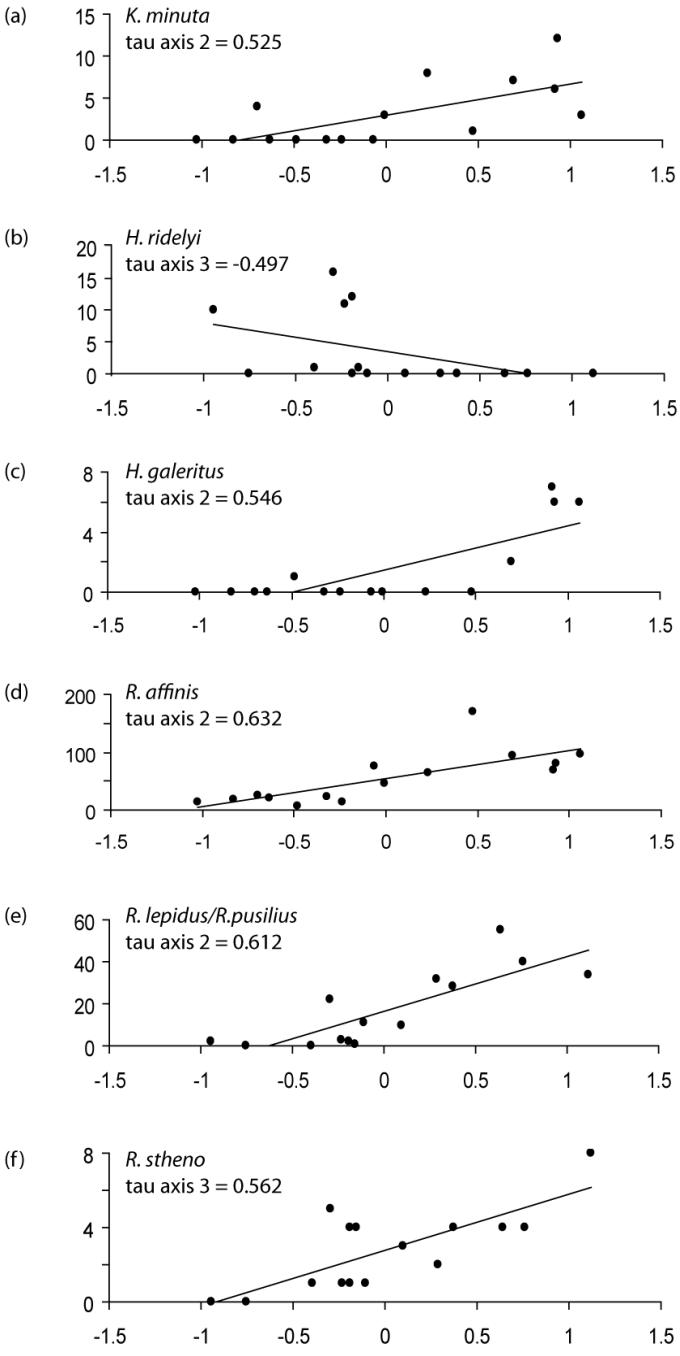
Patterns of assemblage structure

Non-metric multidimensional scaling of raw Sørensen dissimilarity coefficients revealed that a three-axis ordination was chosen as the best solution (P = 0.004), which together explained 86.3% of variation. The final ordination was stable and reliably represented assemblage dissimilarity (stress = 7.26). Of the total variation, axis 1 explained 42.8% of the variation in assemblage dissimilarity, axis 2 explained 26.7%, and axis 3 explained 16.8%. Therefore, axes 1 and 2 represented the greatest portion of the variation in the matrix and were used in subsequent analyses.

The abundance of six species was correlated with NMDS axes scores (Figure 2.8): two tree/foliage–roosting species, *K. minuta* (Tau coefficient = 0.525) and *H. ridleyi* (Tau coefficient = -0.497); and four cave roosting species, *H. galeritus* (Tau coefficient = 0.546), *R. affinis* (Tau coefficient = 0.632), *R. lepidus* (Tau coefficient = 0.612) and *R. stheno* (Tau coefficient = 0.562). However, inspection of abundance-NMDS axes plots (Figure 2.8) revealed that these correlations were statistical artefacts for all but three species: *R. affinis*, *R. lepidus* and *R. stheno*. These plots revealed that the abundance of *R. affinis* was strongly correlated with both axes 2 and 3 and the abundance of *R. lepidus* was strongly correlated with axis 2. Most of the dissimilarity of assemblage was caused by the changes in abundance of *R. affinis* and *R. lepidus*.

Correlation between axes and both latitude and longitude were also tested. Tau correlation indicated latitude correlated weakly with axis 1 (-0.314) and axis 2 (0.314). Longitude was weakly correlated with axis 1 (0.390).

Figure 2.8 Plots to show statistical artifacts of correlation between species abundance and ordination scores in *K. minuta*, *H. ridleyi* and *H. galeritus*. Tau coefficients show at least medium association between ordination axes with the species abundance (Tau coefficient ≥ 0.5). However, distribution of the points revealed artifacts. P values are not reported for each plot as ordination scores are not strictly independent from each other.



Discussion

Broad-scale patterns of assemblage structure can provide valuable insights into past climate change, as well as help to predict the response of biodiversity to future climate changes and the conservation consequences of current and future habitat change. In this chapter, I investigated several aspects of assemblage structure to determine current patterns of forest bat diversity over Peninsular Malaysia and inferred how this may have been shaped by proposed changes in the extent and distribution of tropical rainforest after the Last Glacial Maximum (LGM).

Consequences of past climate oscillation in shaping bat assemblages across the peninsula

I surveyed and analysed assemblages of forest bats at 15 selected sites, all distributed between 1°N and 7°N latitude, and between 100°E and 105°E longitude (Figure 2.1). All of the bats in this study were characterized by ecomorphological traits, including wing shape (broad and short wings) (Arita and Fenton, 1997) and flapping kinematics, that confer slow flight in highly cluttered environments (Aldridge and Rautenbach, 1987; Stockwell, 2001). Similarly, their smaller body size (Stockwell, 2001) and high frequency and/or constant frequency calls (Kingston et al., 2003) also suggest adaptations for hunting in dense vegetation. Current distribution ranges in Southeast Asia (Kingston et al., 2006; Francis, 2008; SAMD, 2009) confirm a tight association between this guild of bats and intact forest, and so it can be assumed that the historical range of these species must have mirrored that of lowland evergreen tropical rainforest.

At the alpha diversity level, species richness of each site was calculated using three indices with different approaches: estimated species richness (S_{obs}), predicted species richness (S_{Shen}) and the reciprocal index (1/D). These three indices revealed a weak decline in species richness from the south of Peninsular Malaysia towards the north, although the trend was only significant when using predicted S_{Shen}, indicating that more sampling might be needed in order to observe the trend based on the empirical data alone. Indeed based on the S_{Shen} index, there were only two sites (PN1 and PG6) for which the species were fully sampled in spite of the large amount of fieldwork undertaken. The observed overall cline in diversity could suggest a northward expansion

of forest, with greater numbers of forest species in the south nearer to the equator. I also found a weaker relationship between species richness and longitude, although this probably reflects the co-variation between longitude and latitude due to the shape of the peninsula (Figure 2.3).

The combined effect of the longitudinal and latitudinal clines in diversity was also evident from the significant relationship between pairwise difference in species richness among sites and corresponding geographical distance. In other words, this confirmed that the biggest differences occurred between the most distant (i.e. southeast and northwest) sites. Interestingly, however, the other measures of beta diversity examined did not reveal an effect of distance, although all showed substantial variation in values. A recent high profile and large-scale study of 500 species of tropical herbivorous insects in Papua New Guinea also found no effect of geographical distance on beta diversity across 75,000 square km of lowland rainforest (Novotny et al., 2007). However, in this study the authors found beta diversity was low overall and proposed that insect diversity is probably similar across large areas because of the relatively uniform climate, soil and other conditions that characterise lowland areas. Such a result suggests that my observed variation in assemblage structure in bats in lowland Peninsular Malaysia is worth further study, and might be of considerable conservation importance.

Overall, my results from species richness analyses do not appear to support the maps of past vegetation at the LGM reconstructed by Cannon et al. (2009). In these models, lowland evergreen forest was more widely distributed than at present, occurring across exposed land in the coastal areas around Borneo and the area that is now covered by the South China Sea. If such a long history of forest in Peninsular Malaysia is correct (with no replacement by savannah at the LGM) then it might be expected that species turnover (beta diversity) will be low across latitude or longitude. Instead my data cannot rule out theories that rainforest in the peninsula was replaced by open grassland, and only expanded again after the LGM (see Wurster et al., 2010). There might also be other possible explanations for clinal variation in assemblage structure and diversity across Peninsular Malaysia. One is that aspects of the observed patterns reflect variation in the width of current or historical land. Woodruff and Turner (2009) estimated species richness of terrestrial mammals in the Malay Peninsula from museum specimen collections and literature distribution ranges, and found that diversity was lowest

between 6°N and 13°N at the narrowest part of the Malay-Thai peninsula. They attributed this to an area effect based on island biogeography theory, which suggests that a reduction in area (here due to the rise in sea level) will promote competition among species, and the eventual loss of some species as long-term equilibrium is reached. Indeed at around 11,000 years BP, the sea level rose (Sathiamurthy and Voris, 2006) to 40 m below present, covering part of east Sundaland and remaining at this level for over half of the interglacial period (Voris, 2000; Sathiamurthy and Voris, 2006). Further increases in sea level disconnected the Malay-Thai peninsula from Sumatra and Borneo and formed the exposed land shape that we see today.

Woodruff and Turner (2009) showed that as well as the decrease at the narrowest part of the isthmus, species richness peaked just before this, at 5°N, and again at 14°N where the land widens into continental Asia. They explained this pattern as due to transitions in the distributions of multiple species. Because the results from my study suggest a dip in diversity at 5°N, they might not be due to the same effect of land area. However, although Woodruff and Turner's study included data on 103 bats species found in the Malay-Thai peninsula (for which data could be obtained from the literature) they made no distinction between open-space species and forest specialists. In my work, open space insectivorous species and fruit bats were excluded from analyses, furthermore all data were obtained from actual field surveys and so considered both species abundance and species richness to provide more insight into the species diversity of forest insectivorous bats. These differences mean that the clines in bat species richness seen in both studies may reflect different issues. While Woodruff and Turner (2009)'s broader scale focus of more species may have greater power in detecting the consequences of dramatic area effects (which affect all terrestrial species equally), my finer scale focus may better reflect the more subtle variation in beta diversity due to recolonization of forest-dependent species over land.

Clines in species diversity could also result from finer-scale and more subtle habitat variability due to present conditions rather than re-colonisation events, as highlighted by the Holdridge life zones model (Holdridge, 1967). In 2002, Wikramanayake et al. (2002) divided the World into 139 eco-regions, and classified Peninsular Malaysia into three eco-regions: rainforest, montane rainforest and peat swamp forest. Lowland evergreen Dipterocarp tropical rainforest occurs below 300m above seal level and, within Peninsular Malaysia, is distributed between 1°N and 6°N

degrees latitude with the vegetation transition to monsoonal forests reported to be at 6°30'N at the so-called Kangar-Pattani Line (Vincent and Yusuf Hadi, 1993; Woodruff, 2003). In my study, I aimed to minimize the impact of habitat variation across the study region; the northernmost site was located at 6.3 °N, all sites were below 300m, and I avoided peat swamp. Nonetheless I cannot rule out the possibility of local conditions influencing assemblage structure. Conditions in Malaysia also differ longitudinally: annual rainfall is slightly higher and temperatures are relatively cooler at the east coast compared to the west coast; (Tija, 1988; Vincent and Yusuf Hadi, 1993), and the central montane part of Peninsular Malaysia averages fewer sunshine hours and is also cooler than coastal areas (Sayang Mohd Deni et al., 2009).

To date, few studies have conducted fieldwork to look at large-scale patterns of species diversity across Southeast Asia. As mentioned, a low rate of species turnover (beta diversity) was detected for herbivorous insect guilds consisting of species from seven groups in Papua New Guinea (Novotny et al., 2007). On Borneo, a study of geometrid moth beta diversity traced species turnover patterns across 700km, and uncovered environmental and temporal factors that shaped the turnover trend (Beck et al. 2007). Beck et al. (2007) also analysed distribution of sphingid moths across the Southeast Asian mainland, and found that species richness peaked in northern Thailand and was lower both further north and towards the south. Like Woodruff and Turner (2009), Beck et al. (2007) proposed a peninsular effect to explain a southward decreases in moth species richness, and they also proposed an influence of environmental factors after finding that diversity was greater in higher altitude areas.

These different results all show that more work is needed in Southeast Asia to gain an overall picture of latitudinal patterns of species diversity, and the processes that caused these patterns. In comparison, a greater number of detailed studies have been conducted in the New World, on groups such as birds (Blackburn and Gaston, 1996), large mammals (McCoy and Connor, 1980) and insects (Stout and Vandermeer, 1975). In a comprehensive survey of bats, Stevens and Willig (2002) studied the latitudinal patterns of species across both South and North America continents, covering three climatic zones (temperate, subtropical and tropical). They revealed that local species richness (alpha diversity) increases and is more variable with decreasing latitude. In their study, Stevens and Willig (2002) also compared 14 indices that differed in sensitivity and showed that some had advantages over others. This and other evaluations

of multiple indices have provided useful guidance for ecologists (Beck and Schwanghart, 2010). For example, it is clear that indices that attempt to predict species richness can be especially useful for rapid diversity assessments because they can provide information when inventories are incomplete.

Although this study is one of the first detailed studies of assemblage structure in a mammal group across peninsular Malaysia, more sampling (both in terms of numbers of bats and the geographical area) is needed in order to confirm the results. One secondary finding that is of particular interest was the highest alpha diversity detected in the extreme south (JH2), which is near to Singapore. This part of Malaysia is under the most intensive pressure for land, and has already suffered from huge loss of forest in recent decades (Peh et al., 2006). Thus the high alpha diversity of forest bats recorded in southern Malaysia is of critical conservation concern, and the rich diversity may expected to decline soon if there is an extinction debt (Tilman et al., 1994; Loehle and Li, 1996; Brook et al., 2003,).

Appendix of supporting information

Table S2.1 All bat species captured in 22 surveyed sites in Peninsular Malaysia. (continued on next page)

FAMILY/*species*	IUCN status[a]	Ensem[b]	Species abundance in surveyed sites: JR 1	JR 2	JR 3	KH 1	KH 2	KH 3	KT 1	KT 2	KT 3	MK 4	MK 5	NS 1	PH 1	PH 2	PH 3	PH 4	PH 5	PH 6	PK 1	PK 2	PK 3	PN 1	P N2	F0 1	F0 2	F 23	F 24
PTEROPODIDAE																													
Balionycteris maculate	NT	F					1								3	1								2					
Cynopterus brachyotis	LC	F		1		1						1																	
Cynopterus horsefieldi	LC	F																		1									
Eonycteris spelaea	LC	C																				1		2					
Macroglossus sobrinus	LC	F														1													
Megaerops ecaudatus	LC	F					1																						
Chironax melanocephalus	LC																							1					
Penthetor lucasi	LC																							1					
MEGADERMATIDAE																													
Megaderma spasma	LC	F																				1	1			1			
NYCTERIDAE																													
Nycteris tragata	LC	F			1	2				1			1	1				1				3	2						2
EMBALLONURIDAE																													
Emballonura monticola	LC	F		2						2														1					
HIPPOSIDERIDAE																													
Hipposideros bicolor 131	LC	C	7	118	136	11	15	1	3	1	4	7		10	11	126	7			2	31			5		5		1	1
Hipposideros. bicolor 142	LC	C				35					27	11		2	3		4		4	170	16	57	38	6				21	5
Hipposideros cervinus	LC	C	14	196	222	10						5		40	7	8				169	24	21		3		24	5	12	2
Hipposideros. larvatus	LC	C		11	5	3	3				36	7		15		100	4		1	220	32		1	27	14	1	26	17	
Hipposideros armiger	LC	C																		2			1	3					
Hipposideros cineraceus	LC	C			1						3		1				1					2							1
Hiposideros diadema	LC	C		7	3	4	1			19	4	2			1	1	1			3				2		1	3		11
Hipposideros doriae	NT	F	1	1	1	2										2											1		
Hipposideros dyacorum	LC	C									2						301			1									
Hipposideros galeritus	LC	C		7	6					1						6				2	1	2	3						
Hipposideros ridleyi	VU	F		1			3			2	1				12	11										10	16		
RHINOLOPHIDAE																													
Rhinolophus affinis	LC	C	40	68	97	25	16	169		3	14	6	1	47	64	80	29		22	94	8	35	24	76		14	23	18	21
Rhinolophus lepidus	LC	C	4		32	55		34		1	1	22		11	2	3	1	1	21	10		49	8	28		2	22	2	40
Rhinolophus luctus	LC	F								1			1	1		1						1	1						
Rhinolophus robinsoni	LC	C		3	3							3	2	13		1				1	3			3					
Rhinolophus stheno	LC	C	1	1	2	4		8			4			1	1	1				3		1	5	4			5	4	4
Rhinolophus trifoliatus	LC	F	4	13	3		4	2		19				1	14	3					4			3		14	22	5	4
Rhinolophus acuminatus	LC	C					2			2						2		1											
Rhinolophus sedulus	NT	F	1	8		3									8	6		1			1					1	13	1	
Rhinolophus macrotis	LC	F																										1	

[a] IUCN red list status: NT, near threaten; VU, vulnerable (SAMD, 2009).
[b]Ensemble to which species belongs: F = predominantly tree cavity and/or foliage roosting narrow-space species; C = predominantly cave roosting narrow-space species

Table S2.1 Bat species in 22 surveyed sites in Peninsular Malaysia.

FAMILY/*species*	IUCN status[a]	Ensemble[b]	Species abundance in surveyed sites																										
			JR1	JR2	JR3	KH1	KH2	KH3	KT1	KT2	KT3	MK4	MK5	NS1	PH1	PH2	PH3	PH4	PH5	PH6	PK1	PK2	PK3	PN1	PN2	F01	F02	F23	F24
VESPERTILIONIDAE																													
Kerivoula hardwickii	LC	F	5	12	5		4		6	2	10	1		7	13	1	3				2		1	10	5			7	
Kerivoula minuta	NT	F		6	3	4	4	1	1					3	8	12				7									
Kerivoula papillosa	LC	F	5	15	1		12	2	8	6	3			3	20	2				5	4		6	3		14	14	11	6
Kerivoula pellucida	LC	F	2	4		1			1	2	1			3	5	5	1			3	1		1			1	9	1	1
Kerivoula intermedia	NT	F	15	1	1					2	2				6	3		2		1	2					47	47		
Phoniscus atrox	NT	F			1										3	2										2	1		
Murina suilla	LC	F		1		1			1	1	2	1		1	10							1	1			2	5	5	6
Murina aenea	VU	F		1		1									1							1						1	
Murina cyclotis	LC	F			1		1		2						1					1	1		1			1		1	
Myotis ridleyi	NT	F		1	3				1					1		1			7		1						6		
Myotis ater	LC	E			1				1							1								1		1	1	3	
Myotis horsefieldii	LC	E		2	1				1																				
Myotis muricola	LC	E								15																			
Glischropus tylopus	LC	E							2	2					2	3											4	4	
Pipistrellus tenuis	LC	E			1														1										
Tylonycteris pachypus	LC	E							1		7																	1	
Tylonycteris robustula	LC	E									1																		
Scotophilus kuhlii	LC	O					1																						
Miniopterus magnater	NT	O							1		1																		
Miniopterus medius/Miniopterus schreibersii	LC	O			1														4										

[a] IUCN red list status: NT, near threaten; VU, vulnerable (SAMD, 2009).

[b] Ensemble to which species belongs: F = predominantly tree cavity and/or foliage roosting narrow-space species; C = predominantly cave roosting narrow-space species

Table S2.2 Pairwise geographical distance (KM) between 15 selected sites for assemblage analysis.

	F01	F02	F23	F24	JR2	JR3	KH1	KH3	KT3	NS1	PH1	PH2	PH6	PN1	PK1
F01	0.000														
F02	7.856	0.000													
F23	52.092	44.293	0.000												
F24	39.710	41.069	64.442	0.000											
JR2	255.527	263.365	306.931	252.851	0.000										
JR3	158.278	166.131	210.243	161.348	99.215	0.000									
KH1	282.669	274.973	230.829	280.604	533.398	439.487	0.000								
KH3	331.687	324.362	281.584	322.652	573.704	483.948	70.538	0.000							
KT3	218.785	212.733	177.148	198.985	441.732	357.842	134.420	140.914	0.000						
NS1	76.283	81.178	117.953	109.495	224.128	126.527	342.956	397.964	293.192	0.000					
PH1	81.681	88.408	126.553	65.975	187.915	101.669	346.171	385.969	256.738	108.015	0.000				
PH2	127.189	124.812	114.703	92.231	314.223	235.934	243.556	267.724	129.279	201.385	134.574	0.000			
PH6	92.659	87.313	61.597	73.118	323.757	234.264	212.115	250.217	127.109	168.491	135.860	58.894	0.000		
PN1	267.608	259.759	215.677	272.000	522.375	425.882	46.366	115.865	159.828	320.748	337.928	250.468	209.620	0.000	
PK1	183.342	175.487	131.769	190.911	438.684	341.556	110.061	174.458	139.823	236.167	256.313	186.472	136.104	84.962	0.000

Table S2.3 Pairwise difference of species richness between 15 selected sites. The values were calculated manually based on the values predicted by Shen Multinomial Model in Table 2.3.

	F01	F02	F23	F24	JR2	JR3	KH1	KH3	KT3	NS1	PH1	PH2	PH6	PN1	PK1
F01	0.0														
F02	3.6	0.0													
F23	2.8	6.4	0.0												
F24	6.6	3.0	9.4	0.0											
JR2	1.3	4.9	1.5	7.9	0.0										
JR3	0.8	2.8	3.6	5.8	2.1	0.0									
KH1	5.3	1.7	8.1	1.3	6.6	4.5	0.0								
KH3	12.9	9.3	15.7	6.3	14.2	12.1	7.6	0.0							
KT3	5.3	1.7	8.1	1.3	6.6	4.5	0.0	7.6	0.0						
NS1	1.1	2.5	3.9	5.5	2.4	0.3	4.2	11.8	4.2	0.0					
PH1	1.1	4.7	1.7	7.7	0.2	1.9	6.4	14.0	6.4	2.2	0.0				
PH2	1.7	5.3	1.1	8.3	0.4	2.5	7.0	14.6	7.0	2.8	0.6	0.0			
PH6	4.7	1.1	7.5	1.9	6.0	3.9	0.6	8.2	0.6	3.6	5.8	6.4	0.0		
PN1	8.7	5.1	11.5	2.1	10.0	7.9	3.4	4.2	3.4	7.6	9.8	10.4	4.0	0.0	
PK1	4.6	1.0	7.4	2.0	5.9	3.8	0.7	8.3	0.7	3.5	5.7	6.3	0.1	4.1	0.0

Table S2.4 Chao-Sørensen corrected similarity between 15 selected sites, based on the capture records of numbers of species and individuals from each site, calculated in EstimateS 8.0.

	F01	F02	F23	F24	JR2	JR3	KH1	KH3	KT3	NS1	PH1	PH2	PH6	PN1	PK1
F01	0.000														
F02	0.982	0.000													
F23	0.764	0.739	0.000												
F24	0.731	0.669	0.817	0.000											
JR2	1.000	0.816	0.951	0.776	0.000										
JR3	0.985	0.828	0.909	1.000	0.971	0.000									
KH1	0.516	0.631	0.928	0.959	0.667	0.854	0.000								
KH3	0.525	0.574	0.552	0.845	0.704	0.791	0.822	0.000							
KT3	0.813	0.736	0.883	0.793	0.641	0.671	0.914	0.394	0.000						
NS1	0.719	0.758	0.988	0.877	0.950	0.983	0.917	0.685	0.795	0.000					
PH1	0.956	0.857	0.956	0.895	0.990	0.992	0.865	0.810	0.775	0.848	0.000				
PH2	0.990	0.756	0.909	0.977	1.000	0.991	0.861	0.880	0.949	0.965	0.822	0.000			
PH6	1.000	0.779	0.896	0.758	0.822	0.859	0.963	0.315	0.814	0.977	0.702	0.839	0.000		
PN1	0.701	0.676	0.937	0.829	0.861	0.964	0.892	0.819	0.949	0.970	0.775	0.929	0.953	0.000	
PK1	0.953	0.692	0.959	0.547	0.930	0.932	0.691	0.552	0.794	0.935	0.786	0.924	0.978	0.862	0.000

Table S2.4 Uncorrected Sørensen similarity between 15 selected sites, based on the capture records of numbers of species and individuals from each site, calculated in EstimateS 8.0.

	F01	F02	F23	F24	JR2	JR3	KH1	KH3	KT3	NS1	PH1	PH2	PH6	PN1	PK1
F01	0.000														
F02	0.962	0.000													
F23	0.621	0.690	0.000												
F24	0.681	0.652	0.798	0.000											
JR2	0.948	0.775	0.847	0.671	0.000										
JR3	0.928	0.745	0.825	0.916	0.948	0.000									
KH1	0.482	0.590	0.840	0.907	0.578	0.838	0.000								
KH3	0.515	0.574	0.550	0.843	0.600	0.687	0.756	0.000							
KT3	0.643	0.683	0.848	0.691	0.589	0.614	0.839	0.370	0.000						
NS1	0.666	0.686	0.927	0.823	0.927	0.963	0.893	0.648	0.737	0.000					
PH1	0.923	0.836	0.838	0.831	0.952	0.873	0.765	0.774	0.703	0.827	0.000				
PH2	0.954	0.745	0.803	0.715	0.986	0.963	0.829	0.753	0.783	0.940	0.797	0.000			
PH6	0.761	0.727	0.888	0.753	0.817	0.846	0.962	0.297	0.794	0.956	0.677	0.828	0.000		
PN1	0.660	0.676	0.930	0.821	0.854	0.960	0.892	0.814	0.917	0.956	0.760	0.906	0.951	0.000	
PK1	0.854	0.656	0.915	0.491	0.912	0.888	0.671	0.498	0.791	0.923	0.760	0.888	0.954	0.862	0.000

CHAPTER THREE

Phylogeography of the intermediate horseshoe bat (*Rhinolophus affinis*) in Peninsular Malaysia and comparisons with Chinese populations

Chapter 3: Phylogeography of the intermediate horseshoe bat (*Rhinolophus affinis*) in Peninsular Malaysia and comparisons with Chinese populations

Chapter summary

The intermediate horseshoe bat *Rhinolophus affinis* was found to be the most widely distributed and common forest-specialist bat species across the Malay Peninsula, and is thus a useful candidate for phylogeographic analyses to assess past habitat change. I studied the colonisation and demographic history of this species, to determine whether it supported a post-LGM population expansion from the south, as suggested by my assemblage analyses. I sampled *R. affinis* from across Peninsular Malaysia, and sequenced 525 base-pairs of the hyper-variable region I of mitochondrial D-loop in 200 individuals. Data were compared to published data from three other subspecies of *R. affinis* from Southern China. Phylogenetic analyses supported monophyly of *R. affinis* in Peninsular Malaysia with a divergence from China around 800,000 years before present (BP), and the time of most recent common ancestor (TMRCA) of the Peninsular Malaysian subspecies around 466,391 years BP. Very high haplotype diversity was detected with 167 haplotypes identified, and demographic analyses suggested no recent population expansion. Median-joining and statistical parsimony networks indicated well mixed haplotypes across regions in Malaysia, and no isolation-by-distance was found. High diversity and an absence of clear population structure suggest strong gene flow or considerable ancestral polymorphism, and do not support a rapid expansion since the LGM. Instead all evidence supports a long history, with a possible origin of the Malaysian subspecies from further north.

Introduction

Phylogeographic analyses

The subject of phylogeography is concerned with the spatial and temporal arrangement of genetic lineages, which may come from within a taxon, or may represent different taxa (Avise, 2009). As such phylogeography combines elements of population genetics at a micro-evolutionary scale (Hickerson et al., 2010), together with the disciplines of phylogenetics, biogeography and historical geography, all of which focus on the macro-evolutionary scale (Avise et al., 1987). Therefore, while phylogeography shares some features with landscape genetics in that both explore genetic lineages from spatial and temporal perspectives, the latter aims to evaluate contemporary environmental processes that influence genetic structure at finer scales (Chan et al., 2011), whereas phylogeography is much more focussed in tracing the historical processes that formed these patterns of genetic variation (Wang, 2010). Combined with a comparative approach, phylogeographic methods can also help to identify similarities or differences in patterns of genetic relationship across species, so providing insights into common processes that have influenced multiple organisms (Taberlet et al., 1998; Hewitt, 2001; Hewitt, 2004; Emerson and Hewitt, 2005).

Because phylogeographic methods aim to capture historical signals of past evolutionary events, they tend to rely on genetic markers with slower mutation rates (Avise et al., 1987; Wang, 2010). Genes or genomes that are characterised by high copy-number, haploidy and uni-parental inheritance offer additional benefits for phylogeographic analyses. Therefore it is not surprising that genes located within organelle genomes such as those of mitochondria (mtDNA) and chloroplasts (cpDNA) – which meet these criteria - have traditionally been the most popular choice for studies of animals and plants, respectively (Avise, 2009). In animals, for example, maternally inherited mtDNA, has a moderate mutation rate (μ) of around 6×10^{-8}, which is slower than that of microsatellite markers yet faster than single-copy nuclear DNA (scnDNA) (Haag-Liautard et al., 2008). In contrast to animal mtDNA, plant mtDNA is characterised by rates of mutation up to 100 times slower. Moreover, plant mitochondrial genome sizes are much larger and more variable than those in animals, leading them to be much less suitable for the analysis of plant phylogeography. The circular cpDNA molecule has faster mutation rates and relatively less size difference

among species (Avise, 2009). With these advantageous characteristics, cpDNA has been widely used as an evolutionary marker in plant phylogeography studies over many years. However, the genetic transmission mode of cpDNA differs from species to species and is not always transmitted purely down the maternal line (Avise, 2009). It is thus essential that the transmission mode of cpDNA is considered for each particular species before this marker can be corrected applied and interpreted. Despite some disadvantages compared to organelle genomes, scnDNA has been successfully applied in the field of phylogeography (Hare, 2001). Notably introns of protein-coding regions (which tend to have faster mutation rates than coding exons) can be useful for resolving population histories, while some sex chromosome genes (e.g. Y-chromosome loci in mammals) are effectively haploid, thus circumventing the problems of recombination (Avise, 2009). Nowadays, a growing number of phylogeographic studies combine multiple types of marker with the aim of simultaneously inferring several aspects of population and lineage history (Godinho et al., 2008; Flanders et al., 2009; Mao et al., 2010b; Polezhaeva et al., 2010).

In vertebrates, mtDNA remains the preferred source of markers for phylogeography studies/analyses, with numerous examples of studies that have examined sections of mtDNA to trace dispersal patterns of species (O'Corry-Crowe et al., 1997; Burbrink et al., 2000, Huang et al., 2010), as well as the colonisation and migration histories of populations (Vigilant et al., 1991; Flanders et al., 2009). Moreover, the D-loop, or control region, has long been considered to be especially informative for revealing evolutionary processes due to its elevated polymorphism resulting from nucleotide variability as well as length variation (Wilkinson and Chapman, 1991). Although high mutation rates can introduce the risks of homoplasious mutations, the D-loop is arguably still the most useful marker for phylogeography and indeed shallow phylogenetic analyses of animal taxa (Avise, 1998; Hewitt, 2001).

Results from genetic analyses are more valuable if they can be related to other sources of information regarding the history and physical make up of a given study area. Unlike landscape genetics, phylogeography analyses focus most heavily on past geographical conditions. Presently, Geographic Information System (GIS) analysis is proving a useful geospatial resource in this context (Hickerson et al., 2010). In particular, data on past environments or ecosystems can offer essential landscape information to complement phylogeographic models in identifying the causes of

reconstructed historical and/or observed contemporary patterns of genetic variation (Flanders et al., 2011). Advances in geospatial resources, together with rapid developments in statistical phylogenetics and demographic modelling, is pushing phylogeography into a new era, which should shed light on the underlying mechanisms of diversification events, as well as inform conservation management decisions (Chan et al., 2011).

Phylogeography studies of bats

The phylogeography of several bat species have been well-studied in Europe and the continents of North and South America. In Europe, these studies have consistently shown evidence of rapid population growth since the last glacial period, with recolonization out of Mediterranean areas (Ruedi and Castella, 2003; Rossiter et al., 2007; Bilgin et al., 2008; Flanders et al., 2009; Furman et al., 2009) and West Asian refugia (Flanders et al., 2009, Rossiter et al., 2007); in line with Hewitt's syntheses on other species (Hewitt, 1999). Moreover, in these studies of temperate species, lower genetic diversity at higher latitudes has also been found, due to the effect of "northern purity", whereas their refugial population tend to be characterised by "southern richness", again supporting Hewitt (1999).

As more markers have become available, studies of bats and other taxa have shown that the typical expansion from refugia in the Balkans, Italy or Iberia is an oversimplification. Indeed, studies that have included more fine-scale sampling have been able to show that populations in traditional refugia might actually contain multiple refugial populations, leading to the idea of "refugia-within-refugia" (Hulva et al., 2004). Moreover, by combining markers with different mutation rates, it has been possible to reconstruct a longer history of colonization. For example, working on the greater horseshoe bat, *Rhinolophus ferrumequinum*, Rossiter et al. (2007) used microsatellites to show that European populations survived the LGM in the Mediterranean and Balkans, and expanded to form suture zones. However, at the same time mtDNA showed almost no variation across Europe, indicating that this population had also undergone an earlier expansion out of the Middle East (Flanders et al., 2009). Other studies have also reported refugia in Asia Minor, including the Caucuses and Turkey (Bilgin et al., 2008). In recent years, there has also been increasing evidence of refugia even further east, including temperate and subtropical areas of China, although the

impact of glaciations were estimated to be less severe than those in Europe (Hewitt, 2004). Our understanding of Asian refugia comes from a range of species, including the plant *Arabidopsis thaliana* (Sharbel et al., 2000; Beck et al., 2008), tawny owl (Brito, 2005) and summer-green trees (Leroy and Arpe, 2007).

In addition to showing northward declines in genetic variability, populations that have undergone expansions can also be identified by their 'star-like' phylogenies, in which there is a common ancestral haplotype with several closely related derived haplotypes. This is the case for *R. affinis* in China (Mao et al., 2010b), in which the estimated most recent common ancestor for the star-like topographies was concurrent with Pleistocene glaciations cycles. Similar topologies have been observed in some other bat species, such as *Carollia perspicillata* and *Carollia sowelli* from the New World tropics (Hoffmann and Baker, 2003) and *Mystacina tuberculata* from New Zealand (Lloyd, 2003).

In comparison to temperate areas, the phylogeography of bats (and other taxa) in the wet tropics has not been well explored. However, current findings have demonstrated greater population variation and network complexity in tropical bats, often with less clear demographic expansions due to higher nucleotide diversity (Carstens et al., 2004; Martins et al., 2007; Martins et al., 2009; Chen et al., 2010). These phenomena are evident in *Cynopterus brachyotis* (Campbell et al., 2004), *Rhinolophus pearsoni* (Mao et al., 2010a) and *Rhinolophus monoceros* (Chen et al., 2006). Here the networks show more even distributions of ancestral haplotypes, with reticulations and often lots of mixing. This is not surprising since the populations and, in some cases the taxa, should be much older in areas that were not directly affected by ice sheets during glacial periods. However, although these tropical populations are older than those in temperate zones, the possibility remains that forest-specialist species have undergone cycles of contractions and expansions if the area of forest in the region decreased during the LGM. More studies are therefore needed in order to test this possibility.

Up until the year 2002, a total of 71 horseshoe bat species, under the single genus *Rhinolophus,* (family Rhinolophidae) had been identified worldwide (2003, Simmons, 2005). *Rhinolophus* species are distributed across the Old World and are particularly diverse in the tropical regions of Asia and Africa (Csorba et al., 2003). The

recently compiled Southeast Asia Mammal Databank (SAMD, 2009) shows that 38 *Rhinolophus* species (around 53% of the total number) occur in Southeast Asia. There has been considerable interest in the timing and mechanisms of diversification of horseshoe bats, as well considerable debate regarding their biogeographical history. A detailed phylogeny has been reconstructed based on Cytochrome *b* sequences (Guillén-Servent et al., 2003) in which the oldest fossil of the genus was used to estimate the split time of the Rhinolophidae from its sister family, the Hipposideridae (Simmons and Geisler, 1998; Gunnell and Simmons, 2005). This phylogeny was used to suggest that the genus originated from Europe during the late Eocene. However, this result conflicted with earlier assessments based solely on morphological data, which suggested a Southeast Asian origin (Bogdanowicz, 1992). More recent phylogeographic inferences based on mitochondrial genes and nuclear introns (Stoffberg et al., 2010) and a combination of morphological and molecular data (Teeling et al., 2005), have not resolved this issue and both Asian and African origins are still proposed (Eick et al., 2005). In most of these studies, the divergence, radiation and colonisation processes of horseshoe bats have been linked to climate or habitat change (Maree and Grant, 1997). Examination of the shallower parts of the tree has focused on mechanisms of species divergence (Kingston and Rossiter, 2004; Mao et al., 2010a; 2010b).

Dispersal and colonisation history of *Rhinolophus affinis*

Rhinolophus affinis is a common species that is widespread across northern India, southern China, mainland Southeast Asia (Thailand, Vietnam and Malaysia) and Indonesia (Csorba et al., 2003; SAMD, 2009; Francis, 2008). To date, nine subspecies of *R. affinis* have been recognised and, with the exceptions of *R. a. himalayanus* and *R. a. hainanus*, all are restricted to Southeast Asia (Csorba et al., 2003).

Studies that have undertaken morphological assessment (Zhou et al., 2005) and reconstructions of population history (Mao et al., 2010b) have revealed differences among three subspecies of *R. affinis* in China: *R. a. himalayanus*, *R. a. macrurus* and *R. a. hainanus*. Of these, the former two are from the mainland, and the latter is an island subspecies from Hainan. Bayesian estimation of the time of the most recent common ancestor (TMRCA) of all three taxa was around 900,000 years ago, a period when the sea level was higher and so suggesting a role of the Qiong Zhou Strait as a geographical barrier mediating the formation of the island subspecies *R. a. hainanus* (Mao et al.,

2010b). This study also suggested that during glaciation periods, when the sea level was low, exposed land bridges aided recolonization events of *R. a. hainanus* back to the mainland, where it formed *R. a. macrurus* and underwent secondary contact with *R. a. himalayanus*.

Other work on *R. affinis* in Southeast Asia linked genetic structure to sea barriers (Maharadatunkamsi et al. 2000). Yet unlike the clear divergence pattern shown in China, the populations of *R. affinis* from 11 islands in the Wallacea region of Indonesia (at the eastern edge of the species range) revealed a different trend. Here, allozyme and morphological data taken from island subpopulations indicated an overall longitudinal decline in heterozygosity from west to east, with lowest diversity in the most isolated islands. Moreover, in contrast to the findings of Mao et al. (2010b), there was no evidence that genetic structure among islands was correlated to sea barriers at the time of the LGM (Maharadatunkamsi et al., 2000).

In Peninsular Malaysia *R. affinis* is represented by just one subspecies, *R. a. superans*, which was first described in 1905 in Pahang (Andersen, 1905). The taxon was later reported in other areas of Peninsular Malaysia, including Pasoh Forest Reserve in Negeri Sembilan, Sungei Siput, Batu Caves, Lenggong in Perak; as well as Krau, Sembilan, Kampung Juara (Tioman Island) and Tanah Rata in Pahang (Zubaid, 1993; Csorba et al., 2003; Kingston et al., 2006; Struebig et al., 2008). Landscape-scale population genetic structure of *R. affinis* population in Pahang was studied by Struebig (2008) based on microsatellite data, who found evidence of two main clusters, suggesting the presence of a potential cryptic species, although further sampling and analyses are needed to confirm this.

Although the aim of my study was to study the intra-species phylogeography of *R. affinis* in Peninsular Malaysia to gain insight into population history and colonisation, it is anticipated that any processes and patterns detected will be applicable to many other bat species that share the same traits and ecological requirements. As mentioned, the study region is home to the highest alpha diversity of insectivorous bats in the Old World, and tropical zones generally host more bat species (Findley, 1993). Similar ecomorphological and behavioural traits are seen in many of the rhinolophid species of Southeast Asia, including *R. sinicus*, *R. lepidus* and *R. stheno* (see Csorba et al. (2003)). The latter two of these taxa were included in a recent study by Rossiter et al.

(2012), who found evidence of correlated signatures of fine-scale gene flow in several forest bats characterised by similar roost habits (although this study did not include *R. affinis*).

Biogeographical history of Sundaland

The Malay Peninsula together with the southern part of Sumatra, Java, Borneo and Greater Palawan are collectively known as the Sundaland part of Southeast Asia, due to the fact that they are all located on the continental Sunda Shelf (Wallace, 1860; Tougard, 2001; Bird et al., 2005). See Figure S3.1 in the Supporting Appendix for a map of the shelf. Sundaland is an important biodiversity hotspot (Myers et al., 2000; Brooks et al., 2002), and is classified as one of the sub-regions of the Oriental biogeographical regions (Tougard, 2001). At the eastern limit of the region is Wallace's line, and at the northern limit is the Isthmus of Kra at 9ºN. A clear turnover of biota has long been recognized at each side of these boundaries (Wallace, 1860). It is generally accepted that the Sunda Shelf was geologically stable throughout the Cenozoic era (Hall and Nichols, 2002), despite its complex geological formation during the Palaeozoic (Burrett et al., 1991), and that of Southeast Asia as a whole (Hall, 1998). Indeed tectonic plate models constructed for Southeast Asia suggest that the Malay Peninsula had formed its present shape, and had connected to mainland Asia over 50 million BP (Hall, 1998). However, there were three subsequent major collision events that occurred beyond the Sunda Shelf that might have impacted on this region. These events were the collision of India and Asia at around 45 million years BP, the collision between the margin of north Australia and the arcs to the north (25 Ma) and the collision of Taiwan (5 Ma) (Hall, 1998; Ali and Aitchison, 2008).

Before the mid-Miocene, the movement of 'terranes' (tectonic fragments) from Gondwana towards the Asian continental margin, acted as stepping stones for terrestrial Gondwanan biota to disperse into Sundaland (Burrett et al., 1991). The biogeography of these terranes has turned out to be fundamental in shaping present-day Sundaic biodiversity. For example, mite harvestmen that originated from the Sibumasu terrane of Gondwana are one example of an ancestral, yet endemic, group of arthropods from Sundaland (Clouse and Giribet, 2010). Other major faunal exchanges occurred between Southeast Asia and India, around 55 Ma, and also between northern Australia and eastern Sundaland (Hall, 1998; reviewed by Ali and Aitchison, 2008).

In addition to geological and tectonic activities, past and present biodiversity in Sundaland will also have been shaped by fluctuations in sea levels (and thus land distribution), as well as climatic elements such as temperature, rainfall and humidity. These factors have played especially important roles during the past one million years when the current shape of Sunda Shelf was fully formed and stable. Some researchers have presented evidence to suggest that Sundaland in the late Pliocene to early Pleistocene was characterized by a drier and seasonal climate (Verstappen, 1997), and this has also been suggested to be the case during the LGM itself (Heaney, 1991; Wikramanayake et al., 2002; Bird et al., 2005; Wurster et al. 2010). During such dry periods, it has been suggested that 'savannah corridors' stretched from the Malay peninsula to southern Borneo and Java, so fragmenting or replacing humid tropical rainforest and providing new habitats to animals (van den Bergh et al., 2001) and, at around 1.9 Ma, humans (Heaney, 1991; van der Kaars and Dam, 1995; Bettis et al., 2004). Work on the taxonomic diversity of forest ant species suggest that the current highlands might have served as rainforest refugia during drier climatic conditions, although this study argued for the persistence of some forest (Quek et al., 2007). Similarly, it has been argued that grasslands replaced forest during glacial maxima in lowland Amazonia, and that this floral replacement contributed to the regional biodiversity by driving isolation and divergence of populations of forest specialists into small upland areas (Haffer, 1969).

Contrary to suggestions that the forest was replaced by grasslands (also see General Introduction) a recently built spatially explicit model that incorporated palaeoclimatic, geographic and geologic information of Sundaland, has argued for a different scenario. Cannon et al. (2009) found that there was evidence of greater continuous coverage of evergreen rainforest in Sundaland during the LGM. In fact, under this model, the larger extent of rainforest was considered normal throughout the last million years, whenever periods of the sea level dropped lower than present day. This study further concluded that Sundaland's tropical forests are currently at a refugial stage, where flora and fauna are retreating and remain in the exposed highlands. If this is the case, rainforest fauna would have been provided with a broad and continuously available habitat with no obvious barriers to dispersal over the fully exposed shelf during low sea level periods since the mid-Pliocene (5.3 to 2.8 Ma), a scenario that has been suggested from data on rodents (Gorog et al., 2004) and primates (Harrison et al.,

2006). Interestingly, the extent of savannah in Amazonia has also been questioned in light of newer data, again indicating that rainforest might have been more resilient to cooler climates than was previously suggested (Colinvaux et al., 1996).

Study objectives

In this study, the widespread distribution of *R. affinis* over Sundaland and Asia, as well as its abundance in the region, provided an ideal model species with which to trace the phylogeographic and colonisation patterns of a forest-specialist mammal in tropical Southeast Asia. By examining mtDNA to characterise the phylogeographic pattern of *R. affinis* populations in Peninsular Malaysia, the following questions and hypotheses were addressed:

i. Characterize the pattern of broad-scale genetic structure and migration model of *R. affinis* populations in Peninsular Malaysia and assess its demographic history. If theories of rainforest loss during the LGM following by post-LGM recovery are correct, I hypothesize that genetic variation (haplotype diversity) will show a clinal signature due to population and range expansion from one or more refugial areas. Conversely, if the rainforest persisted throughout this period, I expect no such cline and instead diversity should be more evenly distributed.

ii. Compare *R. affinis* population genetic diversity and structure in Malaysia to that of China, to incorporate a wider geographical scale.

iii. Estimate and construct maternal genealogies of *R. affinis* populations in Peninsular Malaysia based on statistical parsimony and median-joining methods. Related to (i), I would expect to see evidence of a star-like phylogeny if this species underwent post-glacial population expansion and no such signature if the species was relatively stable during the glacial maximum.

iv. Estimate demographic growth of *R. affinis* in Peninsular Malaysia, again to assess whether there is evidence of population growth consistent with post-LGM recovery.

Material and methods

Sampling sites selection and tissue sampling

I surveyed 28 sites for *R. affinis* individuals across Peninsular Malaysia between February 2008 and September 2009 (Figure 3.1). For 26 sites, bats were captured with harp traps (see Chapter 2). Additionally at site KH3, hand nets were used to capture bats in caves due to practical difficulties of accessing foraging sites. For sampling for DNA analysis, a 3-mm biopsy of wing membrane tissue was removed from each animal using a dermatological punch (Stiefel, UK). From two additional sites, samples were obtained from Dr C. Fletcher of the Forest Research Institute of Malaysia (FRIM): coded as Temenggor Forest Reserve (PK4/PITC) and the FRIM headquarters (FRIM). More details of sampling sites (hereafter referred to as 'populations') are listed in Table 3.1.

DNA extraction and amplification

Genomic DNA for each individual of *R. affinis* was extracted and purified from wing membrane tissue using either the high-throughput Promega Wizard® SV 96 Genomic DNA Purification System (96-well format) or the Promega Wizard® SV Genomic DNA Purification System (250 preps). In each case, digestion using Proteinase K was undertaken overnight, and extractions followed the manufacturer's protocols.

The hypervariable domain I (HV I) of the D-loop of the mitochondrial genome was amplified with primers DL-H 16750 (5'-CCTGAAGTAGGAACCAGATG-3') (Wilkinson and Chapman, 1991) and Thr-L 16272 (5'-CCCGGTCTTGTAAACC-3') (Stanley et al., 1996). This region spans phenylalanine tRNA ($tRNA^{Phe}$) to proline tRNA ($tRNA^{Pro}$) (Clayton, 1982). Polymerase Chain Reactions (PCRs) were undertaken in 30µl volumes containing 1u of Promega GoTaq® Flexi DNA Polymerase, around 3.0ng of DNA template, 0.67µM of each primer, 0.33mM of each dNTP, 2mM of Mg^{2+} and 1x of the manufacturer's buffer. PCR was performed using an Eppendorf Mastercycler Gradient with the following profile: an initial denaturing step of 5 minutes at 95ºC; 35 cycles of amplification, with each cycle consisting of a denaturing step of 30 seconds for 94ºC, an annealing step of 55ºC for 30 seconds and an extension step of 40 seconds for 72ºC. The PCR ended with a final extension step of 72ºC for 10 minutes.

Figure 3.1 Map of Peninsular Malaysia showing 30 localities from which individuals were analysed for sequence-based phylogeographic analyses. To aid with the interpretation of the results, sampling sites (populations) are colour coded as follows: brown-yellow for the west region, green for the central region, blue for the east region, and red-pink for the southern region. For each group, colour tones decrease with latitude (i.e. darker colours for the most north-east samples). These colours are also included in Table 3.1 and Figures 3.4 and 3.5.

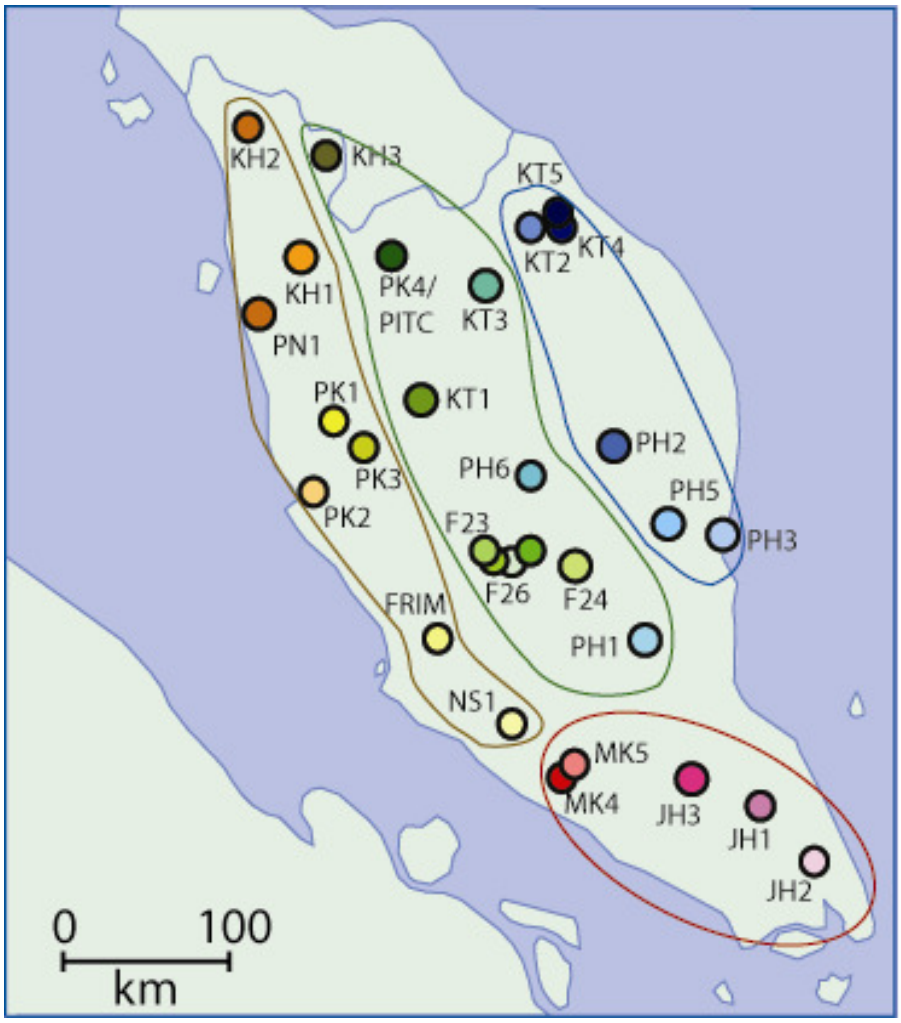


Table 3.1 Localities with samples analysed for sequencing

Site Region	Sampling Locality[a]	Sampling Elevation	Coordinates		n[b]	C[c]
			Longitude	Latitude		
Central	F03	<300m	101.9670	3.7000	1	
	F23	<300m	101.8910	3.7494	5	
	F24	<300m	102.4542	3.6348	6	
	F25	<300m	102.1667	3.7500	2	
	F26	<300m	102.0830	3.6830	6	
	KH3	129m	100.9628	6.1069	6	
	KT1	294m	101.4857	4.6736	5	
	KT3	65-108m	101.9766	5.3398	8	
	PH1	34-56m	102.9011	3.2229	6	
	PK4/PITC	590-810m	101.3600	5.5100	6	
	PH6	70m	102.1882	4.2163	9	
West	*FRIM*	NA	101.6378	3.2369	10	
	KH1	51m	100.7732	5.5012	6	
	KH2	78&178m	100.4840	6.3192	9	
	NS1	<300m	102.0782	2.7050	7	
	PN1	<300m	100.5457	5.1511	6	
	PK1	90m	101.0039	4.5385	8	
	PK2	<300m	100.5550	4.2200	9	
	PK3	18m	101.1757	4.3789	8	
East	KT2	49-257m	102.1681	5.6948	3	
	KT4	94m	102.3784	5.7411	9	
	KT5	48m	102.3384	5.7944	7	
	PH2	140m	102.6813	4.4131	7	
	PH3	6m	103.3575	3.8614	9	
	PH5	<300m	103.0333	3.9167	8	
South	JH1	29-160m	103.5860	2.1862	10	
	JH2	24-29m	103.9139	1.8693	6	
	JH3	97m	103.1589	2.3456	10	
	MK4	<300m	102.3833	2.3833	7	
	MK5	<300m	102.4167	2.4667	1	

[a] Sites in italic are sampling locations of wing punch tissues contributed by Dr C. Fletcher of the Forest Research Institute of Malaysia (FRIM).
[b] Number of samples used in analyses
[c] Colours representing sampling localities, corresponding to Figures 4.1, 4.4 and 4.5.

PCR products were then purified using the QIAquick PCR Purification Kit (Qiagen), and sent for Sanger sequencing, either by Eurofin MWG Operon (Germany) or by 1st Base Pte. Ltd. (Singapore). Both companies used an automated ABI PRISM DNA Sequencer (Applied Biosystems). All PCR products were sequenced in a single direction with primer Thr-L 16272. In cases of failed or low quality sequencing results, this step was repeated at least once with the same primer.

Sequences were edited by eye and aligned using CLUSTAL, implemented in BioEdit (http://www.mbio.ncsu.edu/bioedit/bioedit.html). During alignment and editing, sequences of low quality were filtered. Aligned mtDNA sequences were collapsed into haplotypes using Alignment Transformation Environment (ALTER) (Glez-Peña et al., 2010). Aligned sequences were converted into nexus and phylip file formats for subsequent analyses.

Genetic diversity and demographic history analysis

For the complete dataset, the numbers of polymorphic and parsimony informative sites were calculated using DNA Sequence Polymorphism (DnaSP) version 5.10. Genetic diversity was calculated at the global level (i.e. all populations from the Peninsular Malaysia) and also for the four broad regional groupings: West, Central, East and South regions (Figure 4.1). These areas, although somewhat arbitrary, were chosen because they correspond to natural geographical areas (each side of the main mountain range, the inland area, and the coastal area). It was hoped that comparative genetic analyses of populations from these four regions could thus provide information on broad-scale patterns of structure and variation.

Average pairwise distances among haplotype sequences, within each region and between the four regions, were calculated with MEGA v. 4 (Tamura et al., 2007) using the Kimura-2-parameter model (Kimura, 1980) with a gamma distribution. Gaps were treated as pairwise deletions. Average pairwise differences (k), haplotype diversity (*h*) and nucleotide diversity (π) were calculated for each population as well as at the regional level using the software DnaSP version 5.10 (Librado and Rozas, 2009). Populations with only one individual (e.g. F03 and MK5) were excluded during the

calculation of the three measurements at the population level, leaving 28 populations across Peninsular Malaysia that collectively comprised a total of 198 individuals. Haplotype diversity is defined as the probability of two randomly chosen haplotypes from a sample being different (Nei, 1987), which is analogous to expected heterozygosity for diploid data (Doukakis et al., 2002). Nucleotide diversity (π) describes the average number of nucleotide differences per site between two randomly chosen and randomly mated DNA sequences in the sample (Nei, 1987; Nei and Kumar, 2000).

In order to estimate the demographic history of the study populations, neutrality tests were performed at both the global level (which here means all populations in Peninsular Malaysia) and at the regional level (Central, West, East, and South of Peninsular Malaysia). Pooling sequences from different populations in this way was justified because, based on the results, the data met the assumption for undertaking demographic analyses that population genetic structure is minimal or absent. These tests also assume no recombination, or selection, which are also conditions that are met by mtDNA. For the global analysis, Tajima's test of neutrality, D (Tajima, 1989) was used to assess the null hypothesis of selective neutrality and constant population size (Kimura, 1983) of the D-loop mtDNA region of *R. affinis* based on its DNA polymorphic sites and the average number of nucleotide differences. Beside this, Fu and Li's D* and F* tests (Fu and Li, 1993) were applied to assess population growth of *R. affinis* population in Peninsular Malaysia. These tests focus on DNA polymorphism to detect an excess of old mutations. Fu's F_S (Fu, 1997) test is one of the most powerful tests for population growth when based on non-recombining (Fu, 1997; Ramírez-Soriano et al., 2008) and uni-locus neutral data (Ramos-Onsins et al., 2007). If the global population experienced population expansion or bottleneck events, then the null hypothesis of Tajima's D test will be rejected, and the D value will deviate from zero. Similarly, a significant negative departure of D*, F*, F_S value from zero (e.g. no selective advantage among haplotypes in the population) also signifies past demographic expansion.

To assess the demographic history at a finer scale, I used the R2 statistic, which is a measure of the difference between the average number of nucleotide differences and the number of singleton mutations (Ramos-Onsins and Rozas, 2002). Small R2 values point to a population expansion model. Finally, I also plotted mismatch distributions for

all regions (Rogers and Harpending, 1992), which are distributions of polymorphic sites versus the pairwise number of differences (Librado and Rozas, 2009). I used Harpending's raggedness index (r) to assess the null hypothesis of population growth, which is a measure of smoothness of the curve (Harpending, 1994). Populations at stationary demographic equilibrium are expected to have ragged, bimodal or multimodal distributions so that the null hypothesis can be rejected ($P < 0.05$) whereas populations that have undergone recent demographic expansion are expected to have smoother or unimodal plots and the null model will not be rejected ($P > 0.05$) (Rogers and Harpending, 1992). All of the population demographic history tests and analyses were performed with DnaSP version 5.10 (Librado and Rozas, 2009). Test values were assessed for significance with 10,000 bootstrap replicates using the coalescent simulation tool in the same program.

Population genetic structure analyses

In order to assess dispersal patterns and population structure of *R. affinis* populations in Peninsular Malaysia, I undertook an isolation-by-distance (IBD) analysis. The IBD model of gene flow applies where genetic isolation increases with physical distance due to a decrease in dispersal and mating probability (Wright, 1943). Gene flow is said to be following a stepping stone model. As such, IBD plots are commonly constructed to assess genetic structure among populations based on the Euclidean distances (Wright, 1943), assuming there is no geographical complexity (Jenkins et al., 2010). For my project, I calculated pairwise geographical distances among 23 sampling sites in Peninsular Malaysia (each consisted of a minimum of five samples) using Genetic Analysis using Excel (GENALEX) version 6.4 (Peakall and Smouse, 2006). Recorded coordinates (in the form of latitude and longitude) are shown in Table 3.1. Pairwise genetic distances among the 23 sampling sites were calculated based on aligned haplotypes using MEGA 4 (Tamura et al., 2007) using the Kimura-2-parameter (Kimura, 1980) model of substitution. The correlation between genetic and geographical distances was tested in the software 'Isolation by Distance Web Service' version 3.16 (IBDWS) (http://ibdws.sdsu.edu/) (Jensen et al., 2005). The strength and significance of the relationship between genetic distances and geographic distances was assessed using a Mantel Test and reduced major axis (RMA) regression, based on 10,000 randomizations.

Populations were further classified into two regions based on climatic and geographical characteristics: the Central Montane region that is more humid, mountainous, cooler and less developed (F24, F26, KH1 to KH3, KT1, KT3, NS1, PH2, PH6, PK3) and the "Marginal" region that is lower, flatter, drier, and more developed and thus characterised by forests that show greater fragmentation (JH1 to JH3, KT4, KT5, MK4, PH1, PH3, PH5, PK1, PK2, PN1, FRIM, UKM). IBD tests were repeated within each of these two regions. Here, the Montane region represents undisturbed and more continuous habitat for forest bat species, while the Marginal region is a more disturbed and fragmented habitat. If gene flow in *R. affinis* is influenced by habitat disturbance, I would expect that populations in the marginal areas might show greater levels of differentiation for a given distance than those in the more continuous forest of the Centre.

Phylogenetic analyses

Several methods were used to infer the phylogenetic relationship among populations within Peninsular Malaysia, and also between these and populations further north in China. These methods can provide insights into population origin and historical processes including colonization.

Two network-based genealogical analyses were conducted to assess relationships among all sampled individuals, which are considered particularly suitable for describing intraspecific gene evolution because they can incorporate several phenomena that are common at the population-level but which are not taken into account in conventional phylogenetic approaches (Posada and Crandall, 2001). These phenomena include the coexistence of both ancestral and derived gene copies in the population, reticulate relationships due to recombination events, and multi-furcating relationships.

First, general intraspecific network analysis was performed in NETWORK 4.6.0.0, which implements the median-joining method (Bandelt et al., 1999). A total of 200 sequences from 30 sampling sites were collapsed into 167 haplotypes in NETWORK 4.6.0.0 before proceeding to the three stages of network construction. At

the pre-processing stage, relationships between all haplotypes were first inferred and predicted by a star contraction network with the star radius set to 4. The pre-constructed network was contracted twice at this stage resulting in a total of 155 haplotypes for median-joining (MJ) construction at stage two. The MJ network was constructed with a default character weighting of 10 at each character and an epsilon value (weighted genetic distance) of 10. A frequency criterion of >1 was used at this stage to include sequences for the network skeleton. The connection cost was selected as the network distance calculation method. At the post-processing stage, the Maximum-Parsimony method was applied to identify and suppress unnecessary median vectors and links. The resulting network was then edited in Network Publisher software 1.3.0.0. A regional network was also constructed using the same procedures as described above, based on a total of 229 haplotypes from West Malaysia and China (Mao et al., 2010b), which was ultimately represented by 54 active haplotypes.

Second, I also inferred the mtDNA gene genealogy of *R. affinis* by constructing a network cladogram based on the statistical parsimony method in the software TCS 1.21 (Clement et al., 2000). A parsimony threshold of 95% was used, and gaps in the sequences were treated as a fifth state. Statistical parsimony is an algorithm established by Templeton et al. (1992), which estimates gene genealogies based on the calculation of maximum numbers of mutational steps formed by the most parsimonious connection between two haplotypes at a probability of 95% (significant standard deviations). This algorithm is limited to DNA sequences or segments with low occurrence of recombination. Unlike the previous network, all haplotypes from 200 individuals representing 30 localities were fully demonstrated in this network cladogram. The resulting genealogical network cladogram was then edited in Microsoft Power Point 2007. The colour codes used are listed in Table 3.1.

In addition to networks, three traditional phylogenetic analyses were also used to assess the relationships among haplotypes found across the study area: UPGMA, Maximum-Likelihood and Bayesian (see below for details). For these, the D-loop sequence of *Rhinolophus monoceros* (GenBank ref: DQ314025) was used as an out-group to root the trees. These analyses were applied to the data from Peninsular Malaysia, and also to the combined data from Peninsular Malaysia and China (see Mao et al., 2010b).

A phylogram based on the UPGMA was undertaken in PAUP version 4.0 beta 10 for Windows System (Swofford, 2002). The HKY85 gamma-corrected genetic distance (Hasegawa et al., 1985) was used, using 1000 bootstraps (Felsenstein, 1985). HKY85 is a model of substitution for DNA sequence data (Hasegawa et al., 1985) that allows for unequal base frequencies and an unequal transition to transversion ratio (ti/tv ratio) (Swofford, 2002). As mentioned above, UPGMA phylograms were constructed using only Peninsular Malaysia samples (148 selected haplotypes) and also these with the samples from China (108 selected sequences from Mao et al. (2010b)).

For phylogenies based on Maximum Likelihood and Bayesian methods, it was first necessary to select a best-fit nucleotide substitution model for the data using MrModeltest 2.3 (Nylander, 2004). Model selection was undertaken based on 148 selected aligned haplotypes from 30 sampling sites in Peninsular Malaysia. MrModeltest 2.3 implemented model selection by running several nucleotide substitution models and comparing model fit using likelihood-ratio tests and the Akaike Information Criterion (AIC) (Posada and Crandall, 1998; Posada, 2008).

Using the best fitting model (see Results), Maximum-Likelihood phylograms were undertaken in PhyML 3.0 Online (http://atgc.lirmm.fr/phyml/) (Guindon and Gascuel, 2003). This program has been written to cope with large datasets. Bootstrap analysis was performed with 500 replicates. Bayesian phylogenetic reconstruction was performed using MrBayes (Huelsenbeck and Ronquist, 2001; Ronquist and Huelsenbeck, 2003), run on the high performance computer (BioHPC) at Cornell University (USA) (http://cbsuapps.tc.cornell.edu/mrbayes.aspx). For estimation of posterior probabilities of the Malaysia dataset, four Monte-Carlo Markov Chains (MCMC) were run, each for 4,000,000 generations. During the run, trees were sampled at every 100^{th} generation, and the first 80,000 trees were discarded as burn-in. For inferring relationships between the Peninsular Malaysia and Chinese populations, the same parameters were applied to the whole dataset with 6,000,000 MCMC generations. Nodes with at least 70% bootstrap support in UPGMA and ML phylograms, as well as minimum Bayesian posterior probability of 0.95, were considered as significant and robust. Generated phylograms were opened and edited with TreeView 1.6.6 (Page, 1996) and FigTree version 1.3.1 (http://tree.bio.ed.ac.uk/software/figtree/).

Finally, in order to estimate the time to the most recent common ancestor (TMRCA) for the major mtDNA clades, a Bayesian tree was constructed in the software BEAST ('Bayesian Evolutionary Analysis Sampling Trees') version 1.6.1 (Drummond and Rambaut, 2007). In this program, a total of 241 mtDNA sequences from both Chinese and Peninsular Malaysia populations were analysed based on the HKY+I+G model as well as a relaxed-clock model with an uncorrected lognormal distribution using a substitution rate of 20% per million years. This substitution rate was previously estimated empirically for the control region of the noctule bat (Petit et al., 1999), and has also been used for other horseshoe bat species (Chen et al., 2006). Two independent runs of 15 million generations each were performed, with a burn-in of 15,000 generations and sampled every 1000 steps from each respective run. Results from both runs were combined and assessed for effective sample size (ESS) for each parameter in Tracer version 1.5 (Drummond and Rambaut, 2007). Bayesian trees from both of the runs were also combined using LogCombiner version 1.6.1 (Drummond and Rambaut, 2007) and visualized and edited in FigTree version 1.3.1.

Results

Genetic diversity and demographic history analysis

I obtained a total of 200 HV I of D-loop region sequences of *R. affinis* from 30 sampling sites across Peninsular Malaysia. The sequenced section comprised 525 base-pairs and ranged from positions 6272 and 16750 (Wilkinson and Chapman, 1991). Of these 200 sequences, 167 different haplotypes were recorded, including 144 (86.23%) singletons. A few haplotypes occurred as multiple copies (18 cases of two copies, two of three copies, one of four copies and two of five copies). Overall, haplotype diversity (*h*) was 0.9975. In terms of geographical structure, 13 haplotypes were found in multiple populations, mostly (n = 8) from the same region. Just one haplotype occurred in all five regions. Details of the localities and frequencies of the haplotypes are shown in Table 3.2a. A table summarising the published haplotype data from Chinese and Vietnamese populations of *R. affinis* revealed that the level of diversity was also extremely high in this species in higher latitudes north of Malaysia (Table 3.2b). Mao et al. (2010b) estimated the haplotype diversity (*h*) to be 0.983, therefore almost identical to the value found in this study.

Table 3.2a Haplotypes for D-loop region of *R. affinis* mtDNA that successfully identified based on 200 individuals from 30 sampled populations across Peninsular Malaysia Continued overleaf

Haplotypes observed	Individuals	Populations observed	Number of sequances shared
1	F03041	F03	1
2	F23010, F23023	F23	2
3	F23038, PK301006	F23,PK3	2
4	F23062, PH601049	F23,PH6	2
5	F23065, PH601064	F23,PH6	2
6	F24005	F24	1
7	F24007	F24	1
8	F24021	F24	1
9	F24033	F24	1
10	F24034, F24035	F24	2
11	F25020	F25	1
12	F25021	F25	1
13	F26020	F26	1
14	F26021	F26	1
15	F26023	F26	1
16	F26071	F26	1
17	F26073	F26	1
18	F26084, JH301007, NS1MA, NS10013, PH10106	F26,JH3,NS1,PH1	4
19	FRIM1585 KH20107 KT501025	FRIM,KH2,KT5	3
20	FRIM1632	FRIM	1
21	FRIM1690	FRIM	1
22	FRIM3761	FRIM	1
23	FRIM3763	FRIM	1
24	FRIM3768	FRIM	1
25	FRIM3770	FRIM	1
26	FRIM3827	FRIM	1
27	FRIM3948	FRIM	1
28	FRIM3991	FRIM	1
29	JH10107	JH1	1
30	JH10110	JH1	1
31	JH10121	JH1	1
32	JH10122	JH1	1
33	JH10123	JH1	1
34	JH101180	JH1	1
35	JH10203	JH1	1
36	JH10204	JH1	1
37	JH10524	JH1	1
38	JH10538	JH1	1
39	JH201114	JH2	1
40	JH201117	JH2	1
41	JH201121	JH2	1
42	JH201125	JH2	1
43	JH201135, JH302027	JH2, JH3	2
44	JH202009	JH2	1
45	JH302008	JH3	1
46	JH302014	JH3	1
47	JH302016	JH3	1
48	JH302017	JH3	1
49	JH302018	JH3	1
50	JH302028	JH3	1
51	JH302033	JH3	1
52	JH303035	JH3	1
53	KH10125	KH1	1
54	KH10128	KH1	1
55	KH10135	KH1	1
56	KH102149	KH1	1
57	KH10211, KH20111	KH1, KH2	2
58	KH10252	KH1	1
59	KH20108	KH2	1
60	KH20138	KH2	1
61	KH20143	KH2	1
62	KH20144	KH2	1
63	KH20205	KH2	1
64	KH20214	KH2	1

65	KH20218, KH30206	KH2, KH3	2
66	KH30101	KH3	1
67	KH30203	KH3	1
68	KH30234	KH3	1
69	KH30253	KH3	1
70	KH30255	KH3	1
71	KT10110	KT1	1
72	KT10120	KT1	1
73	KT10132	KT1	1
74	KT10513	KT1	1
75	KT10526	KT1	1
76	KT201015, KT401053	KT2, KT4	2
77	KT201024	KT2	1
78	KT202013	KT2	1
79	KT302005	KT3	1
80	KT302007	KT3	1
81	KT302008	KT3	1
82	KT302013	KT3	1
83	KT302016	KT3	1
84	KT302017	KT3	1
85	KT302024, PH601063	KT3, PH6	2
86	KT302033	KT3	1
87	KT401003	KT4	1
88	KT401013, PK302003	KT4, PK3	1
89	KT401014, KT401078	KT4	2
90	KT401019	KT4	1
91	KT401026, PITC1611	KT4, PITC	2
92	KT401052	KT4	1
93	KT401058	KT4	1
94	KT501008	KT5	1
95	KT50122, PH301009	KT5, PH3	2
96	KT501031	KT5	1
97	KT501044	KT5	1
98	KT501055	KT5	1
99	KT501067	KT5	1
100	MK40106, MK40135	MK4	2
101	MK40129	MK4	1
102	MK40130, MK40136	MK4	2
103	MK40132	MK4	1
104	MK40147	MK4	1
105	MK50104	MK5	1
106	NS1FARPL	NS1	1
107	NS1FARPL2	NS1	1
108	NS10021	NS1	1
109	NS10024	NS1	1
110	NS10709	NS1	1
111	PH10102	PH1	1
112	PH10103	PH1	1
113	PH10105	PH1	1
114	PH10107	PH1	1
115	PH10345	PH1	1
116	PH20133	PH2	1
117	PH20162	PH2	1
118	PH20192	PH2	1
119	PH201128, PH301026, PH301056, PH301061	PH2, PH3	4
120	PH201147	PH2	1
121	PH201151	PH2	1
122	PH201174	PH2	1
123	PH301014	PH3	1
124	PH301017	PH3	1
125	PH301045	PH3	1
126	PH301060	PH3	1
127	PH301079	PH3	1
128	PH501003, PH501051	PH5	2
129	PH501013	PH5	1
130	PH501018	PH5	1
131	PH501022	PH5	1
132	PH501045	PH5	1
133	PH501046	PH5	1
134	PH501055	PH5	1
135	PH601034	PH6	1
136	PH601053	PH6	1

137	PH601062	PH6	1
138	PH602035	PH6	1
139	PH602043	PH6	1
140	PH602044	PH6	1
141	PITC0124	PITC	1
142	PITC0156 PITC1642	PITC	2
143	PITC1699	PITC	1
144	PITC3537	PITC	1
145	PK10102	PK1	1
146	PK10103	PK1	1
147	PK10104	PK1	1
148	PK10107	PK1	1
149	PK10151	PK1	2
	PK10209		
150	PK10173	PK1	1
151	PK101129	PK1	1
152	PK203037 PK203038 PK203040 PK205031 PK205050	PK2	5
153	PK203065	PK2	1
154	PK205046	PK2	1
155	PK205058	PK2	1
156	PK206008	PK2	1
157	PK301002	PK3	1
158	PK301008 PK301048	PK3	2
159	PK301012	PK3	1
160	PK301018	PK3	1
161	PK303001	PK3	1
162	PN10201	PN1	1
163	PN102098	PN1	1
164	PN102109	PN1	1
165	PN102115	PN1	1
166	PN102116	PN1	1
167	PN102117	PN1	1

Table 3.2b Summary information for 121 published haplotypes for D-loop region of R. affinis based on 164 individuals from five localities in China and one locality in Vietnam published data by Mao et al. (2010b). Continued overleaf

Haplotypes observed	Individuals	Populations observed	Number of sequances shared
168	FQX009, SLD001, SLD004, SLD017, SLD022, WLB033 JLH004, JLH006,	Fu Jian (FJ), Guang Dong (GD), Guang Xi (GX), Jiang Xi (JX)	8
169	FQX010	FJ	1
170	FQX011, SLD020	FJ, GD	2
171	FQX012	FJ	1
172	FQX015	FJ	1
173	LF007	GD	1
174	LF020	GD	1
175	SLD002 , SLD016	GD	2
176	SLD003	GD	1
177	SLD005, SLD011, SLD019	GD	3
178	SLD006	GD	1
179	SLD009	GD	1
180	SLD010	GD	1
181	SLD021	GD	1
182	SLD023	GD	1
183	WLB001, WLB032	GX	2
184	NBCP001	Bong area, Cuc Phuong National Park, Ninh Binh Province Vietnam (VN)	1
185	NBCP015	VN	1
186	JLH001	JX	1
187	JLH002	JX	1
188	JLH005, JLH008	JX	2
189	JLH009	JX	1
190	LZ539, JC400, DL608, YG07	Hainan Island (HND)	4
191	LZ540	HND	1
192	LZ541, QE001, QE004, QE006, SK362, DL616	HND	6
193	LZ542	HND	1
194	LZ543, XL002, DL611,YG20, YG23	HND	5
195	LZ544, XL006, XL012, DL164, SL24, DL615 YG09, YG12	HND	8
196	LZ545	HND	1
197	LZ546	HND	1
198	LZ547	HND	1
199	LZ548	HND	1
200	LZ549, DL163, YG18	HND	3
201	LZ550, XMSK198	HND	2
202	LZ551	HND	1
203	XL003, SK364	HND	2
204	XL004	HND	1
205	XL007	HND	1
206	XL009	HND	1
207	XL010	HND	1
208	XL011	HND	1
209	XL014	HND	1
210	XL015	HND	1
211	QE002, DL597	HND	2
212	QE003	HND	1
213	QE005	HND	1
214	QE007, DL609	HND	2
215	XMSK197, XMSK208	HND	2
216	XMSK201	HND	1
217	XMSK205	HND	1
218	XMSK214	HND	1
219	XMSK216	HND	1
220	MC186	HND	1
221	MC187	HND	1
222	MC192, SL25	HND	2
223	NX001	HND	1
224	NX129	HND	1
225	SL19	HND	1

226	SL20	HND	1
227	SL21	HND	1
228	SL22, SL31	HND	2
229	SL23	HND	1
230	SL26	HND	1
231	SL27, JC397	HND	2
232	SL28	HND	1
233	SL29		1
234	SL30, JC396, JC399	HND	3
235	SK360	HND	1
236	SK361	HND	1
237	SK363	HND	1
238	SK365	HND	1
239	SK366, JC394	HND	2
240	JC389	HND	1
241	JC390	HND	1
242	JC391	HND	1
243	JC392	HND	1
244	JC393	HND	1
245	JC395, YG08, YGL432	HND	3
246	JC398	HND	1
247	JC401	HND	1
248	JC402	HND	1
249	JC403	HND	1
250	JC404	HND	1
251	JC405	HND	1
252	JC406	HND	1
253	JC407	HND	1
254	JC408	HND	1
255	LJ009	HND	1
256	HL002	HND	1
257	DL598	HND	1
258	DL602	HND	1
259	DL603	HND	1
260	DL604	HND	1
261	DL605	HND	1
262	DL606	HND	1
263	DL607	HND	1
264	DL610 , YG10,	HND	2
265	DL612	HND	1
266	DL613	HND	1
267	DL614	HND	1
268	DL617	HND	1
269	YG05,YGL431	HND	2
270	YG11	HND	1
271	YG13	HND	1
272	YG14, YG21	HND	2
273	YG25	HND	1
274	YGL433	HND	1
275	YGL434	HND	1

Haplotypes from Malaysia collectively had a total of 147 variable sites (127 sites with two nucleotide substitution and 20 sites with more than three nucleotides substitution). Of these 147 variable sites, 111 were identified as being parsimony informative sites. The transition to transversion ratio was estimated to be 19.81 based on the nucleotide substitution model HKY85 (Hasegawa et al., 1985). Based on the 200 mtDNA sequences, nucleotide diversity (π) was 0.03216 ± 0.00128 (± standard deviation). Average pairwise distance among haplotypes was 0.0389. Like haplotype diversity, estimated nucleotide diversity from Malaysia was remarkably similar to that reported for the Chinese and Vietnamese populations (π = 0.041) (Mao et al., 2010b).

When these haplotypes were grouped into regions, the average pairwise distance within the Central region was 0.0454, the East region was 0.0364, the West region was 0.0368, and the South region was 0.0270 (Table 3.3). The largest pairwise genetic distance was found between West-Central and East-Central regions, and the shortest distance was found between West and South region.

Table 3.3 Average pairwise genetic distances between regions generated by MEGA 4. Values in brackets below the region are the average pairwise distances within the respective region.

Region	**Central** (0.0454)	**West** (0.0368)	**South** (0.0270)	**East** (0.0364)
Central				
West	0.0421			
South	0.0396	0.0325		
East	0.0421	0.0360	0.0335	

Estimates of genetic diversity for each population in Malaysia are reported in Table 3.4. Populations from the Central region exhibited the highest haplotype diversity (h = 0.9961) and nucleotide diversity (π = 0.0400) in spite of having the fewest observed haplotypes (n = 53). The West region was found to have the next highest nucleotide diversity (π = 0.0310), whereas values were lower in the East (π = 0.0310) and South (π = 0.0273) regions. Overall numbers of polymorphic sites and pairwise genetic distances were also lower in the south and east. Therefore, unlike the findings for bat assemblage structure in the Chapter 2, genetic diversity of *R. affinis* based on the mtDNA D-loop did not decrease with increasing latitude or decreasing longitude.

Demographic analyses of Malaysian bats based on Fu's F_S test showed a significant negative departure from zero (Fs = -205.25, $P < 0.0001$), a trend that was also indicated by Fu and Li's D* = -2.0283 ($P < 0.05$) and by F*= -1.8579 ($P < 0.05$). These statistical results reflect an excess of rare mutations in the population and thus indicate that the study population has not experienced a large population expansion. However, the small and significant Fs value of -205.25 ($P < 0.001$) also suggests that the study population may not be truly panmictic. In addition to these tests, Tajima's D indicated a non-significant excess of rare mutations with slightly negative D values (D=-1.0556, $P > 0.05$), which supports the null hypothesis of Tajima's D of a constant population size.

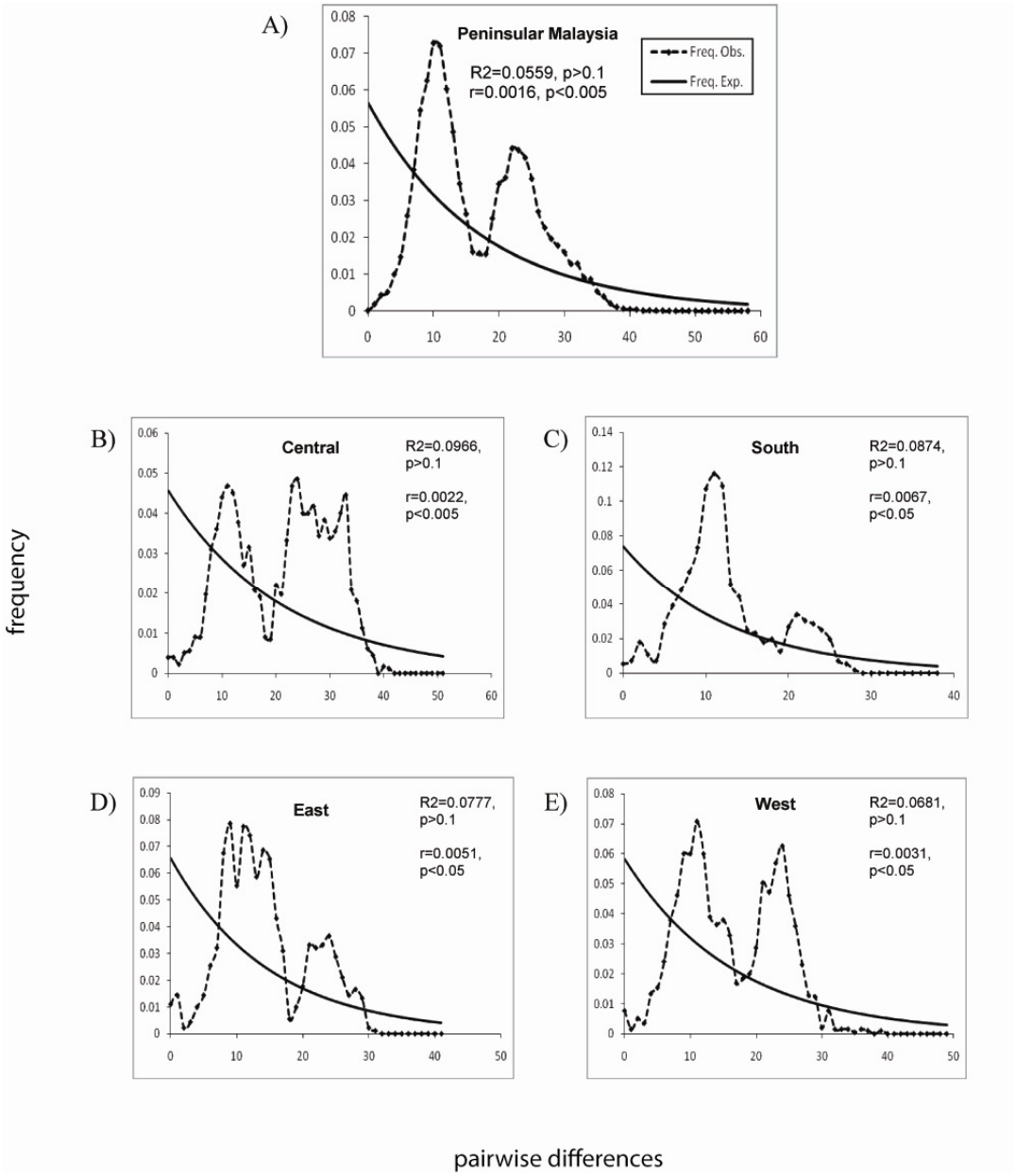
Mismatch distribution plots showed approximately bimodal distributions for all of the four regions (Figure 3.2), with the observed mismatch distribution in the South and East regions peaking at a lower number of pairwise differences than the other regions. A unimodal distribution would occur under a model of growth, so a bimodal distribution is indicative of a more constant population size. I found that the raggedness index was significantly low for the whole Malaysian population (Figure 3.2a) as well as for each of the four regional groupings (Figure 3.2 b-d), suggesting rejection of the null model of population growth. There was also no evidence of recent growth as indicated by the R2 index, which was not significant in all of the regions. Based on all of the tests of population expansion, including both r and R2 indices, it seems that there is good evidence of a constant size for *R. affinis* in Peninsular Malaysia, and certainly no recent severe population growth (R2 = 0.0559, $P > 0.1$).

Table 3.4 Genetic diversity in 30 populations of *R. affinis* based on 525bp of mtDNA D-loop region sequences

No.	Sampling Locality	Latitude (°N)	n^a	$H_o{}^b$	S^c	k^d	h^e	π^f
	Central (Total)		**60**	**53**	**105**	**21.3972**	**0.9961**	**0.0400**
1	KH3	6.1069	6	6	38	18.1333	1.0000	0.0346
2	PK4/PITC	5.5100	6	5	42	21.4667	0.9333	0.0396
3	KT3	5.3398	8	8	53	23.2143	1.0000	0.0443
4	KT1	4.6736	5	5	17	8.1000	1.0000	0.0155
5	PH6	4.2163	9	9	72	25.3889	1.0000	0.0471
6	F25	3.7500	2	2	10	10.0000	1.0000	0.0191
7	F23	3.7494	5	4	39	18.8000	0.9000	0.3588
8	F03	3.7000	1	1	-	-	-	-
9	F26	3.6830	6	6	23	9.3333	1.0000	0.0178
10	F24	3.6348	6	5	37	15.8000	0.9333	0.3015
11	PH1	3.2229	6	6	16	7.5333	1.0000	0.0144
	West (Total)		**63**	**54**	**114**	**16.9160**	**0.9923**	**0.0310**
12	KH2	6.3192	9	9	43	16.8889	1.0000	0.0311
13	PN1	5.1511	6	6	33	14.3333	1.0000	0.0268
14	KH1	5.5012	6	6	26	10.6000	1.0000	0.0202
15	PK1	4.5385	8	7	40	14.9643	0.9643	0.0286
16	PK3	4.3789	8	7	40	15.8929	0.9643	0.0291
17	PK2	4.2200	9	5	22	9.7778	0.7222	0.0180
18	FRIM	3.2369	10	10	75	22.5111	1.0000	0.0423
19	NS1	2.7050	7	6	36	12.7619	0.9524	0.0244
	East (Total)		**43**	**36**	**87**	**14.5415**	**0.9889**	**0.0273**
20	KT5	5.7944	7	7	51	19.8095	1.0000	0.0368
21	KT4	5.7411	9	8	53	16.7222	0.9722	0.0319
22	KT2	5.6948	3	3	29	19.3333	1.0000	0.0369
23	PH2	4.4131	7	7	33	12.0952	1.0000	0.0222
24	PH5	3.9167	8	7	31	12.1429	0.9643	0.0232
25	PH3	3.8614	9	7	29	9.2222	0.9167	0.0169
	South (Total)		**34**	**31**	**65**	**12.5668**	**0.9947**	**0.0240**
26	MK5	2.4667	1	1	-	-	-	-
27	MK4	2.3833	7	5	24	10.7619	0.9048	0.0205
28	JH3	2.3456	10	10	43	12.2222	1.0000	0.0233
29	JH1	2.1862	10	10	41	13.6667	1.0000	0.0261
30	JH2	1.8693	6	6	37	14.4667	1.0000	0.0276

[a] Sample size
[b] Haplotypes observed
[c] Polymorphic sites
[d] Mean pairwise differences
[e] haplotype diversity; [f] nucleotide diversity

Figure 3.2 Mismatch distributions plotted for *Rhinolophus affinis* populations in Peninsular Malaysia to test for demographic growth. Plots were conducted for (A) all regions, (B) the Central region, (C) the Southern region, (D) the Eastern region, and (E) the Western region. Results were assessed using the R2 statistic (Rasmos-Onsins and Rozas, 2002) and the raggedness index *r* (Harpending, 1994), the results of which are shown on each plot.



Population genetic structure

To test for evidence of restricted dispersal among populations, I fitted an isolation-by-distance (IBD) model of gene flow. This model assumes genetic structure will increase with Euclidean distances due to stepping-stone dispersal. Deviations from IBD can thus indicate geographical barriers or an island model of gene flow.

No IBD was detected for *R. affinis* populations across Peninsular Malaysia (r^2 = 7.66 x 10^{-3}, P = 0.7587; Figure 3.3), indicating that adjacent populations are not less differentiated than those further apart. Upper points (higher differentiation based on values of > 0.05) in the IBD plot were seen to involve comparisons within the Central montane region, whereas most lower points (genetic distance of 0.02 to 0.05) tended to represent comparisons with the other regions. These comparisons were separated for closer examination (see Figures 3.3 b and 3.3c). Although genetic distances among most sites within the Central region were approximately the same as those from the other regions, three populations were seen to be associated with higher differentiation suggesting greater isolation (Figure 3.3b); these were populations KT3 (genetic distance with other sites ranged 0.04 to 0.05), PH6 (genetic distance with other sites ranged 0.04 to 0.06) and KT1 (genetic distance with other sites ranged 0.06 to 0.08). Differences in genetic structure within and between regions are also summarized in Table 3.4.

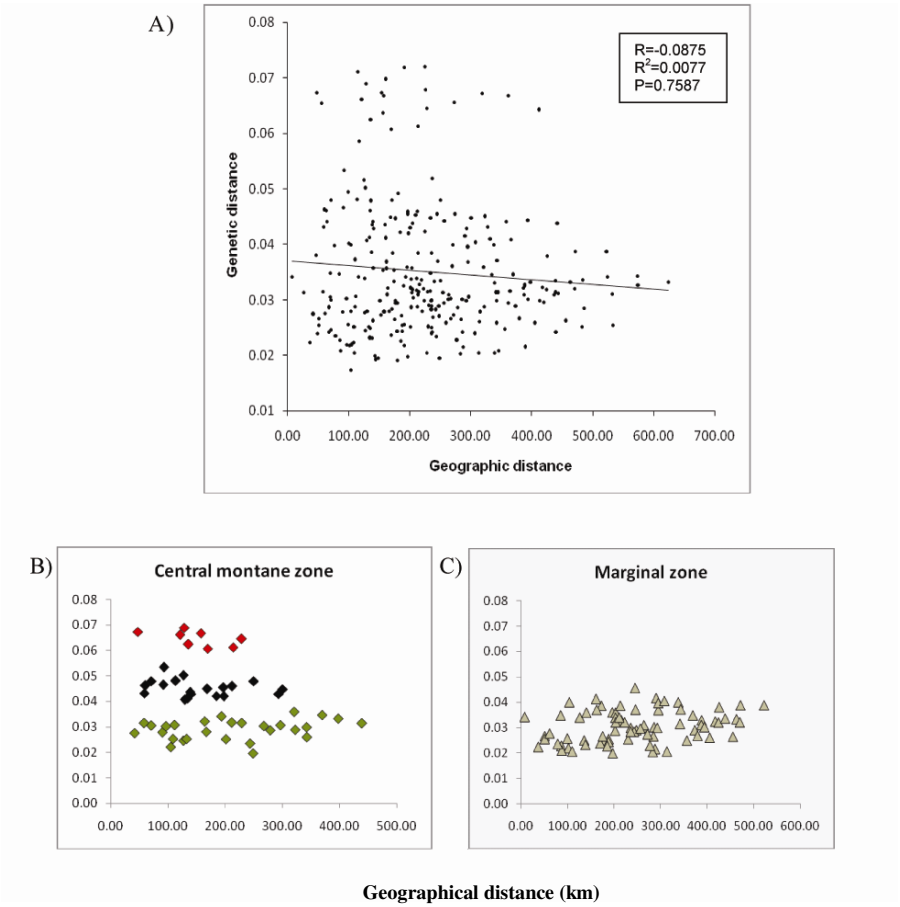


Figure 3.3 Part (A) shows isolation-by-distance (IBD) undertaken for pairwise populations across Peninsular Malaysia. Part (B) shows IBD within the central montane region. These points have been colour-coded to show different levels of differentiation recorded within this region: red diamonds indicate genetic distances between KT1 and other sites from the region, black diamonds indicate genetic distances between PH6 and KT3 and other sites from the region, and green diamonds indicate genetic distances between remaining sites from the region. Part (C) shows IBD within the coastal regions.

Phylogenetic analyses

An unrooted Median-Joining network constructed in NETWORK 4.6.0.0 consisted of 157 contracted haplotypes with 26 active haplotypes (see Figure 3.4a). Most private haplotypes were excluded in this network in order to present the overall intraspecific maternal population structure of *R. affinis* in Peninsular Malaysia. The Median-Joining network showed just a single major haplo-group. Overall, very little phylogeographic structure was evident and haplotype frequencies were relatively even. All four regions were mixed in the network, although only a few haplotypes were shared by more than one region (shown by pi-charts with mixed colours), so that all regions had private haplotypes. At least 14 mutational steps separated the closest related haplotypes up to a maximum of 31 mutational steps between least related haplotypes. In the main group, reticulations were formed between interior inferred haplotypes, although very few interior haplotypes were sampled. The same network showing finer resolution geographical information (Figure 3.4b) highlighted this high level of mixing, with some regional private haplotypes from Figure 3.4a shown to occur in multiple populations.

A statistical parsimony (95% threshold) network for Peninsular Malaysia based on the same set of 200 mtDNA sequences revealed six main sub-networks representing haplo-groups, and nine haplotypes inferred to be outgroups (shown as rectangles) (Figure 3.5). All haplotypes within haplo-groups were connected by up to eight steps. A high level of reticulation among haplotypes was seen, especially in the major haplo-group I and III. The network also indicated a high frequency of private singleton haplotypes (spread among interior and exterior positions) and a high level of variability of maternal lineages in the Central region. Putative ancestral haplotypes (out-group haplotypes, interior common haplotypes and haplotypes with many connections) were seen within all of the regions and often connected to haplotypes from multiple regions. There is some evidence of structure with a few related haplotypes restricted to the same region (e.g. sub-network II) and a high frequency exterior haplotype (PK2) that corresponds to an offshore island population. However, these are exceptional cases, and in general the network shows mixing across the country.

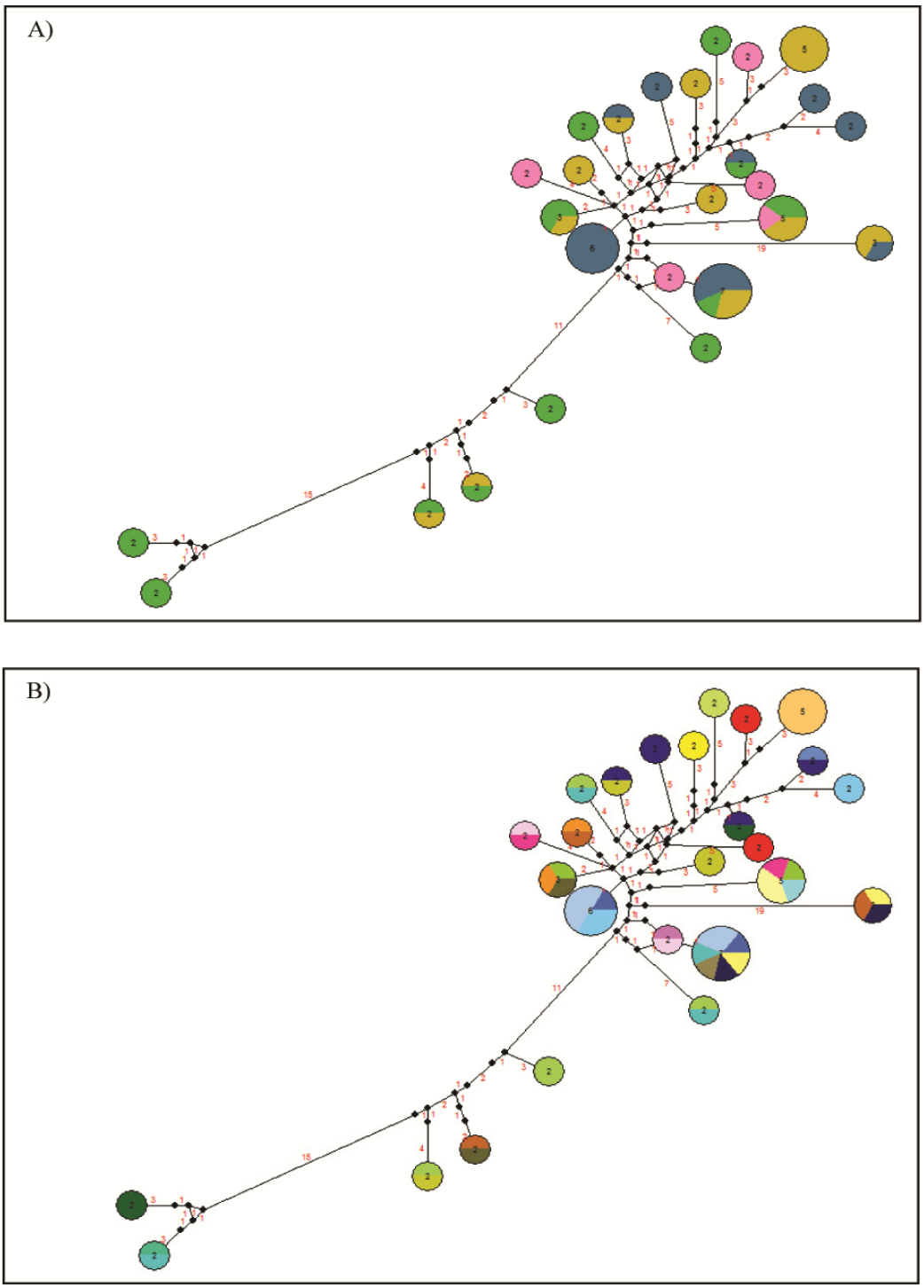


Figure 3.4 Unrooted Median-Joining haplotype networks showing the main intraspecific relationships among detected *R. affinis* haplotypes in Peninsular Malaysia. Each circle represents an active haplotype and small black circles are inferred intermediate haplotypes. Circle size is scaled to haplotype frequency, which is also shown in the circle. Numbers in red indicate mutational steps between connected haplotypes. The network is presented at two levels: (A) regional level (green = Central, yellow = West, blue = East and pink = South) and (B): population level (colours follow those in Table 3.1)

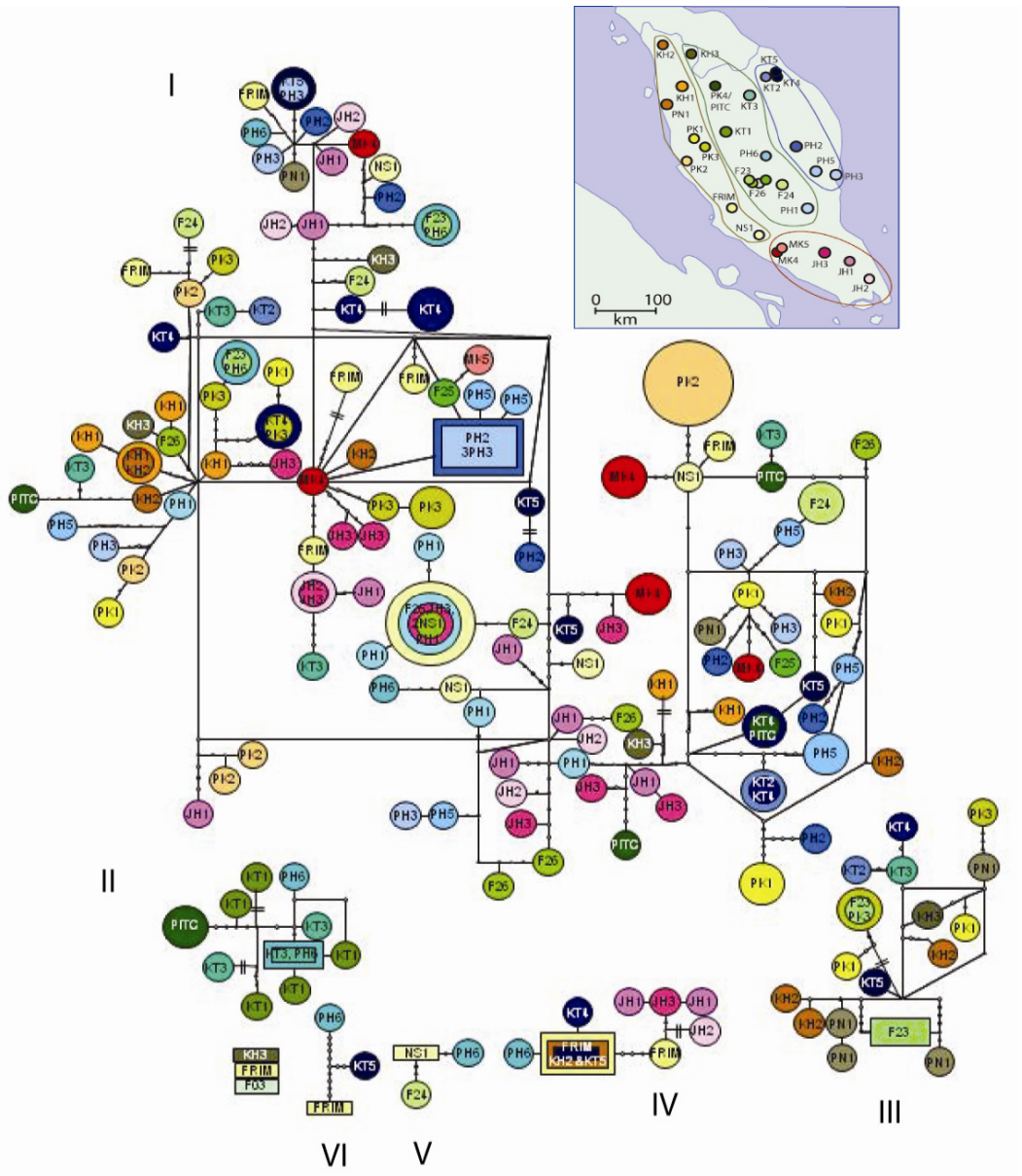


Figure 3.5 Unrooted statistical parsimony network detailing intraspecific relationships between detected haplotypes from sampled *R. affinis*. As in Figure 3.4, each circle represents a haplotype and circle size is proportional to haplotype frequency. Localities of the haplotypes are represented by colour codes following Table 3.1 (see inset map) and abbreviations on the circles are also listed in Table 3.1. Small circles on the branches represent inferred intermediate haplotypes. Breakpoints on the branches are equal to five mutational steps.

To assess the relationship among Malaysian and Chinese haplotypes, an unrooted Median-Joining network was also constructed using data from this study and also Mao et al. (2010b) (see Tables 3.2a and 3.2b). The network comprised 54 active haplotypes selected from 229 haplotypes separated by 1 to 25 mutational steps (Figure 3.6). Haplotypes from China and Peninsular Malaysia formed a single clade with a high level of reticulation, especially among inferred missing intermediate haplotypes that connected south mainland China to Centre and West Peninsular Malaysia. Overall three main sub-clades were recovered corresponding to geographical divisions: Hainan, China mainland and Peninsular Malaysia. The former of these were closely related to each other compared to Peninsular Malaysia.

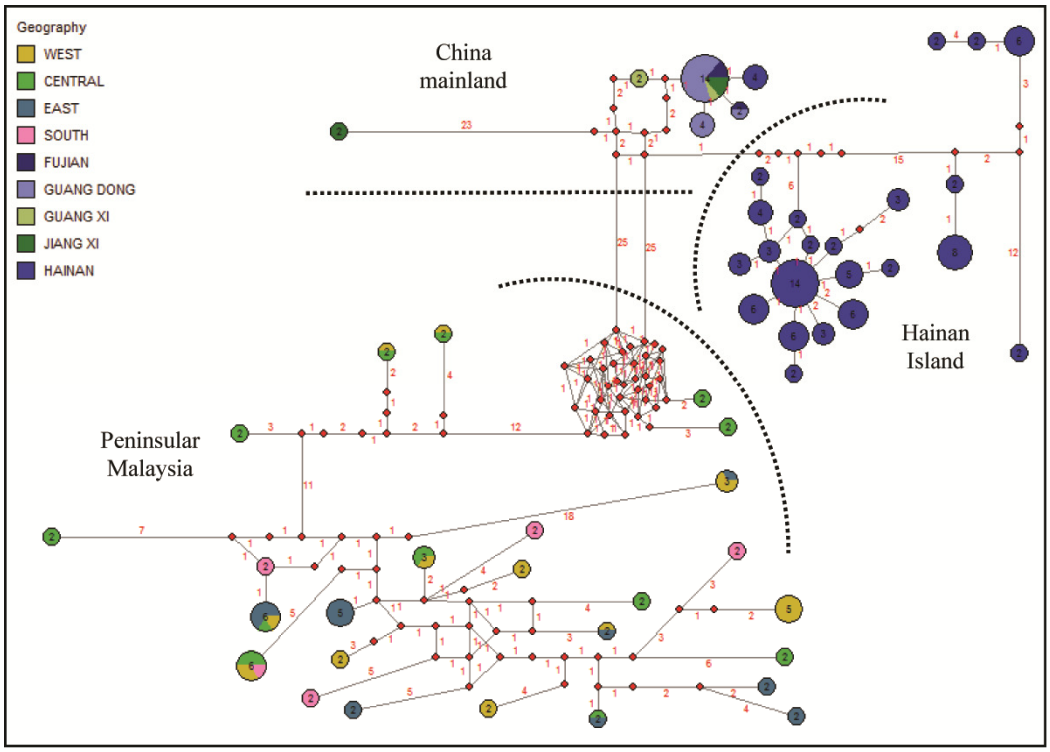


Figure 3.6 Unrooted Median-Joining network showing the intraspecific relationships among haplotypes from *R. affinis* in Peninsular Malaysia and China (published in Mao et al., 2010b). Each of the colour-filled circles represents an active haplotype, and small red circles represent inferred intermediate haplotypes. Circle size is scaled to haplotype frequency, which is also shown in the circle. Numbers in red indicate the numbers of mutation steps between connected haplotypes.

In addition to networks, I undertook phylogenetic tree reconstructions using Bayesian, ML and UPGMA approaches. Tree-based methods have the advantage of allowing estimation of the correct mode of mutation, so in this sense they might be more accurate at modelling the evolutionary process. Prior to Bayesian analysis, MrModelTest 2.3 (Nylander, 2004) indicated that the substitution model that best fitted the data was the Hasegawa-Kishino-Yano + invariant sites + gamma model (HKY+I+G) (-lnL = 4048.429, AIC=8776.858). This model allows a combination of different rates for transitions and transversions, variable substitution rate across sites, and a proportion of invariable sites. The Gamma distribution shape rate = 0.3950, the proportion of invariable sites = 0.4700, and ti/tv ratio = 15.4158.

The Bayesian phylogenetic tree of *R. affinis* from Peninsular Malaysia recovered some clear structure, as indicated by reasonable support (posterior probabilities >0.6) for several nodes (see Figure 3.7). Two well-supported phylogroups were identified, which are named group I and group II. The first of these groups was made up predominantly of haplotypes from the Centre, representing the main phylogeographical signal in the dataset. These populations are all specifically located near the Titiwangsa montane region (see Figure S3.12 in the Supporting Appendix). The latter group is more mixed, although it is mostly made up of western haplotypes and again these are near to Titiwangsa montane region. When a Bayesian tree was reconstructed with the data from Malaysia combined with data from China+Vietnam, both sets of data formed reciprocally monophyletic clades. Within Malaysia, the groups I and II were again recovered, as well as a third well-supported node (Figure 3.8). For the Malaysian data phylogenetic tree reconstruction based on maximum-likelihood analysis recovered broadly the same branching pattern (Figure 3.9). Although in the ML tree the Centre was the first to split from the rest of Peninsular Malaysia, suggesting these share a recent common ancestor with the ancestral haplotypes of all of the other sequences. The same groups were also retained in the tree of Malaysia+ China+Vietnam (Figure 3.10). Overall, support for groupings within Peninsular Malaysia appeared to increase when Chinese haplotypes were added due to the effect of polarization.

The last two trees, reconstructed using UPGMA for Malaysia only and Malaysia+ China+Vietnam (Figures 3.11 and 3.12), recovered similar topologies to the other methods, although the splitting order in Malaysia was more similar to the ML tree. Once again the first Malaysian phylogroup (Group I) diverged from the others and

consisted of haplotypes mostly from the Central and West regions. All of the haplotypes detected from KT1 fell into this ancestral phylogroup with high statistical support. More recently diverged phylogroups consisted of haplotypes from all of the other sampling sites, except for KT1, and showed considerable mixing. In all three methods, the haplotypes from China were seen to split into multiple well supported clades, which are known to correspond to the subspecies *R. a. macrunus* (mainland subspecies), *R. a. hainanus* (island subspecies) which split into two subgroups, and finally *R. himalayanus* (mainland subspecies). These were also reported by Mao et al. (2010b).

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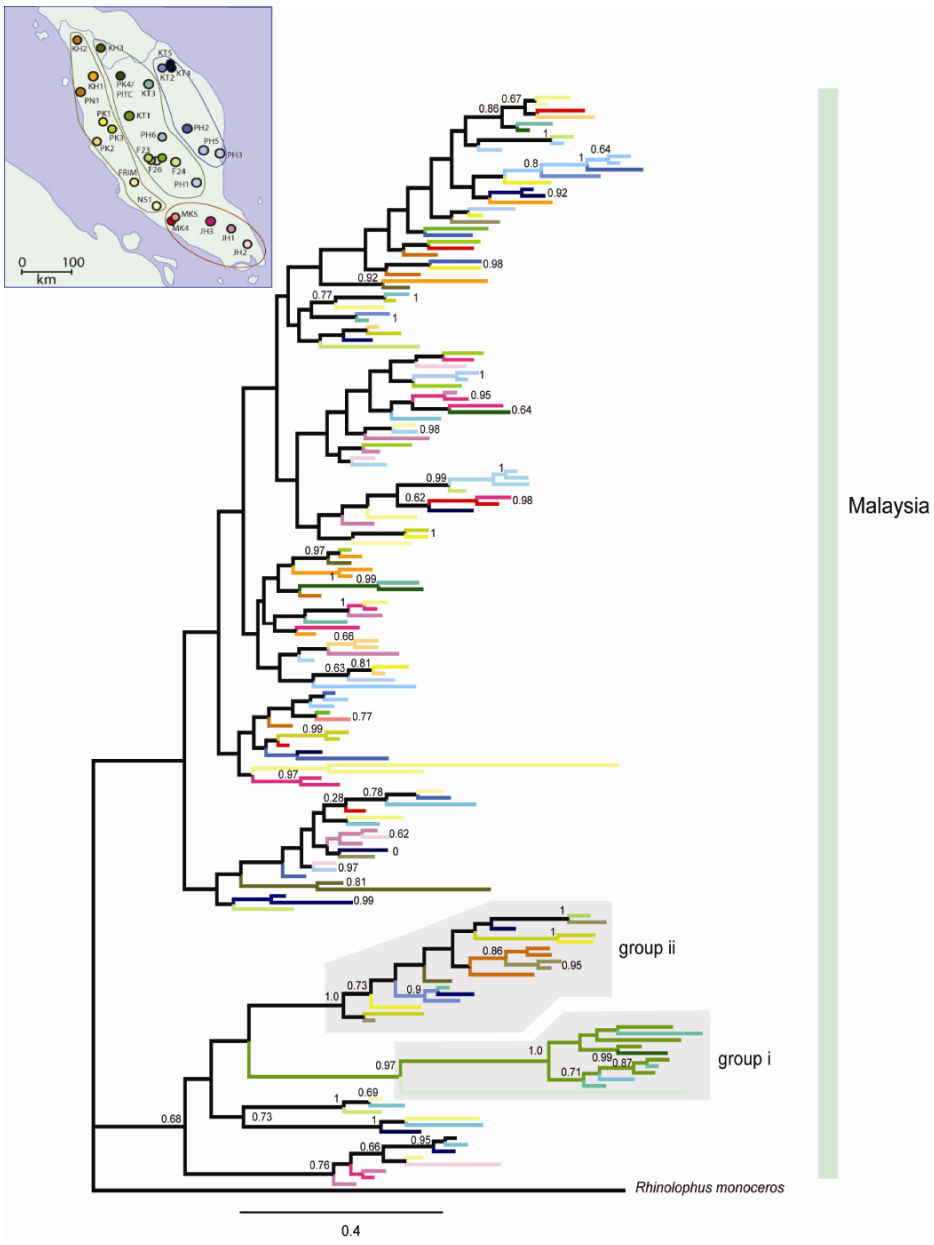


Figure 3.7 Rooted Bayesian tree of *R. affinis* from Peninsular Malaysia based on the D-loop region. Numbers above the branches are Bayesian posterior probability values and are only given where >0.6. Localities of the haplotypes are represented by colour codes following Table 3.1 (see inset map). Group i comprises haplotypes from the Centre particularly along the Titiwangsa montane region (see Figure S3.2) whereas Group ii mainly comprises those from the West and North that are also near to the Titiwangsa montane region.

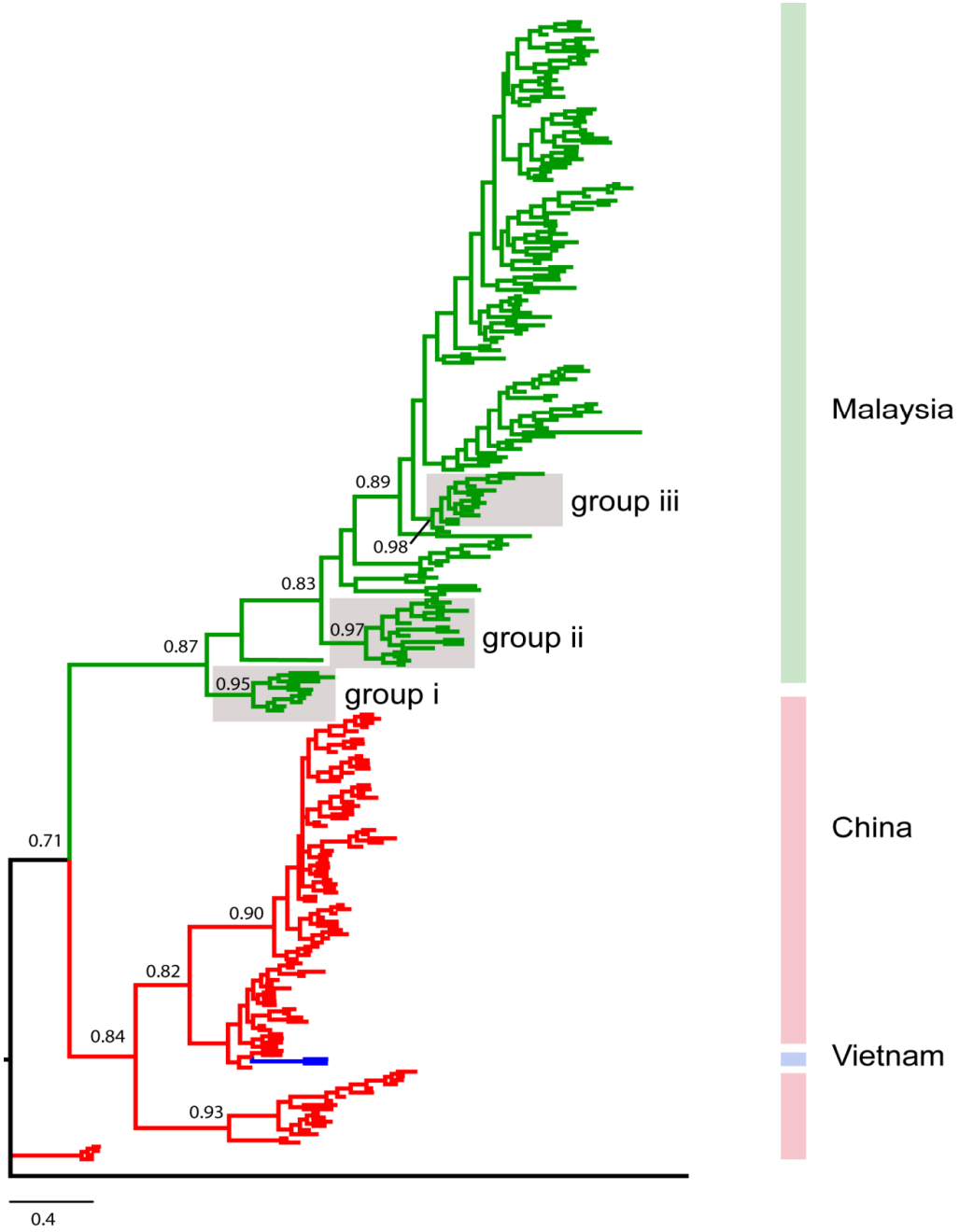


Figure 3.8 Rooted Bayesian tree of *R. affinis* from Peninsular Malaysia, China and Vietnam based on the D-loop region. Numbers above the branches are Bayesian posterior probability values and are only given where >0.7. Lineages from Peninsular Malaysia, China and Vietnam were coloured in green, red and blue respectively. In Malaysia, groups i and ii correspond to those from Figure 3.7, while group iii is newly seen in this analysis.

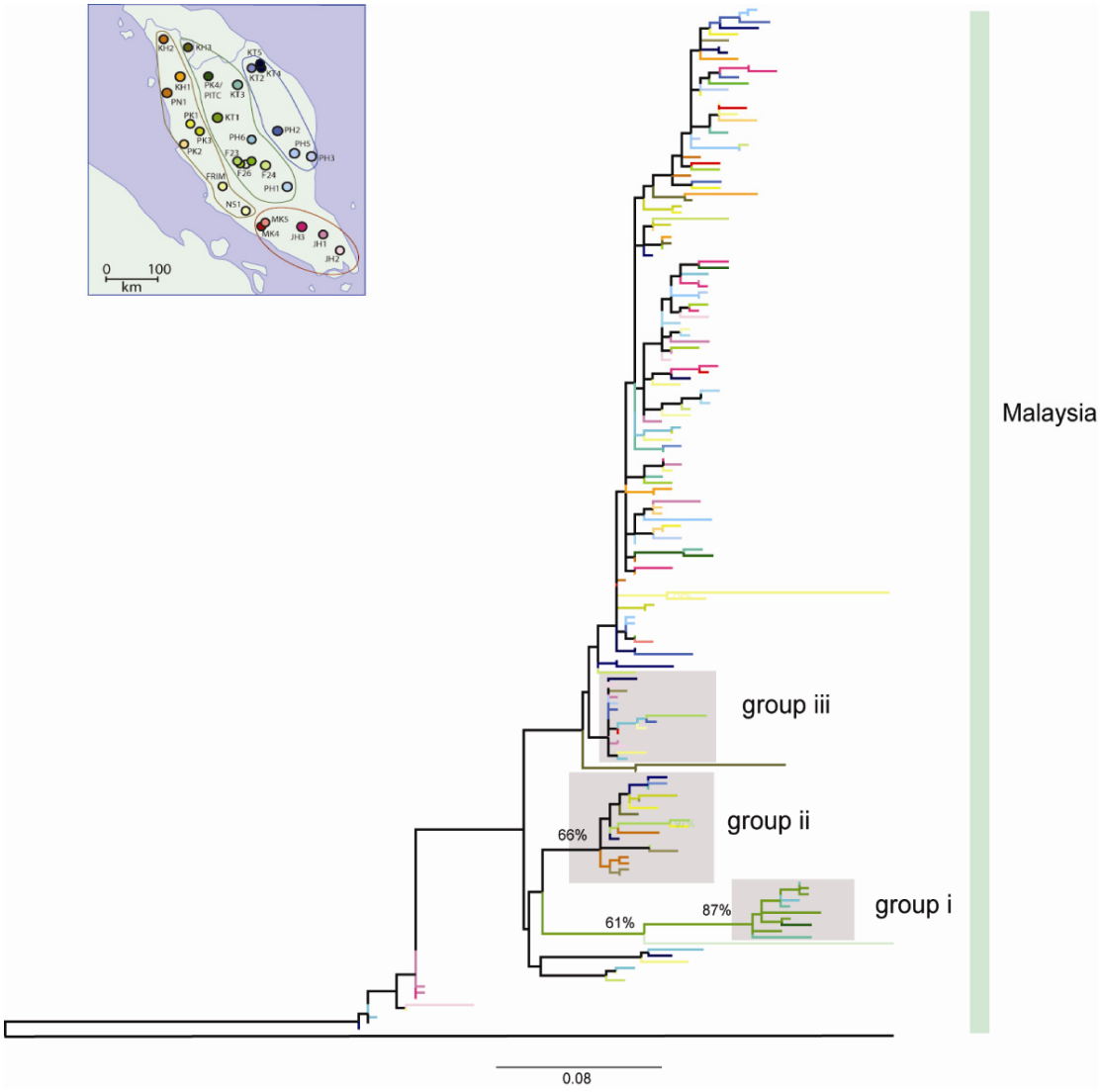


Figure 3.9 Rooted Maximum likelihood tree of *R. affinis* from Peninsular Malaysia based on the D-loop region. Numbers above the branches are Maximum likelihood bootstrap values, and are only shown where >60%. . Localities of the haplotypes are represented by colour codes following Table 3.1 (see inset map). Group i, ii and iii are the same groups recovered by the Bayesian trees (Figures 3.7 and 3.8).

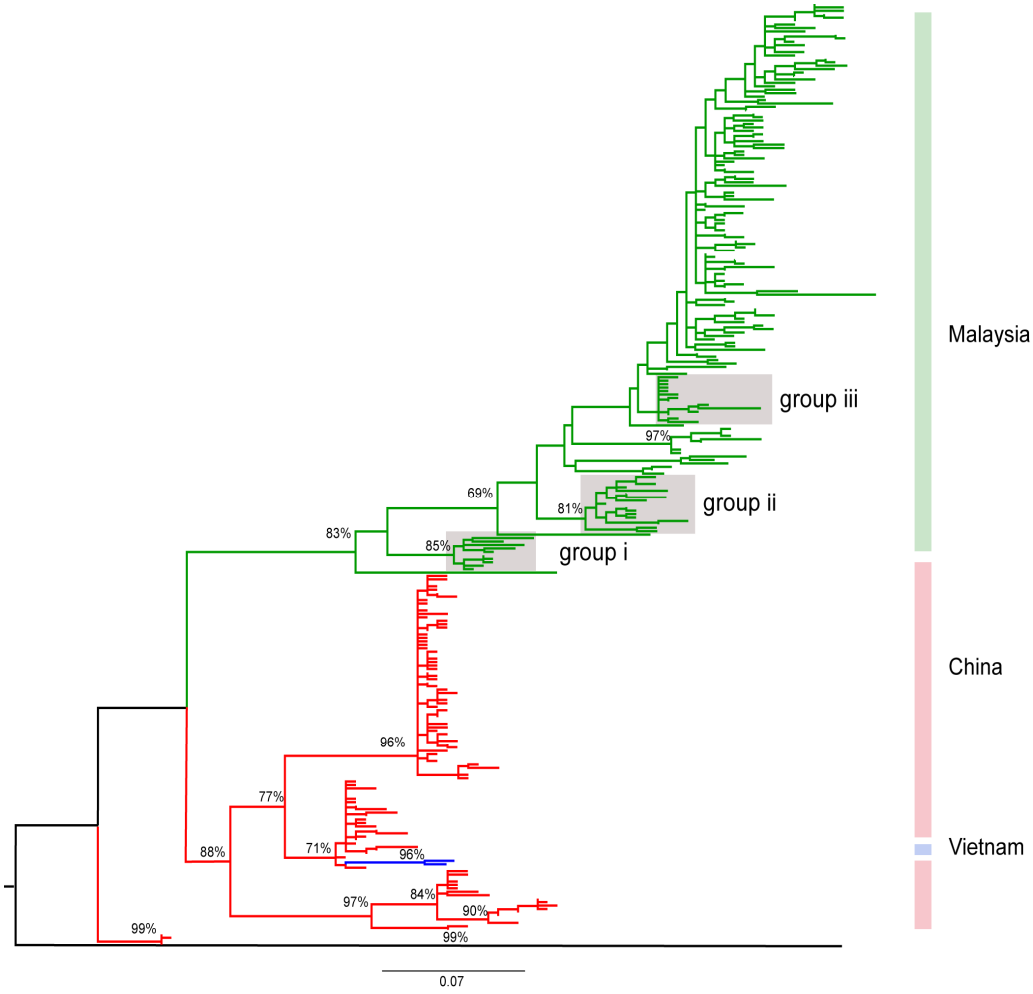


Figure 3.10 Rooted Maximum likelihood tree of *R. affinis* from Peninsular Malaysia, China and Vietnam based on the D-loop region. Numbers above the branches are Maximum likelihood bootstrap values, and are only shown where >60%.. Group i, ii and iii are the same groups recovered by the Bayesian trees

Figure 3.11 Rooted UPGMA tree of *R. affinis* from Peninsular Malaysia based on the D-loop region. Numbers above the branches are bootstrap support values. . Localities of the haplotypes are represented by colour codes following Table 3.1 (see inset map).. Group i comprises haplotypes from the Central region, particularly along the Titiwangsa montane region, whereas Group ii mainly comprises haplotypes from the West and North of peninsula Malaysia near to the montane region.

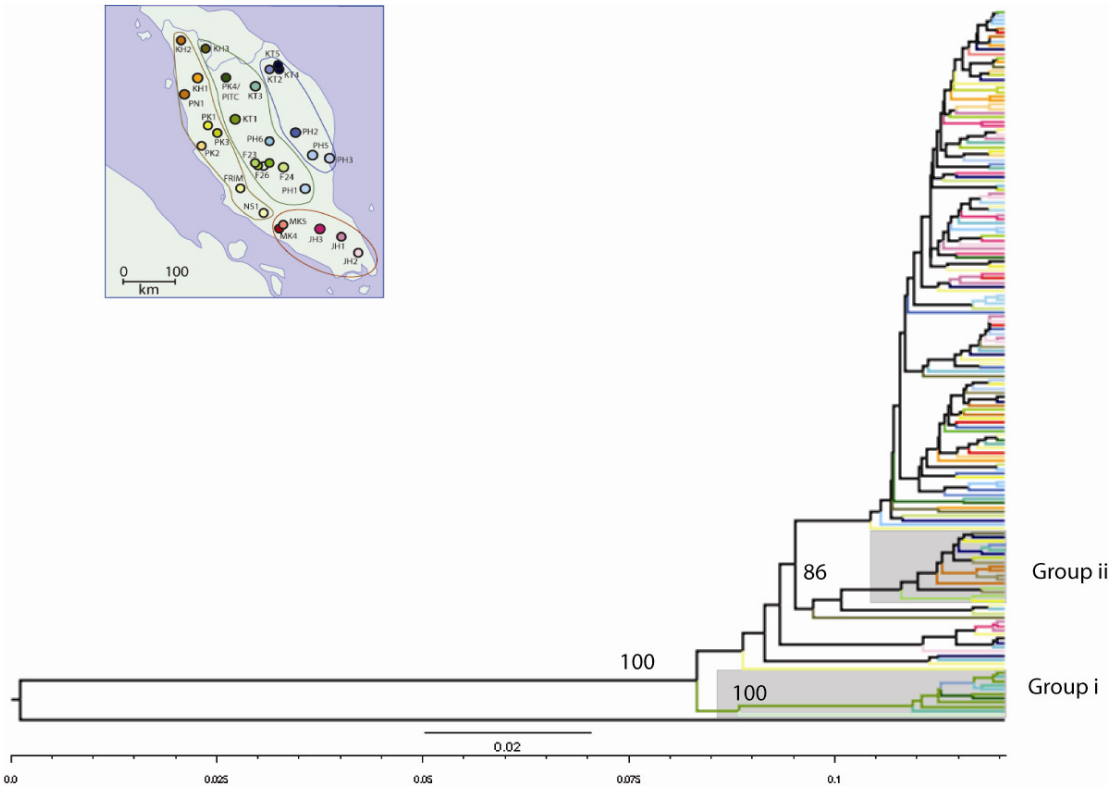
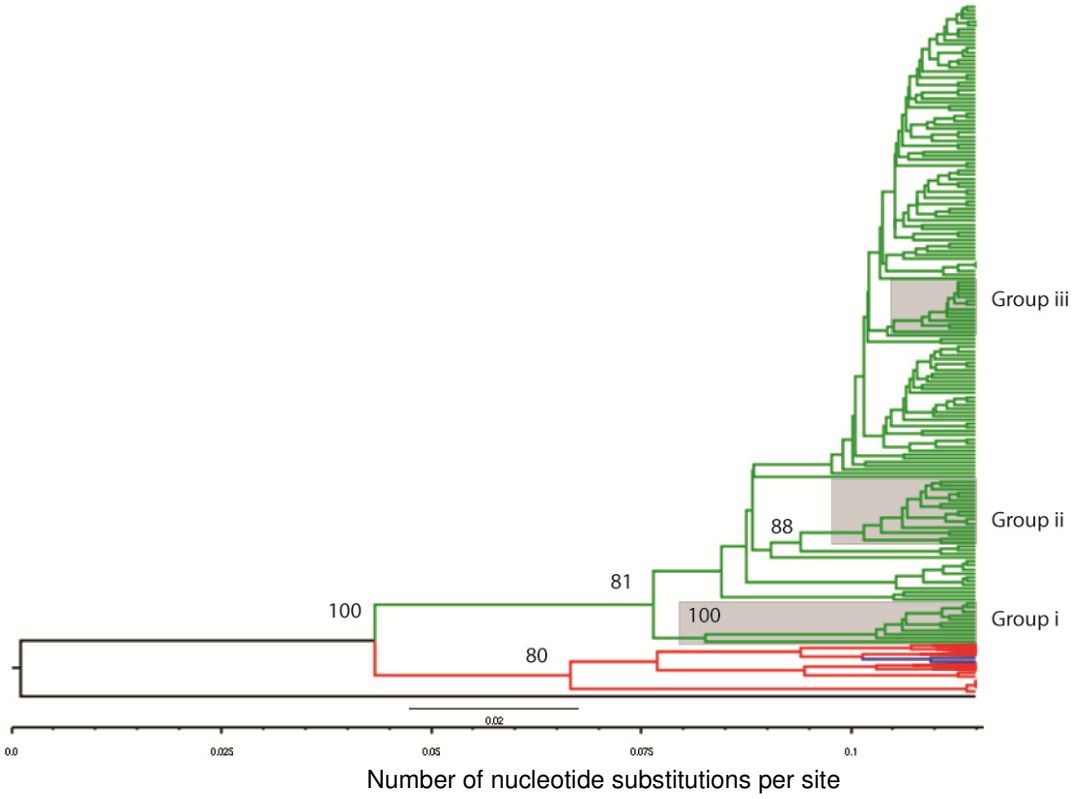


Figure 3.12 Rooted UPGMA tree of *R. affinis* from Peninsular Malaysia, China and Vietnam based on the D-loop region. Numbers above the branches are bootstrap support values. Lineages from Peninsular Malaysia, China and Vietnam are coloured in green, red and blue, respectively. In Malaysia, Group I contains haplotypes from the Centre, particularly along the Titiwangsa montane region; Group ii comprises haplotypes from the West and North near to the montane region, and Group iii comprises haplotypes from coastal areas.



To estimate the approximate timing of the origin of *R. affinis superans* in Peninsular Malaysia, I undertook Bayesian estimation of dating in BEAST. The estimated time to the most common ancestor (TMRCA) was about 466,391 years before present (BP) (95% highest posterior density, HPD: 140,803 to 710,249 years BP; see Figure 3.13 and Table 3.5). Bayesian posterior probabilities supported groups Group i, Group ii and Group iii from Figure 3.7 to Figure 3.10 with Effective Sample Size (ESS) of parameters successfully estimated exceeding 100. The TMRCA for Group i was inferred at 138,203 years BP (95% HPD: 25,626 to 183,440 years BP), while Group ii started to diverge from the main lineage about 334,963 years BP, and was formed about 153,937 years BP (95% HPD: 32,330 to 212,471 years BP). Group iii had the youngest TMRCA at about 109,524 years BP (95% HPD: 15,591 to 135,771 years BP). All three groups had TMRCAs that fell within the same geological time period during the middle to late Pleistocene, and pre-dating the LGM.

Table 3.5 Mean estimate of TMRCA for each phylogroup based on D-loop region.

Clade	mean TMRCA (years BP)	95% HPD	Quaternary stage see Ogg et al. (2008)
PENINSULAR MALAYSIA			
All *R. affinis superans*	466 391	140,803-710,249	Ionian
Group i	138 203	25,626-183,440	Late Ionian
Group ii	153 937	32,330-212,471	Late Ionian
Group iii	109 524	15,591-135,771	Early Late-Pleistocene
CHINA			
All China	598 923	195,030-950,307	Ionian
R. affinis himalayanus	53 262	17,13-34,825	Late Pleistocene
R. affinis macrunus	275 700	58,476-412,496	Late Ionian
R. affinis hainanus	209 591	51,712-311,741	Late Ionian
ALL (PENINSULAR MALAYSIA + CHINA)			
All *R. affinis* sequences	800 190	338,481-1,168,864	End of Early Pleistocene (Calabrian) and beginning of Mid-Pleistocene (Ionian)

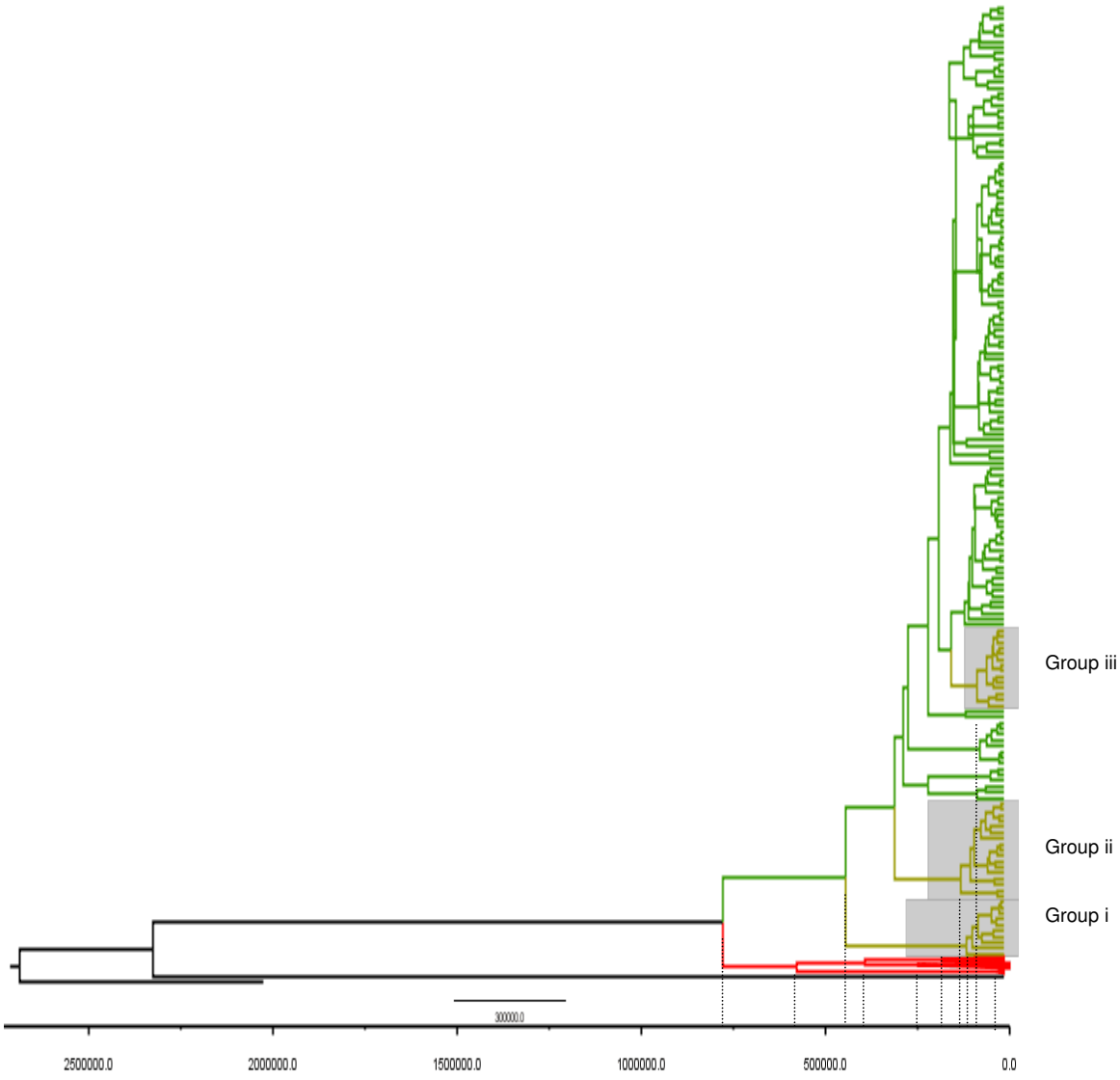


Figure 3.13 Rooted Bayesian tree scaled by estimated TMRCA constructed by BEAST. Estimated divergence times of TMRCA of the main groups are listed in Table 3.5. Lineages from Peninsular Malaysia and China are coloured in green and red, respectively.

Discussion

Genetic variation and demographic history of *R. affinis* in Peninsular Malaysia

The horseshoe bat *Rhinolophus affinis* is a cave-roosting species that is classified as being highly dependent on intact rainforest (Kingston et al., 2006; Francis, 2008). In this study I collected tissue samples from *R. a. superans* populations across Peninsular Malaysia and sequenced the mtDNA control region from all individuals. I then conducted phylogeographic, demographic and population genetic analyses to test for evidence of historical range shifts, and also evidence of responses to more recent habitat loss.

My findings revealed that all populations consistently contained very high variation with a total of 167 haplotypes detected from 200 sampled individuals (Table 3.2a). Haplotype diversity was highest in the Centre, followed by the East, West and South (Table 3.4). Although the higher haplotype diversity of the Centre could also perhaps reflect the fact that more populations were sampled here than in the South and East, this explanation cannot also account for the observed high genetic distances among haplotypes (and thus high nucleotide diversity) in the central montane area (Table 3.4). In general, this geographical pattern does not appear to support the idea that forest has undergone a post-LGM expansion (see Wurster et al. 2010) from refugial regions, in which case diversity might be expected to be lower at higher latitudes in the direction of colonization.

Apart from measuring genetic diversity in populations, phylogenetic relationships were also reconstructed to gain information the status of *R. a. superans,* which has been less studied than other *R. affinis* subspecies from China and Indonesia (Csorba et al. (2003). All of the phylogenetic trees reconstructed in this study support the monophyly of *R. a. superans* with respect to three Chinese subspecies (*R. a. himalayanus, R. a. macrunus and R. a. hainanus*). Previously, Struebig (2008) reported the possibility of a cryptic species of *R. affinis* in the Krau area located in the central region of Peninsular Malaysia (F03, F23 to F26 in Table and Figure 4.1) (2008), however, I found no deep structure in the mtDNA of these populations, and so found no support for cryptic species. Nonetheless I cannot rule out the possibility that multiple

species do occur for the reasons that my sampling effort was not concentrated in this same region as in Struebig's study. Additionally, there are numerous published cases of related species sharing genes due to introgression. For example, it was recently shown that two sister species of horseshoe bat, *R. pearsoni* and *R. yunanensis*, have undergone large-scale male-mediated ncDNA exchange, probably during glacial periods when they were forced into the same refugia (Mao et al. 2010). Introgression of mtDNA is even widely reported from numerous taxonomic groups such as fishes (Aboim et al., 2010; Joyce et al., 2011), mammals (Senn et al., 2010), plants (Lepais et al., 2009) and insects (Kulathinal et al., 2009), while closely related taxa might also share the same haplotype due to incomplete lineage sorting of ancestral polymorphism (Sanderson and Shafer, 2002).

Closer examination of intra-specific relationships among haplotypes, especially in relation to geographical locations, provided additional insights into the historical processes that have shaped *R. affinis* populations in Malaysia. Overall, little structure was observed, with generally low nodal support values and very few well-defined clades. The only obvious phylogeographic signal was a group of mostly Central haplotypes. Almost complete mixing of haplotypes across the study area explains the observed lack of IBD. Although strong support for the monophyly of Malaysian *R. affinis* could suggest recent demographic growth from a single common ancestor, the phylogenetic tree topologies did not show 'starburst' signatures or large numbers of unresolved branches (polytomies), which are characteristic features of population expansions (Avise, 2009). Phylogenetic reconstructions using network methods also supported these results. Networks are considered more useful for showing intra-specific patterns because they allow for the persistence of ancestral haplotypes alongside derived haplotypes. In this study, both the median-joining network and the parsimony network showed that most haplotypes occurred at low frequencies. Additionally, there were no very common, geographically widespread haplotypes, which are normally considered to be ancestral sequences in haplotype networks. Instead, most of the interior haplotypes were inferred, and thus were either extinct or were not sampled in this study. Given the huge haplotype diversity recorded, there is a strong possibility that many haplotypes were not sampled. The network also helped to explain some of the results from the genetic structure analysis, such as high pairwise differentiation in the IBD plot between some populations in the central montane area and other populations (see Figure 3.3). Indeed some haplotypes from the Centre and West regions were positioned far from the

other sequences in median-joining network (Figure 3.5) or even occurred as isolated sub-networks and/or outgroups in the parsimony network analyses (Figure 3.6).

All demographic analyses failed to detect historical growth (R2, Fu's F, Tajima's D, Fu and Li's D* and F*), therefore suggesting a relatively stable population size in Malaysia. For example, the low raggedness index based on the mismatch distribution for the whole country and all the regions, means I could reject the null model of growth (which would give a smoother unimodal curve). The lowest value of the ragged index (which could also be rejected most robustly) was that of the Centre. This may suggest that populations of the montane area have had a more stable history than populations in the coastal areas. Most reviews of demographic analyses suggest that no single test should be used, but rather multiple estimates need to be combined and compared (e.g. Ramos-Onsins and Rozas, 2002). Therefore, based on my results I can reject a rapid historical demographic expansion of *R. affinis* in Peninsular Malaysia.

Combined evidence from multiple analyses all point to a long and stable population of *R. affinis* in Peninsular Malaysia, so arguing against previous hypotheses that the rainforest was replaced by savannah during last glacial maximum about 18,000 years BP (Heaney, 1991; Wurster et al., 2010). Instead the results are consistent with the proposal that lowland evergreen rainforest on the the Sunda Shelf was at maximum coverage during LGM and has since undergone a decrease in area to its current level (Cannon et al., 2009). This model of forest coverage from simulations of climate data also suggested that the forest was at an earlier refugial stage at around 120,000 years BP (Cannon et al., 2009). Interestingly, my molecular dating based on the Bayesian phylogeny suggested that the time to the most recent common ancestors (TMCRA) of the three best-supported clades in the Malaysian population (groups I, II and III) were, respectively, 138,203, 153,937 and 109,524 years BP. Given the potential for some error associated with any molecular dating, it is therefore possible that these clades have undergone diversification following a refugial period. However, the TMCRA of all the Malaysian bats was estimated to be much older at around 466,000 years BP and that of all four subspecies was 800,000 years BP. It is noteworthy that in East Asia the pollen records confirm the occurrence of tropical forest from about 908,000 to 355,000 years BP (Sun et al., 2003).

While climate and associated environmental changes have been demonstrated to be important elements in shaping the distribution and population structure of species in the Northern Hemisphere throughout the Quaternary, the Sunda Shelf has not been directly affected by cycles of growth and decay of ice sheets (Hewitt, 2004, Hewitt, 2000). Nonetheless, sea level fluctuations during the Pleistocene had major effects on population structure in the tropics, by altering terrestrial barriers via submerging or exposing lowlands (Voris, 2000; Sathiamurthy and Voris, 2006). According to a review by Miller et al. (2005), global sea levels have been above the present level during four periods within the past one million years. Assuming a long history, as suggested by the results in this study, the population of *R. affinis* in the Malay Peninsula would have experienced three such high sea stands, at approximately 0.45, 0.36 and 0.13 million years BP (Miller et al., 2005; Woodruff and Turner, 2009). In general, it is unlikely that the resulting respective sea level increases of 20 m, 10-15 m and 30 m would have majorly altered the overall exposed area of the Sunda Shelf (Sathiamurthy and Voris, 2006, Woodruff and Woodruff, 2008; Woodruff and Turner, 2009). However, one locality where a higher sea level might have had a relatively bigger impact is at the narrowest part of the Malay peninsula at the Isthmus of Kra (see General Introduction). Previously, the observed and well-known zone of faunal transition at the Isthmus of Kra has been attributed to the narrowness of the peninsula at this latitude (Woodruff and Woodruff, 2008; 2009). In my study, it is of particular interest that the TMCRA of *R. affinis* in Malaysia (460,000 years BP) corresponds very closly to the period of 450,000 years BP, when the sea level was 20 m higher than it is now and will have submerged the lower coastal and narrowest areas of Malay Peninsula. Thus it is possible that the Malaysian subspecies budded off from a more northerly population at around this time.

Analyses presented in this chapter show that the central part of Peninsular Malaysia (where disturbance is relatively low and land is still largely covered by continuous evergreen tropical rainforest) harbours the most genetically diverse and the most demographically stable populations. Indeed the upland regions in the west (Bintang and Titiwangsa Montane Region) and east (East Coast Montane Region) (see Figure S3.2) both contain ancient lineages from Asia mainland. This region is home to a number of tropical vegetation types, including lowland and highland evergreen rainforests as well as montane rainforest. Since *R. affinis* exhibits modest flexibility with respect to altitude, it seems that upland areas would not have restricted gene flow,

and instead might have acted as a reservoir of diversity for populations in coastal areas, which at times would have been submerged by the sea.

The results from this study strong contrast with those of another study of *R. affinis* in the Wallacea islands at the margins of its South-eastern distribution (Maharadatunkamsi et al., 2000). Using allozyme data, this research group revealed a longitudinal decline of heterozygosity from the west to the eastern end of the range, and attributed this to the 'marginal effect' in which lower genetic variability is seen in peripheral populations than in the central one (Cunha and Dobzhansky, 1954). Current records show *R. a. superans* is restricted to the Malay Peninsula, Sumatra and Mentawai Islands (western part of the peripheral Sunda Shelf), however, no cline in variability was observed. However, although exceptionally high mtDNA diversity, and no obvious cline or pattern of IBD are good evidence that there has been no post-LGM recolonization or population expansion; they cannot be used to make any firm assessment of the extent or nature of gene flow in Malaysia. This is because most of the diversity is likely to be ancient widespread polymorphism rather than mixing by contemporary dispersal or genetic mixing. In other words, the variability reduces the power to quantify differentiation at this marker because nearly all sequences recorded are unique. Much more sampling is needed, or a multi-locus approach.

Detecting the genetic consequences of recent human-induced habitat change is also not straightforward. In the coastal areas, especially along the west coast, massive human alteration to the landscape during the last century has caused large areas of formerly continuous lowland Dipterocarp forest (see Adams and Faure, 1997) to be lost (see Figure 2.1). Forest dependent taxa in these areas may also suffer from micro-environmental changes in remnant habitat due to edge effects so that suitable forest might be even less common than it appears (Bierregaard et al., 1992, Laurance, 1991). It is interesting that some of the lowest pairwise differences were associated with the PH1 population, which has been heavily disturbed by logging and mining in the last decade, and is now a very open and dry landscape compared to the denser more primitive forest that previously covered the area. A similar situation is seen in KT1, which also had low pairwise distance within the population (second lowest among all of the subpopulations as listed in Table 4.4). In contrast, the populations KT3 and PH6 are both from undisturbed forests in the Centre and were found to have relatively very high genetic diversity. Despite these observations it is important to be cautious when relating

population-wise estimates of diversity to habitat condition or human activity. Like with most studies, the number of samples analysed for mtDNA per population was rather low, so there might be considerable noise in the data. Indeed previous work on *R. affinis* from limestone caves at Gunung Senyum Forest Reserve in Pahang, found that this species dominated assemblages in forest fragments up to 11km away from the roost (Struebig et al., 2009). Thus although this species is heavily dependent on forest, it may be able to disperse between neighbouring populations within regions. Microsatellite genotyping of greater numbers of animals per population offers better chances of characterising the extent and nature of gene flow (see Chapter 4).

Appendix of supporting information

Figure S3.1 Map of Sundaland directly adopted from Bird et al. (2005).

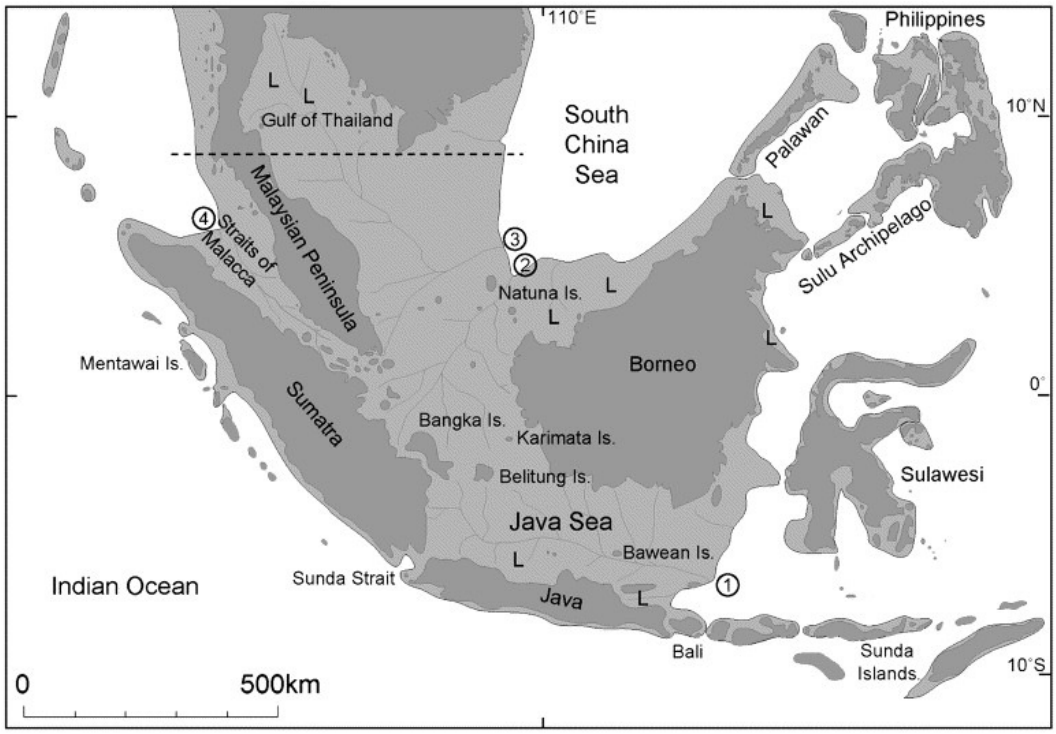


Figure S3.2 Map of Peninsular Malaysia showing the locations of montane regions (listed below).

1. Nakawan Range,
2. Kedah-Singgora Range
3. Bintang Range
4. Keledang Range
5. Titiwangsa/Central Range
6. Benom Range
7. Tahan Range
8. East Coast Range

CHAPTER FOUR

Genetic structure of *Rhinolophus affinis* (intermediate horseshoe bat) and *R. lepidus* (Blyth's horseshoe bat) in Peninsular Malaysia

Chapter 4: Genetic structure of *Rhinolophus affinis* (intermediate horseshoe bat) and *R. lepidus* (Blyth's horseshoe bat) in Peninsular Malaysia

Chapter summary

Multi-locus analyses such as those using microsatellites provide powerful tools for resolving patterns of genetic differentiation and contemporary gene flow, as well as quantifying genetic diversity for conservation assessments. In addition, several studies have demonstrated the use of microsatellites in reconstructing population history and phylogeography. In Peninsular Malaysia, human activity has been the principle cause of loss of forest coverage, and is the main present threat to biodiversity. To investigate the genetic consequences of habitat loss and past climate change, I undertook microsatellite analyses on two species of co-distributed forest-interior horseshoe bat: *Rhinolophus affinis* and *R. lepidus.* For these species I used panels of 14 loci and 10 loci, respectively, to characterise genetic structure and measure diversity. My results revealed that allelic richness was lower in *R. lepidus* populations than in *R. affinis* populations, however, in both species the allelic richness showed a significant negative association with increasing longitude. Due to the shape of Peninsular Malaysia, this result also means that diversity was highest in the north, so conflicting with expectations under a post-glacial expansion from the equatorial regions. Both species showed increasing genetic distance among populations with distance, although significant isolation-by-distance was only seen in *R. affinis*. Bayesian clustering and Principal Coordinate Analyses revealed no clear genetic structure, indicating no major barriers to gene flow and in agreement with isolation-by-distance. Although lower detected genetic diversity in the southern populations cannot be related to greater forest loss in this area, it could mean that these populations will be less able to adapt to long term environmental change.

Introduction

Southeast Asia has a rich biodiversity due to its complex geological and tectonic history since the Tertiary (Morley, 2000). The region encompasses three biodiversity hotspots, which are priorities for conservation due to their high density of endemic species and current state of vulnerability from habitat change (Myers et al., 2000). Human-related activities have been the main cause of global diversity loss since 1992 (World Conservation Monitoring Centre, 1992) and in Southeast Asia these threats include over-logging of forests (Laurance, 1999) and clear felling for plantations of high commercial value crops, such as oil palm (*Elaeis guineensis*) (Koh and Wilcove, 2007). In fact, the conversion to plantations in the region often appears to be initiated by disturbance via logging activities, which reduce the quality of secondary forests. Finally plantations are often transformed into construction projects, including residential areas, small towns or new cities (Forestry Department of Malaysia; personal communication).

Other threats to the regional tropical biodiversity include increasing human populations, weak government and policies, and increasing trade liberalisation, all of which are relatively young issues (past two centuries) but which have already contributed to massive forest loss and fragmentation (Laurance, 1999). Overall this combination of factors have led to deforestation rates of around 54% to 60%, as estimated by the World Conservation Monitoring Center (WCMC) and the Food and Agriculture Organisation (FAO), respectively (Sodhi et al., 2004; FAO, 2005), although these rates do not include loss of secondary forests that have been cleared at a rate of 0.8% per year between 2000 and 2005 (Koh and Wilcove, 2007). Finally, these threats are probably made worse by the local effects of global climate change; for example, Malhi and Wright (2004) reported that since the 1970s, there has been a general decline in precipitation and an increase in temperatures in the tropical rainforests of Southeast Asia.

Genetics can offer a powerful approach for monitoring and assessing how populations and species react to rapid habitat change. Moreover, genes themselves are recognised by the IUCN as one of the main units of diversity and are thus an important element in global biodiversity conservation (Frankham, 1995). Over the past two decades, applications of population genetics to conservation have become hugely

important, with the recognition of the sub-discipline conservation genetics (for reviews on the subject see O'Brien, 1994; Frankham, 1995; Allendorf *et. al.*, 2010; Ouborg et al., 2010). A key role for conservation genetics has been to obtain and use genetic information to improve management of small or captive populations; for example, by monitoring the genetic profiles of founding individuals and subsequent generations in order to minimise inbreeding depression and encourage disassortative mating (Frankham, 1995; Allendorf et al., 2010). Further areas in which conservation genetics has made an impact has been in resolving taxonomic uncertainties, identifying evolutionarily significant units (ESUs) and management units (MUs) within species, and resolving species identities in forensics (Frankham et al., 2002).

To date, most conservation genetics studies have focused on taxa from Europe and northern America as well as Australia, with relatively little work in Southeast Asia despite its extreme biodiversity. Nonetheless there are some recent and notable exceptions from a range of taxa such as the mountain hawk eagle (*Spizaetus nipalensis*) (Hirai and Yamazaki, 2010), intermediate horseshoe bat (*Rhinolophus affinis*) (Mao et al., 2009) and venus clam (*Cyclina sinensis*) (Feng et al., 2010). Focusing on the regional endemic murine rodent species, *Leopoldamys neilli*, (Latinne et al., 2011) used mitochondrial and nuclear markers to identify management units. The authors found six allopatric lineages from 20 localities in Thailand, suggesting very low levels of gene flow among its limestone karst habitat, and highlighting the importance of protecting karst in order to preserve this taxon's unusual intra-species diversity. Similarly, a study of one species of cyprinid fish endemic to Sarawak, *Tor douronensis*, reported two main genetic clusters that were consistent with geographical barriers (Nguyen, 2008). Conclusions led the author to identify these populations as independent ESUs, and suggest that they should be conserved separately via managing their respective river systems.

The growth of conservation genetics can largely be linked to the discovery and growing application of microsatellites in population genetics (Litt and Lutty, 1989; Jarne and Lagoda, 1996; Zane et al., 2002). Unlike older methods based on allozymes, microsatellites offer opportunities for non-lethal and non-invasive sampling of endangered species because they can be amplified by PCR from small amounts of starting material (Palsbøll 1999). A second major advantage of microsatellites is their rapid mutation rate of about 10^{-3} mutations per locus (compared to an evolutionary rate

for DNA sequence of 10^{-8} to 10^{-9} per year for point mutations) so that they can be applied to examine processes that have occurred in recent evolutionary time, in the order of hundreds of generations (Ülo et al., 2008). Consequently, microsatellites are useful for resolving landscape-scale spatial genetic structure and contemporary gene flow (Guillot et al., 2009). At even finer scales, microsatellites are useful for studying social organisation including segregation patterns between kinship groups or the genetic consequences of kinship or sex-specific philopatry (Peakall et al., 2003). At the same time, however, there can be uncertainties regarding the origins of allelic polymorphism at such loci given their stepwise and rapid rate of mutation, and it has been suggested that there are biases in reported genetic diversity due to the selective use and reporting of only the most highly polymorphic markers for analyses (Estoup et al., 2002; Zane et al., 2002; Hoffman and Amos, 2005; Ülo et al., 2008).

In spite of the popularity of microsatellite loci for applications in conservation and population genetics, in recent years several studies have also demonstrated their usefulness for reconstructing phylogeographic processes. For example, among vertebrates microsatellites have been used to identify population growth in species as diverse as humans (Patin et al., 2009), birds (McKay et al., 2010) and tiger salamanders (Bos et al., 2008) as well contact zone between populations that have come from different glacial refugia (Coyer et al., 2011). Microsatellites have also already been applied to understand the phylogeographic history of several bat species, such as the Mexican free-tailed bat (Russell et al., 2011), *Pipistrellus* bat species complex (Hulva et al., 2010) and the greater horseshoe bat (Flanders et al., 2009).

Comparative studies of phylogeography have proven particularly useful in identifying common processes and refugia that have influenced multiple taxa; however, most such studies have relied on meta-analyses (Hewitt, 2001; 2004; Provan and Bennett, 2008; Médail and Diadema, 2009). Recently there have been increasing numbers of investigations that have collected data on more than one species and compared patterns of genetic structure. For instance, McGovern et al. (2010) found similar population divergence times in populations of two co-distributed marine mollusc species, which was thought to be due to common responses to past climate change. Similarly, Bell et al. (2011) compared multi-locus sequence data for five co-distributed frog species endemic to the Australian tropical rainforest, and successfully traced historical forest contraction. In terms of studies on bats, Meyer et al. (2009) compared

two species of *Phyllostomus* from Central America and reported less population subdivision in the more mobile species. More recently, Rossiter et al. (2012) compared gene flow across continuous populations of seven forest bat species with different roosting behaviours as well as social organisations. At a landscape-scale, the authors found more genetic structure in tree-roosting bat species which also have lower mobility, yet almost no structure for cave roosting colonial species that are thought to disperse over greater distances.

Rhinolophus affinis and *Rhinolophus lepidus* across Peninsular Malaysia

In this study I used microsatellites to compare the population genetic structure of two horseshoe bat species, *Rhinolophus affinis* (intermediate horseshoe bat) and *R. lepidus* (Blyth's horseshoe bat) (Figure S4.1) across Peninsular Malaysia. In this region, this genus of bats is represented by up to 18 species (Kingston et al., 2006), with one new species, *Rhinolophus chiewkweeae* reported by Yoshiyuki and Lim (2005). Of these, *R. convexus* is categorised as rare, *R. sedulus* and *R. robinsoni* as near threatened, and the remaining 11 species as not at risk globally (Francis, 2008). However, all 18 species are critically dependent on forest, and are therefore under enormous threat. Additionally, some of these species roost in karst limestone caves, and so also face threats from mining (see SAMD, 2009). The similar habitat requirements of all *Rhinolophus* species mean that any detected patterns of genetic structure in the two focal species are also likely to occur in their relatives.

R. affinis is widely distributed and abundant in peninsular Malaysia and was first described from Java by Horsefield in 1823. Since then, it has been recorded over a wide area, from India in the west, through South China and Vietnam to Borneo and its offshore islands. To date, eight subspecies are recognised: *R. a. andamanensis*, *R. a. hainanus*, *R. a. himalayanus*, *R. a. macrunus*, *R. a. nesites*, *R. a. princeps*, *R. a. superans* and *R. a. tener* (Csorba et al., 2003, Simmons, 2005; SAMD, 2009). In Peninsular Malaysia, this is a cave roosting species that forages in intact forest, though it can also be found in secondary forests (Kingston et al., 2006). In the study region it frequently shares roosts with other related members of the Rhinolophidae (e.g. *R. lepidus*) and Hipposideridae (e.g. *H. bicolor* and *H. larvatus*) (personal observation).

Blyth's horseshoe bat (*Rhinolophus lepidus*) was firstly reported by Blyth in 1844 with four more subspecies being recognised afterwards that have since been included in the species: *Rhinolophus monticola* in Masuri, northwest of India in 1905, *Rhinolophus refulgens* in Gunung Igar, Malaya in 1905, *Rhinolophus refulgens cuneatus* in Sukaranda, Northeast of Sumatra in 1918 and *Rhinolophus feae* in Biapo, Burma in 1907 (Csorba, 2002; Francis, 2008). It is widely distributed in a few countries in Asia included India, Afghanistan, Pakistan, Nepal, Myanmar, Thailand, peninsular Malaysia and Sumatra Island in Indonesia (Csorba et al., 2003). Like *R. affinis*, *R. lepidus* is considered a forest specialist that has been recorded in lowland and hill forests (Kingston et al., 2006). *R. lepidus* usually roosts in boulders crevices and caves (Csorba et al., 2003; Kingston et al., 2006; Francis, 2008) although it sometimes occurs in houses (Medway, 1982; Csorba et al., 2003), tunnels and other manmade structures (Csorba et al., 2003).

Little conservation work has been undertaken on the focal species, however, a preliminary comparative study of several bat species (including *R. affinis*) across undisturbed forest, disturbed forest and agricultural areas in Peninsular Malaysia identified sperm abnormalities in agricultural areas, probably caused by chemical emissions and so providing indirect evidence of human impacts (Siti-Tafzilmeriam et al., 2006). Yet despite the focus on protecting tropical rainforests for animal conservation in general, bats can be equally adversely impacted by disturbance at their roost sites (Russo et al., 2004). In the case of *R. affinis*, *R. lepidus* and related species, there is a specific threat of the loss of suitable roosts in limestone karst due to mining and tourism. In Southeast Asia, karst covers about 400,000 square kilometres, relatively less area than tropical rainforest, with the largest karst area found in Indonesia, Thailand and Cambodia (Day and Urich, 2000). This may explain why conservation policies of countries in the region, including Peninsular Malaysia, overlook limestone caves. Nonetheless, caves formed from natural mechanical and chemical erosion in the region serve as unique habitats and thus hold high biodiversity value (Clements et al., 2009). The ability of insectivorous bats to echolocate means that they often roost deep in caves, sometimes in colonies of thousands of individuals, and the importance of protecting caves for the Old World bats has been highlighted in several studies (Suyanto and Struebig, 2007; Struebig et al., 2009). Indeed, Struebig et al. (2009) examined the influence of karst on local assemblage structure across nine fragmented sites in Pahang,

Malaysia, and found that *R. affinis* and *R. lepidus* were the dominant species in bat assemblages up to 11km from the cave roosts.

Study objectives

In this study I undertook analyses of microsatellites to characterize the population genetic population structure of two co-distributed and ecologically similar species across Peninsular Malaysia: *R. affinis* and *R. lepidus*. Several questions and hypotheses were addressed to assess the effects of ancient climate change and recent human activity on these taxa and their forest habitat.

i. To determine the pattern of population genetic structure of both species across the study region. I hypothesize that if the species have undergone contractions and periods of isolation consistent with fragmentation of wet forest during the LGM, then there would be evidence of deep genetic structure. If forest persisted across Malaysia, I would expect little or no deep structure.

ii. To test for latitudinal trends in allelic richness. I hypothesized that if the populations have undergone a post-LGM expansion from the equatorial areas, there would be a northward decrease in allelic diversity in both species. Alternatively, no such pattern would be seen if there has been no such expansion.

iii. To test for evidence of restricted gene flow among isolated forests as expected if gene flow has been affected by forest loss and fragmentation.

Methods and Materials

Sample collection

Bats were surveyed at 28 forest sites across Peninsular Malaysia between February 2008 and September 2008. Individuals were captured either by using four-bank harp traps placed along foraging paths or by hand-netting roosting bats in caves. Tissue samples from bats from two sites not visited in this study were obtained from colleagues. In total, tissue samples from individuals of *Rhinolophus affinis* and/or *R. lepidus* were obtained from 17 sites (hereafter referred to as 'populations'), as shown in Figure 4.1 and listed in Table 4.1. For additional details of the geographical characteristics of the localities see Table 2.1 (Chapter 2). For DNA analyses, a 3-mm wing membrane biopsy was taken from all individuals of *Rhinolophus affinis* and *R. lepidus*, as described in Chapter 2.

Table 4.1 Samples for microsatellite analyses for *Rhinolophus affinis* and *R. lepidus*

Sampling locality *	Sampling elevation	Coordinates		Sample size	
		Longitude	Latitude	***R. affinis***	***R. lepidus***
KH2	78&178m	100.4840	6.3192	5	
KH3	129m	100.9628	6.1069	8	4
KT2	49-257m	102.1681	5.6948		1
TG2/GT	58-441m	102.6000	5.5500		6
PK4/PITC	590-810m	101.3600	5.5100		2
KH1	51m	100.7732	5.5012	8	17
PN1	<300m	100.5457	5.1511	45	10
PK1	90m	101.0039	4.5385	8	
PH2	140m	102.6813	4.4131	31	
FRIM	NA	101.6378	3.2369	5	2
PH1	34-56m	102.9011	3.2229	11	
SL2/UKM	NA	101.7814	2.9197		2
NS3/BRB	249-605	102.0700	2.8000		6
NS1	<300m	102.0782	2.7050	17	7
MK4	<300m	102.3833	2.3833		18
JH1	29-160m	103.5860	2.1862	28	4
JH2	24-29m	103.9139	1.8693	34	
			Total	200	79

* For sites in italics, wing tissue biopsies were contributed by C. Fletcher.

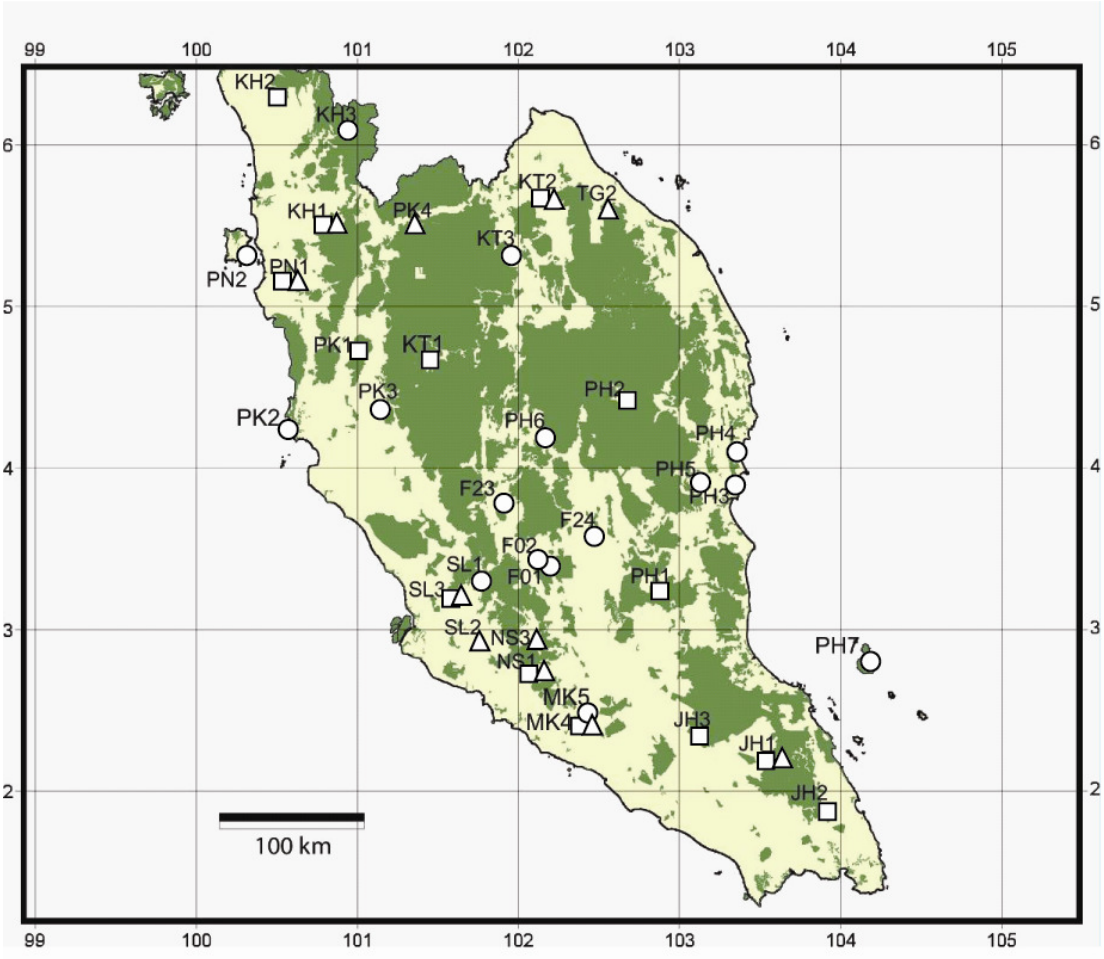


Figure 4.1 Map of Peninsular Malaysia showing the coverage of lowland tropical rainforest in 1997 as shown in green (Forestry Department of Peninsular Malaysia, 1997). Populations from which *Rhinolophus affinis* tissue samples were obtained for microsatellite analysis are shown as squares, populations from which *R. lepidus* samples were obtained for microsatellite analysis are shown as triangles. Sites shown as circles either did not contain these species, or were sampled in the second field season so were not included due to time constraints.

DNA extraction and microsatellite genotyping

Genomic DNA for each individual from both of the species was extracted using Promega Wizard Purification Kits. *R. affinis* individuals were genotyped at 13 polymorphic microsatellite markers while *R. lepidus* individuals were genotyped at 10 markers. Primers sequences were obtained from the literature (Table 4.2), and the forward primer of each primer pair was fluoro-labeled with a FAM, HEX or TAMRA tag. For optimization, each marker was first tested across a temperature gradient, to determine the best annealing temperature for the species/primer combination. For genotyping, I then designed panels of around three or four markers for each species, based on non-overlapping PCR products.

For *R. lepidus*, PCRs contained a single primer set and were undertaken in reaction volumes of 15µl, containing 5-25ng of genomic DNA, 0.5U of Roche FastStart Taq DNA Polymerase, 0.25mM of labeled forward primer, 0.25mM of unlabeled reverse primer, 2mM of $MgCl_2$ and 0.2mM of each dNTP. I used a thermal profile of an initial denaturation step at 95ºC for 15 minutes; 35 amplification cycles each comprising a denaturation step (95ºC for 30 seconds), an annealing step (50ºC to 61ºC for 30 seconds) and an extension step (72ºC for 30 seconds); and a final extension at 72ºC for 10 minutes. Amplified products were pooled into panels and genotyped on an ABI PRISM® 3700 Sequencer. Products were genotyped with Genotyper version 3.6.

R. affinis genotyping was conducted a few months later, when multiplex kits were available. Therefore, for *R. affinis* individuals, I was able to amplify all primers together from each panel using QIAGEN® Multiplex PCR Kits. PCRs were undertaken in volumes of 10µl and contained 1× of QIAGEN® multiplex, 10X primer mix (0.2 µM of each primer) and 1-10ng genomic DNA. PCR reactions were undertaken at thermal profile suggested by the kit, which was 15 minutes of an initial activation step at 95ºC; 35 amplification cycles each comprising a denaturation step at 94ºC for 30 seconds, an annealing step (57 to 63 ºC) for 90 seconds and an extension at 72ºC for 60 seconds; and a final extension step at 60ºC for 30 minutes. Amplified products were genotyped on an ABI PRISM® 3730xl Sequencer and analysed with GeneMapper® version 4.0.

Table 4.2 Details of the loci used in this study for (a) *Rhinolophus affinis* and (b) *R. lepidus*.

	Locus	Tm (°C)[a]	Allele size range (bp)	GenBank accession	Reference[b]
a)	A26	58	230-280	EU737082	1
	A62	59	110-160	EU737084	1
	B12	55	100-140	EU737085	1
	B63	55	160-220	EU737086	1
	B71	60	180-250	EU737087	1
	D6	60	180-230	EU737088	1
	D41	56	150-190	EU737095	1
	E7	56	260-300	EU737089	1
	E45	58	210-270	EU737090	1
	E93	58	200-270	EU737092	1
	E95	55	100-160	EU737094	1
	E119	56	140-220	EU737093	1
	F77	56	260-310	EU737096	1
b)	Rferr01	61	110-134	AF160200	2
	Rferr11	54	172-196	AF160210	2
	Rferr14	60	227-241	AJ560195	3
	Rferr27	50	135-207	AJ560170	3
	RHA8	54	137-176	JF750631	4
	RHA101	56	131-153	JF750632	4
	RHA104	55	281-316	JF750633	4
	RHA105	56	172-190	JF750634	4
	RHA118	54	223-251	JF750636	4
	RHD107	51	214-242	DQ102694	5

[a] Tm is annealing temperature

[b] Details of the loci including primer sequences were obtained from references (1) (Mao et al., 2009), (2) (Rossiter et al., 1999), (3) (Dawson et al., 2004), (4) (Struebig et al., 2011) and (5) (Puechmaille et al., 2005)

Analysis of genetic diversity

Microsatellite alleles were sized and scored using Genotyper v. 3.6 or GeneMapper v. 4.0. To reduce potential sources or error, individuals with missing data were removed prior to subsequent population genetic analyses. All analyses genetic diversity and structure were undertaken separately for both species.

To test for deviation from Hardy-Weinberg equilibrium (HWE) for each locus in each population, I calculated F_{IS} using the Robertson and Hill (1984) estimator in the software Genepop on the Web (Raymond and Rousset, 1995). I also tested for linkage disequilibrium (LDE) for each pair of loci in each population using the same software. For both tests, the exact P-value was estimated using a Markov Chain (settings 10,000 dememorizations, 10,000 batches and 10,000 iterations). To avoid possible type I errors that can arise from undertaking multiple tests, Bonferroni corrections were carried out following Rice (1989) in order to adjust the nominal alpha level. The effective number of alleles (Ne), observed heterozygosity (H_O) and expected heterozygosity (H_E) were calculated using GENALEX 6.41 (Peakall and Smouse, 2006). The numbers of alleles (Na) and allelic richness (Rs) for all loci for all populations were calculated in FSTAT 2.9.3.2 (Goudet, 1995) with 10 000 permutations. Estimation of allelic richness (Rs) involved rarefaction in order to obtain a value that is independent of the sample size for a locus (N) so allowing unbiased comparisons among samples (Goudet, 1995). Values of allelic richness (Rs) for each population were used to compare allelic diversity across longitudinal and latitudinal gradients within Peninsular Malaysia.

Analysis of genetic structure and gene flow

(i) F-statistics and Isolation-by-distance

Genetic differentiation among populations was first determined using F-statistics and their analogues. Pairwise vales of F_{ST} (Weir and Cockerham, 1984) were calculated among all populations for which at least five individuals were genotyped (11 populations for *R. affinis* nd 8 for *R. lepidus*) using FSTAT 2.9.3.2 (Goudet, 1995) with 1000 permutations for each species' data set. Bonferroni adjustment was performed used to correct for multiple tests. Because F_{ST} is calculated based solely on the probability of allelic identity and does not consider allele size (Hardy and Vekemans,

2009) it can be biased downwards if stepwise mutation is important (Allendorf and Luikart, 2007). Therefore, R_{ST} among populations was also calculated in the software SPAGeDi v. 1.3 (Hardy and Vekemans, 2002). R_{ST} is analogous to F_{ST} but was proposed to estimate genetic differentiation based on allele size rather than allele identity, and is therefore suited to hypervariable loci such as microsatellites in which allelic variation probably results from a stepwise mutation process (Rousset, 1996, Slatkin, 1995). Comparisons of values of F_{ST} and R_{ST} can thus provide information on the relative importance of the processes of drift versus mutation. When genetic drift is more important in contributing to the genetic differentiation, both of the values are expected to be similar, whereas R_{ST} values should be greater than F_{ST} values if there is a contribution of stepwise mutation to the differentiation. To make the comparison as valid as possible, I specifically compared R_{ST} to a form of permuted R_{ST} ($_pR_{ST}$), in which alleles were randomized with respect to their size, so that any possible influence of stepwise mutation is removed. If observed R_{ST} is significantly larger than $_pR_{ST}$, R_{ST} is the most suitable estimator in describing genetic structure, while no significant difference suggests pR_{ST} or F_{ST} is sufficient. Since drift is likely to occur more rapidly than stepwise mutation, higher R_{ST} values are also thought to provide evidence of differentiation due to phylogeographic separation rather than to recent isolation (Hardy and Vekemans, 2002; Rossiter et al., 2007).

To examine whether genetic structure among populations in Peninsular Malaysia was consistent with a stepping-stone model of gene flow, plots of isolation-by-distance (IBD) were constructed from pairwise geographical distance versus genetic distance. Only populations with a minimum of five samples were included. Pairwise geographical distances were calculated using the software GENALEX v. 6.41 (Peakall and Smouse, 2006) based on the spatial coordinates listed in Table 4.1. Pairwise genetic distances were calculated as $F_{ST}/(1- F_{ST})$ in the software SPAGeDi v. 1.3 (Hardy and Vekemans, 2002). Using the software Isolation By Distance Web Service (IBD-WS) v. 1.53 (Jensen et al., 2005), these sets of distances were used for IBD analysis for populations from the whole of Peninsular Malaysia as well as for populations with each of the four regions (see Chapter 3). Strength and significance of the IBD relationships was assessed by a Mantel Test (10,000 randomizations) and by reduced major axis (RMA) regression. RMA regression analysis was performed to calculate the slope and intercept of the relationship between the genetic and geographical distance.

(ii) Clustering analyses

The same data were further analysed for genetic structure using STRUCTURE v. 2.3.3 (Pritchard et al., 2000; Falush et al., 2003; Pritchard et al., 2007; Hubisz et al., 2009). The program implements a Bayesian clustering of multi loci genotypes to determine the most likely number of groups present (Pritchard et al., 2007). This method makes no assumptions about original population membership and instead groups individuals into different numbers of clusters in order to minimise deviations from HWE and Linkage equilibrium. For each number of clusters in the model, the probability is calculated. In the outputted bar graphs, each bar represents an individual, which is assigned a given number of clusters in proportion to the probability of membership to each of these clusters. This estimated membership coefficient of a bar for an individual will sum to 1. Assignment to more than one cluster can indicate genetic admixture. In my study I ran STRUCTURE with 100,000 iterations of the Monte Carlo Markov Chain (MCMC) and discarded the first 30,000 iterations as burn-in. Based on the correlated allele frequency model, I repeated the analysis for values of K from one to nine, and undertook five replicate runs per value of K. To avoid overestimating K, I followed the recommendations of Pritchard et. al. (2010) for identifying the smallest value of K that is able to recover the major structure, and which shows a relatively stable and constant α throughout the run. For the value of K that showed the highest likelihood, so maximised Pr(X|K), replicate runs were combined in the software CLUMPP (Jakobsson and Rosenberg, 2007). Finally, individuals were displayed graphically in latitudinal order in DISTRUCT 1.1 (Rosenberg, 2004). These analyses were also all repeated with the same data but where all bats were assigned to the original subpopulations regardless of spatial locality of the subpopulations.

(iii) Principal Coordinate Analysis (PCoA)

For each species, the pairwise genetic distances among populations were also analysed with a Principle Coordinate Analysis (PCoA) implemented in GENALEX v 6.41 (Peakall and Smouse, 2006). This multivariate method is related to the more common Principle Coordinate Analysis and provides a powerful way of visualizing the major genetic relationships and patterns in the dataset by finding the main axes of variation in

multidimensional space. PCoA provides a complimentary method to clustering and tree-based approaches of recovering population relationships.

Results

Genetic diversity

The full results from genetic diversity analyses for *Rhinolophus affinis* and *R. lepidus* are shown in Tables 4.3a and Table 4.3b, respectively. For *R. affinis*, estimated values of F_{IS} showed that no populations consistently deviated from Hardy-Weinberg equilibrium (HWE) across multiple loci. However, in three loci (Locus A62, D41 and E93), multiple populations (seven, two and two populations, respectively) showed significant deviations from HWE. In these cases, the F_{IS} was high indicating an excess of homozygotes, and suggesting possible presence of null alleles (Allendorf and Luikart, 2007). Nonetheless, the presence of null alleles in this marker was not previously recorded for *R. affinis* populations in China (Mao et al., 2009). For *R. lepidus*, again no populations consistently deviated from HWE across multiple loci. Four out of 10 microsatellite loci (Rferr11, RHA101, RHA104 and RHA105) were characterised by some high F_{IS} values in a few populations but these were not significant (Table 4.3b). No evidence for linkage disequilibrium was found for either species.

In terms of diversity, for *R. affinis* mean allelic richness per population ranged from 5.5 to 6.5 (Table 4.3a) whereas the same values were much lower (1.75 to 1.88) for *R. lepidus* (Table 4.3b). In both species, allelic richness per population appeared to increase with latitude, however, this was not significant (see Figures 4.2a and 4.2c). On the other hand, allelic richness showed a weak but significant decrease with longitude in both species (*R. affinis*: $r^2 = 0.2531$, $P = 0.0082$ and *R. lepidus*: $r^2 = 0.2205$, $P = 0.0080$) (see Figures 4.2d and 4.2d, respectively. These trends indicate a gradual decrease in the number of alleles of both species from west to east. Due to the shape of Peninsular Malaysia, this also indicates a southerly decline in the number of alleles.

Table 4.3 Genetic data for (a) *R. affinis* and (b) *R. lepidus*. Total number of individuals selected in the analysis (N), number of alleles (Na), number of effective alleles (Ne), unbiased expected heterozygosity (H_E), observed heterozygosity (H_O) and polymorphism (P). F_{IS} is shown to indicate deviation from Hardy–Weinberg equilibrium. * $P < 0.05$ after Bonferroni correction.

(a) *Rhinolophus affinis*

Population	NS1	JH1	PK1	KH2	KH3	KH1	FRIM	JH2	PH2	PN1	PH1
N	17	28	8	5	8	8	5	34	31	45	11
Locus A20											
Na (6)	2	3	2	3	2	3	2	4	3	5	3
Ne	1.262	1.075	1.600	1.515	1.133	1.684	1.724	1.236	1.431	1.864	1.449
Rs	1.771	1.357	1.992	3.000	1.625	2.750	2.000	1.900	2.117	2.552	2.312
H_E (unbiased)	0.214	0.071	0.400	0.378	0.125	0.433	0.467	0.194	0.306	0.469	0.325
H_O	0.118	0.071	0.500	0.400	0.125	0.500	0.600	0.206	0.290	0.489	0.364
F_{IS} estimate	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
Locus A26											
Na (14)	6	8	7	4	6	7	6	9	7	10	6
Ne	2.524	3.187	5.333	3.333	3.765	4.571	4.167	3.860	3.348	3.479	3.457
Rs	3.767	4.480	5.698	4.000	4.964	5.589	6.000	4.871	4.289	4.263	4.450
H_E (unbiased)	0.622	0.699	0.867	0.778	0.783	0.833	0.844	0.752	0.713	0.721	0.745
H_O	0.647	0.643	0.875	0.600	0.750	0.875	0.800	0.853	0.742	0.689	0.727
F_{IS} estimate	NS	NS	NS	NS	NS	NS	NS	NS	NS	-0.0036	NS
Locus A62											
Na (17)	8	8	5	5	6	7	6	10	13	13	5
Ne	6.021	4.709	4.414	3.125	3.765	4.571	5.000	5.709	8.042	8.198	2.495
Rs	5.687	4.901	4.705	5.000	4.749	5.589	6.000	5.406	6.479	6.464	3.815
H_E (unbiased)	0.859	0.802	0.825	0.756	0.783	0.833	0.889	0.837	0.890	0.888	0.628
H_O	0.118	0.286	0.125	0.400	0.375	0.375	0.400	0.118	0.129	0.267	0.091
F_{IS} estimate	0.8287*	0.5137 *	1.0000 *	0.3500	0.3857	0.4881	0.5000	0.7937 *	0.7698 *	0.6026*	0.8173 *
Locus B12											
Na (12)	8	8	7	6	9	6	5	8	7	9	5
Ne	5.352	5.227	5.818	4.167	7.111	4.000	4.545	5.453	5.460	6.418	3.967
Rs	5.481	5.114	5.920	6.000	6.992	4.831	5.000	5.082	5.044	5.560	4.415
H_E (unbiased)	0.838	0.823	0.883	0.844	0.917	0.800	0.867	0.829	0.830	0.854	0.784
H_O	0.706	0.893	0.750	0.800	0.875	0.375	0.200	0.824	0.774	0.867	0.909
F_{IS} estimate	0.2420	NS	NS	NS	NS	0.3571	0.8750	NS	NS	NS	NS
Locus B63											
Na (14)	9	10	7	5	7	8	6	10	11	14	6
Ne	6.352	5.141	5.565	3.125	5.120	6.400	4.545	5.048	7.392	6.308	4.102
Rs	5.892	5.101	5.760	5.000	5.677	6.456	6.000	5.110	6.047	5.820	4.932
H_E (unbiased)	0.868	0.820	0.875	0.756	0.858	0.900	0.867	0.814	0.879	0.851	0.792
H_O	0.882	0.893	0.875	0.600	0.750	0.875	1.000	0.853	0.774	0.756	0.818
F_{IS} estimate	NS	NS	NS	NS	NS	NS	NS	NS	0.0655	NS	NS
Locus B71											
Na (6)	3	4	4	3	2	4	3	4	4	5	4
Ne	2.165	2.299	2.844	2.273	2.000	2.560	2.174	3.007	3.027	2.443	2.305
Rs	2.505	2.916	3.500	3.000	2.000	3.554	3.000	3.432	3.358	2.862	2.908
H_E (unbiased)	0.554	0.575	0.692	0.622	0.533	0.650	0.600	0.677	0.681	0.597	0.593
H_O	0.294	0.464	0.625	0.600	0.000	0.500	0.400	0.647	0.516	0.467	0.455
F_{IS} estimate	0.7258	NS	NS	1.1429	NS	NS	NS	NS	NS	NS	NS
Locus D6											
Na (14)	8	8	9	6	7	8	6	11	12	10	8
Ne	4.446	4.978	8.000	5.000	4.923	6.400	5.000	5.838	7.308	6.795	6.050
Rs	4.804	4.838	7.214	6.000	5.615	6.456	6.000	5.553	6.160	5.734	5.972
H_E (unbiased)	0.799	0.814	0.933	0.889	0.850	0.900	0.889	0.841	0.877	0.862	0.874
H_O	0.765	0.857	1	1.000	0.625	0.750	1.000	0.794	0.935	0.844	0.818
F_{IS} estimate	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
Locus D41											
Na (10)	4	3	4	3	3	5	3	7	6	7	4
Ne	2.359	1.931	2.909	2.778	2.032	2.783	1.852	2.227	2.433	3.418	2.782
Rs	2.801	2.315	3.742	3.000	2.624	3.874	3.000	3.350	3.197	4.002	3.374
H_E (unbiased)	0.594	0.491	0.700	0.711	0.542	0.683	0.511	0.559	0.599	0.715	0.671
H_O	0.294	0.357	0.375	0.400	0.375	0.375	0.400	0.265	0.387	0.556	0.182

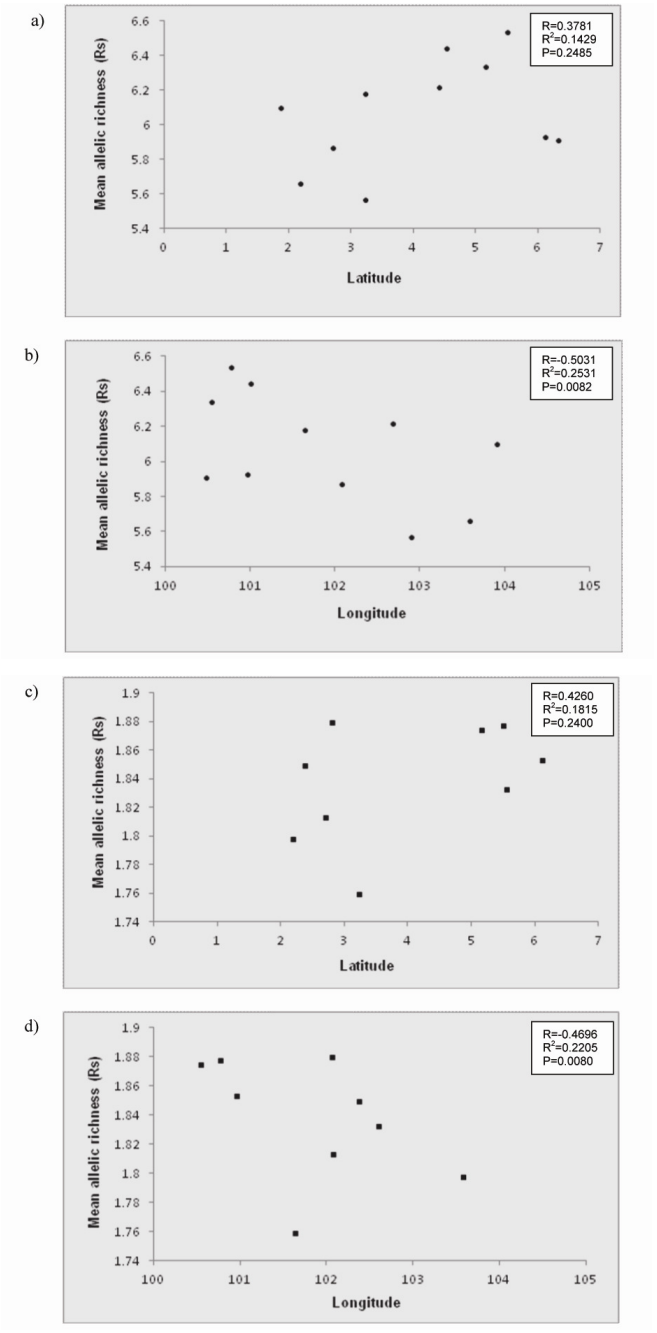
F_{IS} estimate	0.5194	0.6127	NS	NS	NS	0.1786	NS	0.5113 *	0.1587	0.2644	0.6015 *
Locus E7											
Na (8)	5	5	5	2	4	4	5	6	6	7	3
Ne	4.624	2.975	3.282	1.724	2.844	3.459	3.125	3.853	3.274	3.835	2.659
Rs	4.539	3.635	4.125	2.000	3.500	3.838	5.000	4.407	3.884	4.203	2.958
H_E (unbiased)	0.807	0.676	0.742	0.467	0.692	0.758	0.756	0.752	0.706	0.748	0.654
H_O	0.588	0.714	0.750	0.600	0.500	0.875	0.600	0.647	0.613	0.733	0.364
F_{IS} estimate	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
Locus E45											
Na (12)	11	10	7	6	7	7	5	11	10	10	8
Ne	5.026	7.362	4.414	4.545	5.565	5.120	4.167	6.985	6.025	7.271	6.541
Rs	5.808	6.100	5.428	6.000	5.999	5.677	5.000	6.128	5.745	6.009	6.146
H_E (unbiased)	0.825	0.880	0.825	0.867	0.875	0.858	0.844	0.870	0.848	0.872	0.887
H_O	0.706	0.714	0.625	0.600	0.875	0.875	0.800	0.647	0.871	0.800	1.000
F_{IS} estimate	NS	0.1193	NS	NS	NS	NS	NS	NS	0.2617	NS	NS
Locus E93											
Na (17)	10	12	9	5	8	9	5	13	12	14	7
Ne	6.149	7.193	7.529	4.545	4.741	7.529	3.846	6.901	7.145	8.420	5.762
Rs	5.887	6.136	7.054	5.000	5.964	7.054	5.000	6.148	6.099	6.515	5.660
H_E (unbiased)	0.863	0.877	0.925	0.867	0.842	0.925	0.822	0.868	0.874	0.891	0.866
H_O	0.824	0.786	0.750	1.000	0.875	0.750	0.800	0.706	0.581	0.778	0.273
F_{IS} estimate	NS	NS	0.1607	NS	NS	0.1161	NS	0.0989	0.2002 *	NS	0.6625 *
Locus E95											
Na (17)	13	13	9	7	7	9	6	13	13	14	9
Ne	9.031	8.082	7.529	6.250	5.333	6.737	5.000	8.170	8.940	8.901	7.333
Rs	7.043	6.440	7.054	7.000	5.698	6.831	6.000	6.424	6.783	6.544	6.563
H_E (unbiased)	0.916	0.892	0.925	0.933	0.867	0.908	0.889	0.891	0.903	0.898	0.905
H_O	0.882	0.786	0.875	1.000	0.813	0.875	1.000	0.882	0.839	0.911	0.818
F_{IS} estimate	NS	NS	NS	NS	NS	0.0268	NS	NS	NS	NS	NS
Locus E119											
Na (23)	9	11	7	6	8	8	5	14	15	13	6
Ne	3.420	3.891	4.414	5.000	4.741	4.129	2.500	3.772	3.572	4.677	3.103
Rs	4.915	5.148	5.428	6.000	5.964	5.875	5.000	5.268	5.144	5.459	4.308
H_E (unbiased)	0.729	0.756	0.825	0.889	0.842	0.808	0.667	0.746	0.732	0.795	0.710
H_O	0.529	0.786	0.875	1.000	0.875	1.000	0.600	0.735	0.677	0.844	0.727
F_{IS} estimate	0.2800	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
Locus F77											
Na (8)	4	5	4	4	4	4	5	6	7	5	4
Ne	3.124	3.246	2.510	2.778	3.282	2.844	4.167	3.596	3.051	3.295	3.103
Rs	3.654	3.779	3.250	4.000	3.831	3.500	5.000	4.035	4.021	3.724	3.419
H_E (unbiased)	0.701	0.705	0.642	0.711	0.742	0.692	0.844	0.733	0.683	0.704	0.710
H_O	0.412	0.679	0.500	0.600	0.500	0.500	1.000	0.588	0.516	0.467	0.364
F_{IS} estimate	NS	NS	NS	NS	NS	NS	NS	0.1625	0.3142	0.2843*	0.4133
Mean allele/locus	7.1429	7.4258	6.1429	4.2143	5.7143	6.3571	4.8571	9.0000	9.0000	9.7142	5.5714
Mean Rs	5.8685	5.6600	6.4427	5.9091	5.9275	6.5340	6.1818	6.1013	6.2152	6.3374	5.5665
Mean H_E	0.679	0.659	0.737	0.698	0.683	0.732	0.717	0.691	0.701	0.724	0.676
Mean H_O	0.518	0.595	0.633	0.640	0.567	0.633	0.640	0.584	0.576	0.631	0.527
P	0.9333	0.9333	0.9333	0.9333	0.9333	0.9333	0.9333	0.9333	0.9333	0.9333	0.9333

(b) *Rhinolophus lepidus*

Population	NS1	JH1	KH1	MK4	PN1	KH3	NS3	PK4	FRIM	TG2	SL2
N	7	4	17	18	10	4	6	2	4	6	2
LocusRferr01											
Na (8)	6	4	7	7	8	5	7	4	3	6	4
Ne	5.444	3.556	5.352	4.985	6.061	4.000	5.538	4.000	2.667	5.143	4.000
Rs	1.879	1.821	1.838	1.822	1.879	1.857	1.894	2.000	1.833	1.879	2.000
H_E (unbiased)	0.879	0.821	0.838	0.822	0.879	0.857	0.894	1.000	0.833	0.879	1.000
H_O	1.000	0.750	0.882	0.722	0.900	0.750	0.833	1.000	0.500	1.000	1.000
F_{IS} estimate	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
Locus Rferr11											
Na (13)	7	5	11	8	7	5	7	2	3	5	4
Ne	3.920	4.000	7.918	5.143	5.556	3.200	6.000	2.000	2.667	3.789	4.000
Rs	1.802	1.857	1.900	1.829	1.863	1.786	1.909	1.667	1.833	1.803	2.000
H_E (unbiased)	0.802	0.857	0.900	0.829	0.863	0.786	0.909	0.667	0.833	0.803	1.000
H_O	0.714	0.500	0.765	0.944	0.900	0.750	0.667	1.000	1.000	1.000	1.000
F_{IS} estimate	NS	NS	NS	NS	NS	NS	0.2222	NS	NS	NS	NS
Locus Rferr14											
Na (8)	3	4	7	7	6	5	5	3	1	4	3
Ne	2.174	3.200	4.031	4.765	3.922	4.571	4.235	2.667	1.000	3.273	2.667
Rs	1.600	1.786	1.776	1.813	1.784	1.893	1.833	1.833	1.000	1.758	1.833
H_E (unbiased)	0.600	0.786	0.776	0.813	0.784	0.893	0.833	0.833	0.000	0.758	0.833
H_O	0.6000	1.000	0.688	0.778	0.800	1.000	1.000	0.500	0.000	0.500	0.500
F_{IS} estimate	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
Locus Rferr27											
Na (24)	10	6	14	13	11	4	7	1	3	4	3
Ne	8.167	4.571	9.660	8.627	8.100	2.909	6.400	1.000	2.667	6.250	2.667
Rs	1.945	1.893	1.925	1.911	1.928	1.750	1.964	1.000	1.833	1.933	1.833
H_E (unbiased)	0.945	0.893	0.925	0.911	0.928	0.750	0.964	0.000	0.833	0.933	0.833
H_O	1.000	0.750	1.000	0.706	1.000	0.750	1.000	0.000	1.000	0.800	1.000
F_{IS} estimate	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
Locus RHA8											
Na (19)	9	7	14	11	11	7	8	4	4	8	4
Ne	7.200	6.400	9.966	6.231	7.111	6.400	6.545	4.000	4.000	7.200	4.000
Rs	1.923	1.964	1.927	1.863	1.868	1.964	1.924	2.000	2.000	1.939	2.000
H_E (unbiased)	0.939	0.964	0.927	0.863	0.917	0.964	0.924	1.000	1.000	0.939	1.000
H_O	1.000	1.000	0.824	0.833	0.875	1.000	0.667	1.000	1.000	1.000	1.000
F_{IS} estimate	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
Locus RHA101											
Na (15)	4	5	10	8	10	4	6	3	3	5	3
Ne	3.429	4.571	6.644	5.505	7.714	3.556	5.143	2.667	2.667	3.600	2.667
Rs	1.773	1.893	1.892	1.845	1.921	1.821	1.879	1.833	1.833	1.788	1.833
H_E (unbiased)	0.773	0.893	0.881	0.845	0.922	0.821	0.879	0.833	0.833	0.788	0.833
H_O	0.167	0.250	0.429	0.313	0.556	0.250	0.500	1.000	0.500	0.333	0.500
F_{IS} estimate	0.7111	0.7500	0.3617	0.5992	0.2593	0.8148	NS	NS	NS	0.5033	NS
Locus RHA104											
Na (16)	8	6	11	9	9	5	7	4	3	7	4
Ne	4.900	4.571	8.036	7.136	6.897	4.000	5.538	4.000	2.667	6.000	4.000
Rs	1.857	1.893	1.906	1.894	1.900	1.857	1.894	2.000	1.833	1.909	2.000
H_E (unbiased)	0.857	0.893	0.906	0.886	0.900	0.857	0.894	1.000	0.833	0.909	1.000
H_O	0.857	0.750	0.600	0.588	0.600	1.000	1.000	1.000	0.500	1.000	1.000
F_{IS} estimate	NS	NS	0.3538	0.3320	0.2535	NS	NS	NS	NS	NS	NS
Locus RHA105											
Na (10)	5	3	7	7	6	4	4	2	3	4	3
Ne	2.649	2.133	4.923	4.777	4.348	3.556	3.000	2.000	2.667	2.057	2.667
Rs	1.670	1.607	1.823	1.821	1.811	1.821	1.727	1.667	1.833	1.561	1.833
H_E (unbiased)	0.670	0.607	0.823	0.815	0.811	0.821	0.727	0.667	0.833	0.561	0.833
H_O	0.571	0.750	0.875	0.706	0.600	0.250	0.833	0.000	0.500	0.500	1.000
F_{IS} estimate	NS	NS	NS	NS	NS	0.8148	NS	NS	NS	NS	NS
Locus RHA118											
Na (11)	8	3	10	10	9	6	7	4	3	8	3
Ne	5.158	1.684	8.500	5.684	7.364	5.333	5.538	4.000	2.667	6.545	2.667
Rs	1.868	1.464	1.909	1.848	1.915	1.929	1.894	2.000	1.833	1.924	1.833
H_E (unbiased)	0.868	0.464	0.909	0.848	0.915	0.929	0.894	1.000	0.833	0.924	0.833
H_O	0.857	0.500	0.941	0.889	0.889	1.000	1.000	1.000	0.500	0.833	1.000
F_{IS} estimate	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS

Locus RHD107											
Na (9)	5	5	7	8	8	4	8	3	NA	7	2
Ne	3.630	3.200	4.857	6.113	5.714	3.600	4.500	2.667	0.000	5.538	2.000
Rs	1.780	1.786	1.818	1.860	1.868	1.867	1.848	1.833	NA	2.000	1.845
H_E (unbiased)	0.780	0.786	0.818	0.860	0.868	0.867	0.848	0.833	0.000	0.894	1.000
H_O	0.857	1.000	0.706	0.833	1.000	1.000	0.833	1.000	0.000	0.667	1.000
F_{IS} estimate	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
Mean allele/locus	6.5000	4.8000	9.8000	8.8000	8.5000	4.9000	6.6000	3.0000	2.6000	6.1000	3.3000
Mean Rs	1.8130	1.7976	1.8773	1.8496	1.8743	1.8531	1.8798	NA	1.7590	1.8327	NA
Mean H_E (unbiased)	0.811	0.796	0.870	0.849	0.879	0.855	0.877	0.783	0.683	0.839	0.917
Mean H_O	0.762	0.725	0.771	0.731	0.812	0.775	0.833	0.750	0.550	0.763	0.850
P	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	0.9000	0.8000	1.0000	1.0000

Figure 4.2 Parts (a) and (b) show mean allelic richness (Rs) for populations of *R. affinis* with latitude and longitude, respectively. Parts (c) and (d) show mean allelic richness (Rs) for populations of *R. lepidus* across latitude and longitude, respectively.



Analysis of population structure

(i) F-statistics and Isolation-by-distance

For both species, estimated global values of R_{ST}, as well as pairwise R_{ST} among populations were not significantly larger than the corresponding permuted analogues pR_{ST} (values not shown) indicating stepwise mutation has not contributed to the observed differentiation, and thus F_{ST} is adequate to describe genetic distance. Closer examination of pairwise F_{ST} among *R. affinis* populations showed the greatest distance (F_{ST} = 0.0448, P < 0.001) was found between KH2 and JH1, which are the northernmost sampling site and second southernmost sampling site in this study (see Table 4.4a). However, the southernmost of all sampling sites (JH2) showed no significant differentiation with KH2 (F_{ST} = 0.0372, P > 0.05). Other significant pairwise differences were detected between KH3 and JH1 (F_{ST} = 0.0123, P < 0.05), PN1 and PH1 (F_{ST} = 0.0162, P < 0.05), PN1 and JH1 (F_{ST} = 0.0147, P < 0.01) and PN1 and JH2 (F_{ST} = 0.0145, P < 0.001). When all values of pairwise genetic distance were plotted against corresponding geographic distances, significant isolation-by-distance (IBD) was detected (r^2 = 0.243, slope 0.0001; P = 0.0013 (Figure 4.3a).

In the case of *R. lepidus*, pairwise F_{ST} values among subpopulations ranged from -0.0126 (PN1 and KH3) to 0.0734 (NS1 and JH1), however, none were significant. In general, the range of pairwise differences among *R. lepidus* populations was wider compared to that of pairwise differences among *R. affinis* populations. An IBD plot of genetic distance versus geographic distance for *R. lepidus* revealed a positive relationship, however, this was not significant (Figure 4.3b).

Table 4.4 Pairwise estimated of F_{ST} and R_{ST} calculated for a) 11 populations of *R. affinis* and b) 8 populations of *R. lepidus* across Peninsular Malaysia. Significant comparisons are denoted by * for P < 0.05, ** for P < 0.01 and *** for P < 0.001.

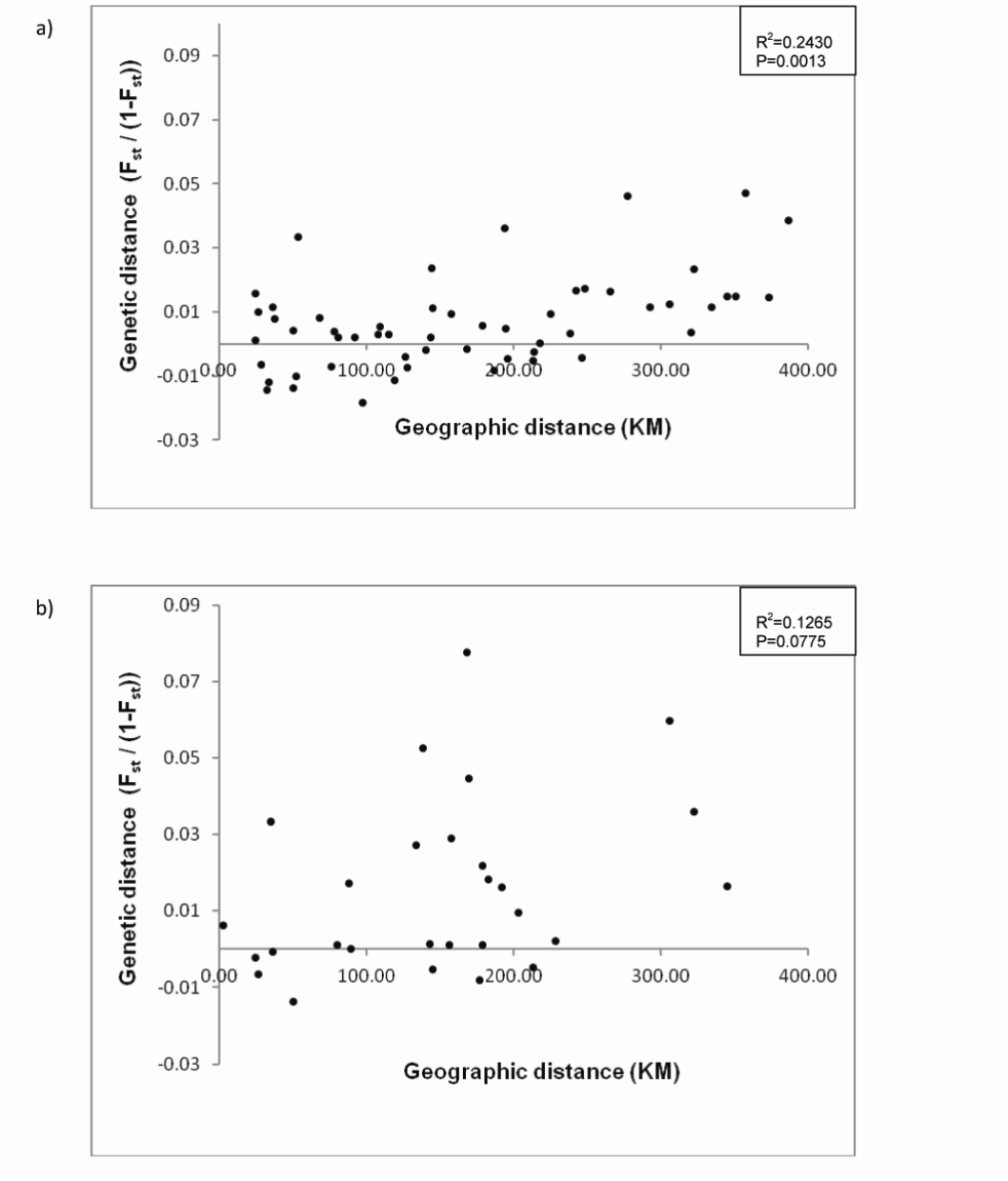
a)

R_{ST} \ F_{ST}	NS1	JH1	PK1	KH2	KH3	KH1	FRIM	JH2	PH2	PN1	PH1
NS1		-0.0015	-0.0041	0.0348	0.0111	0.0092	-0.0141	-0.0046	0.0039	0.0058	0.0022
JH1	-0.0139		0.0113	0.0448**	0.0123*	0.0229	0.0002	-0.0065	0.003	0.0147**	0.0022
PK1	-0.0157	-0.0056		0.008	-0.012	-0.0147	-0.0072	0.0035	-0.0084	-0.0102	-0.0053
KH2	-0.0156	0.0066	-0.0377		0.0322	0.0114	0.023	0.0372	0.017	0.0154	0.044
KH3	-0.0078	0.0002	-0.0489	-0.0459		0.0013	-0.0188	0.0113	0.0047	0.0041	0.0093
KH1	-0.0131	-0.0011	-0.0063	-0.0359	-0.0271		0.003	0.0146	-0.0024	0.0098	0.0163
FRIM	-0.0659*	-0.0192	-0.0182	0.0181	-0.0131	0.0089		-0.0044	-0.0114	-0.0073	-0.002
JH2	0.0077	-0.0024	-0.0181	0.0022	-0.0217	0.0127	-0.0089		0.0021	0.0145***	0.0053
PH2	0.0198	0.0058	-0.0136	-0.0145	-0.0291	-0.0117	0.0192	-0.0047		0.0031	0.0077
PN1	-0.0012	0.003	-0.0331*	-0.0343	-0.0312*	-0.0111	-0.0073	0.0002	0.0044		0.0162*
PH1	-0.0131	-0.0168	-0.0433	-0.0285	-0.0311	-0.0223	-0.006	-0.0177	-0.025*	-0.0207*	

b)

R_{ST} \ F_{ST}	NS1	JH1	KH1	MK4	PN1	KH3	NS3	TG2
NS1		0.0734	0.0288	0.0325	0.0227	-0.0039	0.0079	0.0013
JH1	0.0356		0.0345	0.0246	0.0176	0.0563	0.0427	0.0499
KH1	0.0255	-0.0273		0.0158	-0.0067	-0.0009	0.0014	0.0091
MK4	0.0237	0.0154	-0.0064		-0.005	0.0006	-0.0014	0.0016
PN1	0.0292	-0.0146	0.0183	0.013		-0.0126	-0.0064	0.0037
KH3	-0.0208	0.1292	0.0612	0.0217	0.0499		0.0013	0.0179
NS3	0.0449	-0.034	0.0276	0.0752	0.0971	0.1639		0.0169
TG2	-0.0336	0.0527	0.0149	-0.0199	0.0017	-0.0593	0.1093	

Figure 4.3 Plot of genetic distance against geographical distance for pairwise population comparisons across peninsular Malaysia, undertaken for (a) *R. affinis* and (b) *R. lepidus*.



(ii) Clustering analyses

In addition to IBD analyses, wider patterns of genetic structure were also analysed without geographical information using a Bayesian clustering method. Mean probabilities were generated for one to nine genetic clusters (K values), each based on averaging the results from five replicate runs. K was plotted against ln P(D) to determine the most probable number of clusters in the data. Following this method, it appeared that the most probable number of clusters for *R. affinis* in Peninsular Malaysia was K = 3, whereas for *R. lepidus* in Peninsular Malaysia it was K = 1 (see Figure 4.4a and b, respectively). For both species, these results were the same and the overall broad patterns were similar in runs where population membership was considered.

Following grouping of runs in CLUMPP, the results remained the same. For *R. affinis*, cluster membership of all individuals at K = 3 is shown in Figure 4.5a, whereas for *R. lepidus* the structure at K = 2 (the second highest probability) (Figure 4.5b). To assess whether any detected clustering follows a clinal pattern, individuals on these plots are shown in order of increasing latitude. Overall I found very little evidence of population structure, with all populations showing strong admixture (mixed cluster membership). Cluster membership coefficients (Q) of all individuals did not exceed 0.5 for a particular genetic cluster. However, for *R. affinis*, there was some weak subdivision present, which showed some association with location. For example, JH1 and NS1 both had the same signature of admixture, whereas isolated smaller forest fragments such as PH1, FRIM and PK1 formed independent clusters from their neighboring sites. Although located in continuous primary forest, the southernmost and northernmost site, JH2 and KH2 formed independent clusters as well. KH3 is the site where hand net capturing was conducted for both of the species. For *R. lepidus*, in the bar graph for K = 2 individuals showed no differences across all populations (Figure 4.5b) with cluster membership split equally (Q = 0.5). This is to be expected based on the fact that K=1 had the highest probability (Figure 4.4b).

Figure 4.4 Average likelihood values for increasing number of clusters (values of K) shown for (a) *R. affinis* and (b) *R. lepidus*. In both plots, filled circles represent model runs in which there was no consideration of original population membership, whereas squares represent model runs in which original population membership information was included.

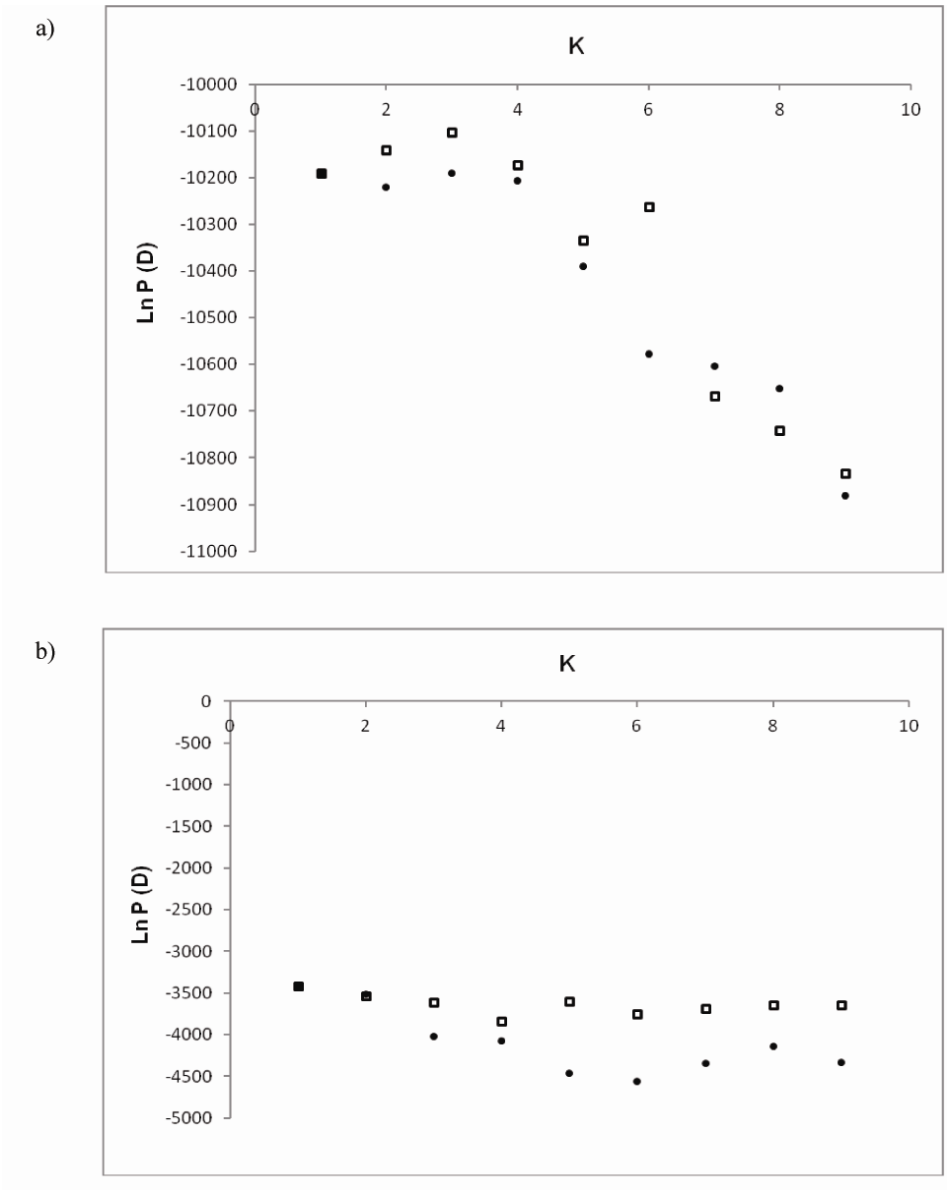
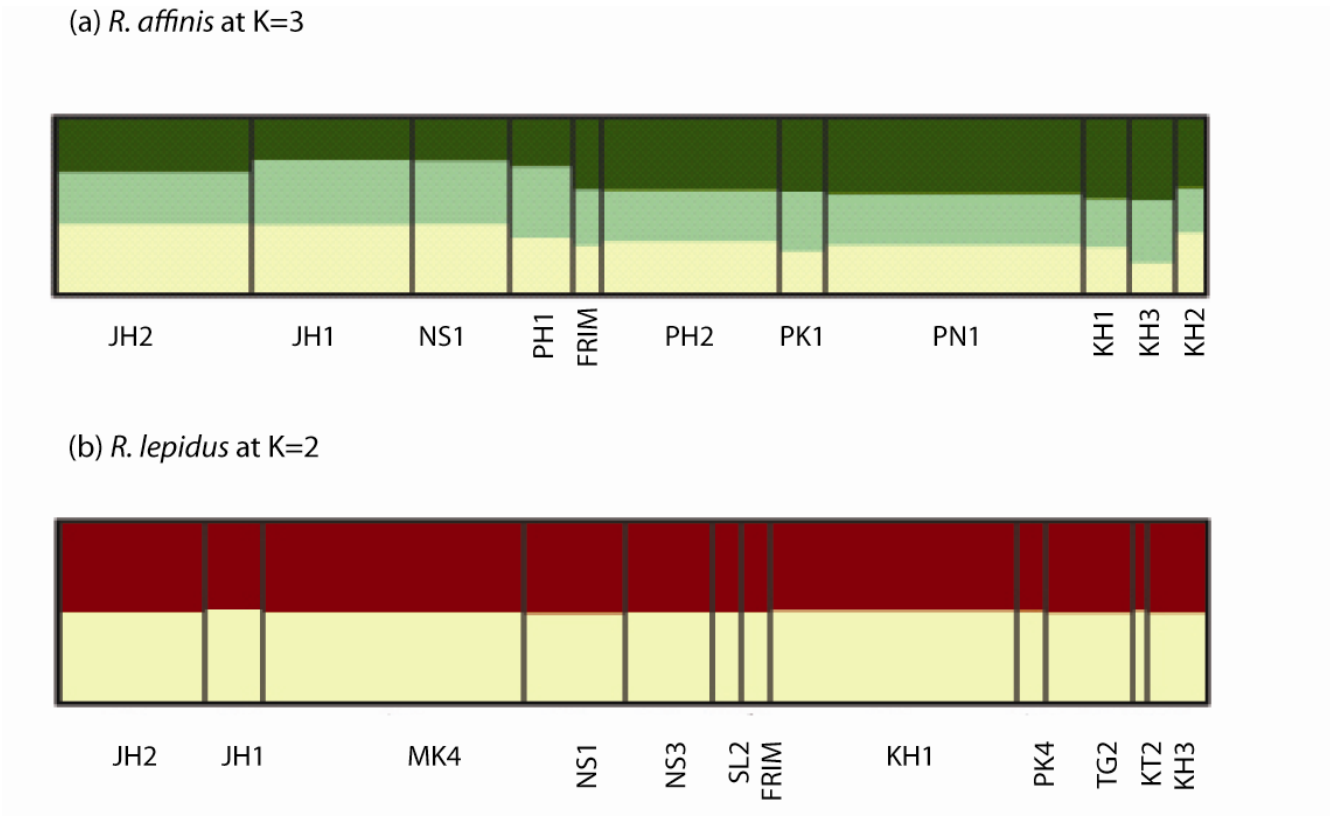


Figure 4.5 Graphical representation of population structure in the Peninsular Malaysian for (a) *R. affinis* at K = 3 and (b) *R. lepidus* at K = 2. Although no population membership information was used in assigning individuals, they have been sorted into their original populations and ordered by increasing latitude for display purposes. Plots were produced in DISTRUCT and are based on multiple replicate runs combined in CLUMMP. In these plots, each individual is depicted as a vertical line (not visible) that is subdivided into K colour sections. The length of the section represents the estimated membership coefficient (Q value) for that cluster.

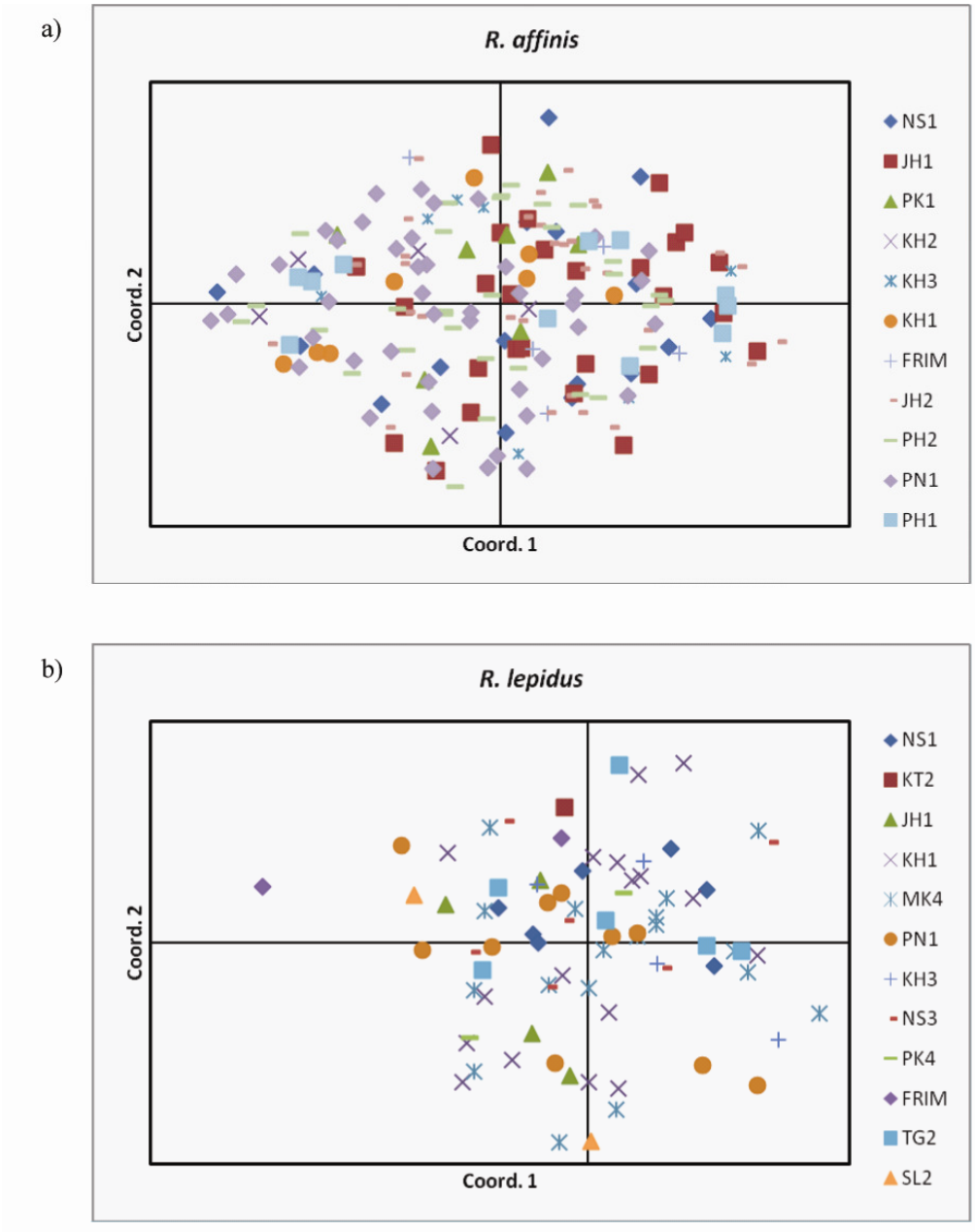


(iii) Principal Coordinate Analysis (PCoA)

Principal Coordinate Analyses (PCoA) were conducted based on pairwise genetic distances among individuals within each of the respective species. For *R. affinis*, coordinates (= axes) 1 and 2 together explained 40.89% of the total inertia (coordinate 1 = 21.82% and coordinate 2 = 19.08%) (see Figure 4.6a). Overall the PCoA pattern showed that individuals from all of the populations were generally intermingled, although representation of different populations appeared to differ along coordinate 1. In particular, most of the individuals from the southern part of Peninsular Malaysia tended to be distributed at the left of coordinate 1, showing segregation from individuals from the smaller forest fragments in the south (JH1 and PH1) that were mainly distributed at the right of coordinate 1. Most of the individuals from other populations were distributed evenly across coordinate 2.

For *R. lepidus* population, the first two coordinates explained a total of 36.94% of the total inertia (coordinate 1 = 18.50% and coordinate 2 = 18.44%). Individuals from most populations were distributed rather evenly along both coordinates, however, there was evidence that individuals from some populations were more concentrated towards the left of coordinate 1 (e.g. JH1), whereas others were more concentrated towards the right (e.g. KH1 and MK4). The most obvious outlier was an individual from FRIM, which occurred at the far left.

Figure 4.6 Two dimensional plots of first two coordinates based on a Principal Coordinate Analysis of genetic distances for all of the sampling locations for (a) *R. affinis* and (b) *R. lepidus.*



Discussion

In this study I utilised microsatellites data to analyse the genetic structure of two forest specialist species, *Rhinolophus affinis* and *R. lepidus*, in Peninsular Malaysia. In both species, plots of allelic richness (Rs) versus longitude showed significant negative correlations, with more alleles in the west (Figures 4.2a and c). Due to the shape of the Malay Peninsula, longitudinal declines also reflect greater diversity in the north. These results agree with those of Chapter 3, in which mtDNA diversity was also found to be higher in the north. Latitudinal declines in microsatellite allelic richness have been observed in several vertebrate species, such as the Anadromous brook char (Castric and Bernatchez, 2003), the European nine-spined stickleback (Shikano et al., 2010) and the common frog both in Sweden (Johansson et al., 2006) and across Europe (Palo et al., 2004). The opposite trend of northerly (and westerly) increases in microsatellite allelic richness detected in this study could reflect reductions in the south due to the effects of habitat loss. Alternatively higher diversity in the north might also be due to a longer or more stable population history as suggested by network and demographic analyses of mtDNA, or to admixture with the divergent population from north of the Isthmus of Kra (Chapter 3). As with mtDNA, the direction of the observed clines in microsatellite allelic richness do not agree with a scenario of expansion of the forest from the equator following the Last Glacial Maximum (e.g. Wurster et al., 2010). Finally, in the case of *R. affinis*, high diversity in the north could also be due to a larger effective population size, since this species ranges much further north. However, this seems less likely for *R. lepidus*, which is more restricted to the wet tropics (Csorba et al., 2003).

Weak isolation-by-distance (IBD) was revealed in *R. affinis* but not in *R. lepidus*. This difference might in part be due to the greater number of pairwise comparisons at the equivalent distances in the former taxon, thus providing more power to detect IBD in *R. affinis*. However, comparisons of the two IBD plots show that for a given pairwise geographical distance, the genetic distances are often higher in *R. lepidus* (Figure 4.3b), possibly reflecting smaller sample sizes. Indeed, the estimation of differentiation or fixation indices is known to be vulnerable to large sampling errors in population with small sample sizes (Nei and Chesser, 1983). Previously among bats, IBD has also been reported in the

Neotropical phyllostomid bat *Carollia perspicillata* but not in related species *Uroderma bilobatum* (Meyer et al., 2009). More commonly IBD has been observed in temperate species such as *Pipistrellus pipistrellus*, *P. pygmaeus* (Racey et al., 2007) and *Plecotus auritus* (Burland et al., 1999). To date, there have only been two published studies that have looked at IBD in other horseshoe bats (*Rhinolophus*). Chen et al. (2008) worked on the subtropical bat species *R. monoceros* and reported a very similar pattern of IBD and the same range of F_{ST} values as *R. affinis* based on microsatellite data across 400 kilometres in Taiwan (Chen et al., 2008). In comparison, in the Asia-Europe widespread temperate greater horseshoe bat, *R. ferrumequinum*, the gradient of IBD was steeper in the UK population than in the continental European population, probably reflecting stepwise colonisation of the species that led to drift in the north, with subsequent population isolation following habitat fragmentation (Rossiter et al., 2007).

Interestingly - and in disagreement with my finding of microsatellite-based IBD in *R. affinis* - I discovered no such pattern of IBD from maternally inherited mtDNA (Chapter 3). In the social organisation systems of most mammals, including bats, females tend to remain in their natal site more than males (philopatry), which disperse at sexual maturity (Greenwood, 1980). This phenomenon causes maternally inherited alleles to accumulate locally and is therefore expected to promote greater maternal population subdivision than paternal subdivision (Storz, 1999). As a consequence of these sex-biased behaviours, patterns of genetic diversity and subdivision in wild populations are known to often differ from theoretical expectations based on classically defined demic groups in which mating and dispersal is random (Chesser, 1991). In previous work on bats, for example, haplotypes that were private to single populations were used to infer strong female natal philopatry and male-mediated gene flow (Worthington-Wilmer et al. 1994, 1999; Kerth et al. 2000; Chen et al. 2008). In my study, mtDNA diversity was extremely high due to the long population history of the species in Peninsular Malaysia. The consequent accumulation of ancestral polymorphisms has led to nearly all sampled haplotypes being private to their source population, so causing difficulties in estimating genetic structure among populations. Therefore, given the extreme haplotype variability relative to the sample sizes, the mtDNA data were unsuitable for detecting sex-biased philopatry and dispersal. Indeed in this situation, microsatellites analyses have more power in detecting genetic structure because

numerous loci are considered. Since the microsatellite markers are sexually neutral, it is unlikely that the detected IBD structure is specifically due to female philopatry in the focal species, but rather it probably reflected restricted gene flow in general.

Apart from IBD, I detected some evidence of slight clustering in *R. affinis* but not in *R. lepidus*, although even in the former species none of the individuals were unambiguously assigned to a single cluster with a high membership coefficient. As such, I found no evidence for the presence of cryptic species of *R. affinis*, as suggested from an intensive study of central Peninsular Malaysia (Struebig, 2008). Instead, all individuals showed evidence of admixture, and any population structure was only evident as slight changes in the relative membership of the different clusters (Figure 4.5a). Admixture in populations usually arises due to the presence of descendants of immigrants or, in other words, gene flow (Excoffier and Heckel, 2006). Admixture inferred from mixed membership of clusters has been reported in some other species, including fishes (e.g. sailfin silverside; Walter et al., 2009)) and mammals (e.g. red squirrel; Grill et al., 2009) as well as in other bats (e.g. greater horseshoe bat; Rossiter et al., 2007). Nonetheless in such cases, admixture is seen in populations that are located between clearly define clusters. In my study, geographically widespread admixture that showed slight differences among some populations could either reflect a signature of ancient multiple refugial populations or perhaps introgression (e.g. Mao et al., 2010a), which has not yet been completely eroded by gene flow. According to Pritchard (2007), the STRUCTURE program will face difficulties in analysing genotype data of populations in which IBD is the main process, and that this will cause admixed cluster memberships in individuals and overestimation of the number of clusters. Even so, STRUCTURE has been shown to be less prone to overestimating number of clusters in a population in comparison to similar clustering approaches implemented in GENELAND and BAPS (Frantz et al., 2009).

The contrasting patterns of structure observed between the two focal horseshoe bat species show similarities to results from a study conducted on the co-distributed Old World fruit bats, *Cynopterus sphinx* and *Rousettus leschenaulti* (Chen et al., 2010). In this study, the former species showed strong structure across southern China, whereas in the latter species, no population division was recorded, with equal assignment to clusters using

STRUCTURE. Of particular relevance here is the fact that the haplotype network for *R. leschenaulti* reported by Chen et al. (2010) showed evidence of a long population history with a high incidence of private haplotypes, whereas that of *C. sphinx* showed a signature of expansion. Therefore the lack of structure described in this Chapter for both *R. affinis* and *R. lepidus* could occur for the same reasons as in *R. leschenaulti.* Chen et al. (2010) also suggested that these differences might be caused by greater vagility in *R. leschenaulti* due as its adaptation to roosting in large cave colonies, compared to *C. sphinx*, which roosts in small groups in caves (Chen et al., 2010). A similar argument was also proposed by Rossiter et al. (2012) who found that for two genera of forest bats, species that roost in caves tended to show higher gene flow over a landscape spatial scale than species that roost in trees. Both species of horseshoe bat are cave roosters, so might also be able to fly sufficiently far to prevent structure. Actual limits of dispersal are not known; however, Struebig et al. (2009) studied the assemblage composition in a limestone area in Pahang, Peninsular Malaysia, and found that individuals of *R. affinis* and *R. lepidus* could forage in forest up to 11 km away from their cave roosts.

In summary, all analyses of population genetic structure applied in this study, including estimation of F_{ST}, IBD plots, Bayesian clustering and PCoA analysis, gave results that were consistent with an IBD model in which the probability of dispersal and mating decreases with physical distance (Wright, 1943). To date, IBD estimation has been commonly applied to measure genetic structure among populations based on Euclidean distances regardless of geographic complexity (Jenkins et al., 2010). Historically, the main potential barrier to gene flow in the peninsula is the Titiwangsa Central mountain range; however, pairwise F_{ST} showed no consistent increase in genetic differentiation between western and eastern bat populations. In fact, the Titiwangsa mountains are <1800m and are covered by continuous forest, so they might not be expected to act as an effective barrier. Instead, there is good evidence that upland areas harbour populations with the highest genetic richness in both species, and in general, these species appear to occur across a range of altitudes within their distributions (Csorba et al., 2003).

As well as containing relatively low levels of allelic richness, the southern and eastern populations of both focal species have also been under the greatest threats from

human activities. Around half of the forest sites sampled on the east coast are subject to logging or mining activities, while most have some level of disturbance. *R. lepidus* has even been recorded roosting in man-made tunnels in Singapore (B. Lee, personal communication) suggesting an ability to cope with human activity, although the health and future viability of such populations in the face of ongoing forest loss, are not known. Struebig et al. (2011) recorded an effect of fragment size on genetic diversity of Malaysian bat populations but they focused on smaller forest fragments than those investigated here. Nevertheless, it is likely that larger fragments will show more delays in any negative impacts, and these forest patches might further decrease in size. The break up of a large continuous population into several small demes can in theory cause a 'Wahlund effect' in which a deficit in heterozygosity (significant inbreeding coefficients or F_{IS}) occurs due to the presence of cryptic structure (Allendorf and Luikart, 2007). Wahlund effects might also be particularly likely in species such as bats, if sampling is near to caves roosts that serve multiple family groups or colonies. However, in the case of my study, although an excess in homozygosity was recorded for some markers in some populations of *R. affinis* and *R. lepidus* (e.g. notably in the former taxon in populations JH1, JH2, PH2 and PH1), all positive deviations from HWE appeared to be due to marker characteristics rather than any effect of population processes.

Appendix of supporting information

Figure S4.1 Two focal species studied: (a) *Rhinolophus affinis*, intermediate horseshoe bat and **(b)** *Rhinolophus lepidus*, lesser horseshoe bat

(a)

(b)

CHAPTER FIVE

General Discussion

Chapter 5: General Discussion

In this project, I conducted intensive field surveys to study the assemblage structure of insectivorous forest-dependent bats in Peninsular Malaysia and found some evidence for uneven species richness, with lowest diversity in the north and greatest in the south. These results contradict published findings based on literature surveys of mammal species, as reported by Woodruff and Turner (2009), and also findings of Hughes et al. (2011), who inferred diversity from ranges of species inferred from presence-only data, either collected in the field or contributed by third-parties. Reasons for these discrepancies might reflect the fact that in my study, species richness was calculated based on forest bats only and also took account of sampling effort. In this study I also attempted to control for site elevation, fragment size and, moreover, most of my data were collected by harp-trapping of foraging bats in forest. For these reasons the trends I found were perhaps less biased by local conditions such as roost availability (for example, based on museum and other records, the presence of cave roosting bats may be biased upwards in areas where there are caves).

A clinal pattern in species diversity, with greatest diversity in the south, could be considered consistent with theories that the forest over much of the Malay Peninsula was replaced by savannah during the Last Glacial Maximum, which subsequently expanded from refugial areas (e.g. Wurster et al. 2010). On the other hand, recent recolonization of former grassland by forest trees and forest-interior species might also be expected to lead to a correlation between beta diversity and geographical distance. Although I found a significant association between pairwise differences in diversity and geographical distance, this seemed to reflect the extremes in alpha diversity between north and south, and overall there was no evidence of an effect of distance on beta diversity among sites. More importantly, post-glacial range expansion of forest-interior bats was not supported by the genetic data (see below). For these reasons, although the observed geographical variation in species richness could be interpreted as supporting historical changes in forest coverage from the LGM, this seems less likely when viewed alongside all the other evidence.

In Chapter 3, I undertook analysis of demographic and population genetic structure that was applied to one of the most widely distributed bat species, *Rhinolophus affinis*. In recent years phylogeography has proven to be a powerful method for reconstructing the past responses of taxa to ancient changes in climate and habitat distribution. The results of phylogenetic trees and networks, as well as demographic analyses, all suggest that within Peninsular Malaysia this species has had a very stable population history, which would long pre-date the LGM. Highest haplotype diversity was observed in the central upland areas, where populations also show evidence of having been the most stable. These results provide evidence against that of Wurster et al. (2010), who claimed that at the time of the LGM, forest across the peninsula was replaced by open savannah. While *R. affinis* is widespread, suggesting some ecological flexibility, it is typically found in or near to forest and it would almost certainly not be able to survive in large expanses of open grassland due to its ecomorphological adaptations for feeding in cluttered vegetation. In fact, none of the approximately 80 known extant species of horseshoe bat are associated with open grassland habitats (Csorba et al., 2003). Thus my data indicate that this species has not undergone dramatic changes in population size, and might have occurred in Peninsular Malaysia since before the LGM. Although the presence of extant rainforest species in some parts of Southeast Asian has previously been used to argue for a long history of rainforest (e.g. Meijaard, 2003), the same sorts of arguments have not been made using genetic evidence. This is in direct contrast to northern Europe and other temperate areas, where haplotype data are commonly used to support past population contraction and expansion (e.g. Hewitt, 2004).

Phylogenetic analyses that included other subspecies from China allowed estimation of the time to the most recent common ancestor (TMRCA) for *R. affinis* (see Chapter 3). A dated phylogeny suggested that this taxon was estimated to have formed about 800,000 years BP, when according to the literature, East Asia probably had a warmer climate than at present, and so was also covered by humid evergreen tropical vegetation. In Peninsular Malaysia, the TMRCA of *R. a. superans* was estimated to be about 460,000 years BP, when the sea level was about 20 metres higher than the present sea level and the Isthmus of Kra was narrower than it is now. These findings therefore suggest that past sea level changes might have contributed to the divergence of the focal subspecies in the same way

that they appear to have driven subspecies diversification of *R. affinis* in China (Mao et al. 2010b).

In the final empirical study (Chapter 4) I used microsatellite markers to examine patterns of population genetic structure, allelic richness and gene flow in two of the most abundant horseshoe bat species in Peninsular Malaysia: *R. affinis* and *R. lepidus*. Here I found that genetic distance correlated with geographical distance in both taxa, although significant isolation-by-distance was only detected in *R. affinis*. My other analyses also failed to detect any deep population structure. All of the results thus point to restricted dispersal. Also, no effects of human-induced fragmentation of the forest were seen at the spatial scales examined, in contrast to the landscape-scale study of Struebig et al. (2011) that focused on smaller fragments in which the effects of genetic drift would be more rapidly seen. If either of the focal species in this Chapter had been dramatically impacted by habitat change at the LGM (in particular if the rainforest was only able exist as distant patches separated by open savannah – Wurster et al. 2010) then some population genetic structure might still be expected to be visible (see Rossiter et al. 2007). Therefore, the absence of strong genetic differentiation from Bayesian clustering and also the PCoA adds additional weight to the conclusion that there has been a long population history in the peninsula. Future work to verify my results could involve extending the comparative phylogenetics approach to include other co-distributed forest specialist species. More in-depth sampling might also provide evidence of the potential presence of cryptic species of *R. affinis*, as suggested by Struebig (2008).

Following interpretations from the mtDNA results and other microsatellite-based analyses, the observed northward (and westward) increases in allelic richness, as seen in both horseshoe bat species, were unexpected. If I rule out southerly colonization events, which seem highly unlikely, then a more plausible explanation for more alleles in the north is a longer population history and/or recurrent gene flow from the Thai populations north of the Isthmus of Kra. From the phylogenetic results of *R. affinis*, then some population divergence would be expected to occur further north. Unfortunately sampling of populations from Thailand was not possible in my study; however, this could form a valuable part of any future work on these species.

Several aspects of my findings have conservation implications. First, the greatest forest bat diversity was found in the southern areas of Johor, Melacca and the tip of Pahang. Human activity in Sundaland over the past two centuries has led to massively modification of the natural landscape, and rates of deforestation have been particularly high in the southern part of the peninsula (Peh *et al.* 2006). However, as mentioned in Chapter 2, high species diversity cannot - unfortunately - be taken to signify that such species are in any way resilient to change. The loss of taxa can lag behind the loss of the habitat by many years (extinction debt) as seen clearly in Singapore where most forest bats have disappeared. My study suggests action needs to be taken now to preserve the remaining diversity in southern Peninsular Malaysia before a new equilibrium is reached. Such a priority is even more important in light of the fact that my results support the suggestions of Cannon et al. (2009) that there has been no contraction of forests at the LGM. Thus the species diversity in the south of Malaysia, as well as the genetic diversity in the north, will have accumulated over many thousands of years and possibly over the course of multiple glacial maxima. While the species are being conserved, new advances in genome sequencing mean that the potential now exists for documenting some of this extraordinary population genetic diversity before it is lost (Allendorf et al., 2010).

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