THE UNIVERSITY OF CHICAGO

PHYLOGEOGRAPHY AND PHENOTYPIC EVOLUTION OF *MACACA FASCICULARIS* IN SOUTHEAST ASIA

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CHAPTER 1

Introduction

When we think about islands, we think about those tropical getaways in exotic localities. But what is really fascinating about islands are the unique evolutionary processes that occur on these diverse, isolated regions, giving rise to a phenomenon that has been named the Island Rule. The Island Rule refers to an evolutionary process whereby large-bodied animals become smaller and small-bodied animals become larger on islands. These processes likely occur due to a combination of resource limitation and ecological release on islands. Extreme cases are often cited as examples of the Island Rule, with pygmy mammoths on the Channel Islands of California, pygmy hippopotamuses on the Mediterranean islands, or the large-bodied Komodo dragons residing on Southeast Asian islands. Although these certainly are examples of the Island Rule, they do not provide enough evidence to establish that these island processes constitute a universal rule. So scientists have extensively debated the validity of this rule by analyzing large datasets across mammals and other vertebrates.

The literature thoroughly discusses various aspects of the Island Rule, including the hypotheses proposed to explain why these processes occur, the universality of this rule, the effects of island size on body size, and whether there is convergence toward an optimal body size on islands. However, other facets of the Island Rule are rarely studied. This is why I spent the five years of my PhD, in addition to some time during the latter part of my undergraduate years, beginning to fill in the gaps culminating in this dissertation.

The two major themes throughout my dissertation work address issues regarding the Island Rule that have been studied surprisingly rarely. Most studies focus on the relationships across species on islands, but the Island Rule applies first and foremost between very closely related species or even within species. Investigators should therefore really be honing in on the evolutionary processes occurring within a single species or between species that are known to share a very recent common ancestor. So the first major point is that my dissertation focuses on a single species, *Macaca fascicularis* (long-tailed macaques, also known as crab-eating macaques) (Figure 1.1). *M. fascicularis* are Old World monkeys that display significant sexual dimorphism in body size, with males being larger than females. They are also female philopatric so the males disperse at sexual maturity. This species has a particularly wide geographical distribution throughout Southeast Asia and occurs on the mainland and islands in the region, making it an ideal model organism for a study of the Island Rule. There are thousands of islands in Southeast Asia, allowing me to take island size into account in my analyses. The region is also composed of two types of islands: continental islands which are those that were once connected to the mainland when sea levels were low during glaciation periods and oceanic islands, which are those that had never been connected to the mainland. The presence of both continental and oceanic islands allows me to distinguish any patterns due to island type (Figure 1.2).

Figure 1.1. Images I took of *M. fascicularis* on the island of Bali in Indonesia.

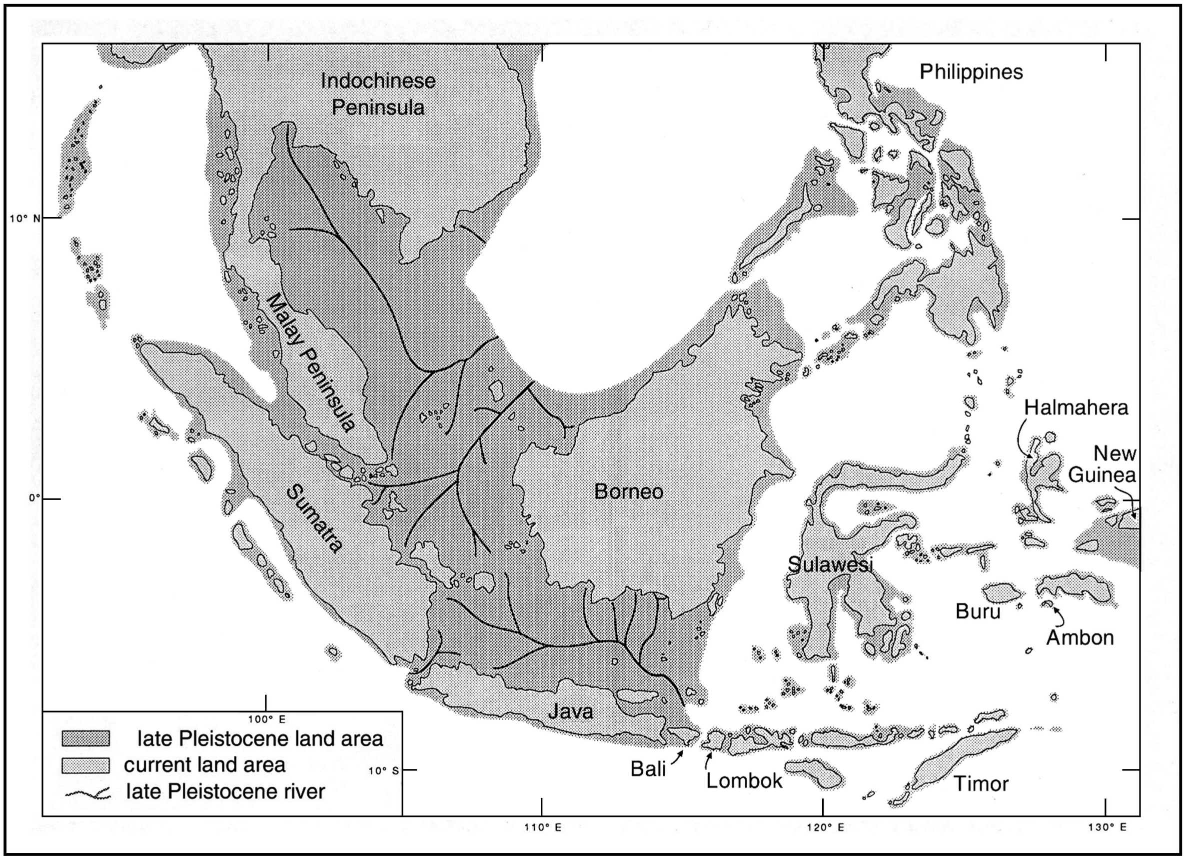

Figure 1.2. Map of Southeast Asia from Heaney (1991).

The second issue I address throughout my research is the importance of having a reliable phylogeny documenting the pattern of divergences among populations within a species when analyzing the Island Rule. Some studies of the Island Rule have included interspecific phylogenies to control for phylogenetic non-independence, and their conclusions disagree with studies that do not control for phylogenetic relatedness. Because the Island Rule applies within species or very closely related species, it is necessary to control for the within species relationships in scaling analyses. Additionally, an intraspecific phylogeny is crucial for understanding population-level relationships within and between islands.

Therefore, in my first research chapter, Chapter 2, I have applied next-generation sequencing techniques to the mitochondrial genomes (mitogenomes) of museum specimens to reconstruct an intraspecific phylogeny of *M. fascicularis*. Successful extraction and sequencing using dried tissue samples from many individuals collected throughout Southeast Asia reliably established the relationships between populations. Although a few previous studies had been conducted with similar aims, I set out to expand the sample size significantly (eventually more than tripling the available number of individual mitogenomes) and to include specimens from known localities throughout the Philippines and small Indonesian islands, which had not previously been included. This phylogeny, which was of considerable value for exploring the colonization history and phylogeographical context of *Macaca fascicularis*, was also further used to control for phylogenetic non-independence in the analyses performed in subsequent chapters.

Chapter 3 tests for the validity of the Island Rule in *M. fascicularis* by analyzing body size data that I collected from museum specimens in relation to island size. Most previous studies of the Island Rule and body size analyzed variation across species. So this chapter is particularly important because it comprehensively analyzes differences in body size of a single species that resides on multiple islands of various sizes and types. Analyses also took sex and island type (continental *versus* oceanic) into account, and the data were tested for phylogenetic non-independence using the intraspecific phylogeny inferred in Chapter 2. If there is dwarfing in body size in this species, I expected it to be limited to specimens from continental islands because those on oceanic islands were likely introduced by humans in the recent past, thus not allowing enough time for those specimens to evolve smaller body size. Furthermore, I hypothesized that specimens from small islands would display smaller body size because both food resources and predators are more limited on small islands than large ones. This chapter also discusses certain problematic issues with previous studies of body size and the Island Rule that should be avoided in future studies. A notable example is widespread use of pairwise comparisons of species in the absence of adequate information regarding ancestral relationships.

Compared to the number of studies that have set out to test the universality of the Island Rule with respect to body size, the number of studies analyzing absolute and relative brain size in relation to the Island Rule is extremely limited. In fact, such allometric scaling analyses largely began in the context of recent attempts to explain the tiny brain of the Late Pleistocene LB1 hominin specimen known as *Homo floresiensis,* which was discovered on the island of Flores in Indonesia. In Chapter 4, I analyze the brain size of *M. fascicularis* from islands and the mainland in Southeast Asia, using both raw and phylogenetically corrected data. My expectation was that there would be no significant decrease in the absolute or relative brain size of this species on small islands because brains are organs that have crucial functional consequences if reduced. The results have implications for better understanding of evolutionary processes on islands affecting factors other than body size alone.

In my final research chapter, Chapter 5, I decided to turn my efforts toward conducting an analysis that had not previously been applied to studies concerning the Island Rule: 3D geometric morphometric data analysis. I wanted to determine the cranial morphological differences, if any, in *M. fascicularis* between islands and between islands and the mainland. Because other studies have shown that there are particular changes associated with mastication in island-dwelling mammals, my expectation was to find cranial differences in the regions of the skull associated with chewing. This aspect of my research yielded some baffling yet thought-provoking results, but they do provide evidence for shape differences in specimens from islands and the mainland.

Taken as a whole, my research comprehensively analyzes the relevance of the Island Rule to *M. fascicularis* using a combination of genetic and morphological data. My efforts reveal that the Island Rule, if it can still be regarded as such, involves complex processes that still need to be teased apart, despite all of the research that has already been conducted on this topic. It is my hope that my dissertation research effectively closes some of the gaps in the study of the Island Rule and will inspire other investigators to continue filling in those gaps.

CHAPTER 2

Tracing the phylogeographic history of Southeast Asian long-tailed macaques through mitogenomes of museum specimens

INTRODUCTION.

Macaca species belong to one of the most widely distributed primate genera, occurring on two continents and multiple islands. Fossil evidence indicates that this highly successful genus originated in Africa around 7 million years ago (Ma) (Delson, 1980), after which macaques expanded into Asia approximately 5.5 Ma (Alba, 2014; Delson, 2000). Twenty-two species subdivided into seven species groups (Figure 2.1) are currently recognized based on distribution, morphology, behavior and genetics (Li et al., 2009; Tosi et al., 2003). Based mostly on morphological data, three of these groups are monospecific: *M. sylvanus*, which is the only extant macaque in northern Africa and southern Europe, *M. fascicularis*, and *M. arctoides*. The remaining four groups are polyspecific, with six species in the Sulawesi group, five in the *M. silenus* group, three in the *M. mulatta* group, and five in the *M. sinica* group (Zinner et al., 2013a). However, classification into species groups has changed over time in tandem with extensive debate. Originally, *M. mulatta*, *M. cyclopis*, *M. fuscata*, and *M. fascicularis* were all included in the *M. fascicularis* species group (Fooden, 1976). Groves (2001) and Zinner et al. (2013a) then combined *M. mulatta*, *M. cyclopis*, and *M. fuscata* in a *M. mulatta* species group. Groves (2001) also added *M. arctoides* to the *M. fascicularis* species group, but Zinner et al. (2013a) subsequently classified *M. arctoides* and *M. fascicularis* as two separate monotypic groups.

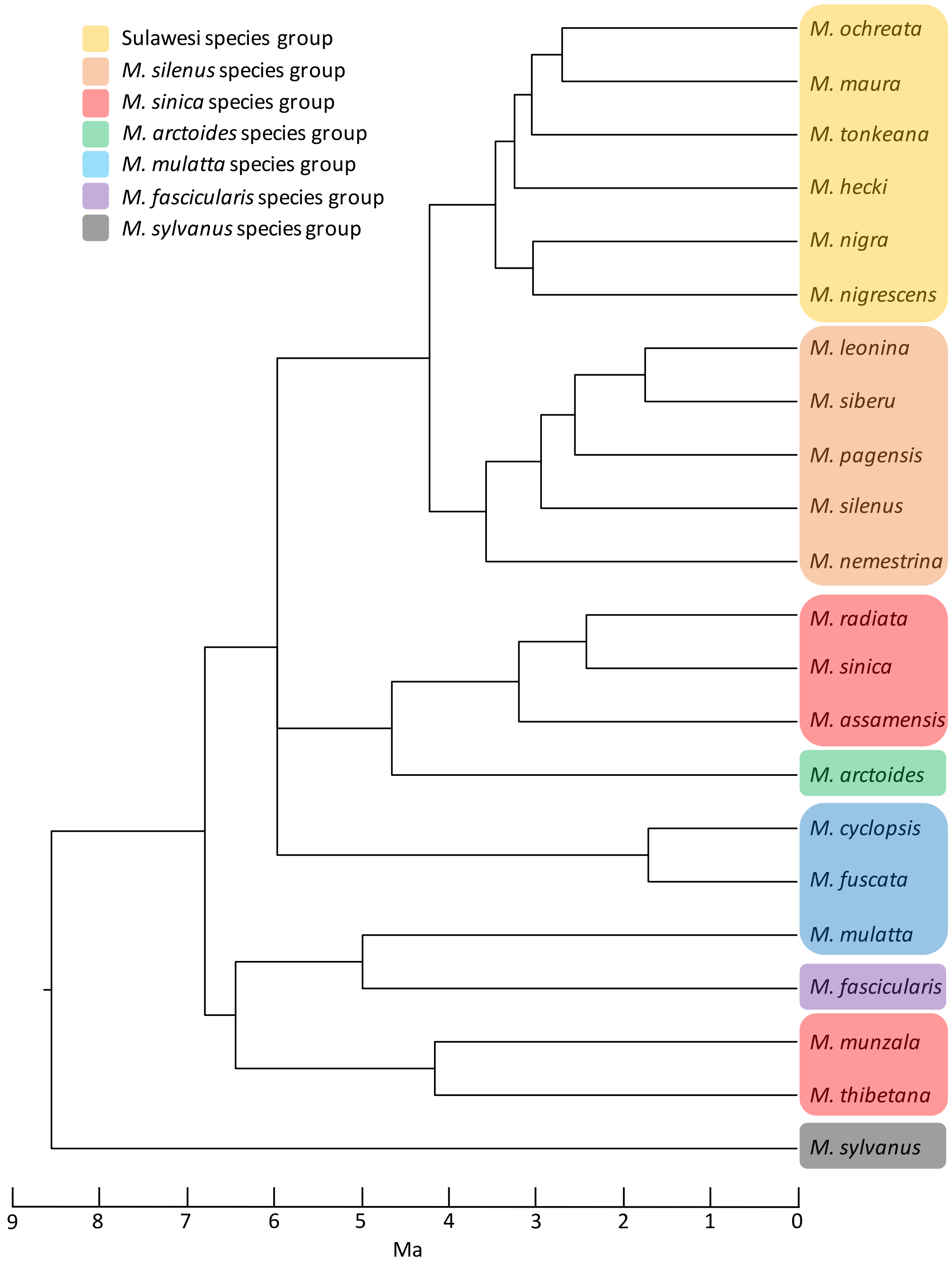

Figure 2.1. The consensus tree for the genus *Macaca* downloaded from the 10kTrees Project (Arnold et al., 2010). I selected all species currently recognized within Macaca to be included in the reconstruction based on 10,000 phylogenies sampled from a Bayesian tree inference. Branch lengths are proportional to time in million of years (Ma). Species are color-coded by species groups recognized primarily on morphological grounds.

A study investigating species-level relationships within the genus *Macaca* using 358 loci (Li et al. 2009) concluded that *M. fascicularis* is the sister group to a clade containing *M. fuscata* and *M. mulatta*. However, in the consensus tree inferred using 10kTrees (Arnold et al., 2010), basing analyses on an entire set of trees rather than on the assumption that a single topology is correct, *M. fascicularis* is most closely related to *M. mulatta* (Figure 2.1). In fact, the molecular phylogeny in Figure 2.1 appears to be the most complete one to date for the genus *Macaca*, including all recognized species. It suggests that some species groups should be re-classified based on this molecular data. Specifically, *M. arctoides* should be included in the *M. sinica* species group while *M. munzala* and *M. thibetana* should be removed. The *M. mulatta* species group should be broken up into two groups, with one containing *M. cyclopsis* and *M. fuscata* and another species group containing *M. munzala, M. thibetana, M. mulatta,* and *M. fascicularis.* Detailed studies have demonstrated that there have been major hybridization events between *M. fascicularis* and *M. mulatta*, most likely reflecting overlapping geographical distribution on the Indo-Chinese peninsula (Fooden, 1964; Fooden, 2000; Bonhomme et al., 2009; Stevison and Kohn, 2009; Kanthaswamy et al., 2009; Tosi et al., 2002). In a study comparing the genomes and divergence rates of these two species, Yan et al. (2011) found that approximately 30% of the *M. fascicularis* genome originates from *M. mulatta.* This gene flow appears to be non-maternal in origin as mitochondrial analyses show no signs of such hybridization. *M fascicularis* and *M. nemestrina* have overlapping geographical distributions on the Southeast Asian islands, but no cases of natural hybridization between these two species have been reported.

Here, I am particularly interested in *M. fascicularis* because this species occupies the second largest area after *M. mulatta* and is also the most geographically diverse. Ten subspecies have been recognized within the species *M. fascicularis*, but there are few visible differences aside from pelage color, tail length and the shape of the cheek whiskers (Groves, 2001; Ong and Richardson, 2008). Seven of these subspecies are allopatric, with localized distributions on small islands (*M. f. atriceps, M. f. condorensis, M. f. fuscus, M. f. karimondjawae, M. f. lasiae, M. f. tua, M. f. umbrosus*), while the remaining three are widely distributed throughout Southeast Asia (*M. f. fascicularis*, *M. f. philippinensis*, *M. f. aureus*). *Macaca f. aureus* is geographically isolated from *M. f. fascicularis* and *M. f. philippinensis,* but there is a putative area of intergradation of *M. f. fascicularis* and *M. f. philippinensis* on some of the Philippine islands, including southern Negros and Mindanao (Fooden, 1995; see Figure 2.2). These three widely distributed subspecies have a high level of genetic variation, presumably reflecting their large ranges.

The range of *M. fascicularis* includes the Southeast Asian mainland (Malay peninsula, Myanmar, Thailand, Cambodia, Vietnam, Laos, and Bangladesh) along with islands of varying sizes and different types, i.e. continental or oceanic (Figure 2.2). Faunas differ fundamentally between the Sunda islands (Sundaland) — the biogeographical region encompassing the continental shelf that was exposed as a continuous landmass during Pleistocene glacial periods — and oceanic islands, those that have never been connected to the mainland (Wallace, 1863). These two biogeographical regions are separated by what is called the Wallace Line. At the southern end, this line separates Bali and Lombok islands at the Strait of Lombok, which is only ~24 km wide. In the north, this separation occurs at the 129-370 km wide Makassar Strait between Borneo and Sulawesi (formerly Celebes) and extends east into the ~201 km wide and ~1500-2500 m deep strait in the Pacific Ocean between Mindanao and the Sanghir Islands (Bergman et al., 1996; George, 1981; Wallace, 1863). The islands to the east of the Wallace Line make up Wallacea.

Huxley (1868) corroborated this division but drew the line directly north so that the Philippines, except for Palawan and its associated islands, lie to the east of the Wallace Line. Although there appears to be a disjunction in faunal occurrences on either side of Huxley's line, *M. fascicularis* is an exception as one of the few species of non-volant mammals that occurs widely on both sides of Wallace's Line.

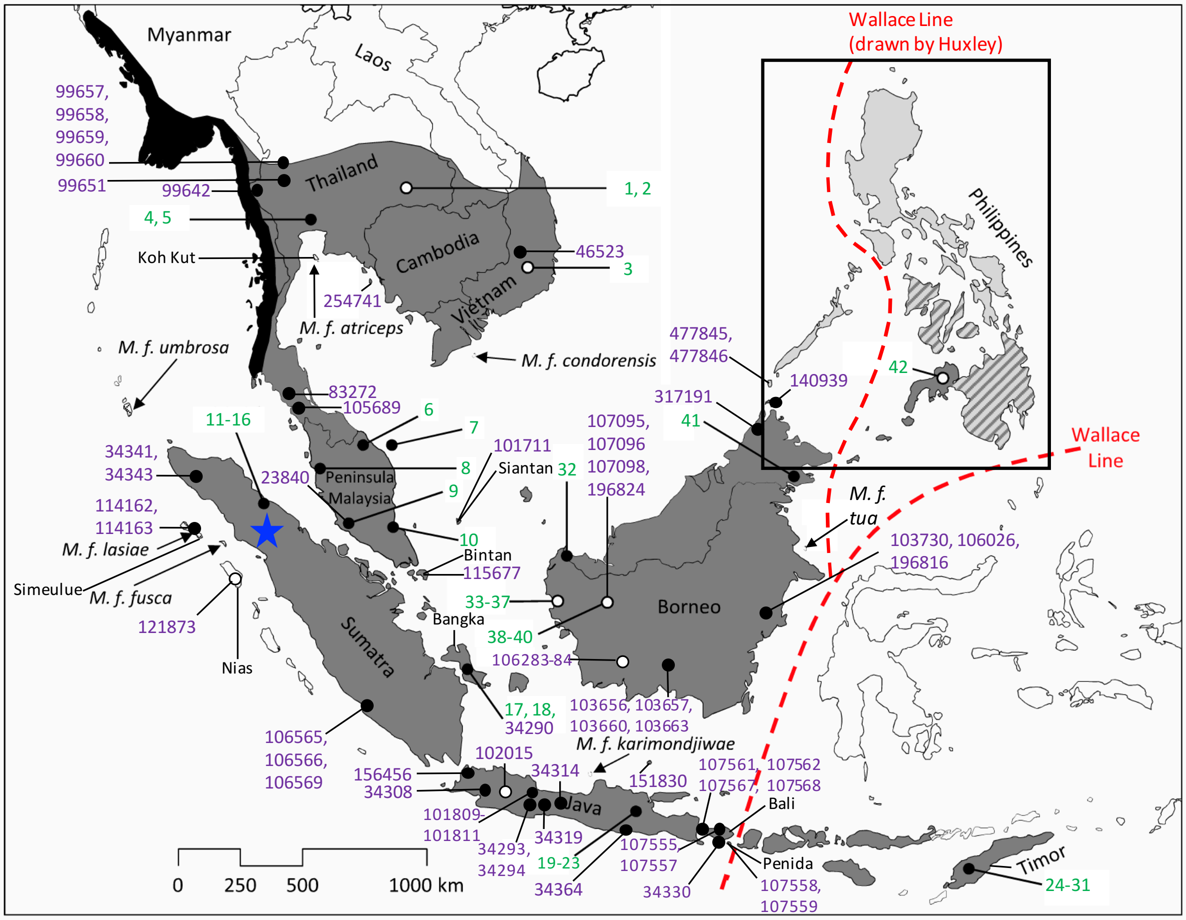

Figure 2.2. (a) A map of Southeast Asia derived from Liedigk et al. (2015) marked with specimens from the current study and Liedigk et al.'s (2015) study. The black, dark grey and light grey regions indicate the ranges of *M. f. aureus*, *M. f. fascicularis* and *M. f. philippinensis.* The region colored in light and dark grey lines is the putative area of intergradation of *M. f. fascicularis* and *M. f. philippinensis* based on studies by Fooden (1995; 2006). White circles represent approximate location whereas black circles represent exact location. ID numbers of *M. fascicularis* museum specimens in green correspond to samples sequenced in Liedigk et al. (2015) and those in purple are the newly sequenced samples in this study. Lake Toba is marked with a blue star.

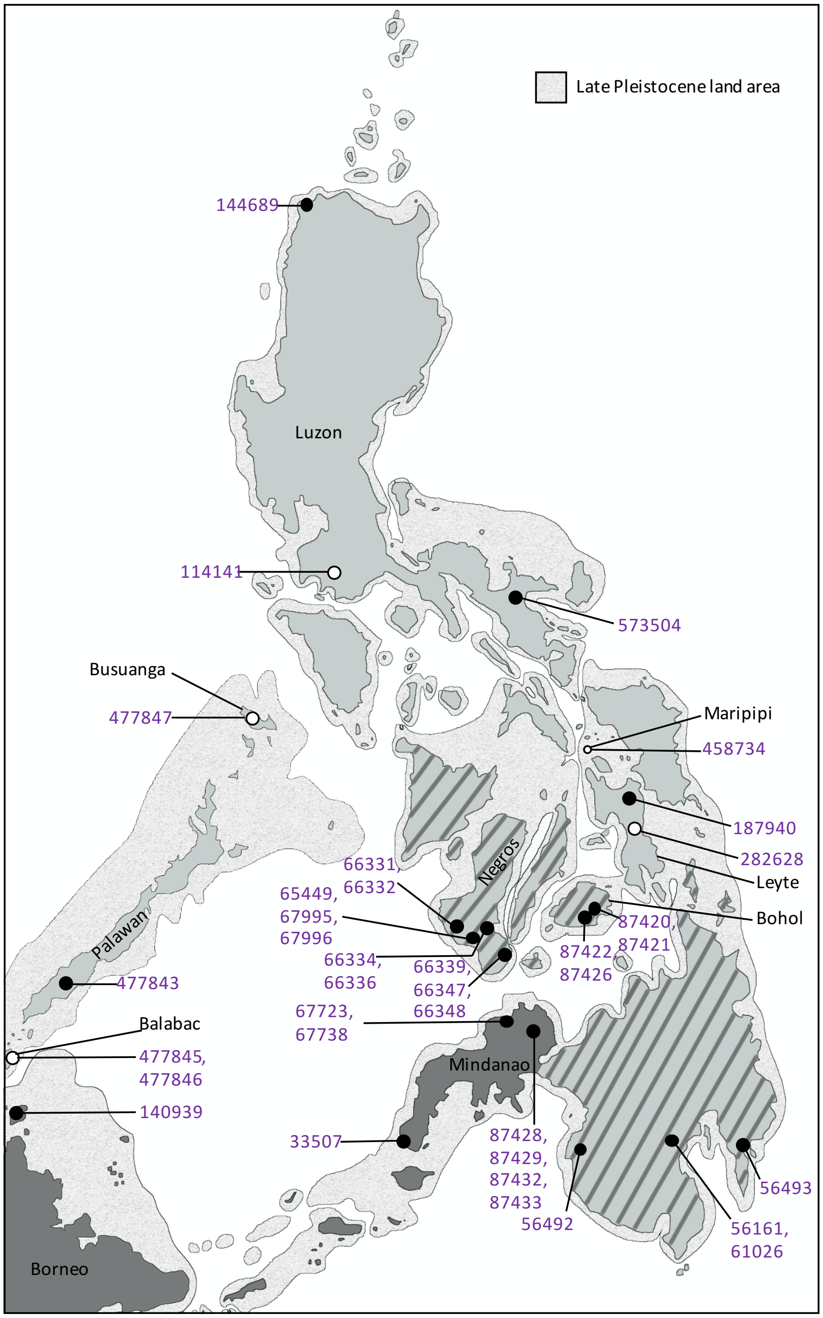

Figure 2.2. (b) A map of the Philippines (Philippines region in rectangular box from Figure 2.2a magnified), derived from Heaney (1986), marked with specimens from the current study. The circles and colored ID numbers of *M. fascicularis* specimens are labeled according to the description in map (a). All ID numbers correspond to those in Figure 2.3 and Table 2.1.

It has been inferred that *M. fascicularis* first colonized Sundaland during global cooling in the Pliocene (~5.3-2.6 Ma) (Delson, 1980), and that subpopulations became isolated on those islands once sea levels rose. During the Pleistocene glacial periods, *M. fascicularis* was able to expand its range even further throughout the continental shelf islands (Delson, 1980; Fooden, 2006), as is evidenced by early fossil remains of *M. fascicularis* on Java (Aimi and Aziz, 1985; Fooden,

2006) and the extensive genetic diversity in Sunda shelf populations in comparison to those on the mainland (Smith et al., 2007). Time-calibrated molecular phylogenies indicate a time frame within the last 5 million years for the arrival of both *M. fascicularis* and other primates on the larger Sunda shelf islands (Chan et al., 2010; Chatterjee, 2006; Liedigk et al., 2015; Tosi and Coke, 2007). Tosi and Coke (2007) inferred from a 1.5kb fragment of mtDNA and two Y-chromosome loci, using a maximum likelihood analysis, that *M. fascicularis* colonized Sumatra in multiple waves throughout the Pleistocene, starting at about 1.2 Ma and continuing until the most recent glacial maximum. This particular study analyzed the mtDNA fragment and Y-chromosome loci separately. mtDNA analyses indicated that separation of macaques on the Sunda shelf islands from the mainland occurred approximately 1.2 Ma, and there was later a bifurcation in the Y-chromosome loci at around 0.4 Ma. The separation based on mtDNA is the deepest intraspecific bifurcation within *M. fascicularis*. Moreover, the mtDNA-based phylogeny reconstructed by Tosi and Coke (2007) clustered Sumatran individuals with conspecifics on other islands, whereas the chromosome-based phylogeny indicates that the Y-chromosomal loci of Sumatran individuals cluster with those of mainland individuals as well as with other insular individuals. The authors reconciled this difference with the explanation of secondary contact. Overall, they found that *M. fascicularis* individuals from Sumatra are split into some that cluster with other islands and some that cluster with the Southeast Asian mainland in general.

The most recently published intraspecific phylogeny for *M. fascicularis* is based on entire mitogenomes (Liedigk et al., 2015). Using five fossil calibrations and a relaxed molecular clock approach, they found the divergence between the mainland-Sumatran clade and the clade containing other insular individuals to be ~1.7 Ma. However, Liedigk et al.'s (2015) results differ from those of Tosi and Coke’s (2007) study in that all Sumatran individuals represented cluster with the mainland samples rather than the insular individuals. Their mitogenome phylogeny again indicates the presence of two separate clades. Clade A includes all mainland and Sumatran lineages in a paraphyletic array, while Clade B includes the lineages from all other islands in monophyletic groups except for their only Philippine individual, which was nested within the Borneo clade. The exact origin of the Philippine individual was unclear, although it was marked as a specimen from Mindanao on their map.

Even though *M. fascicularis* is widespread throughout most of Southeast Asia, very few studies have examined the timing of colonization of oceanic islands (Fooden, 2006), such as the Philippines. Additionally, intraspecific relationships of *M. fascicularis* have been resolved only for the mainland, the larger Sunda islands and a single Philippine island (Tosi and Coke, 2007; Liedigk et al., 2015). A genetic study of *M. fascicularis* in the Philippines concluded that those populations had naturally colonized the islands in two major waves separated in time (Smith et al., 2014). On the other hand, Fooden (2006) and Heaney et al. (2016) hypothesized that the lineages on the Philippines east of the Huxley-Wallace Line were introduced by humans approximately 4000 to 3500 years ago. Because *M. fascicularis* are able to swim well, it is thought that they could have swum between islands that are in close proximity (Gumert and Malaivijitnond, 2012). The most recent study (Liedigk et al., 2015) included only one Philippine individual (specific locality unknown), which appears to have diverged from the Borneo lineages approximately 0.21 Ma, considerably earlier than the hypothesized date for human introduction.

The goals in this study are to expand previous knowledge of phylogenetic relationships within *M. fascicularis* substantially in order to determine intraspecific relationships in this region, and to clarify when *M. fascicularis* migrated to the various islands, particularly the Philippines and the small Sunda islands. To achieve this objective, I collected tissue samples from museum specimens of *M. fascicularis* from many distinct localities throughout the Southeast Asian region (Figure 2.2), focusing particularly on the Philippines and small Sunda islands, and sequenced their entire mitochondrial genomes (mitogenomes) using high-throughput sequencing. The far more comprehensive intraspecific phylogeny thus obtained provides a more secure basis for a better understanding of colonization of this extensive geographical region by long-tailed macaques.

METHODS.

All molecular lab work along with associated computational processing was conducted at two major locations: (1) Malhi ancient DNA lab and the Malhi Molecular Anthropology lab at the University of Illinois at Urbana-Champaign (UIUC), and (2) the Pritzker Lab for Molecular Systematics and Evolution at The Field Museum. To minimize contamination, at each extraction and amplification step a clean-room facility (the Malhi ancient DNA lab) and negative experimental controls were used. No animals were sacrificed for this study.

Mitochondrial DNA (mtDNA) is widely used in population genetic studies because its sequences evolve relatively rapidly and are inherited maternally, such that they lack recombination (Brown et al., 1979). Because evolution on islands may occur comparatively quickly (Evans et al., 2012), mtDNA is an excellent tool for studying diversity within populations of Southeast Asian mammals. Specifically, I examined the mitogenome of *M. fascicularis* (16,544 bp) using next-generation sequencing, because the mitogenome provides better phylogenetic resolution and precision compared to traditional localized mtDNA markers and can help to overcome inadvertent amplification of pseudogenes of nuclear mitochondrial origin (numts). Using different regions of the mitogenome can yield incongruent results concerning divergence dates, taxonomy and phylogeography (Pacheco et al., 2011; Rohland et al., 2007). Additionally, there are numerous copies of the mitogenome in each cell whereas there is only one copy of the nuclear genome, making the mitogenome easier to sequence, especially with low-quality samples such as in ancient DNA (Briggs et al., 2009; Krause et al., 2010; Rowe et al., 2011; Mason et al., 2011; Guschanski et al., 2013).

Sample collection. Recent tissue samples from Southeast Asian *M. fascicularis* are difficult to obtain, especially from museum collections, because the most recent specimens collected from the region (late 1990s) were not prepared for DNA extraction, and current regulations now restrict tissue collection and importation from live primates. Samples for DNA sequencing in this project were therefore collected from fragments of dried tissue of 50- to 150-year-old wild *M. fascicularis* specimens at The Field Museum of Natural History (FMNH) in Chicago, American Museum of Natural History (AMNH) in New York, Smithsonian Institution National Museum of Natural History (NMNH) in Washington, and the Naturalis Biodiversity Center (RMNH) in Leiden, Netherlands. Using a scalpel and tweezers, which were sanitized between specimens using heat, dried tissue fragments comparable in size to 1-2 grains of rice were carefully excised either from bone surfaces or within the braincase of both male and female skulls of museum specimens. Efforts were made to collect tissue samples from as many localities as possible.

Tissue samples representing 196 specimens were obtained from the mainland and from 22 Southeast Asian islands (see Figure 2.2 for geographical localities of successfully sequenced specimens in this study).

DNA extraction. DNA was extracted from the tissue samples following the ancient DNA extraction protocol established for the Malhi Lab. To eliminate contamination problems, all extraction work was performed in the sterile ancient DNA lab at UIUC. Dried tissue samples were first digested overnight in a rotating 37°C incubator in 4 mL 0.5M EDTA, 150 µl of 33.3 mg/ml proteinase K and 300 µl of 10% N-lauryl sarcosine. All DNA extractions were accompanied by negative controls to permit detection of contamination during the extraction process. Once the samples were digested, DNA was extracted using a Qiagen extraction kit following the manufactures protocol and eluted to 100 µl per sample.

Because DNA from dried tissue is fragmented and often degraded, it is important to confirm that usable DNA is present in order to avoid wasting substantial funds on samples whose DNA cannot be sequenced. Accordingly, I designed primers for a 200 bp section of *M. fascicularis* cytochrome *b* (cyt*b*) based on previously published mitogenomes (Liedigk et al., 2015). These primers (F: 5'- TACGCAAATCCAACCCAATC -3'; R: 5'- GGTGATGTGTGCAATTGAGG - 3') were successfully tested on *M. fascicularis* tissues excised from frozen cadavers in Dr. Callum Ross's biomechanics laboratory at the University of Chicago and on three ancient DNA samples. I set up each sample of extracted DNA with negative and positive controls in a modified PCR reaction using these primers. These samples were then taken in an airtight container to the Malhi Molecular Anthropology laboratory two blocks away and placed in an

Eppendorf Mastercycler (thermal cycler) for Polymerase Chain Reaction (PCR) amplification using an optimized 52°C annealing temperature and 35 cycles.

All PCR-amplified samples along with negative and positive controls were run on agarose gels in The Pritzker Lab to confirm that the 200 bp of cyt*b* sequences from the museum specimens were successfully amplified. If they were successfully amplified, I then proceeded with the preparation of genomic libraries; if not, I started afresh and repeated the digestion and extraction processes in hopes of extracting usable DNA.

Genomic libraries and high-throughput sequencing. Genomic libraries with Illumina platform-specific oligonucleotide adapters unique to each library were created in the ancient DNA lab using 50 µl of extracted DNA per sample and the NEBNext Ultra DNA Library Prep Kit for Illumina following the TruSeq DNA Sample Preparation V2 protocol by Illumina. Due to the particular nature of DNA extracted from museum specimens, certain modifications were made to the protocol. First, the DNA extract was not sheared because the DNA is already highly fragmented. The concentration of DNA in each extract was expected to be low, so the adapters were diluted 1:20 (Cui et al., 2013). Adapter dimers often form during ligation, and this is problematic because this dimer can bind to the flow cell and be sequenced, but the output is only the sequence of the adapter present. To avoid this, multiple AMPure Bead XP clean ups were conducted.

PCR setup of the genomic libraries using unique NEBNext Multiplex Dual Index primers was also conducted in the Malhi ancient DNA laboratory. Dual index primers were used to avoid cross-contamination when multiplexing samples on a single HiSeq lane (Kircher et al., 2012). The samples were transferred to thermal cyclers in an airtight container in the Malhi Molecular Anthropology lab for amplification. NEBNext High Fidelity 2X PCR Master Mix was used to amplify the libraries because its proof-reading properties limit nucleotide misincorporations resulting from cytosine deamination (Ginolhac et al., 2011). The amplified genomic libraries were cleaned using the Qiagen MinElute Purification Kit.

The cleaned libraries were amplified until the final concentrations of all libraries reached 100 ng/µl. The enriched mitogenome samples were then assessed for fragment size and quantification using the Agilent 2100 Bioanalyzer or the AATI Fragment Analyzer. Nucleic acid concentrations were determined using a Qubit 2.0 Fluorometer. Finally, the samples were pooled and shotgun sequenced at the Keck Biotech Center at UIUC, using an ILLUMINA HiSeq2500, which generated lanes of 100-bp single-end reads for 24 samples per lane.

Alignment and assessment. The Trimmomatic program was used to trim DNA sequences and remove adaptors. This step removes the sequences that entered the sample after the clean room preparation and reduces false variant discovery by ignoring reads that are below standard quality due to DNA damage (Kircher, 2012). BOWTIE 2 (Langmead and Salzberg, 2012), was used to assemble each sample against a *M. fascicularis* reference mitogenome (GenBank ID: KJ567052.1; Liedigk et al., 2015), as this program allows for alignments around insertion/deletions (INDELs). The program SAMtools was utilized for sorting, indexing, and quantifying contamination by examining informative sites (Malström et al., 2007) and removing potential duplicate reads that could result from PCR amplification.

Table 2.1. List of all *M. fascicularis* specimens sequenced in this study along with pertinent information. Under the "Island type" heading, mainland specimens are indicated with an "M", oceanic island specimens are indicated with an "O", and continental shelf island specimens are indicated with a "C".

Museum	Specimen #	Year	Sex	Locality	Island	Island type	Collector
FMNH	33507	1929	F	San Ramon	Mindanao	O	F.C. Wonder
FMNH	46523	1937	M	Annam: Darlac Province, Ban Me Thuot	Mainland	M	W.H. Osgood
FMNH	56161	1946	M	Davao City Province, Mt. McKinley, east slope, 2500 ft	Mindanao	O	H. Hoogstraal
FMNH	56492	1947	F	Maguindanao Province, Upi, Burungkot, 1500'	Mindanao	O	Alcasid/Anonuevo
FMNH	56493	1946	M	Davao Oriental Province, Mati, Camansi	Mindanao	O	H. Hoogstraal
FMNH	61026	1946	M	Davao del Sur, Mt. Apo, east slope, Mainit, 3800 ft.	Mindanao	O	M. Celestino
FMNH	99642	1967	M	Kanchanaburi Prov: Ban Wangphato	Mainland	M	J. Fooden
FMNH	99657	1967	M	Kamphaeng Phet Prov: Ban Mae Na Ree, Khlong Suan Mak	Mainland	M	J. Fooden
FMNH	99658	1967	F	Kamphaeng Phet Prov: Ban Mae Na Ree, Khlong Suan Mak	Mainland	M	J. Fooden
FMNH	99659	1967	F	Kamphaeng Phet Prov: Ban Mae Na Ree, Khlong Suan Mak	Mainland	M	J. Fooden
FMNH	99660	1967	F	Kamphaeng Phet Prov; Ban Nam Lai Tai, Upper Khlong Khlung	Mainland	M	J. Fooden
FMNH	140939	1991	M	Sabah; Banggi Island, Karakit	Banggi	C	Shukor Md Nor
FMNH	67723	1950	F	Zamboanga del Norte Province, Katipunan, Sigayan	Mindanao	O	D.S. Rabor
FMNH	67738	1950	F	Zamboanga del Norte Province, Katipunan, Canibongan	Mindanao	O	D.S. Rabor
FMNH	65449	1948	F	Negros Oriental Province, Amio, Kabongakan	Negros	O	D.S. Rabor
FMNH	66331	1948	M	Negros Oriental Province; Inubungan, Santa Catalina	Negros	O	D.S. Rabor
FMNH	66332	1948	F	Negros Oriental Province; Inubungan, Santa Catalina	Negros	O	D.S. Rabor
FMNH	66334	1949	M	Negros Oriental Province; Bais, Mabaha	Negros	O	D.S. Rabor
FMNH	66336	1949	M	Negros Oriental Province; Bais, Mabaha	Negros	O	D.S. Rabor
FMNH	66339	1949	M	Negros Oriental Province; Lake Balinsasayao	Negros	O	D.S. Rabor
FMNH	66347	1949	M	Negros Oriental Province; Lake Balinsasayao	Negros	O	D.S. Rabor
FMNH	66348	1949	M	Negros Oriental Province; Lake Balinsasayao	Negros	O	D.S. Rabor
FMNH	67994	1950	M	Negros Oriental Province; Tolong, Kandomao	Negros	O	D.S. Rabor
FMNH	67996	1950	M	Negros Oriental Province; Tolong, Balangbang	Negros	O	D.S. Rabor
FMNH	105689	1973	M	Trang Province; Ban Palian, 4 km E (Khao Rang Kai), 75 m.	Mainland	M	J. Fooden
FMNH	87420	-	M	Philippines: Bohol I: Bohol Province; Sandayong	Bohol	O	D.S. Rabor
FMNH	87421	-	M	Philippines: Bohol I: Bohol Province; Sandayong or Cantaub	Bohol	O	D.S. Rabor
FMNH	87422	-	F	Philippines: Bohol I; Bohol Province; Cantaub	Bohol	O	D.S. Rabor
FMNH	87426	-	F	Philippines: Bohol I: Bohol Province; Cantaub	Bohol	O	D.S. Rabor

Table 2.1 (continued)

Museum	Specimen #	Year	Sex	Locality	Island	Island type	Collector
FMNH	87428	-	F	Misamis Occidental Province; Mt. Malindang, Masawan	Mindanao	O	D.S. Rabor
FMNH	87429	-	M	Misamis Occidental Province; Mt. Malindang, Masawan	Mindanao	O	D.S. Rabor
FMNH	87432	-	F	Misamis Occidental Province; Mt. Malindang, Masawan	Mindanao	O	D.S. Rabor
FMNH	87433	-	M	Misamis Occidental Province; Mt. Malindang, Masawan	Mindanao	O	D.S. Rabor
FMNH	99651	1967	M	Thailand: Uthai Thani Province; Kata Taek	Mainland	M	J. Fooden
AMNH	101809	1932	M	Cheribon, Madgalenka	Java	C	J.J. Menden
AMNH	101810	1932	M	Cheribon, Madgalenka	Java	C	J.J. Menden
AMNH	101811	1933	M	Cheribon, Lingertjati	Java	C	J.J. Menden
AMNH	102015	1933	M	Indonesia: Java: Tjerimai.	Java	C	J.J. Menden
AMNH	103656	1935	M	Kalimantan Tengah, Sampit-Parit (Tjempaga)	Borneo	C	J.J. Menden
AMNH	103657	1935	F	Kalimantan Tengah, Sampit-Parit (Tjempaga)	Borneo	C	J.J. Menden
AMNH	103660	1935	F	Kalimantan Tengah, Sampit-Parit (Tjempaga)	Borneo	C	J.J. Menden
AMNH	103663	1935	F	Kalimantan Tengah, Sampit-Parit (Tjempaga)	Borneo	C	J.J. Menden
AMNH	103730	1935	M	Kalimantan Timur, Badang, Sungai Kajan	Borneo	C	Baron V von Plessen
AMNH	106026	1935	M	Kalimantan Timur, Badang	Borneo	C	Baron V von Plessen
AMNH	106283	1935	M	Indonesia: Borneo: Kalimantan Tengah, Riam, Kotawaringin	Borneo	C	J.J. Menden
AMNH	106284	1935	M	Indonesia: Borneo: Kalimantan Tengah, Riam, Kotawaringin	Borneo	C	J.J. Menden
AMNH	106565	1936	M	Boekit Sanggoel, Benkoelen	Sumatra	C	J.J. Menden
AMNH	106566	1936	M	Boekit Sanggoel, Benkoelen	Sumatra	C	J.J. Menden
AMNH	106569	1936	M	Boekit Sanggoel, Benkoelen	Sumatra	C	J.J. Menden
AMNH	107095	1939	F	Kalimantan Barat, Perboeah, Landak River	Borneo	C	J.J. Menden
AMNH	107096	1939	F	Kalimantan Barat, Perboeah, Landak River	Borneo	C	J.J. Menden
AMNH	107098	1937	M	Kalimantan Barat, Perboeah, Landak River	Borneo	C	J.J. Menden
AMNH	107555	1938	F	Bratan	Bali	C	Baron V von Plessen
AMNH	107557	1937	M	Bratan	Bali	C	Baron V von Plessen
AMNH	107558	1937	M	Penida, S. Noesa	Penida	C	Baron V von Plessen
AMNH	107559	1938	M	Penida, S. Noesa	Penida	C	Baron V von Plessen
AMNH	107561	1938	M	Gilimanoek	Bali	C	Baron V von Plessen
AMNH	107562	1938	M	Gilimanoek	Bali	C	Baron V von Plessen
AMNH	107567	1938	M	Gilimanoek	Bali	C	Baron V von Plessen
AMNH	107568	1938	M	Gilimanoek	Bali	C	Baron V von Plessen
AMNH	187940	1961	M	Mt. Lobi, Dagami, Bo Patok	Leyte	O	Alcasid Celestino
NMNH	83272	1896	M	Trang: Trong	Mainland	M	W.L. Abbott

Table 2.1 (continued)

Museum	Specimen #	Year	Sex	Locality	Island	Island type	Collector
NMNH	101711	1899	M	Anambas Islands	Siantan	C	W.L. Abbott
NMNH	114141	-	F	Batangas	Luzon	O	-
NMNH	114162	1901	F	Pulo Simalur	Simeulue	O	W.L. Abbott
NMNH	114163	1901	M	Pulo Simalur	Simeulue	O	W.L. Abbott
NMNH	115677	1902	M	Rhio Archipelago, Pulo Bintang	Bintan	M	W.L. Abbott
NMNH	121873	1903	M	Unknown	Nias	O	W.L. Abbott
NMNH	144689	1907	F	Ilocos Norte Province: Nagpartian	Luzon	O	E.A. Mearns
NMNH	151830	1907	F	Unknown	Bawean	C	W.L. Abbott
NMNH	156456	1909	F	Bantam, Tjihara	Java	C	W.M. Palmer
NMNH	196815	1912	M	Unknown	Borneo	C	H.C. Raven
NMNH	196816	1912	F	Karang Mumus River	Borneo	C	H.C. Raven
NMNH	196824	1912	F	Karang Tigan	Borneo	C	H.C. Raven
NMNH	254741	1929	M	Trat	Koh Kut	C	H.M. Smith
NMNH	282628	1945	M	Unknown	Leyte	O	Carl Mohr
NMNH	317191	1960	M	Sabah: Ranau	Borneo	C	R.E. Kuntz
NMNH	477843	-	F	Mt. Mantalingajar	Palawan	O	-
NMNH	477845	-	M	Unknown	Balabac	O	-
NMNH	477846	-	M	Unknown	Balabac	O	-
NMNH	477847	-	F	Unknown	Busuanga	O	-
NMNH	573504	1988	M	Camarines Sur Province, Mt. Isarog	Luzon	O	L.R. Heaney
AMNH	16676	1901	M	Central Park Zoo	-	-	-
NMNH	458734	-	-	Philippines: Leyte Island: Maripipi	Maripipi	O	P.D. Heideman
RMNH	34314	1929	F	Tjandiroto bij Soembing	Java	C	Thomas/Wroughton
RMNH	34364	-	F	Wonokoio, Dampit, Malang, Java, Indonesia	Java	C	Thomas/Wroughton
RMNH	34308	1931	M	Gunung Salak	Java	C	Thomas/Wroughton
RMNH	34294	1931	F	Teringin bij Bandjar, Tiisaga	Java	C	Thomas/Wroughton
RMNH	34293	1931	F	Tjeringin, Tjisaga, W. Java, Indonesia	Java	C	Thomas/Wroughton
RMNH	34319	1929	M	Batoeraden, 700 m., Mt. Slamet	Java	C	Thomas/Wroughton
RMNH	23840	1965	F	Kuala Lumpur	Mainland	M	-
RMNH	34343	1931	F	Parlak, Atjeh	Sumatra	C	H.J.V. Sody
RMNH	34341	1931	M	Parlak, Atjeh	Sumatra	C	H.J.V. Sody
RMNH	34290	-	M	Unknown	Bangka	C	H.J.V. Sody
RMNH	34330	1930	M	Sendang	Bali	C	H.J.V. Sody

Phylogenetic reconstruction. To reconstruct the phylogenetic relationships of *M. fascicularis* I included mitogenome sequences for 95 samples successfully sequenced as part of this current study (Table 2.1), 40 *M. fascicularis* mitogenome sequences from Liedigk et al. (2015), and 17 mtDNA genome sequences from other macaques (10 individuals from six species) and non-macaque primate taxa (seven individuals from different genera) obtained from GenBank as outgroups to expand the phylogenetic perspective (please contact Lu Yao, Robert D. Martin or Corrie S. Moreau for complete list of specimens).

All sequences were aligned with MAFFT version 7 (Katoh, 2013) and corrected by hand in Mesquite (Maddison and Maddison, 2011). Taking the same approach as Liedigk et al. (2015), I removed poorly aligned positions and indels. For maximum likelihood and Bayesian reconstructions, I used RAxML 8.2.4 (Stamatakis, 2006), and MrBayes 3.2.2 (Ronquist and Huelsenbeck, 2003), respectively, through the CIPRES Science Gateway V3.3 (Miller et al., 2010). The parameters for MrBayes included four Markov Chain Monte Carlo (MCMC) runs, a default 0.2 temperature, and the GTR+I model. All analyses were run for 10 million generations with a parameter sampling frequency of 1000 generations, with the first 10% of samples discarded as burn-in.

In order to estimate divergence dates, I implemented BEAST 1.8.3 (Drummond and Rambaut, 2007; Suchard and Rambaut, 2009) with the Bayesian MCMC method and relaxed molecular clock model (Drummond et al., 2006) using CIPRES Science Gateway V3.3 (Miller et al., 2010). I assumed a Birth-Death Process prior for branching rates, as was previously done in Liedigk et al. (2015). I used the same five fossil calibration points as in Liedigk et al. (2015) with a lognormal distribution prior for all nodes: 1) *Homo - Pan* divergence at 6.5 Ma with 95% CI of 0.5 Ma (Vignaud et al., 2002; Brunet et al., 2005; Lebatard et al., 2008), 2) *Pongo – Homo+Pan clade* divergence at 14 Ma with 95% CI of 1.0 Ma (Kelley, 2002), 3) *Theropithecus – Papio* divergence at 5 Ma with 95% CI of 1.5 Ma (Leakey, 1993; Delson, 2000), 4) African – Asian macaques at 5.5 Ma with 95% CI of 1.0 Ma (Delson, 2000, Alba et al., 2014), and 5) hominids – cercopithecids at 27.5 Ma with 95% CI of 3.5 Ma (Zalmout et al., 2010; Pozzi et al., 2011; Stevens et al., 2013). A total of four replicates was run for 700,000,000 generations, and the tree and parameter sampling was performed every 5000 generations. I used Tracer to check that a 10% burn-in is sufficient and all ESS values suggested convergence. I used TreeAnnotator 1.8.3 to reconstruct a consensus topology based on the distribution of trees, and I visualized the phylogeny with FigTree 1.4.2 (Rambaut). I also conducted a pairwise distance analysis in PAUP (Swofford, 2002) to check if any specimens were driving an increase in diversity

RESULTS.

From a total of 196 tissue samples collected, I successfully extracted DNA from 151 (77%), of which the mitogenomes of 95 *M. fascicularis* individuals were successfully sequenced by shotgun sequencing using the Illumina HiSeq2500 (see Table 2.1 for list of specimens sequenced here). All raw sequences will be available on Genbank upon publication or upon request from Lu Yao, Robert D. Martin or Corrie S. Moreau. The mitogenomes of 56 samples for which I had built libraries were not fully sequenced and had relatively low-quality scores and were excluded from the analyses. The bioanalyzer results for the overall set of samples were not indicative of how well the libraries would perform during shotgun sequencing. On seven HiSeq lanes, I obtained an average of 674,364 (49,852-2,064,668) reads with an average 40.95x (3.28 – 125.03) coverage for the mitogenomes that were successfully sequenced per sample. All mitogenomes that I generated ranged between 15,185 and 16,564 bp. It is important to note that extraction and sequencing success rates differed between museums where they were collected (Table 2.2).

Museum	# sampled	# successful extractions	% extracted	# successfully sequenced	% sequenced
FMNH	71	55	77	34	62
AMNH	51	34	67	28	82
NMNH	54	44	81	22	50
RMNH	20	19	95	11	58
Total	**196**	**152**	**78**	**95**	**63**

Table 2.2. Number of *M. fascicularis* samples collected and percentage of successful specimens sequenced for samples from four museum collections. FMNH = The Field Museum of Natural History in Chicago, IL; AMNH = American Museum of Natural History in New York, NY; NMNH = Smithsonian Institution National Museum of Natural History in Washington, DC; RMNH = Naturalis Biodiversity Center in Leiden, Netherlands. % sequenced represents the % of successfully sequenced samples of the successfully extracted samples.

All steps that I took to check for contamination gave negative results. At the extraction stage, all negative controls were found to be clean, and the positive controls using DNA extracted from the fresh cadavers of *M. fascicularis* indicated that the extraction process was successful when amplifying the 200 bp of Cyt*b.* After shotgun sequencing, the BLAST results for each sequence revealed that the sequences of interest matched those of *M. fascicularis* mitogenomes.

In total, I aligned 95 newly generated *M. fascicularis* mitogenomes, 40 *M.fascicularis* mitogenomes previously sequenced by Liedigk et al. (2015), and 17 mitogenomes of outgroup taxa. The latter two sets of mitogenomes were downloaded from Genbank with the accession numbers provided in Liedigk et al. (2015). The final alignment had a total length of 16,873 bp (available upon publication or upon request from Lu Yao, Robert D. Martin or Corrie S. Moreau). One specimen from Thailand (NMNH 251661) that was sequenced, which is not included in the 95 specimens, was the only specimen found to be an outgroup to all *M.*

fascicularis samples. Although no other specimens from the same locality were sequenced to test whether or not this one specimen is representative of its source population, the specimens from nearby localities do not cluster with this single specimen from Nakhon Si Thammarat in Thailand. Therefore, this specimen was removed from the reconstruction and further analyses.

Figure 2.3. Newly reconstructed phylogeny of 135 M. fascicularis museum specimens. Samples starting with MFAS are from Liedigk et al. (2015) while samples beginning with museum specimen numbers were newly sequenced for this study. (continued on Page 27)

Figure 2.3 (continued). Green = mainland, orange = Sundaland, blue = oceanic islands. Clade A is indicated in yellow, and Clade B is indicated in pink. All significant nodes are marked with a red circle (ML bootstrap support of >95% and Bayesian posterior probabilities of 1.0).

Nearly identical phylogenies with strongly supported nodes (ML bootstrap values: >95%, Bayesian posterior probabilities: 1.0) were reconstructed based on maximum-likelihood and Bayesian analyses (available upon publication or upon request from Lu Yao, Robert D. Martin or Corrie S. Moreau). The newly reconstructed phylogeny included samples from regions with known localities that had not previously been sampled at the mitogenome level. They include DNA of *M. fascicularis* from small Sunda islands such as Bali, Bintan, Banggi, Penida, Siantan, Bawean, Nias, Simeulue, and Koh Kut and from the Philippine islands, which include Mindanao, Negros, Bohol, Maripipi, Luzon, Leyte, Palawan, Busuanga, and Balabac islands. The phylogeny splits the specimens into two major clades, with Clade A of Liedigk et al. (2015) containing all mainland specimens, northern Sumatran specimens, specimens from Bintan, Siantan and Koh Kut islands, and a single specimen from Mindanao. Clade B contains all other insular specimens including those from southern Sumatra.

Based on the analyses in this study, the divergence dates for most divergences are earlier than had been previously noted (Figure 2.3; Table 2.3; Nexus file will be available upon publication or through request from Lu Yao, Robert D. Martin or Corrie S. Moreau). At the root, Hominidae and Cercopithecidae separated 41.11 Ma (95% credibility interval [CI]: 33.80-49.42). Within the hominids, *Homo* and *Pan* split 6.75 Ma (6.53-7.20), and *Pongo* diverged from *Homo* + *Pan* 17.59 Ma (14.30-20.59). Within Cercopithecidae, *Colobus* diverged first 28.79 Ma (23.51-34.36) followed by the divergence of *Chlorocebus* 18.17 Ma (15.00-21.54). Papionini split from

Macaca 15.19 Ma (12.54-17.93), and within Papionini, *Theropithecus* and *Papio* diverged 5.99 Ma (5.11-7.14). Within the macaques, the African and Asian macaques diverged 8.29 Ma (6.84-9.78). *M. silenus* and *M. tonkeana* diverged next from the other Asian macaques 7.47 Ma (6.17-8.79). These two species split from each other 5.09 Ma (4.08-6.10). *M. thibetana* split from the group including *M. fascicularis, M. mulatta* and *M. arctoides* 5.69 Ma (4.67-6.71). *M. fascicularis* then diverged from *M. mulatta* and *M. arctoides* 4.70 Ma (3.87-5.55) and these latter two species split 4.22 Ma (3.45-5.02).

Clades	**Liedigk et al. (My)**	**Current Study (My)**
Hominidae - Cercopithecidae	28.60 (25.31-31.78)	41.11 (33.80-49.92)
Pongo - Homo+Pan	13.82 (12.68-14.86)	17.59 (14.30-20.59)
Homo - Pan	6.32 (5.73-6.89)	6.75 (6.53-7.20)
Colobus - other Cercopithecidae	19.89 (16.17-23.87)	28.79 (23.51-34.36)
Chlorocebus - Papionini+*Macaca*	12.81 (10.59-15.22)	18.17 (15.00-21.54)
Papionini - *Macaca*	10.90 (8.92-12.90)	15.19 (12.54-17.93)
Theropithecus - Papio	4.77 (3.87-5.72)	5.99 (5.11-7.14)
M. sylvanus - Asian macaques	6.10 (5.23-6.92)	8.29 (6.84-9.78)
M. silenus+M. tonkeana - other Asian *Macaca*	5.49 (4.69-6.34)	7.47 (6.17-8.79)
M. silenus - M. tonkeana	3.70 (2.80-4.54)	5.09 (4.08-6.10)
M. thibetana - M. fascicularis+M. mulatta+M. arctoides	4.16 (3.47-4.85)	5.69 (4.67-6.71)
M. fascicularis - M. mulatta+M.arctoides	3.42 (2.83-4.01)	4.7 (3.87-5.55)
M. mulatta - M. arctoides	3.02 (2.42-3.60)	4.22 (3.45-5.02)
Clade A - Clade B	1.70 (1.36-2.04)	2.26 (1.86-2.67)
Clade A splitting events	0.96 (0.78-1.16)	1.22 (1.01-1.43)
N. Sumatran specimens - mainland specimens	-	0.31-0.52 (0.28-0.62)
Clade B splitting events (Timor specimens - rest of Clade B)	0.93 (0.74-1.12)	1.23 (1.02-1.46)
Javan and surrounding insular specimens - rest of Clade B (excluding Timor specimens)	0.87 (0.70-1.05)	1.15 (0.95-1.36
Nias and Simeulue specimens - Javan specimens	-	1.11 (0.92-1.31)
S. Sumatran+Bangka specimens - Borneo specimens	-	0.65 (0.53-0.79)
S. Sumatran specimens - Bangka specimens	-	0.44 (0.35-0.54)
Philippine specimens - Borneo specimens	0.21 (0.15-0.28)	0.60 (0.50-0.71)

Table 2.3. Comparison of major divergence dates for *M. fascicularis* inferred in the Liedigk et al. (2015) study and in the present study.

Within *M. fascicularis,* Clades A and B diverged early at 2.26 Ma (1.86-2.67). Clade A's splitting events began approximately 1.22 Ma (1.01-1.43). Within this clade, the individuals are not grouped into monophyletic clades by geographic localities. The northern Sumatran specimens that I sequenced form a cluster, which also contains lineages from mainland Malaysia, Thailand, and Bintan and Siantan islands, diverging from mainland populations between 0.31-0.52 Ma (0.28-0.62). There are four other insular specimens within Clade A, and those include specimens from Koh Kut, Siantan, Bintan, and Mindanao islands. The divergence of the specimen in Mindanao from Thailand specimens is the most recent of the four, at 2.4 kya (0-9 kya).

Liedigk et al. (2015) had found Clade B to contain distinct monophyletic lineages based on geographic regions, but the results in this study demonstrate that this is not the case, especially for specimens from Borneo, the Philippines, and Java and its surrounding islands. The Timor specimens that Liedigk et al. (2015) sequenced diverged as a single clade within Clade B 1.23 Ma (1.02-1.46). The specimens from Java and its surrounding islands diverged as two major groups. The first group, which contains solely specimens from Java and its surrounding islands, diverged 1.15 Ma (0.95-1.36). The second group, which also includes specimens from Nias, Simeulue and Mauritius islands, then diverged 1.11 Ma (0.92-1.31). The Mauritius lineage split off 0.17 Ma (0.12-0.22), while the monophyletic group of specimens from Nias and Simeulue islands diverged from the Javan specimens earlier at 1.09 Ma (0.90-1.29). The split of the lineages from these two islands to the west of Sumatra occurred 1.04 Ma (0.85-1.23). After the paraphyletic Javan clade, a group containing specimens from Borneo, Bangka and southern Sumatra diverged within Clade B 0.83 Ma (0.69-0.98). The Bangka and southern Sumatra lineages diverged from a western Borneo lineage 0.65 Ma (0.53-0.79), and the former lineages split 0.44 Ma (0.35-0.54). The clade containing specimens from Borneo and the Philippine islands began splitting 0.60 Ma (0.50-0.71). The Philippine specimens do not form a monophyletic clade. One group containing lineages from Banggi, Balabac and Mindanao islands diverged from other Bornean specimens 0.46 Ma (0.38-0.54). Additionally, there are Borneo specimens nested within this particular group. The remaining Philippine specimens form a monophyletic group with the exception of a single specimen from northern Borneo, which diverged from two Mindanao specimens 0.08 Ma (0.05-0.12). The lineages from Negros Island diverged from other Philippine specimens 0.18 Ma (0.14-0.22).

These divergence dates are earlier than previously noted in other studies of *M. fascicularis*, but the results of the pairwise distance analysis in PAUP (Swofford, 2002) show that no specimens stood out in the analysis (available upon publication or through request from Lu Yao, Robert D. Martin or Corrie S. Moreau).

DISCUSSION.

DNA extracted from museum specimens is usually highly degraded (Mason et al., 2011; Liedigk et al., 2015; Burrell et al., 2015), but with the development of next-generation sequencing techniques it has become faster and less costly to sequence such fragmented DNA. Although many studies use DNA-capture techniques to target the region of interest, such as the mitogenome, I have found that shotgun sequencing the genomes will yield high coverage of entire mitogenomes if the genetic libraries are of high quality. An additional advantage of shotgun sequencing is that it is possible to sequence the nuclear genomes at low coverage (approximately 1.3x if multiplexing mammalian samples), which can be used for further studies. But, as past studies have emphasized, there are only two copies of the nuclear genome in a cell in comparison to the hundreds to thousands of copies of mitogenomes in each cell, so it is inherently more difficult to achieve coverage of the nuclear genome than the mitogenome when sequencing ancient DNA (Hagelberg and Clegg, 1991; Mason et al., 2011; Rowe et al., 2011; Guschanski et al., 2013). As I have found, next-generation sequencing does not achieve high-quality and/or high-precision results for every specimen. Few studies have looked at extraction and sequencing success of specimens across museums and collectors (Bailey et al., 2015). Because I collected tissues from the same species in the same region from four different natural history museums, this study sheds some light on variation in the success of sequencing museum specimens across various collections (Table 2.1). The highest success rate for extractions (95%) was for specimens from the collections at RMNH, and the lowest extraction success rate (67%) was for specimens housed at AMNH. The success rate for sequencing the entire mitogenome differed from the extraction rate, with the highest success rate (82%) in AMNH specimens and the lowest success rate (50%) for NMNH specimens. The different rates of successful extraction and sequencing among various museums may be due the effects of various chemicals used for cleaning the skeletal materials on the preservation of dried tissues. Unfortunately, how the specimens were cleaned and prepared when they were first collected is not recorded. These results are not indicative of how well DNA from other species is sequenced across museums.

Although the relationships amongst individuals in the expanded intraspecific phylogeny (Figure 2.3) is in close agreement with the results reported by Liedigk et al. (2015), I did not find neatly monophyletic lineages in Clade B and the divergence dates are earlier than previously inferred (Table 2.3). Because the only major difference between my analyses and those of Liedigk et al. is sample size, I believe this may account for the disparity between the two sets of results. Pozzi et al. (2014) demonstrated that extensive taxon sampling can recover phylogenetic relationships that are consistent with morphological and nuclear data in primates. This is consistent with previous studies that raise the concern that incomplete or biased taxon sampling results in phylogenetic error (Zwickl and Hillis, 2002; Hillis et al., 2003; Plazzi et al., 2010; Townsend and Leuenberger, 2011; Nabhan and Sarkar, 2012). So the non-monophyletic relationships I recovered, particularly in Clade B, may be the result of increased and more complete taxon sampling of *M. fascicularis* throughout Southeast Asia. Moreover, various studies indicate that under-sampling taxa in phylogenetic analyses will lead to underestimation of divergence dates, while more complete taxon sampling will recover earlier node ages (Milne, 2009; Crête-Lafrenière, 2012; Schulte, 2013), especially when using a relaxed molecular clock model (Soares and Schago, 2015). This may be the case for this study, as I have more than tripled the sample size of the most recent intraspecific study of *M. fascicularis* (Liedigk et al., 2015).

The inferred intraspecific phylogeny revealed a number of biogeographic patterns as a result of increased taxon sampling. In particular, it emerged from my overall phylogeny based on mitogenomes that the polyphyletic clustering of individuals on Sumatra can now be explained on a geographical basis: Northern Sumatran individuals fall within the mainland cluster (Clade A), whereas southern Sumatran individuals are nested within the insular cluster with populations from Borneo (Clade B). This corroborates the suggestion made by Liedigk et al. (2015) that this could be why Tosi & Coke's (2007) samples from southern Sumatra clustered in a different clade from their own samples from northern Sumatra. Inclusion of museum samples from both the northern and southern ends of Sumatra in this study permitted clear resolution of this issue. Accordingly, Sumatran *M. fascicularis* have two distinct geographic origins. The northern stock most likely originated from the Indo-Chinese Peninsula in the east while the southern lineages appear to be of insular origin, by way of Java and Borneo (through Bangka Island). This is in accord with the Y-chromosomal data from Tosi & Coke (2007), which revealed that both haplogroups are present on Sumatra.

Although this split in populations on Sumatra is rare in vertebrates (Leonard et al., 2015), it appears that orangutans also exhibit this phylogeographic pattern within species on Sumatra (Nater et al., 2011, 2015). The Sumatran orangutans (*Pongo abelii*) currently reside only in northern Sumatra, although the subfossil record indicates that their distribution had been widespread across Asia, including the entirety of Sumatra (Delgado and VanSchaik, 2000). This species is particularly diverse in comparison to the other species (*Pongo pygmaeus*) on the island of Borneo (Steiper, 2006). In a series of genetic studies, Nater et al. (2011, 2015) found that an extant population of *P. abelii* diverged more recently from *P. pygmaeus* at ~2.09 Ma than from the other populations of *P. abelii* on Sumatra ~3.50 Ma. This particular *P. abelii* population from Batang Toru is the only extant group located to the south of Lake Toba, a large lake that is the site of the recent Toba supereruption approximately 73 kya along with four other major eruptions within the last 1.2 million years (Chesner et al., 1991). Williams et al. (2009) demonstrated that the supereruption resulted in climatic cooling and prolonged deforestation, which had significant consequences for the organisms in the region, one of which was to seal off the region north of Lake Toba from the rest of Sumatra (Ambrose, 2003). This boundary at Lake Toba has led to a split that is seen in other taxa. For example, the mountain agile gibbon (*Hylobates agilis*) occurs only south of Lake Toba while the white-handed gibbon (*Hylobates lar*) occurs only to the north (Whittaker et al., 2007; Thinh et al., 2010). The split I see within *M. fascicularis* may also be the result of the Toba eruptions as the northern Sumatran lineages are north of Lake Toba while the southern Sumatran lineages are well to the south of the lake. While northern and southern Sumatran lineages diverged approximated 2.26 Ma according to my study, which is earlier than the set of major Toba eruptions, it could be possible that the survival of *M. fascicularis* was affected by the Toba supereruption on Sumatra. Once the environment began thriving again, new *M. fascicularis* lineages could migrate to the northern and southern ends of Sumatra via Malaysia and Borneo, respectively, but were still separated by the supereruption site. Although the populations may have met once the forest around Lake Toba was restored after the eruptions, it is unlikely that there would be merging in the mitochondrial genome as *M. fascicularis* are female philopatric (Gerber et al., 2016; Ruiter and Geffen, 1998; Melnick and Hoelzer, 1991). Future studies should examine the nuclear DNA, with a focus on the Y chromosome, to look for patterns of male dispersal in this species.

The hypothesis that the Toba eruption is the cause of the split in the northern and southern Sumatran specimens is, however, questionable because there is no evidence of this division in the mitochondrial DNA of most other Sumatran vertebrates, including rodents, carnivores and birds (Leonard et al., 2015). Leonard et al. (2015) analyzed 28 vertebrate taxa distributed throughout the continental shelf region in Southeast Asia, and 16 of those taxa had Bornean populations that were basal to Sumatran and Malay Peninsular populations. The divergence dates varied between taxa. All other taxa in their analyses, except for *M. fascicularis*, were either unresolved or had no geographical structure, although this may be due to the fact that only a few mitochondrial genes were analyzed. If the Toba eruption had such a great impact in the distribution of organisms on the Sunda Shelf, it would be expected that many more species, especially terrestrial vertebrates, would display the divide in Sumatra as is seen in long-tailed macaques, orangutans and gibbons.

The majority of the island-living individuals sampled are nested within Clade B, the insular cluster. However, there are four exceptions. The population from Koh Kut, a continental shelf island off the coast of Thailand, is sister to all Thai samples north of the island. The Thai samples that were collected close to the Malaysia-Thailand political border form a sub-cluster within the clade of populations from the Malay Peninsula and northern Sumatra. Another exception is a single individual from Mindanao (FMNH 56161) that clusters within the populations from northern Thailand. There could be two explanations for this. First, that particular lineage was recently introduced to Mindanao by way of human migration ~2,400 years ago. Second, this specimen may have been labeled incorrectly at some point in the past because it is the only specimen out of 12 specimens I had sequenced from Mindanao that nests within Clade A. The final exceptions are the lineages from Bintan and Siantan Islands, both of which are small continental shelf islands off the coast of the Malay Peninsula. These populations likely originated from the mainland stock.

But the majority of insular lineages are clustered within Clade B. As expected, the lineages from islands near Java, such as Bali, Penida and Bawean islands share a common ancestor with lineages from east Java. The Javan specimens form two major groups in Clade B, one of which includes the specimens from Nias and Simeulue Islands to the west of northern Sumatra. This is unexpected as Nias and Simeulue are not geographically close to Java. Additionally, they are considered to be oceanic islands, but whether or not they were connected to the continental shelf during glacial periods in the Pleistocene is unclear. The Nias basin is ~500 m. deep (Deighton et al., 2014), and there do not appear to be any non-volant species that are strictly endemic to the island (Barbour, 1912), so natural colonization of Nias Island may have been possible. The Simeulue basin is deeper at ~1000 m (Milsom, 2005) with very few endemic species, including three snake species, one bird species, one pig species, and a subspecies of *M. fascicularis, M. f. lasiae* (Whitten et al., 2000). Because the strait between these islands and Sumatra is narrow, certain populations may have been able to migrate naturally from the continental shelf by swimming (Gumert and Malaivijitnond, 2012).

The Philippine samples in Clade B appear to have originated from Borneo, which supports previous hypotheses for Philippine colonization by way of Borneo (Abegg and Thierry, 2002; Smith et al., 2014). However, most node support between individuals from the Philippine islands is lacking in statistical significance (Figure 2.3). A very recent date of introduction, as is the case with these lineages from the Philippines, could explain the absence of statistically significant node support within certain Philippines macaque groups (Guschanski et al., 2013; Liedigk et al., 2014, 2012; Roos et al., 2011; Zinner et al., 2013b).

In their study using both short tandem repeat (STR) and mtDNA sequences, Smith et al. (2014) suggested that there were at least two paths of dispersal by *M. fascicularis* into the Philippines from Borneo: (1) through Palawan to the northern Philippine islands, and (2) through the Sulu archipelago to the southern Philippine islands. The newly inferred phylogeny indicates that dispersal event (1) likely led to the distribution of *M. fascicularis* to Luzon in the north but also resulted in the distribution of *M. fascicularis* to some of the more southern islands such as

Negros, Bohol, Leyte, and perhaps even Mindanao. The potentially human-mediated dispersal to these southern islands may have been directly through Palawan or through Luzon and Samar (and the adjacent islands). Analyzing samples from Mindoro and Panay Islands would help elucidate the dispersal passage to these more southern islands.

Dispersal event (2) of Smith et al. (2014) is also consistent with the results in this study, as individuals from Mindanao cluster with some of the Borneo individuals; but they are monophyletic within a clade that includes an individual from Balabac, an island associated with Palawan. Additionally, the single individual Liedigk et al. (2015) sequenced from the Philippines appears to be from the southern Philippines, based on its genetic makeup, and clusters with the Mindanao and Borneo individuals from this particular dispersal event. Since I was only able to sequence the mitogenome of one individual from Palawan and no individuals from the islands in the Sulu archipelago — although they do occur in that region (Musser and Heaney, 1985) — future analysis of more individuals from those islands would be useful in determining whether the majority of current lineages in Mindanao had arrived by way of Palawan, through the Sulu archipelago, or both.

In their study, Liedigk et al. (2015) pointed out that the divergence date of approximately 0.93 Ma for the Timor clade that they had sequenced is inconsistent with the hypothesis that lineages to the east of the Wallace line were introduced within the last 4000 years (Fooden, 2006; Heaney et al., 2016). However, based on genetic data, modern humans had colonized the Timor region by 37 ka (Gomes et al., 2015), so *M. fascicularis* could have been introduced by humans much earlier. This study estimates an even earlier divergence date of Timor individuals from all other insular individuals of approximately 1.23 Ma for this clade from the rest of Clade B, although the most recent common ancestor within the Timor clade is estimated to be 0.032 Ma. The divergence date and placement of this clade in the phylogeny implies an unexpected history for this lineage. Liedigk et al. (2015) suggested that the Timor lineage was the sister group in Clade B because the Timor haplotypes had originated from an area somewhere in Sundaland that they had not sampled in their study. However, my thorough sampling throughout Sundaland (Figure 2) does not suggest a locality for the origin of the Timor clade. Still, this does not eliminate the possibility that the Timor lineage had naturally colonized the island. Future studies that include specimens from islands near Timor, such as Lombok, Sumbawa, Flores, and Sumba may help illuminate the biogeographic history of this clade.

It must be borne in mind that the inferred phylogeny is based on mtDNA alone. It therefore reflects population-level cladogenic events attributable to the sedentary nature of female macaques (Pusey and Packer, 1987; de Ruiter and Geffen, 1998) and cannot reveal any influences exerted by the typical wide dispersal of males. Analyses of the nuclear genome could help further clarify the dispersal of *M. fascicularis* throughout Southeast Asia and would elucidate the genetic selection throughout the colonization of this region.

CONCLUSION.

In this study, I have elucidated biogeographic patterns of *M. fascicularis* throughout Southeast Asia by using thorough sampling of museum specimens from the region, and in the process, I have identified avenues that still need to be pursued in order to fully understand this widespread species:

1. It is necessary to examine the nuclear DNA, especially the Y chromosome, to determine patterns of male dispersal in this species.
2. I was not able to determine the origins of the Timor specimens, and including specimens from islands near Timor, such as Lombok, Sumbawa, Flores, and Sumba could illuminate the biogeographic history of this clade.
3. Although I have sampled widely throughout the Philippines, it appears that there may be two major migration routes into this region. Analyzing samples from Mindoro, Panay, Palawan, and the islands in the Sulu archipelago would help determine dispersal passages through this chain of oceanic islands.

I have shown here that the use of next-generation sequencing, particularly shotgun sequencing, on museum specimens proves to be a successful way of targeting entire mitogenomes at high coverage due to the nature of ancient DNA. The newly reconstructed intraspecific phylogeny demonstrates that lineages within this species diverged earlier than previously inferred, that there is a geographical separation in Sumatran macaques, and that there is a colonization pattern in the Philippines. The results of this study demonstrate the importance of a large geographic sampling for a widespread species because it expands knowledge about the relationships and divergence dates within that species. *M. fascicularis* is commonly studied for both evolutionary and biomedical purposes, and I hope these findings will be of use to future studies that use this species as a model.

CHAPTER 3

Evaluating the occurrence of island dwarfing in *Macaca fascicularis*

INTRODUCTION.

Mammals originated approximately 200 Ma (Meredith et al., 2011; O'Leary et al., 2013) from a small-bodied common ancestor. They evolved to range across almost eight orders of magnitude in body size (Baker et al., 2015), showing that certain lineages underwent far greater evolutionary size changes in comparison to others. Cope's Rule postulates that species within taxonomic groups generally tend to evolve toward larger body size (Cope, 1896; Alroy, 1998). This pattern is observed clearly in the fossil record of some mammalian lineages extending over long evolutionary time periods (Cope, 1896; Alroy, 1998; Van Valkenburgh et al., 2004; Bokma et al., 2014; Puttick and Thomas, 2015). However, it is hypothesized that there is an exception to this general pattern on islands.

The Island Rule has captured scientists' attention for over a century. Forsyth Major (1902) and Bate (1903) first noted that large-bodied Pleistocene mammals had reduced body size on Mediterranean islands, but these studies are rarely mentioned in the literature. Instead, the widely cited source is Foster (1964), who studied 116 species of mammals from islands in western North America and Europe to test whether insular forms are larger, smaller, or the same size as the presumed mainland ancestor. He concluded that there is a general trend on islands for small-bodied species, particularly rodents and some marsupials, to become larger simply due to absolute or relative absence of predators, while large-bodied species, such as carnivores, lagomorphs and artiodactyls, undergo reduction in body size because food resources are severely limited. On the other hand, insectivores (which are predominantly small-bodied) did not appear to exhibit any shift in body size on islands. Van Valen (1973) dubbed the general phenomenon recognized by Foster the "Island Rule" and claimed that it is fairly universal compared to other biological rules. Lomolino (1985) subsequently modified the "Island Rule" by demonstrating that it does not involve categorical body size differences but rather reflects a "graded trend from gigantism in the smaller species of insular mammals to dwarfism in the larger species". In other words, the larger the body size of the mainland counterpart, the greater the degree of dwarfism of that species or closely related species on islands, and conversely the greater the degree of gigantism for small-bodied mainland species. It is the dwarfing in body size that captures my attention because it opposes the general tendency for animals to evolve larger size and it deals with striking examples, such as donkey-sized elephants.

A number of hypotheses have been proposed to explain why a decrease in body size may occur on islands. MacArthur and Wilson (1967) showed that, as island area decreases, so usually does species richness. So ecological release due to decreased species richness, and therefore competition, on islands (Heaney, 1984; Harcourt, 1999), accompanied by reduced predation (Sondaar and Boekschoten, 1967; Dayan and Simberloff, 1998), may account for size reduction. In Southeast Asia, carnivore species numbers decline as island area decreases, indicating that carnivore extinction is more likely on small to medium islands compared to large islands (Heaney, 1984). Large body size reduces predation risk (Sinclair et al., 2003; Brown and Sibly, 2006; Kandler and Smaers, 2012), so a lower risk on smaller islands would be expected to favor decreased body size. In fact, some small islands completely lack predators (MacArthur and Wilson, 1967; Kurtén, 1972).

Another explanation for insular dwarfing is limitation of food resources on islands, because large mammals need more energy than small mammals. On an island with limited resources, small-bodied individuals might be expected to fare better because they need less food to reach breeding size. With large-bodied species, under these conditions selection for smaller size is to be expected in a food-stressed environment to increase fitness (Hessee et al., 1951; MacArthur and Wilson, 1967; Sondaar, 1977; Case, 1978; Heaney, 1978; Lawlor, 1982; Lomolino, 1985). From this it can be predicted that food availability should be most restricted on small islands because of the particularly limited area available for resources.

Various ecological factors, such as food resources or predation, have been shown to be correlated with island size (Heaney, 1978; Harcourt, 1999; Burness et al., 2001; Lomolino, 1985, 2005; Lomolino et al., 2013; Meiri et al., 2005a, 2005b; White and Searle, 2007). With a large dataset for tri-colored squirrels from various islands in Southeast Asia, Heaney (1978) demonstrated that there is a curvilinear relationship between body size and island area: mammals residing on smaller islands undergo greater body size changes. However, his review of the literature showed that there are striking exceptions to this relationship, as seen in grizzly bears on British Columbian islands or mice on very small islands off the coast of British Columbia (Redfield, 1976), thus suggesting that there may be some variability in the “Island Rule”. Lawlor (1982) was one of the first to question the generality of the “Island Rule” in a study of lagomorph and rodent populations on islands off the coast of Baja California and Mexico. He found that his dataset did not indicate a trend in mammal body size on islands nor reveal the expected relationship between body size and island area. He concluded with a cautionary message for future studies concerning island evolution: to be careful when making island-mainland comparisons and generalizing about insular body size trends.

More recently, a series of papers have debated whether or not the "Island Rule" is universal. On the one hand, Lomolino (2005) and Lomolino et al. (2013) used an extensive dataset of vertebrate taxa from around the world to support the case that the rule is indeed a general phenomenon. A number of studies have debated whether there actually is an optimal body size for each clade with a particular ecological strategy and what that optimal size may be (Brown et al., 1993; Damuth, 1993; Lomolino, 2005; Raia et al., 2010; Itescu et al., 2014). Damuth (1993) explicitly defined this optimal size as 1 kg while Brown et al. (1993) and Kelt and Van Vuren (1999) stated that it is just 100 g. Lomolino et al. (2013) found that the resulting convergence applies to mammals specifically in species-poor environments, allowing them to converge on an intermediate body size that is absent in that particular environment. On the other hand, Meiri et al. (2004, 2005a, 2005b, 2006, 2008a, 2011) demonstrated that the Island Rule is not universal and applies only to a few clades within mammals, including carnivores, heteromyid rodents, and artiodactyls (Meiri et al., 2008a).

In order to directly test the Island Rule, many studies compare pairs of insular and adjacent mainland populations of the same species (e.g. Lawlor, 1982; Lomolino, 1985, 2005; Meiri, 2008a; Itescu et al., 2014), which is the most economical way to test for the rule across many species. However, there are two problems with this method. First, a mean body size for a population or even species is usually calculated for the data. This is problematic because the mean may be based on samples of varying sizes. Small sample sizes could result in skewed mean values, which would then lead to confounding results regarding body size evolution on islands. Additionally, a mean value does not necessarily capture the wide range of variation in body size that is found within populations, and the extent of size variation is even greater in large-bodied mammals (Meiri et al., 2009; Hallgrímsson and Maiorana, 2000). So it is essential to account for intraspecific variation in the dataset (Ives et al., 2007; Felsenstein, 2008; Stone et al., 2011), yet this is not often done in broad studies using the method of pairing insular and mainland populations. The other problem with the pairing method is that it is often accompanied by the assumption that a chosen mainland population represents an ancestral form and is the direct relative of the insular population or species. Although insular forms are usually descendants of mainland forms, it is theoretically possible that a small-bodied ancestral population migrated to an island and remained small while the mainland lineage evolved larger body size. The inferred mainland-island population relationships are usually corroborated using a combination of taxonomy, distribution data, phylogenetics, and advice from experts. However, published phylogenetic studies rarely furnish the level of relatedness that is needed to tease apart relationships for studies of the Island Rule. As a result, although the investigators can be confident at some level that selected island-mainland pairs reflect true sister relationships, it remains possible that certain groups may be paired inappropriately. Related to this point, when using this method it is often assumed that taxonomic groups are monophyletic based on geographic locality, but this may not be the case, as can be evidenced by intraspecific phylogenies (e.g. Chapter 2).

Meiri et al. (2008a) applied phylogenetic comparative methods to a large dataset, and this may account for the discordance between reported results as Lomolino (1985, 2005) and Lomolino et al. (2013) did not control for phylogenetic relatedness in their analyses. Meiri et al. (2008a) state that, because Lomolino's study (2005) did not correct for phylogenetic relatedness, its patterns were influenced by pseudo-replication of the species with large sample sizes in the study. This directly highlights a fundamental problem that arises with certain studies of the Island Rule because they use raw data without phylogenetic correction. Felsenstein (1985) stressed the need to account for relatedness when using comparative methods because of the potential problem of phylogenetic inertia. He was concerned with differences *between* species, but the Island Rule applies between very closely related species or even *within* species (Lomolino, 1985; Boback and Guyer, 2003; Meiri, 2007). Thus, it is important to be wary of phylogenetic non-independence within species or between very closely related species when studying the Island Rule. Quite apart from that consideration, an intraspecific phylogeny is very useful for inferring when populations colonized various islands and the patterns of colonization that occurred.

Studies of the Island Rule have rarely focused on primates. Lomolino's studies (1985; 2005; 2013) on mammals found that the Island Rule applies to all primates while Meiri et al.'s phylogenetic analyses (2006; 2008a) determined that primates are not one of the groups to which the Island Rule applies. In 2007, using the paired-species approach Bromham and Cardillo conducted a study using 39 extant island primate species and subspecies that reportedly confirmed the Island Rule for primate body size. Welch (2009) broadly corroborated this finding, although his study showed that results depended on which measurement was used as a proxy for body size. When skull length or body mass were used, the Island Rule was found to hold for primates.

As stated above, the Island Rule may potentially apply within species, so it is important to analyze body size differences between individuals or populations on islands and/or the mainland within individual species, which has rarely been done for primates. Fooden and Albrecht (1993) analyzed insular variation in long-tailed macaques (*Macaca fascicularis*) using skull length as a proxy for body size. They concluded that specimens from the smaller islands surrounding the mainland and large Southeast Asian islands (Borneo, Sumatra and Java) tend to have shorter skull length. Yet a study by Schillaci et al. (2008), which directly tested the Island Rule in *M. fascicularis*, revealed that this species does not show differences in body size on islands of various sizes when either body length or skull length is used as an indicator of body size. However, their overall sample size was relatively small and mainland specimens were not included in the analyses with island area.

Here, I test the Island Rule in *M. fascicularis* (long-tailed macaques) using a large sample size that I was able to obtain for this single species. Instead of resorting to the conventional short-cut of testing the Island Rule by pairing insular and mainland populations, I test the rule directly by regressing body size on island size. Because of the possibility that phylogenetic relatedness might influence the outcome, the data for *M. fascicularis* are analyzed both without controlling for phylogenetic inertia and using appropriate methods to counter the effects of phylogenetic non-independence.

MATERIALS AND METHODS.

Southeast Asia was chosen to conduct this particular study because the region is geographically very diverse, with thousands of islands covering an extensive range of sizes and types (both oceanic and continental). The islands in this region range from just a few square kilometers to giant landmasses such as Borneo (743,330 km^2) and Sumatra (473,481 km^2). The extensive variation in island area present in this region is advantageous because it provides me with considerable scope for taking island size into account. Additionally, being able to sample such a large number of islands is a major asset because abundant data can be collected from specimens collected from many islands of similar sizes. These islands all fall into two categories: oceanic or continental. Oceanic islands are those that had never been connected to the mainland, while continental islands are those that lie on a continental shelf, such that they have been connected with the mainland, forming a single landmass, when sea levels were low during glaciation periods (Gillespie and Clague, 2009). The Wallace-Huxley Line, which separates Bali and Lombok islands to the south and Palawan and the rest of the Philippines to the north, sets the eastern limit to the group of continental islands in Southeast Asia, consisting of Sumatra, Borneo, Java and over 900 smaller islands. Beyond the Wallace-Huxley Line lies Wallacea, a group of oceanic islands including the 7,107 islands of the Philippines and numerous Indonesian islands (Figure 3.1). Having both types of islands is important in order to be able to distinguish any patterns due to natural colonization of islands (in the case of continental islands) in comparison to patterns that may have evolved after human introduction of a species to islands (hypothesized to be the case for some species of large mammals on most oceanic islands; Heaney et al., 2016). Long-tailed macaques were selected because they are widespread, large-bodied representatives of the order Primates (average adult male body mass: 5.36 kg; average adult female body mass: 3.59 kg; Smith and Jungers, 1997; Fooden, 1995).

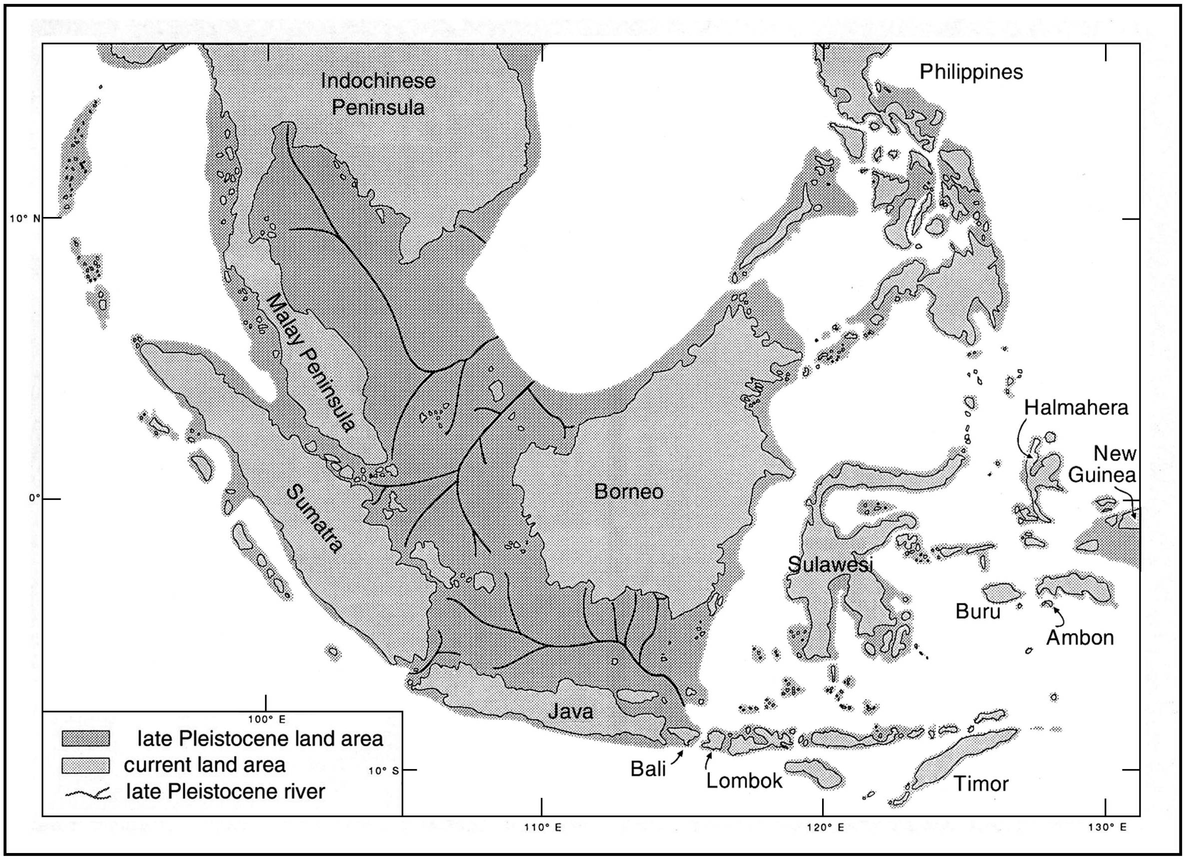

Figure 3.1. Map of Southeast Asia from Heaney (1991), indicating exposure of the continental shelf above sea-level during Pleistocene periods.

Data collection. Skulls of wild-caught *M. fascicularis* were examined and measured. The specimens concerned are housed at the Field Museum of Natural History in Chicago, the U.S. National Museum of Natural History in Washington, D.C., the American Museum of Natural History in New York, the Natural History Museum in London, the Raffles Museum of Biodiversity Research (now the Lee Kong Chian Natural History Museum) in Singapore, and the Naturalis Biodiversity Center in Leiden. All specimens were identified as adults by the presence of complete eruption of the third molars. I measured as many male and female skulls from each locality as were available.

Standard cranial dimensions of the skulls were measured with a digital sliding caliper. Dimensions recorded included maximum skull length (SL) and maximum skull width (SW) because the product of these measurements is known to be a suitable predictor for body mass (e.g. for primates; Martin, 1990). This method was used in place of using body mass or overall body size directly because museum collections of mammals generally do not include body mass data or postcranial skeletal remains to complement the skull. For the phylogenetic analysis, I used the intraspecific phylogeny based on mitochondrial DNA sequences from Chapter 2.

Statistical analyses. I used ordinary least squares regression with log-transformed data to examine estimated body size (skull length x skull width) in relationship to island size, using the statistical program R (R Development Core Team, 2010). Because island size is measured with negligible error, ordinary least squares is an appropriate analysis to apply to these data. I set the mainland size as an arbitrarily large area, 10 million km^2, which exceeds the size of any island in Southeast Asia. Because macaques are sexually dimorphic in body size (Leutenegger, 1982), I separated the analyses by sex. I also performed an ANCOVA to test whether sex or island type (mainland, oceanic or continental) influences the relationship between body size and island area. An ANOVA was then used to determine whether removing the interaction of either sex or island type with island area significantly affects the fit of the model.

I utilized an intraspecific phylogeny that includes 92 of the *M. fascicularis* individuals that I measured in this study (Chapter 2) to test and correct for non-independence in the ordinary least squares analysis. To this end, I used a generalized least squares approach that accounts for intraspecific phylogenetic effects by estimating Pagel's λ (Pagel, 1999), which transforms the branch lengths to best fit the data to a Brownian motion model of evolution.

RESULTS.

The dataset included estimated body sizes for 224 *M. fascicularis* specimens, consisting of 142 males and 82 females. These data will be available upon publication, which is scheduled for 2018. If you would like to request access to the data before publication, please contact Lu Yao or Robert D. Martin.

Sex	n	Intercept	Slope	r^2	p
Male	142	1.944	-0.015	0.041	0.068
Female	82	1.979	-0.006	0.006	0.346

Table 3.1. Ordinary least squares intercepts and slopes for the regression of estimated body size of *M. fascicularis* on island size.

Ordinary least squares regressions. Slopes and intercepts for the ordinary least squares regressions are displayed in Table 3.1. *M. fascicularis* does not exhibit statistically significant dwarfing in body size for males or females on islands, as is also indicated by the relatively flat regression lines (Figure 3.2). Indeed, the regression actually has slightly negative slopes instead of the expected positive slopes.

Somewhat unexpectedly, phylogenetic analysis of the data for 92 specimens of *M. fascicularis* yielded a value of $\lambda=0$ (95% confidence interval: 0, 0.118; p=1), indicating that there is no phylogenetic signal within this species. When estimated body sizes are displayed against the intraspecific phylogeny (Figure 3.3), there is no visually evident pattern in body size attributable to phylogeny. I therefore continued with the analyses for this species without controlling for phylogenetic relatedness.

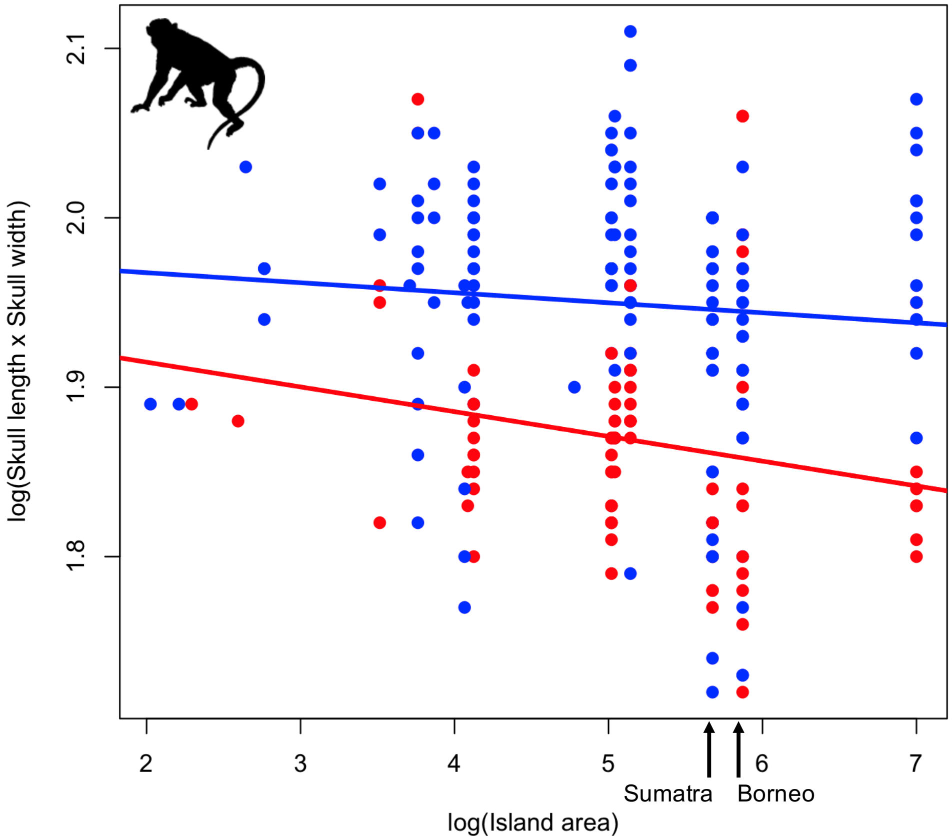

Figure 3.2. Log (skull length x skull width) regressed against log (Island area) using ordinary least squares for 224 individuals of *M. fascicularis*. Males (n=142) are indicated in blue and females (n=82) are indicated in red. Mainland "area" was arbitrarily given a large size of 10 million km^2, resulting in a logarithmic value of 7.

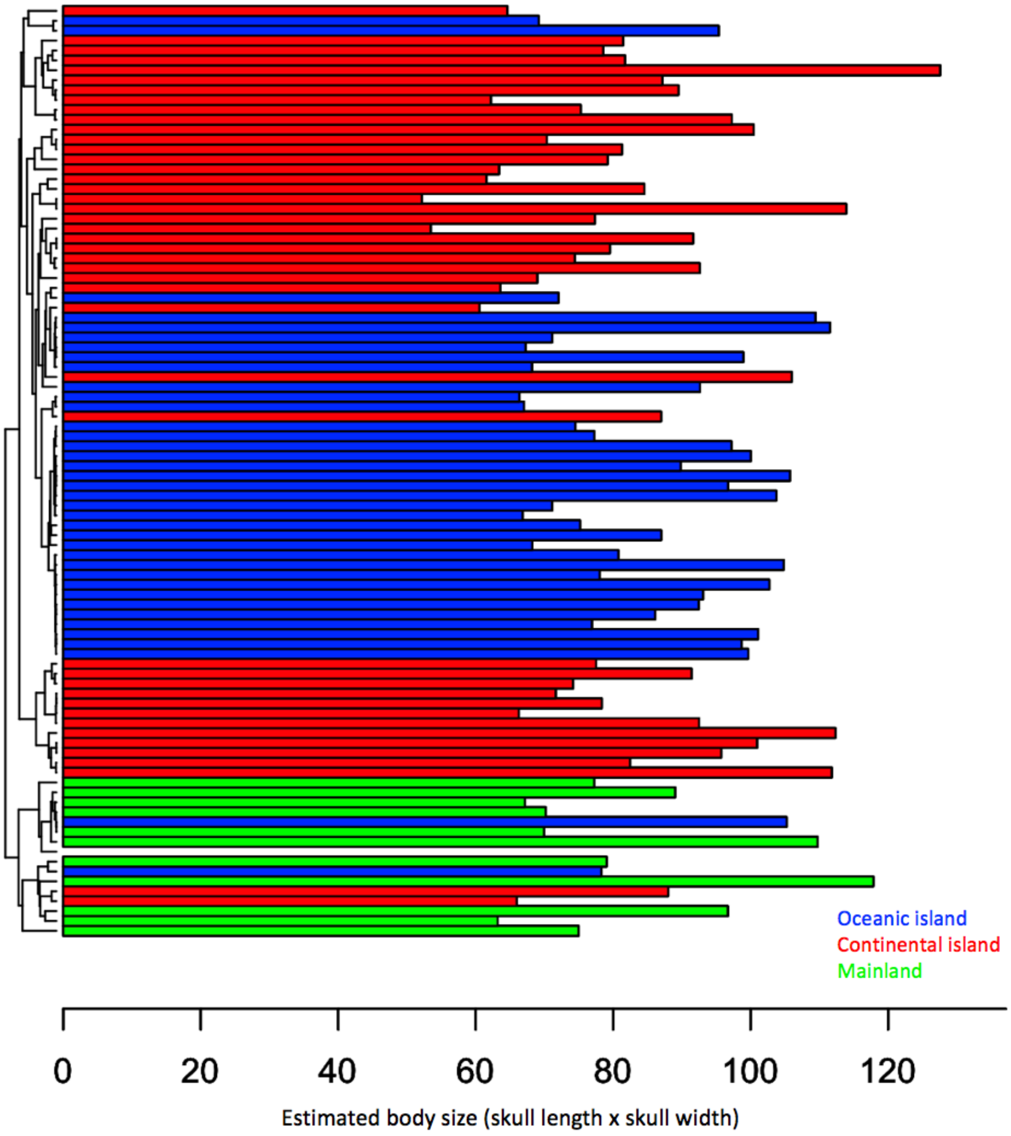

Figure 3.3. Estimated body size (skull length x skull width) mapped onto an intraspecific phylogeny of 92 specimens of *M. fascicularis*.

ANCOVA. ANCOVA results are shown in Table 3.2. The analysis shows that the body sizes within *M. fascicularis* differ by sex when analyzing body size and island size. Males tend to have larger body sizes than females. The interaction effect of sex and island size is not statistically significant. When island type (oceanic island, continental island or mainland) was selected as the categorical factor, the interaction of island type with island size was not statistically significant either. As expected based on the ANCOVA results, ANOVA testing to see whether removing the interaction significantly affects the fit of the regression demonstrated that neither interaction of sex nor interaction of island type (oceanic vs. continental) with island size affects the regression of estimated body size against island size for *M. fascicularis* (sex: F=0.6796, p=0.4106; island type: F=1.4655, p=0.2332).

Macaques: body size~island area * sex

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
Island area	1	0.023	0.23	4.136	0.0432 *
Sex	1	0.332	0.332	59.77	3.77E-13 ***
Island area*sex	1	0.0038	0.0038	0.68	0.4106
Residuals	220	1.2221	0.0056		

Macaques: body size~island area * island type

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
Island area	1	0.023	0.2298	3.291	0.071
Island type	2	0.154	0.0077	1.103	0.334
Island area*Island type	2	0.0205	0.0102	1.465	0.233
Residuals	218	1.5221	0.007		

Table 3.2. ANCOVA results testing the interaction effects of sex with island type and island size in *M. fascicularis*.

DISCUSSION.

These results suggest that the Island Rule does not apply to *M. fascicularis* and is thus not as prevalent in primates as reported by Bromham and Cardillo (2007) and Welch (2009). But the results do corroborate the results in Schillaci et al. (2009), which that there is no evidence of the Island Rule in this species. Meiri et al. (2004, 2008a, 2011) showed that the Island Rule applies only to certain taxa across mammals, and does not include primates, so these results are in line with theirs. Lomolino (2006) says that "Exceptions are not just possible but are expected…" for ecogeographical rules such as the Island Rule, and the results show that *M. fascicularis* is one of these exceptions.

Bromham and Cardillo (2007) and Welch (2009) reported that the Island Rule applies to primates, especially when skull length or body mass are used as indicators of body size. *M. fascicularis* was included in their analysis, comparing Simeulue and Malaysian mainland populations. The analyses, which use skull length as one component for body size estimation, indicate that the Island Rule is not pervasive amongst primates. The *M. fascicularis* that were analyzed do not display significantly smaller body sizes on islands. Rather, regressions of body size on island size have negative slopes, with females having a steeper slope than males, indicating that there is a trend for individuals on the mainland to have somewhat smaller body sizes than their insular counterparts. *M. fascicularis* is well above the expected optimal intermediate size for islands, so there is no expectation for insular gigantism in this species. Thus, there is no evidence of insular dwarfing within the species *M. fascicularis*, directly contradicting the findings reported in previous studies (Bromham and Cardillo, 2007; Welch, 2009).

There is a statistically significant difference between the regressions for males and females. I therefore recommend that analyses should be performed separately for each sex if a species is sexually dimorphic in body size, because one sex could display a difference in body size on islands in comparison to that on the mainland while the other does not. However, few studies thus far have taken sex differences in body size into account (Meiri et al., 2004; Itescu et al., 2014). Most studies use the mean body size for a single sex or even a mean value for an entire population or species that was previously published in literature, so those data do not capture the significant body size differences between sexes (Bromham and Cardillo, 2007; Lomolino, 2005; Lomolino et al., 2013; Meiri et al., 2006; Welch, 2009).

The conclusion from this study may differ from those of Bromham and Cardillo (2007) and Welch (2009) because I did not average the data for each population nor pair populations that are not the closest mainland and insular relatives. For example, in their study, *M. fascicularis* from Simeulue was paired with *M. fascicularis* from mainland Malaysia. Based on Chapter 2, these two populations are very distantly related, with a separation of approximately 2.26 mya. It might be thought that Sumatra should be considered the mainland for Simeulue because it is the nearest continental shelf island. However, the intraspecific phylogeny in Chapter 2 shows that the closest continental island relatives of the specimens from Simeulue are actually from Java, not Sumatra. So, if the aim is to pair mainland and insular populations, it is important to determine phylogenetic relationships at the intraspecific level in order to properly match populations.

Appropriate analysis of the *M. fascicularis* data indicated that correction for phylogenetic non-independence is not necessary in this particular case. However, having an intraspecific phylogeny at hand can be extremely useful for interpreting the results in ways other than controlling for phylogenetic inertia. It can, for instance, be used as a tool to determine directionality of migration to or from islands, and this can be applied in various ways. Notably, in the *M. fascicularis* regression, individuals from Borneo and Sumatra appear to have smaller body sizes than individuals from the mainland or smaller islands. This is particularly interesting because substantial dwarfing in body size would not be expected to occur on islands of such large size on the basis of current theory. Meiri et al. (2008b) showed that mammals from Borneo tend to have smaller body sizes than those on the mainland or on other islands. They suggest this may be because the soil on Borneo is much less fertile than soils on the mainland and other Sunda Shelf islands (MacKinnon et al., 1996), resulting in decreased forest productivity and thus organismal biomass on Borneo (Waterman et al., 1988). For example, *Presbytis* monkeys have been shown to have lower biomass on Borneo than on the mainland, Sumatra, or Java. The less fertile Bornean soils may account for the smaller body size in *M. fascicularis* on this giant island, but it does not explain why macaques are also smaller on Sumatra, which has rich volcanic soil. The intraspecific phylogeny from Chapter 2 may provide some clarification here. That phylogeny shows that southern Sumatran lineages of *M. fascicularis* diverged from a Bornean stock approximately 0.65 mya. The sample of Sumatran *M. fascicularis* in my dataset includes 17 individuals from the south and only two individuals from the north. With so many individuals stemming from Bornean lineages with small body sizes, this may possibly explain why the Sumatran specimens in this study also have small body sizes. The two northern Sumatran specimens are intermediate in body size compared to the individuals from southern Sumatra, so a larger sample size for northern Sumatran *M. fascicularis* individuals would be needed to establish whether or not the small body sizes of Sumatran *M. fascicularis* is a result of their descent from small-bodied Bornean lineages.

Most studies follow the convention established by Lomolino (1985) of testing for gigantism or dwarfism in insular mammals by analyzing the slope in a plot of the ratio of insular body size to mainland body size against mainland body size. As noted in the introduction, this may lead to problematic results because the determination of the nearest mainland population may be incorrect and the mean body size for each population (or species) does not capture all of the body size variation in that particular population. For this study, using the method of pairing an insular population or individual with a mainland population or individual is impossible because of the complex geographic history of Southeast Asia. As a result of the extensive glaciation periods (Delson, 1980), it is unclear which region should be considered the mainland. When sea levels were high, what is currently consider as mainland Asia can be designated as the mainland, but when sea levels were low the continental islands in Indonesia were connected to the mainland, so should they be designated as the mainland instead? Additionally, which island should be considered the "mainland" when examining a region such as the Philippines?

Isolation time of the long-tailed macaques on Southeast Asian islands is an important factor that might potentially explain why there does not appear to be significant dwarfing in body size in this species. Time of isolation on islands is rarely studied, but an analysis of dwarfed mammoths from the Channel Islands show that they had been isolated for at least 40,000 years (Agenbroad, 1998), demonstrating that this period of time is sufficient for body size evolution to occur. Although no minimum isolation time has been shown to be necessary for insular body size changes, Millien (2006) first showed that evolutionary rates of morphological change (reduction and enlargement) in insular mammals can be up to three times faster than the rates for mammalian populations on the mainland. Evans et al. (2012) subsequently calculated clade maximum rate (CMR; a measure of maximum body mass increases or decreases in each clade) and found that rates of body size reduction can be more than ten times that of body size enlargement and up to 30 times greater on islands. Evans et al. (2012) and Uyeda et al. (2011) also noted that fast rates of evolutionary change are continuous over short bursts of time; the rates and their direction likely vary over long periods. Based on the dated intraspecific phylogeny in Chapter 2, the insular clade of *M. fascicularis* split from the mainland clade approximately 2.3 mya, and *M. fascicularis* had certainly began colonizing Southeast Asian islands by at least 1.23 mya. This time of isolation is more than enough time for insular dwarfing to occur, considering that the process occurs at a fast rate and in a relatively short period of evolutionary time. However, it is possible that there were a number of gene flow events throughout the numerous glaciation periods during the Pleistocene, and such exchange could perhaps have eliminated evidence of body size dwarfing on islands. The phylogeny in Chapter 2 is based solely on mitogenomes, and nuclear genes would be necessary to determine the extent of gene flow between insular and mainland populations.

The intraspecific phylogeny I used in the study (Chapter 2) reveals a complicated biogeographical history for *M. fascicularis* in the region. This more complete phylogeny clearly shows that the populations on the mainland and the islands are not all monophyletic as indicated by previous studies (Liedigk et al., 2015; Tosi and Coke, 2007). In fact, one of the major conclusions in Chapter 2 is that there is a deep division between northern and southern Sumatran lineages, with northern Sumatran lineages stemming from mainland Asia and southern Sumatran lineages stemming from a Borneo stock. If it is assumed that Sumatra can be regarded as the mainland in this case, without an intraspecific phylogeny at hand it would be unclear whether a population from northern or southern Sumatra should be selected to pair with a given insular population. This major split in Sumatran lineages not only re-emphasizes the problem of selecting a mainland counterpart to insular populations but also highlights the problem of assuming monophyly for each island when averaging body sizes for specimens from a single island or even from the mainland. Investigators should therefore be much more cautious and rigorous in pairing insular populations with mainland populations. When the underlying relationship is unclear, different methods should be used to study insular body size processes.

CONCLUSION.

My analyses show that insular dwarfing does not apply to *M. fascicularis*, and the Island Rule may not apply as to primates generally as previously thought. Instead of using the conventional short-cut method for testing the Island Rule by pairing insular and mainland populations for many species of mammals, I analyzed the data for *M. fascicularis* by regressing estimated body size against island area. This method bypasses the issues of averaging data for each population and properly determining which population should be considered the closest geographical mainland population. Additionally, it is also important to separate the data by sex, especially when it is known that the species concerned is sexually dimorphic in body size, because the Island Rule may apply differently to males and females. This study is unique in that I present a large dataset for a single species and test for the necessity to control for intraspecific relationships. The analyses indicated that it is not imperative to correct for phylogenetic non-independence in this particular case, but this does not mean that future studies will not need to do so. I suggest that future studies conduct their analyses using raw data and test whether phylogenetic control is necessary. Moreover, having an intraspecific phylogeny available for the species in question would yield additional benefits for answering questions that result from the analyses or for determining island-mainland or island-island relationships between populations.

CHAPTER 4

Evolution of brain size in long-tailed macaques on Southeast Asian islands

INTRODUCTION.

The brain is a complex organ that varies in size relative to body size amongst different species of mammals. Jerison (1973) demonstrated that in the class Mammalia brains have increased in size over time, confirming Marsh's Law (Marsh, 1874), which recognized a general trend toward larger brain size and more physically complex brains over evolutionary time in the fossil record. Recent analyses of relative brain size in living and fossil mammals have confirmed Jerison's findings for primates (Martin, 1990), cetaceans (Marino *et al.*, 2004), carnivores (Finarelli & Flynn, 2007), bats (Yao et al., 2012), and early mammals (Rowe et al., 2011). However, there has also been debate about the possibility of *decrease* in both absolute and relative brain size over time on islands ever since the skeletal remains attributed to *Homo floresiensis* were first reported (Brown et al., 2004).

The remains attributed to *Homo floresiensis* were discovered in Late Pleistocene deposits in a cave, Liang Bua, on the island of Flores, Indonesia. LB1, the most complete specimen, is unique in that it is an adult with very short stature and has by far the smallest absolute and relative endocranial volume ever reported for *Homo* (Brown et al., 2004). This remains true despite a recently revised analysis that pushed the dating of the specimens back from 18 kyr (35-14 kyr) to 100-60 kyr ago (Sutikna et al., 2016). The endocranial volume of *H. floresiensis,* known exclusively from the single fairly complete skeleton LB1, is ~400 cc (Brown et al., 2004; Falk et al., 2005; Kubo et al., 2013). This is less than a third of the size of the average ~1350 cc brain of

H. sapiens (De Miguel & Henneberg, 2001) and well below the size of the average ~980 cc brain of *H. erectus* (Falk et al*.,* 2005), which are the two other *Homo* species known from the region. This tiny absolute brain size is also especially small relative to estimated body size. The initial hypothesis that was proposed to explain the tiny brain of the LB1 specimen was dwarfing of a *H. erectus* population due to long-term isolation on the island of Flores (Brown et al., 2004). Insular dwarfing is one of the trends incorporated into the Island Rule, which states that on islands, large-bodied species evolve smaller body size (dwarfism) due to a combination of limited food resources and reduced predation while small-bodied species evolve larger body size (gigantism) due to reduced predation and competition (Foster, 1964; Van Valen, 1973; Lomolino, 1985). This evolutionary trend has been shown to apply among very closely related species or within species (Lomolino, 1985; Boback and Guyer, 2003; Meiri, 2007). Prior to the discovery of the LB1 skeleton, however, the Island Rule had rarely been applied to brain size, much less with reference to such an extremely small brain size as seen in LB1.

Since the discovery of the remains attributed to *H. floresiensis*, several researchers have sought examples of reduction in absolute or relative brain size in island-living birds (Boerner and Krüger, 2008), bats (Safi et al., 2005, Niven, 2005), bovids (Köhler and Moyà-Solà, 2004), hippopotamuses (Weston and Lister, 2009), primates (Montgomery et al., 2010), and elephants (Roth, 1992, Palombo, 2001). Before presenting the results of this study, I therefore outline the major claims to date and discuss problems inherent in these analyses, which raise questions regarding conclusions drawn for the case of *H. floresiensis*.

Köhler & Moyà-Solà (2004) demonstrated that *Myotragus*, an island-living extinct bovid, on Mallorca had an unusually small brain and associated sense organs compared to putatively related mainland species, with *Rupicapra* designated as the most closely related genus. The small brain size in *Myotragus* was attributed to reduction through adaptation for more efficient energy use in an insular ecosystem, an argument that was subsequently extended to explain the tiny brain of *H. floresiensis*. However, the inference that *Myotragus* is a dwarfed form is not supported by direct evidence. The fossil record for *Myotragus* extends back ~6 my, but there is no evidence for body size reduction over that time; all the fossils are uniformly small. The body size of the presumed common ancestor of *Myotragus* and *Rupicapra* is unknown, and there is no evidence of intraspecific reduction in body size or brain size. Moreover, *Myotragus* differs markedly from *Rupicapra* in skull morphology, with extreme differences in orbit size, orbit orientation, snout dimensions and mandibular morphology. Molecular evidence has yielded an explanation for this marked morphological difference, demonstrating that *Myotragus* is more closely related to sheep (*Ovis*) than to *Rupicrapra* (Lalueza-Fox et al., 2002). So the restricted comparison of *Myotragus* and *Rupicapra* as an example for brain size dwarfing is inappropriate. Furthermore, when the smallest group of Cretan cervids, *Candiacervus*, were analyzed, the results showed that in this case reduction in brain size on islands is by no means as extreme as concluded in the study on *Myotragus* (Palombo et al., 2008).

A separate study by Weston & Lister (2009) examined hippopotamuses, comparing the two African mainland species (the common hippopotamus *Hippopotamus amphibius* and the pygmy hippopotamus from Liberia, *Choeropsis liberiensis*) to two extinct small-bodied island species discovered on Madagascar (*H. madagascarensis* and *H. lemerlei*; both known only as subfossils). *H. amphibius* is though to be the probable ancestor of the two recently extinct insular species, *H. madagascarensis* and *H. lemerlei* (Stuenes, 1989). Together, these three species of the genus *Hippopotamus* form a monophyletic clade. The actual time of dispersal to Madagascar is unknown, but the oldest fossil evidence — giving a minimum age — dates to the Quaternary Period (Burney et al., 2004; Mahé, 1972; Stuenes, 1989). *C. liberiensis* is more distantly related to *Hippopotamus* species, having diverged over 5 mya (Boisserie, 2005). The study by Weston & Lister seemingly provided an empirical demonstration that it is possible for dwarfed insular mammals to evolve smaller brains. After controlling for body size, the average cranial size of *H. amphibius* was reportedly greater than that of the insular species. However, the underlying assumption that the mainland *H. amphibius* has the ancestral body size remains untested. Madagascar hippos have simply been assumed to be dwarf forms that had been subject to body size reduction, but it is possible that their ancestors were similarly small. This is a definite possibility, given that the earliest known hippopotamid from ~21 mya, *Morotochoerus ugandensis*, is small-bodied (Orliac et al., 2010). It is accordingly possible that some hippo species, such as those on Madagascar, stayed small while *H. amphibius* evolved large body size. More importantly, however, the analysis of relative brain size conducted by Weston & Lister used data from both adults and subadults of the mainland *H. amphibius*. Inclusion of immature specimens in studies of relative brain size is inappropriate. It is well known that in mammals brain growth is completed early, well before maturity, so subadults will have unusually large brain sizes for their body size. Removal of all subadults from the plot effectively eliminates the reported difference in relative brain size between *Hippopotamus amphibius* and the two species on Madagascar.

A third study that refers directly to *H. floresiensis* but had no connection to islands was conducted by Safi et al. (2005), who argued that the evolution of relative brain size in certain bat lineages involved reduction rather than enlargement. However, no attempt was made to test for brain size change with reference to the fossil record, a prerequisite for reliable determination of an evolutionary trend. Moreover, the analytical procedure used by Safi et al. was not appropriate to test directionality of evolution in this context (Martin & Isler, 2010). In a study of six fossil endocasts from bats of the genus *Hipposideros* from the Oligocene and Miocene epochs (20-35 mya), Yao et al. (2012) generated 3-dimensional reconstructions and showed that relative brain size in comparison to extant bats yielded no evidence for brain size decrease. Yet the findings reported by Safi et al. had suggested brain size reduction in the family Hipposideridae. Furthermore, a phylogenetic analysis conducted by Yao et al. indicated instead that in *Hipposideros* relative brain sizes have *increased* significantly over time, fitting the general trend that Marsh identified for fossil mammals (Yao et al., 2012).

Contrary to these studies reporting brain size decrease on islands, Palombo (2001) inferred an *increase* in the relative brain size for a small-bodied, fossil elephant, *Elephas falconeri* on the Mediterranean island of Sicily, derived by dwarfing from mainland *Elephas antiquus*. She proposed that the relative brain size is not smaller because the brain cannot function below a critical volume.

The above studies focus on various insular mammal taxa to examine the possibility that dwarfing in brain size could take place on islands. But any attempt to explain the small brain size of *H. floresiensis* really requires consideration of patterns in insular primate taxa. Two studies on primate body sizes in relation to the Island Rule (Bromham and Cardillo, 2007; Welch, 2009) reported that primates have smaller body sizes on islands in comparison to their mainland counterparts, thus implying that the small body size of *H. floresiensis* might be explained by insular dwarfing. However, Chapter 3 has provided evidence that the Island Rule is not universal among primates when looking within species. Therefore, without a clear phylogenetic placement for *H. floresiensis* in the hominin phylogeny, there is no convincing indication that the body size of *H. floresiensis* is a result of insular dwarfing. To date, no study has examined brain size evolution in insular primates specifically, although Montgomery et al. (2010), using ancestral-state reconstructions, found a general trend for primate brains to increase over evolutionary time. They showed that both absolute and relative brain size typically increase in primates, although there are specific exceptions along a number of branches, including those leading to *Microcebus, Callithrix, Cercocebus, Mandrillus,* and *Gorilla*. They inferred that — based on the patterns of brain size decrease in those specific branches of the primate phylogeny and depending on the body mass and phylogenetic status of *H. floresiensis* — there are scenarios in which it is possible that the small brain of the LB1 specimen was a result of evolutionary brain size decrease. However, the study by Montgomery et al. does not distinguish between insular and non-insular primate lineages.

Here, I evaluate relative brain size within a primate species, the long-tailed macaque (*Macaca fascicularis*), which is widespread throughout the Southeast Asian region and hence provides a directly relevant test case. It is important to note that few studies of the Island Rule and insular brain size patterns take phylogenetic non-independence into account, and have failed to consider whether phylogenetic comparative methods may lead to a discordance in results as in the case of body size (Meiri et al., 2008a; Lomolino, 1985, 2005; Lomolino et al., 2013). Because the Island Rule applies either to species that are closely related or to relationships within species (Lomolino, 1985; Boback and Guyer, 2003; Meiri, 2007), I accordingly conducted the analyses using both raw data and intraspecific phylogenetically corrected data.

MATERIALS/METHODS.

Southeast Asia was chosen for the location of this study for two major reasons (Figure 4.1). First, there is a dense array of islands of various sizes, ranging from <1 km^2 to >700,000 km^2. Having an extensive spectrum of island areas is advantageous because it allows us to take island size into account and sample multiple specimens from separate islands of similar sizes. The Southeast Asian region also encompasses islands of various types, notably both continental and oceanic islands. Continental islands lie on a continental shelf, so they were connected to the mainland when sea levels were low during glaciation periods, whereas oceanic islands are those that had never been connected to the mainland (Gillespie and Clague, 2009). The continental islands in Southeast Asia are those to the west of the Wallace-Huxley Line, which includes Palawan but excludes the rest of the Philippines to the north, runs between Borneo and Sulawesi, and separates Bali from Lombok to the south. The oceanic islands in the region lie to the east of this line. Having both types of islands allows us to be able to distinguish any patterns due to natural colonization of islands in the case of continental islands in comparison to patterns due to human-mediated dispersal to islands, which is hypothesized to be the case for oceanic islands (Heaney et al., 2016). Moreover, Southeast Asia is where the conundrum surrounding *H. floresiensis* first arose, so studying this region will provide information regarding general patterns that occurred with evolution of brain size in this specific region. Cranial specimens of *Macaca fascicularis* were chosen for sampling because the species is a relatively large-bodied representative of the order Primates and is widespread throughout Southeast Asia.

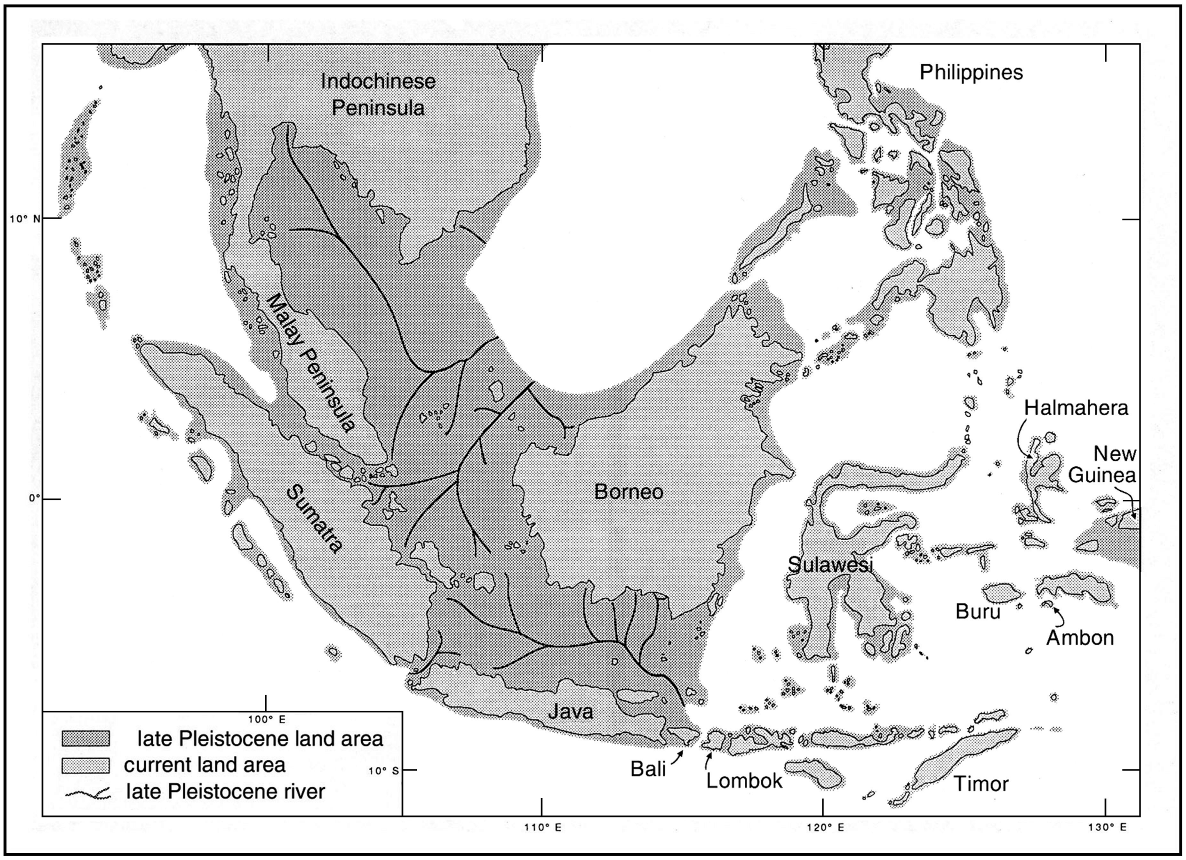

Figure 4.1. Map of Southeast Asia from Heaney (1991) indicating exposure of the continental shelf above sea-level during Pleistocene periods.

Data collection. I examined and measured adult skulls of extant *Macaca fascicularis*. The specimens measured are housed at the Field Museum of Natural History in Chicago, the U.S. National Museum of Natural History in Washington D.C., the American Museum of Natural History in New York, the Raffles Museum of Biodiversity Research (now the Lee Kong Chian Natural History Museum) in Singapore, and the Naturalis Biodiversity Center in Leiden. I took both linear and volumetric skull measurements from as many available adult specimens of each sex as were available. Adult status was determined by the presence of complete eruption of the third molars.

I measured skull length and skull width using digital calipers. Skull length represents the maximum length measured from the most anterior point of the premaxilla to the posterior-most point of the cranium. Skull width was measured between the widest points on the zygomatic arches. The product of skull length and width has been shown to be a suitable predictor of body mass (e.g. Martin, 1990) and is needed as a proxy to estimate for body mass because body size or postcranial data are usually not available for museum specimens. I collected volumetric data from the endocranial cavities of skulls by filling them with polypropylene beads and measuring the volume with measuring cylinders. In order to correct for phylogenetic non-independence, I used the intraspecific phylogeny from Chapter 2.

Statistical analyses. I used both ordinary least squares (OLS) and reduced major axis (RMA) regressions with log-transformed data to examine brain size in relation to estimated body size (skull length x skull width) using the statistical program R (R Development Core Team, 2010). Because both the values for both endocranial volume and estimated body size include error, it has been argued that RMA is be more appropriate than OLS because it allows for error in both the X and Y variables (Harvey and Pagel, 1991; Sokal and Rohlf, 1995). However, other studies have argued that, because RMA does not distinguish between biological and observational error and the model accounts only for observational error, it should not be used to estimate allometric regressions (Smith, 2009; Hansen and Bartoszek, 2012). As interpretations differ, I report here results for both OLS and RMA. Regression analyses were conducted after separating the data by sex to account for sexual dimorphism, and by island type (oceanic or continental) compared to the mainland.

To test and correct for non-independence in the regressions, I utilized an intraspecific phylogeny that includes 93 of the *M. fascicularis* individuals that I measured in this study (Chapter 2). I used R to perform the phylogenetic regressions using a generalized least squares approach (GLS). These analyses account for intraspecific phylogenetic effects by estimating Pagel's λ (Pagel, 1999), which is a probabilistic measure of phylogenetic signal that uses Brownian motion to approximate shared evolutionary history reflected in trait correlations. Because Hansen and Bartoszek (2012) warn against using phylogenetic corrections with RMA, I applied phylogenetic analyses only to the OLS regressions.

In order to test whether sex or island type influences the relationship between brain size and body size in *M. fascicularis*, I tested for differences in the intercepts and slopes based on each categorical factor in R. I then regressed the residuals from the endocranial volume against estimated body size regression on log transformed island size data to establish whether there are any significant trends in relative brain size against island size. I set the mainland size to an arbitrarily large area, 10 million km^2, which greatly exceeds the size of any island in Southeast Asia. These analyses were performed with both raw data and phylogenetically corrected data.

RESULTS.

The raw data analyses included a total of 221 specimens of *M. fascicularis.* This dataset included 81 female individuals and 140 male individuals. 202 of those specimens are from 21 different islands through Southeast Asia (n=95 from continental islands, n=107 from oceanic islands). 19 specimens are from the mainland. The phylogenetic data include a total of 93 specimens of *M. fascicularis*, including 58 males and 34 females. All 21 islands and mainland areas represented by the raw data are included in the reduced sample of phylogenetic data (n=45 from continental islands, n=39 from oceanic islands, n=9 from the mainland). All data will be available upon publication, which will most likely be in 2018. Before then, the data will be available upon request from Lu Yao or Robert D. Martin.

Phylogenetic analysis of the data for 93 specimens of *M. fascicularis* yielded a value of λ=0.437 for OLS, indicating that there is a phylogenetic signal within this species. Therefore, it is necessary to conduct the analyses for this species by controlling for phylogenetic relatedness. So results using both raw data and phylogenetically corrected data are presented.

Table 4.1a. Regression results separated by sex

						Ordinary Least Squares Regression		Reduced Major Axis Regression	
	Categories	**n**	**r**	**p**	**λ**	**Intercept**	**Slope**	**Intercept**	**Slope**
Raw	**Male**	140	0.577	9.069E-14	-	1.176	0.331	0.702	0.574
	Female	81	0.588	7.608E-09	-	1.167	0.337	0.726	0.573
GLS	**Male**	58	0.577	9.069E-14	0.3769	1.400	0.217	-	-
	Female	34	0.588	7.608E-09	0.4336	1.198	0.332	-	-

Table 4.1b. Regression results separated by island type

						Ordinary Least Square Regression		Reduced Major Axis Regression	
	Categories	**n**	**r**	**p**	**λ**	**Intercept**	**Slope**	**Intercept**	**Slope**
Raw	**Continental**	108	0.690	2.220E-16	-	1.127	0.360	0.807	0.526
	Oceanic	95	0.656	5.196E-13	-	1.134	0.351	0.780	0.534
	Mainland	18	0.078	0.751	-	1.700	0.062	0.771	0.543
GLS	**Continental**	45	0.690	2.220E-16	0.3474	1.316	0.258	-	-
	Oceanic	39	0.656	5.196E-13	0.4223	1.140	0.347	-	-
	Mainland	9	0.078	0.751	0.5727	1.780	0.026	-	-

Table 4.1. OLS and RMA regression results of log(endocranial volume) against log(skull length x skull width) for data from museum specimens of *M. fascicularis*. a: separated by sex; b: separated by island type.

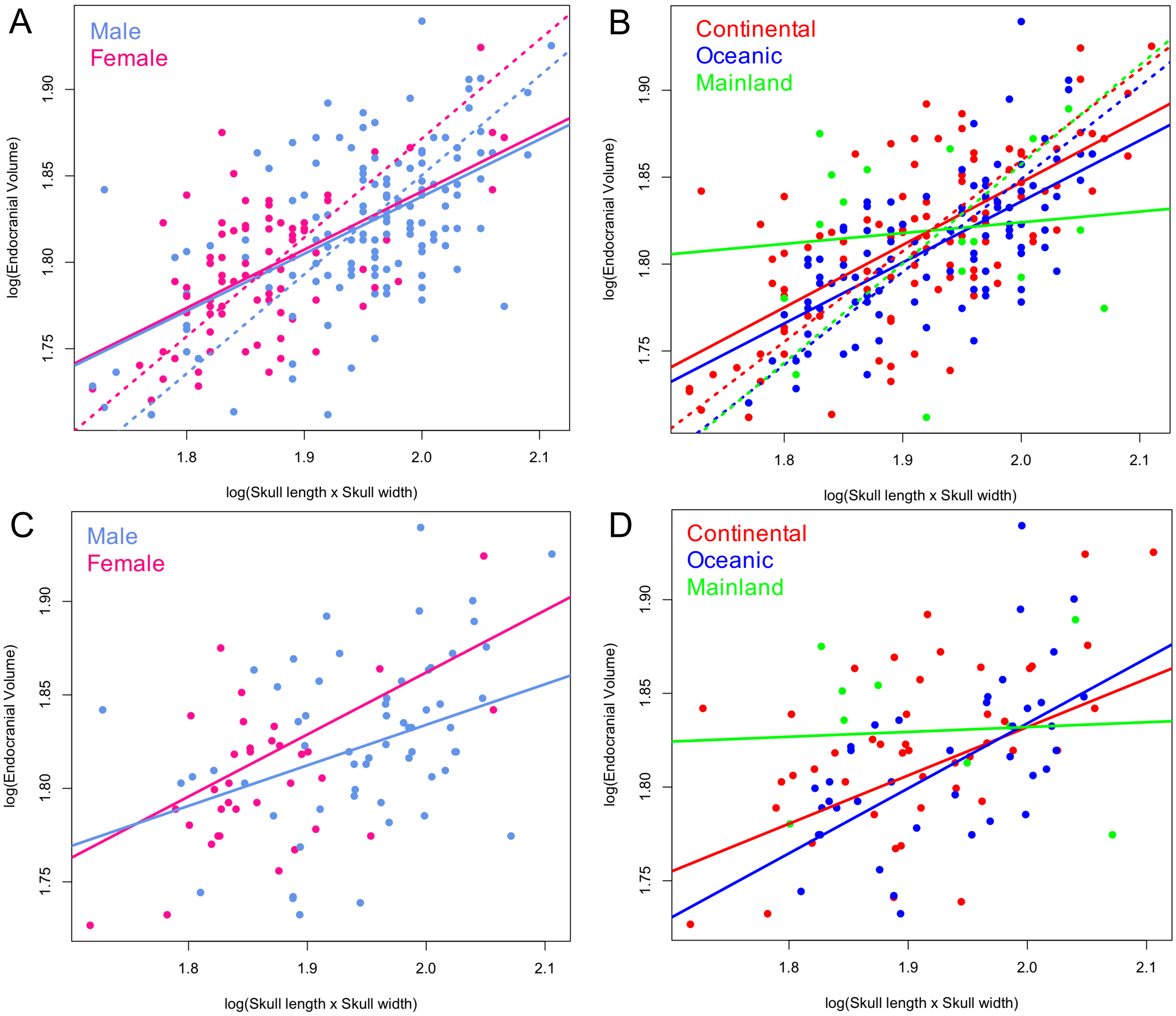

Figure 4.2. Log(endocranial volume) regressed against log(skull length x skull width) of museum specimens of *M. fascicularis*. A and B represent raw data regressions (male, n=140; female, n=81; continental island specimens, n=107; oceanic island specimens, n=95; mainland specimens, n=18) and C and D represent phylogenetically corrected OLS regressions (male, n=58; female, n=34; continental island specimens, n=45; oceanic island specimens, n=39; mainland specimens, n=9). A: Regressions separated by sex with solid lines representing OLS regressions and dotted lines representing RMA regressions; B: Regressions separated by island type with solid lines representing OLS regressions and dotted lines representing RMA regressions; C: phylogenetic regression separated by sex; D: phylogenetic regression separated by island type.

Slopes and intercepts for the OLS and RMA regressions using both raw data and phylogenetically corrected data for *M. fascicularis* are shown in Table 4.1. The correlation coefficients indicate that endocranial volume and estimated body size have a statistically significant relationship for each group except for the mainland macaques. The regressions separating the data by sex and by island type (Figure 4.2) do not visibly reveal that any specific group of individuals has smaller relative brain sizes. With OLS regression using raw data, endocranial volume scales to estimated body size similarly for all categorical variables except in the case of the mainland macaques. In other words, mainland specimens have quite similar absolute brain sizes and differ primarily through variation in body size. The phylogenetically corrected results show that, at large body sizes, males tend to have smaller relative brain sizes than females. When separated by island type, there are no major differences in the regressions. I also plotted absolute brain size against the intraspecific phylogeny, and it is clear that there is no visible pattern in brain size according to the phylogeny (Figure 4.3).

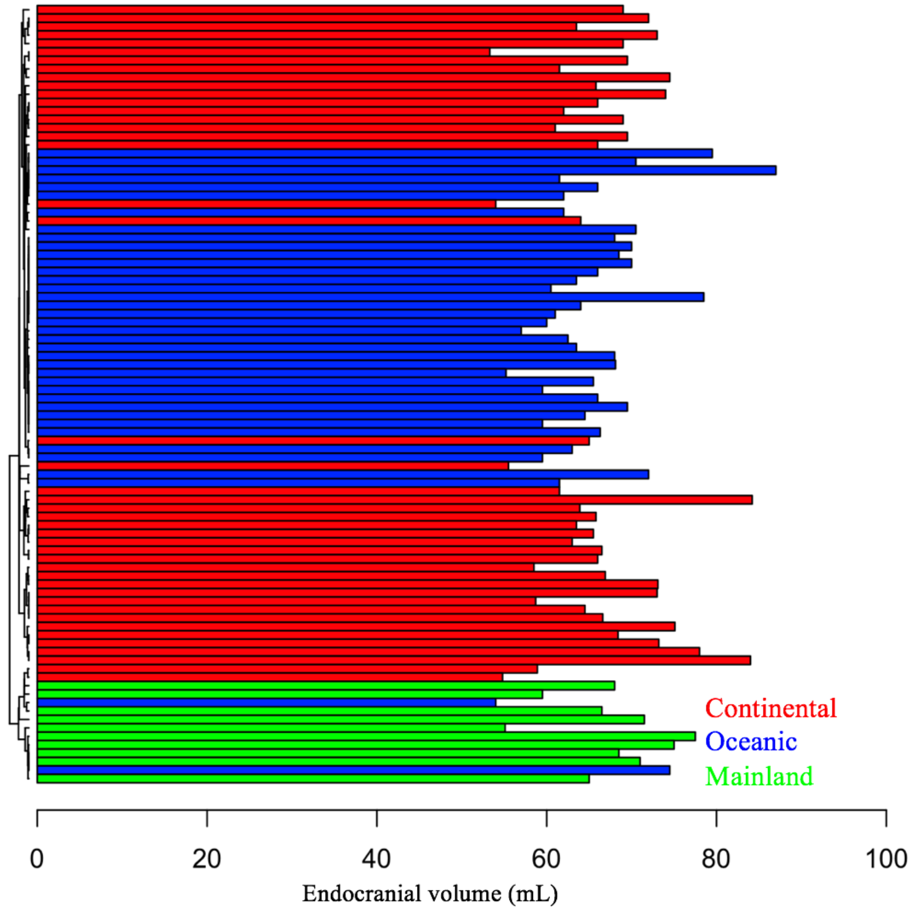

Figure 4.3. Endocranial volume (mL) plotted against the intraspecific phylogeny of *M. fascicularis.* Mainland specimens are indicated in green, specimens from continental islands are indicated in red, and specimens from oceanic islands are indicated in blue.

Testing whether the slopes and intercepts differed between sexes and amongst island types, I found only two instances of significant differences using raw data (Table 4.2). When an OLS regression is used, there are statistically significant differences in slope depending on island type, and when a RMA regression is used males and females show a significant difference in intercepts. The former finding is expected because the mainland macaques are the only group with a slope close to zero in the OLS regression. The difference in intercepts in the RMA regression indicates that there is a significant difference in relative brain size for males and females. Female macaques have larger relative brain sizes than male macaques. However, the OLS regression shows no difference between males and females in the scaling of endocranial volumes to estimated body size.

	OLS		**RMA**	
Categorical Factor	**Intercept**	**Slope**	**Intercept**	**Slope**
Sex	0.701	0.930	0.0003***	0.982
Island type	0.227	0.013*	0.068	0.926

Table 4.2. P-values testing for a common intercept and/or slope for sex and for island type using raw data.

The OLS regression of brain size residuals from the regression analyses against island size (Figure 4.4 and 4.5, Table 4.3) indicates that all slopes are close to zero. Nonetheless, when the RMA residuals are used the slopes are statistically significantly different from zero for female macaques and for macaques of both sexes from continental and oceanic islands. When the OLS residuals are used, continental island macaques do not have statistically significant smaller relative brain sizes on islands. In other words, all female individuals and island-dwelling individuals of both sexes have slightly smaller relative brain sizes on smaller islands. However, values for the coefficient of determination (r^2) for relative brain size against island size are extremely low, indicating that only a very small fraction of the variance in relative endocranical volume can be "explained" by island size.

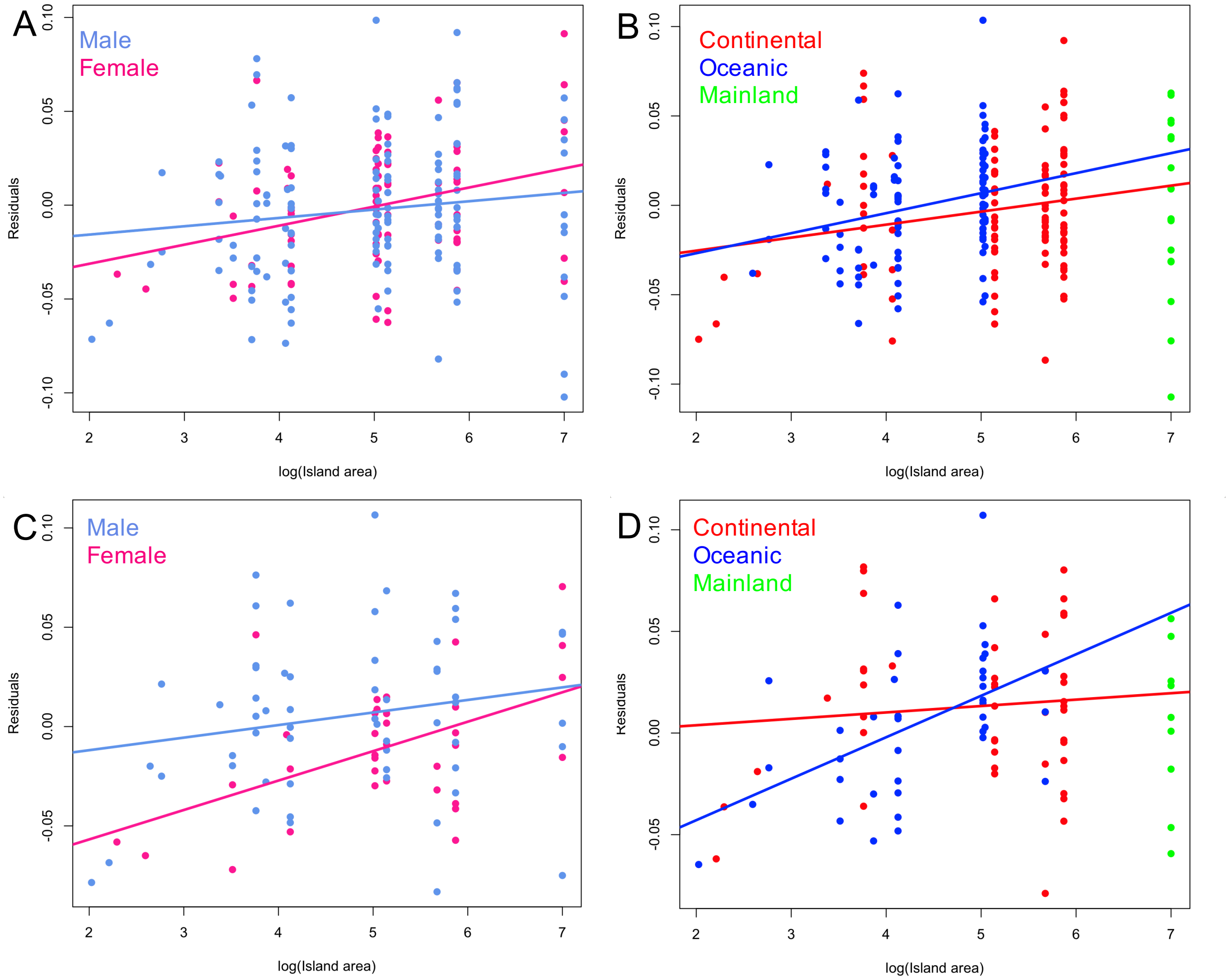

Figure 4.4. Ordinary least squares regression of log(endocranial volume) – log(skull length x skull width) OLS residuals against log(island size) for data from museum specimens of *M. fascicularis*. A and B show the results using raw data to calculate the residuals while C and D display the results using phylogenetically corrected data to calculate the residuals A: separated by sex (male, n=140; female, n=81); B: separated by island type (continental island specimens, n=108; oceanic island specimens, n=95; mainland specimens, n=19); C: separated by sex (male, n=58; female, n=34); D: separated by island type (continental island specimens, n=45; oceanic island specimens, n=39; mainland specimens, n=9).

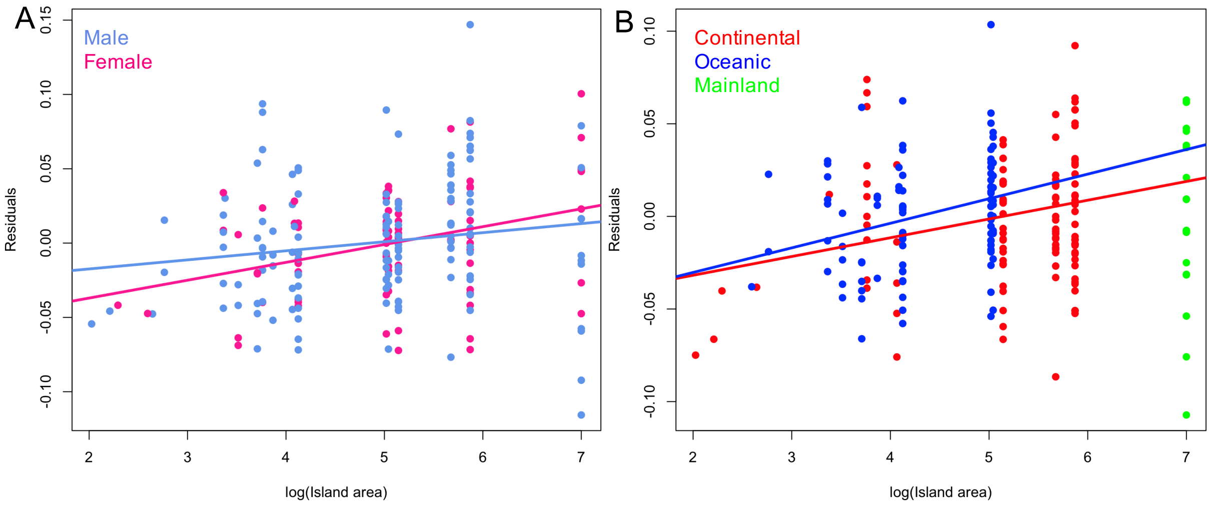

Figure 4.5. Ordinary least squares regression of log(endocranial volume) – log(skull length x skull width) RMA residuals against log(island size) for data from *M. fascicularis*. A: separated by sex (male, n=140; female, n=81); B: separated by island type (continental island specimens, n=108; oceanic island specimens, n=95; mainland specimens, n=19).

Table 4.3a. OLS residuals using raw data

		n	r^2	p	Intercept	Slope	Slope t-value	p (t-value)
Sex	**Male**	140	0.017	0.1208	-0.025	0.004	1.561	0.121
	Female	81	0.102	0.004**	-0.052	0.01	2.997	0.004**
Island type	**Continental**	108	0.035	0.051	-0.040	0.007	1.972	0.051
	Oceanic	95	0.063	0.015*	-0.049	0.011	2.490	0.015*

Table 4.3b. OLS residuals using phylogenetically corrected data

		n	r^2	p	Intercept	Slope	Slope t-value	p (t-value)
Sex	**Male**	58	0.037	0.151	-0.025	0.006	1.457	0.151
	Female	34	0.263	0.002**	-0.086	0.015	3.383	0.002**
Island type	**Continental**	45	0.008	0.556	-0.003	0.003	0.593	0.556
	Oceanic	39	0.064	0.008**	-0.084	0.02	3.582	0.001**

Table 4.3c. RMA residuals using raw data

		n	r^2	p	Intercept	Slope	Slope t-value	p (t-value)
Sex	**Male**	140	0.026	0.058	-0.030	0.006	1.912	0.058
	Female	81	0.113	0.002**	-0.061	0.012	3.173	0.002**
Island type	**Continental**	108	0.058	0.012*	-0.052	0.01	2.55	0.012*
	Oceanic	95	0.073	0.008**	-0.057	0.013	2.702	0.008**

Table 4.3. Ordinary least squares results for the regression of residuals of log(endocranial volume) on log(skull length x skull width) against log(island area) for data from *M. fascicularis*. Mainland area is considered to be 10 million km2. A: OLS residuals using raw data; B: OLS residuals using phylogenetically corrected data; C: RMA residuals using raw data.

Additionally, it seemed that specimens from the smallest islands might be driving the regression results, so I repeated these analyses after removing the specimens from islands smaller than 1,000 km^2 (Figure 4.6). This caused the statistical significance to decrease for all regressions, and for the residuals calculated using an RMA regression, continental island macaques no longer displayed a statistically significant smaller relative brain size. It may therefore be concluded that there is a limited degree of insular dwarfing in relative brain size. However, this is predominantly evident for macaques living on islands smaller than 1,000 km^2, as far as can be determined from the limited number of specimens from small islands in the dataset.

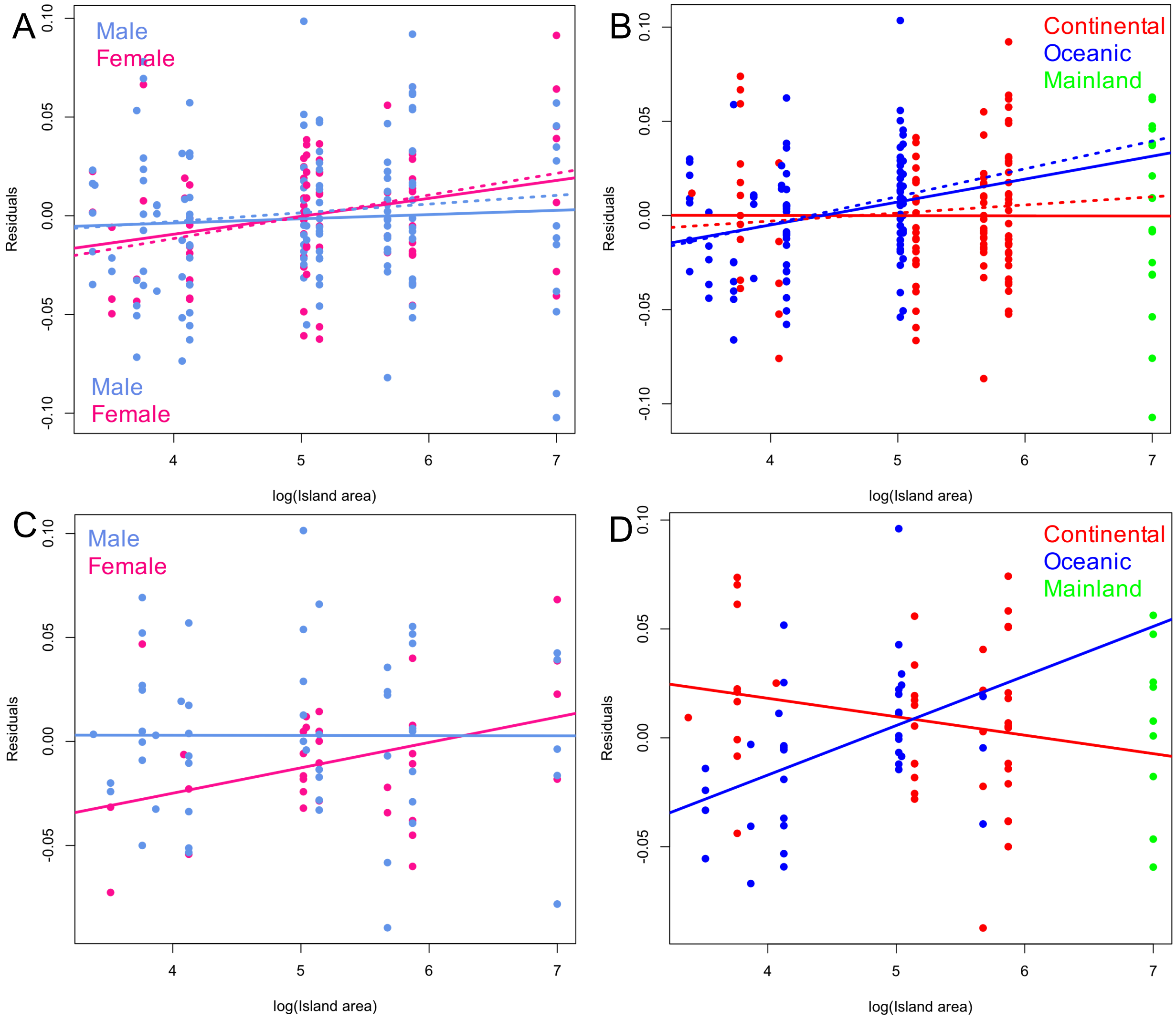

Figure 4.6. Continued on Page 77.

Figure 4.6 (continued). Ordinary least squares regression of log(endocranial volume) –log(skull length x skull width) residuals against log(island size) with *M. fascicularis* specimens from islands smaller than 1,000 km^2 removed. A and B show results using raw data while E and F show results using phylogenetically corrected data. All solid regression lines are based on OLS analyses and all dotted regression lines are based on RMA analyses. A: separated by sex (male, n=135; female, n=79); B: separated by island type (continental island specimens, n=104; oceanic island specimens, n=92; mainland specimens, n=19); C: separated by sex (male, n=53; female, n=32); D: separated by island type (continental island specimens, n=42; oceanic island specimens, n=35; mainland specimens, n=9).

DISCUSSION.

As expected, macaques with larger body sizes tend to have larger absolute brain sizes. In Figure 4.2A, a plot of estimated brain size (endocranial volume) against the proxy measure of body size (skull length x width), in which males and females are distinguished, yields different results, depending on whether ordinary least squares regression (OLS) or reduced major axis (RMA) is taken as the best-fit line. With OLS, brain size seemingly scales with body size in a very similar way in males and females, with no detectable difference between them. By contrast, analysis with RMA reveals a statistically significant difference, with females tending to have larger brains than males at any given body size. The RMA slope values for males and females are virtually identical, and the line for females shows an upward grade shift relative to males. In short, RMA analyses indicate that females have relatively larger brains than males, whereas OLS analyses do not. However, with respect to scaling of brain size in relation to island type (Figure 4.2B), OLS and RMA agree in indicating that there is no overall distinction. The only difference between the two kinds of analysis is that there is no difference across the board with RMA, whereas OLS unexpectedly indicates that brain size does not scale to body size on the mainland. The difference in the OLS and RMA regressions for mainland macaques may be due to a combination of a small sample size and extensive variation in the brain and body sizes of mainland macaques. It is really necessary to sample more individuals from the mainland in order to fit a better regression for mainland macaques. The results were different yet again following correction for phylogenetic relatedness, indicating that female long-tailed macaques have marginally smaller relative brain size than males, while certain groups of macaques tend to have slightly smaller relative brain sizes on small islands. So results vary depending on the statistical regression technique applied to the raw data and whether or not phylogenetic correction is used.

As discussed briefly in the methods section, several studies have questioned the appropriateness of using RMA instead of OLS (Smith, 2009; Hansen and Bartoszek, 2012). On the other hand, it can be argued that in some cases, as when regressing endocranial volume on body size, it is necessary to allow for error in both variables as neither is measured without error. The long-running debate about the suitability of different approaches to determination of best-fit lines is still unresolved, so I report both OLS and RMA values here to permit direct comparison. As already emphasized in discussing Figure 4.2 and Tables 4.1 and 4.2, these two regression methods can yield different results in certain respects.

Felsenstein's foundational 1985 paper emphasized the importance of allowing for phylogenetic non-independence in comparative studies between species, and later studies went a step further to discuss the importance of accounting for phylogenetic relatedness in intraspecific comparisons (Ives et al., 2007; Felsenstein, 2008; Stone et al., 2011). As yet, however, very few studies have applied phylogenetic comparative methods to studies regarding the Island Rule (Meiri et al., 2006, 2008a, 2011; Bromham and Cardillo, 2007; Welch, 2009), and even fewer have applied any correction for phylogenetic relatedness intraspecifically (Itescu et al., 2014; Chapter 3). As I have demonstrated in this study of brain size in *Macaca fascicularis*, there is a detectable phylogenetic signal in the data, and different results are obtained, depending on whether intraspecific phylogenetic correction is applied to the data or not. When regressing endocranial volume on estimated body size, a difference in results is particularly apparent when analyzing males and females using OLS regressions. The raw data shows that the intercepts and slopes are not significantly different between the two sexes, but the phylogenetically corrected regressions show that for larger-bodied macaques, males have smaller relative brain sizes than females. Additionally, when residuals for relative brain size are examined in relation to island size, regressions of OLS residuals against island size for female macaques and macaques of both sexes on oceanic islands were statistically significant using raw data. The slopes of the regressions for these three groups of macaques were also statistically significant from zero. When I corrected for phylogenetic non-independence, the significance of these regressions increased.

I urge caution in the interpretation of these statistical results. Although the regressions of relative brain size on island size are statistically significant for some groups of macaques, the r^2 values are extremely low, indicating that very little of the variance in residuals of endocranial volume against estimated body size can be explained by island size. The low r^2 values are most likely a result of variation in relative brain sizes between islands, and the statistical significance of the regression is partially driven by the very limited numbers of specimens available from particularly small islands. Adding additional data from specimens from small islands could provide us with a better understanding of the degree of variation in the relative brain sizes of macaques living on islands less than 1000 km^2. I also wish to note that, although the slopes of the brain size residuals against island size for certain groups were statistically different from zero, they are only barely so. Thus, if there is indeed a decrease in relative brain size in populations living on smaller islands, that decrease is very small indeed.

Although this study shows that there is a possibility that relative brain size may decrease slightly on islands, it provides little, if any support for the proposal that *Homo floresiensis* underwent dwarfing in relative brain size. In the case of *H. floresiensis*, it is not a just a matter of whether relative brain size decreases in insular organisms but also to what degree. Martin *et al.* (2006a, b) argued that the brain of LB1 is too small to be due to dwarfing with the expected degree of reduction in brain size to match the smaller body size. If existing brain-to-body-height scaling of the *Homo* genus is used to predict body height of LB1 based on its brain size, *H. floresiensis* would theoretically be a creature only about one-foot-tall, far below the approximately three feet tall LB1 specimen actually discovered. Even though there may be a marginal decrease in the relative brain size of some *M. fascicularis* on small islands, it by no means equivalent to the degree of brain size decrease attributed to *H. floresiensis*.

There is no doubt that brains are energetically expensive (Isler and van Schaik, 2006, 2009; Aiello and Wheeler, 1995; Navarrete et al., 2011; Martin, 1981; Martin and Isler, 2010). It is therefore logical to posit that in a food-stressed environment, such as that usually inferred for small islands, reduction in size of this organ would be a possible solution to achieve significant energy savings. However, mammals are able to balance energy budgets in ways other than altering the metabolic needs of the brain (e.g. allocating less energy to body growth and maintenance), so, although a very slight decrease in brain size may be expected as a result of being energetically expensive, a significant reduction in brain size when animals evolve on islands would only be expected in cases of severe limitation in food resources. Because of the importance of brain function, the brain would be one of the last tissues to be cannibalized during energy shortage, so I should see a significant decrease in body size or even in reproduction before any significant decrease occurs in the relative brain sizes of insular populations. From this point of view as well, it is unlikely that island dwarfing is the explanation for the small brain size of *H. floresiensis.*

A leading alternative hypothesis that attempts to explain the small brain of *H. floresiensis* is that *H. floresiensis* may be more closely related to australopithecines than originally believed. In an analysis of the feet of the type specimen, LB1, it is clear that the ape-like feet are remarkably long relative to the lengths of the femur and tibia, showing unique proportions that otherwise do not occur among hominins. The limb proportions and ape-like foot morphology suggest a different biomechanical and kinematic pattern of locomotion that is quite different compared to modern human bipedalism. These and other characteristics point to a species that is more divergent from any other member of the genus *Homo* (Jungers *et al.*, 2009). A possible interpretation is that *H. floresiensis* may be evidence for an "out-of-Africa" event that occurred prior to *H. erectus* (Jungers & Baab, 2009). The problem with this hypothesis is the lack of any fossil evidence across the entire geographical expanse from Africa to Indonesia for a species similar to *H. floresiensis.* The discovery of more hominid fossils along the path from Africa to Asia will hopefully shed light on this particular hypothesis. If *H. floresiensis* really is a highly unusual early "out-of-Africa" species, then it would radically change the current timeline of *Homo* and/or *Australopithecus* evolution.

Another hypothesis is based on the proposal that pathology may play a role in the morphology of *H. floresiensis.* A large number of different pathologies have now been proposed, ranging from cretinism (Obendorf et al., 2008) to Laron Syndrome (Hershkovitz et al., 2007); but the originally proposed pathology was microcephaly (Henneberg and Thorne, 2004). Microcephalic syndromes are neurodevelopmental diseases that result in a significant decrease in brain size. Weber *et al.* (2005) compared a computer model of LB1's braincase with a group of microcephalic *H. sapiens* braincases and showed that the skull of LB1 is the average size for microcephalics. Later morphometric analyses of LB1 along with microcephalic *H. sapiens* resulted in contradicting conclusions, with one study supporting LB1 as a microcephalic *H. sapiens* (Vannucci et al., 2011) and others arguing that LB1 is a distinct species (Falk et al., 2007; Baab et al., 2013). One commonly used argument against pathology is that more than one *H. floresiensis* specimen has been discovered, but with current evidence this cannot be applied to brain size as all other specimens lack a skull. A second individual is represented by an isolated mandible similar in overall size to that of LB1. Some or all of the other individuals represented by fragmentary specimens may be small-bodied like LB1. However, the endocranial volumes of these other individuals are unknown and should not be simply assumed to be small. More exploration to discover additional hominin fossils in the Southeast Asian region is necessary to resolve the ongoing controversy surrounding *H. floresiensis.* However, the results of the present study show that there is no sound basis for attributing the tiny brain of LB1 to island dwarfism.

A major limitation of the study is the fact that only a single representative primate species, the long-tailed macaque, was sampled. In order to completely understand how the Island Rule applies to brain size, it will be necessary to study other large-bodied insular mammals. Without studying a wider variety of species, it can always be claimed that others may display significant reduction in relative brain size on islands. It is hence important for future studies to explore brain size patterns on islands in other mammals, but care should be exercised in selecting which populations or species to study. Especially when comparing different species, it is essential that the phylogenetic relationships should be clearly understood so that appropriate populations are compared.

CONCLUSION.

The study shows that female long-tailed macaques tend to have larger relative brain sizes than males, and that there is slight insular dwarfing in relative brain size in certain groups of macaques, including female macaques and macaques from oceanic islands. I also show that results can vary depending on the statistical method used and whether or not intraspecific phylogenetic relatedness is taken into account. Although I demonstrate that certain groups of long-tailed macaques from particularly small islands tend to have smaller relative brain sizes, the size decrease is very minimal. Due to the functional importance of the brain, it is unlikely that there would be a large degree of dwarfing in relative brain size on islands. So the tiny brain of *H. floresiensis,* which is especially small for its body size, cannot be explained by dwarfing alone.

CHAPTER 5

Exploring cranial morphology of Southeast Asian *Macaca fascicularis* to better understand evolution on islands

INTRODUCTION.

The “Island Rule” is derived from the widely cited Foster’s Rule (Foster, 1964), which proposed explanations for a reported general trend on islands to the effect that large-bodied species of mammals undergo reduction in body size on islands because food resources are severely limited, while small-bodied species become larger simply due to absolute or relative absence of predators and competitors. Van Valen (1973) then named this apparent universal phenomenon the “Island Rule”. Lomolino (1985) later showed that the Island Rule is a graded trend from dwarfism in large-bodied species to gigantism in small-bodied species, without any necessary categorical size differences.

Various explanations for the Island Rule have been proposed, but here I outline the major hypotheses for dwarfing (reduction in body size). One of the main explanations is that there is a severe limitation of food resources on islands, particularly small islands. Because large animals need a higher total energy intake than small mammals, small-bodied individuals would be expected to survive to reproduction more easily because they need less food resources to reach the body size threshold required for breeding. Large-bodied species may be expected to evolve smaller body size to increase reproductive fitness under food-stress conditions, such as on small islands (Hessee et al., 1951; MacArthur and Wilson, 1967; Sondaar, 1977; Case, 1978; Heaney, 1978; Lawlor, 1982; Lomolino, 1985). Another hypothesis is based on the fact that species richness decreases with smaller island area (MacArthur and Wilson, 1967). The decreased species richness on islands (Heaney, 1984; Harcourt, 1999), together with accompanying reduced predation (Sondaar and Boekschoten, 1967; Dayan and Simberloff, 1998), provides the ecological release necessary for a reduction in body size in large animals. Because large body size helps reduce predation risk (Sinclair et al., 2003; Brown and Sibly, 2006; Kandler and Smaers, 2012), large-bodied species are expected to experience dwarfism in body size on islands due to a reduced level or even complete absence of predation.

As body size is reduced, anatomical parts must also be reduced to some degree. Yet such accompanying morphological changes seem to be the subject of intensive analysis only when an extreme case is discovered, as with the case of the small brain size of *Homo floresiensis* (e.g. Köhler and Moyà-Solà, 2004; Weston and Lister, 2009). Aside from absolute and relative brain size, tooth size scaling has also been analyzed in relation to body size dwarfing (Gould, 1975; Marshall and Corruccini, 1978; Shea and Gomez, 1988; Martin, 1992). In general, postcanine teeth are relatively large in species that have recently undergone rapid dwarfing, but in cases of dwarfing with a long evolutionary history, which may be the case with callitrichines (marmosets and tamarins), studies have determined that relatively small teeth may be present (Plavcan and Gomez, 1993; Montgomery and Mundy, 2013).

Differences in the sizes and perhaps shapes of cranial parts may be a result of a marked change in diet on many islands, where resources are limited (Foster, 1964; Lomolino, 1985) and perhaps vegetation is tougher in comparison to the mainland, especially on Mediterranean islands (Alcover et al., 1981; Alcover, 2000). However, food resources are unlikely to be very limited on large islands, such as Madagascar, Borneo or Sumatra. In a study of 81 species of rodents and carnivores from many geographic regions, Evans et al. (2007) found that diet is directly correlated with tooth crown complexity, so diet evidently has an effect on tooth shape. On islands where foods tend to be tougher, insular taxa show greater degrees of hypsodonty (increased crown height) than their mainland counterparts (van der Geer et al., 2010). *Myotragus balearcus*, an extinct, small-bodied bovid from Majorca and Minorca, apparently evolved the capacity for continuous growth of incisors because tough foods wore down the front teeth to a considerable degree (Alcover et al., 1981). Raia et al. (2003) suggested that greater hypsodonty was a result of increased food intake in *Elephas falconeri* from Sicily. Mass-specific metabolic rate is inversely allometrically related to body size (Kleiber, 1947), so a small-bodied mammal would need relatively more food per unit body weight. Because *E. falconeri* accordingly ate more food in proportion its body size, there would have been more rapid tooth wear offset by increased hypsodonty.

As suggested by van der Geer et al. (2010), the different material properties of food, such as increased toughness, on some islands may have an effect on the morphology of the teeth in relation to mastication. However, studies of organisms on islands have not focused on cranial morphological differences due to changes in mastication. Studies not associated with islands show that greater forces combined with more chewing cycles are necessary to fragment tougher foods (Ravosa et al., 2007; Menegaz et al., 2009; Ravosa et al., 2015). With primates, however, it appears that food material alone cannot be used to predict the morphology of the mandible (Daegling and Grine, 2006). In fact, even if food material properties can be consistently associated with dietary categories in primates, Ross et al. (2012) state that those food material properties do not necessarily indicate a particular mandibular morphology. It is therefore unclear whether food resources on islands with different food material properties in comparison to foods on the mainland would actually have an impact on morphology associated with mastication, especially in primates.

Some small-bodied insular endemics from various regions have relatively short muzzles as a result of paedomorphosis (van der Geer et al., 2010). Research into the mechanical requirements of mastication has shown that shorter snout length is associated with greater bite forces (Radinsky, 1987). If island-living organisms truly do have shorter snout lengths, at least some of the masticatory muscles in those organisms must be packed differently in comparison to their mainland counterparts. The major muscles involved in mastication are the temporalis, masseter, medial pterygoid and lateral pterygoid muscles. Herring (2007) showed that there is asymmetrical muscle usage during mastication, and the forces and loadings involved result in bone deformations in the jaw bones and elsewhere on the skull. If greater masticatory loading is associated with tougher foods, as is seen in rabbits and perhaps other herbivores (Ravosa et al., 2015), then there may be morphological changes in the skull in island-living mammals if there are foods with different material properties on islands in comparison to the mainland.

Few studies have been conducted on allometry in small-bodied island-living animals (Prothero and Sereno, 1982; Boerner and Krüger, 2008; Köhler and Moyà-Solà, 2004; Palombo et al., 2008; Weston and Lister, 2009), and analyses of morphological modifications associated with body size changes on islands are extremely limited (Vannucci et al., 2011; Villano et al., 2009). In order to establish whether populations dwelling on islands are subject to evolvution of unique cranial characteristics, I apply here 3-dimensional geometric morphometric analyses to explore morphological changes in the skull of long-tailed macaques (*Macaca fascicularis*) in Southeast

Asia. *M. fascicularis*) was chosen as the study species because these primates are extremely widespread in this region, allowing for a large sample size including numerous individuals from islands of various types and sizes.

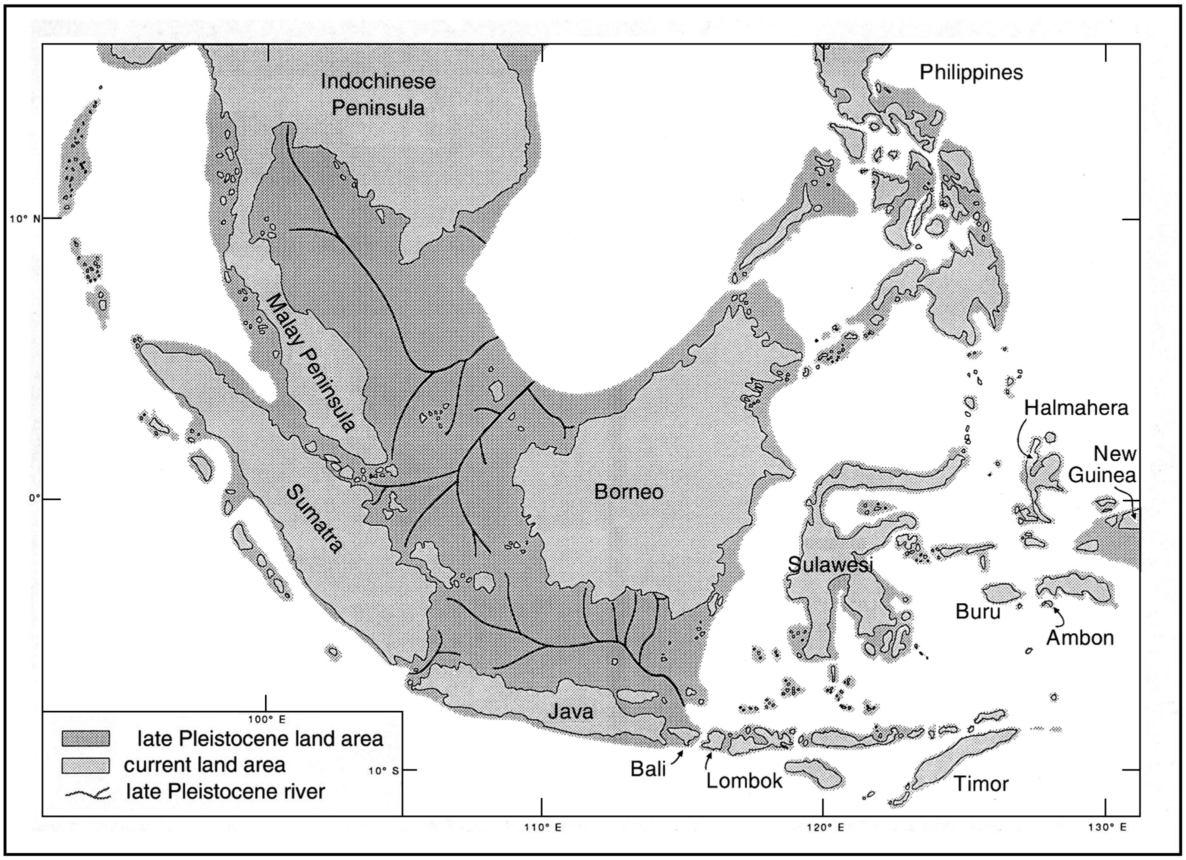

Figure 5.1. Map of Southeast Asia indicating exposed areas of the continental shelf above sea level during Pleistocene periods.

MATERIALS AND METHODS.

Southeast Asia is a geographically diverse region with thousands of islands ranging from less than 1 km^2 to over 700,000 km^2, making this an ideal region for studying evolution on islands. Oceanic islands are those that have never been connected to the mainland (Gillespie and Clague, 2009). In Southeast Asia, the oceanic islands are those to the east of the Huxley's Wallace Line, which separates Bali and Lombok to the south and Palawan and the rest of the Philippines to the north (Figure 5.1). Continental islands are those that connect to the mainland via the continental shelf when sea levels are low during glaciation periods (Gillespie and Clague, 2009). In Southeast Asia, the continental islands are those that make up Sundaland, the region to the west of the Huxley Wallace Line.

I collected data from specimens housed at six natural history museums around the world, including The Field Museum of Natural History (Chicago, IL), American Museum of Natural History (New York, NY), Smithsonian Institute National Museum of Natural History (Washington, DC), Natural History Museum (London, UK), Naturalis Biodiversity Center (Leiden, Netherlands), and Raffles Museum of Biodiversity Research, (now the Lee Kong Chian Natural History Museum (Singapore). All data were collected from mature adults, with fully erupted third molars. I stabilized the specimens using Play-Doh, a water based clay, and collected distinct cranial landmarks (Figure 5.2) from the superior region of the skull (38), the inferior region of the skull (43), and the mandible (36) using a Microscribe 3D digitizer.

MorphoJ (Klingenberg, 2011) was used to check for outliers and to standardize and extract shape information from the data using Procrustes superimposition. Procrustes superimposition allows for all the individual shapes to be centered to a common centroid, scaled to the same centroid size and rotated to minimize the summed squared distances between landmarks. Although it is possible to stitch together the landmarks from the superior and inferior regions of the skull, I performed the Procrustes superimposition separately for each set of landmarks — superior, inferior, and mandibular — in order to test whether the results differ based on which part of the skull is used for morphometric analyses.

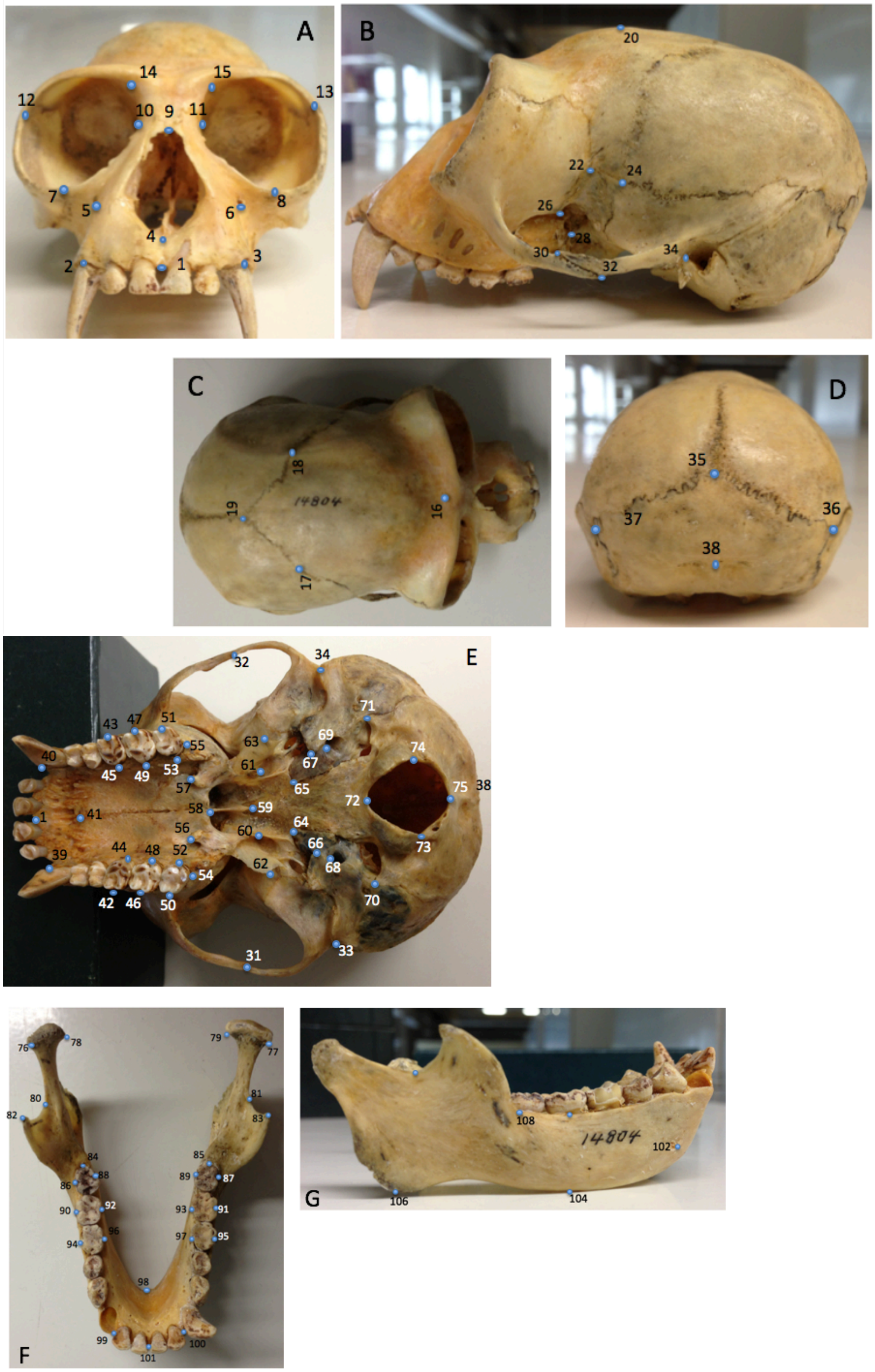

Figure 5.2. Geometric morphometric landmarks microscribed from museum specimens. A-D: 38 superior landmarks. E: 43 inferior landmarks. F, G: 36 mandibular landmarks.

Previous studies of island dwarfing in body size in long-tailed macaques generated contradictory results (Villano et al., 2009; Chapter 3). I therefore used a new measure, centroid size, that had not been used in previous studies to estimate body size to test for body size dwarfing in a large sample of long-tailed macaques from Southeast Asian islands. I regressed centroid size against island size in R (R Development Core Team, 2010) using ordinary least squares. Because *Macaca fascicularis* shows marked sexual dimorphism in body size (Leutenegger, 1982), the data were separated by sex.

To explore differences in morphology, principal components analyses (PCA) were performed in MorphoJ (Klingenberg, 2011). The data were categorized first by sex and then by island size and island type. Land mass size was categorized into five bins: small islands (<10,000 km^2), average islands (10,000-100,000 km^2), large islands (100,000-200,000 km^2), giant islands (>200,000 km^2), and mainland (arbitrarily set at 10,000,000 km^2). Land mass types include continental islands, oceanic islands, and the mainland. To test for differences among groups, I performed canonical variate analyses (CVA) in MorphoJ, classifying by island type and island size. Finally, to check whether or not cranial shape in the various island type groups and island size groups differ according to sex, I conducted a two-way MANOVA using adonis in the Vegan package (Oksanen et al., 2016) in R (R Development Core Team, 2010), which executes a non-parametric multivariate analysis of variance.

RESULTS. In total, landmarks were collected from the superior skull region of 152 specimens, from the inferior skull region of 120 specimens, and from the mandible of 124 specimens. Every landmark was present on all specimens analyzed (landmark data will be available upon publication or upon request from Lu Yao or Robert D. Martin).

Centroid size. Centroid size, which is a proxy for body size, was calculated in MorphoJ and then regressed against island size in R (Figure 5.3). OLS analysis indicates that there is no statistically significant pattern in centroid size in comparison to island size for either males or females using any set of landmarks (Table 5.1). Instead, male centroid size showed little variation across all island sizes.

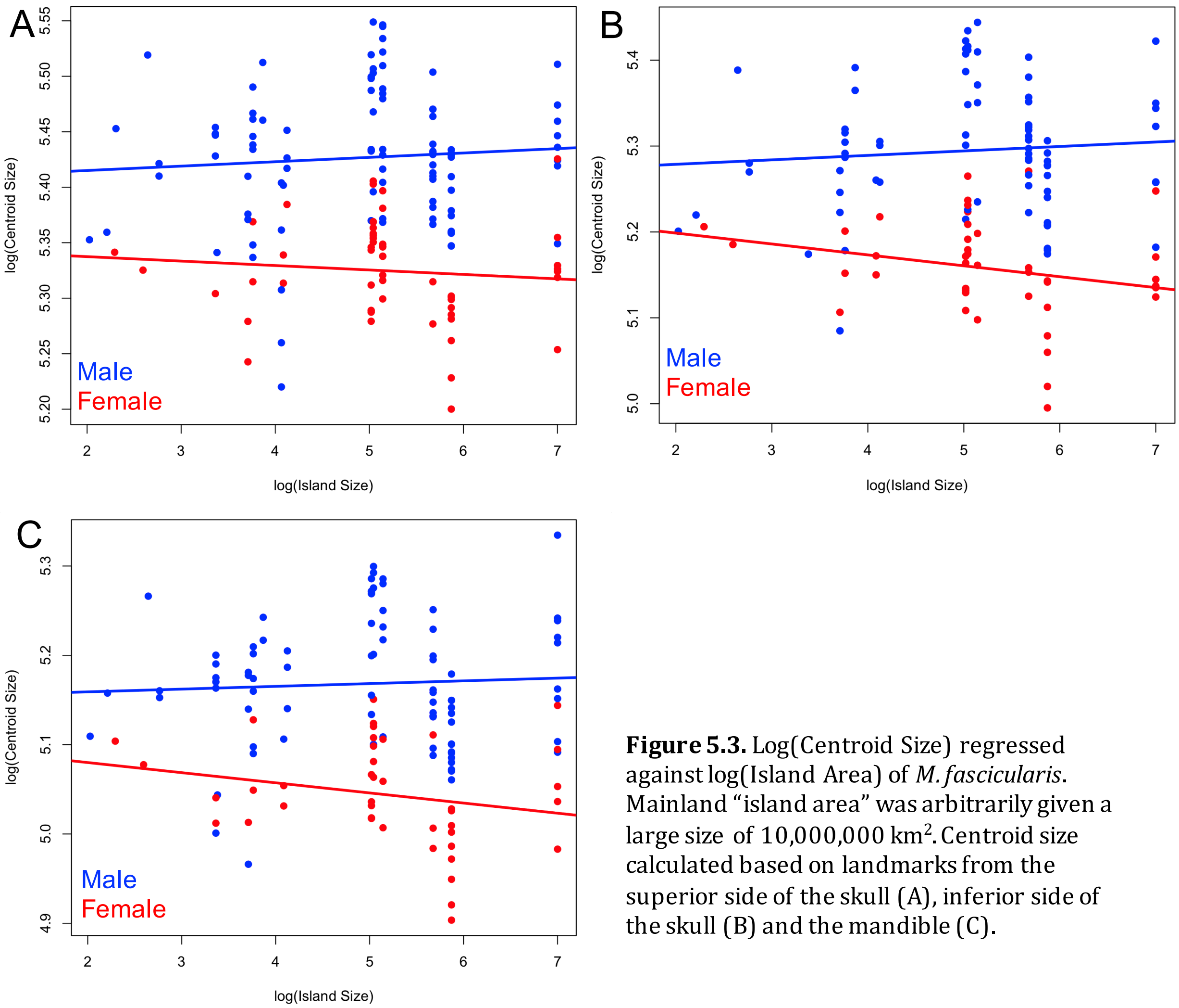

Figure 5.3. Log(Centroid Size) regressed against log(Island Area) of *M. fascicularis*. Mainland "island area" was arbitrarily given a large size of 10,000,000 km^2. Centroid size calculated based on landmarks from the superior side of the skull (A), inferior side of the skull (B) and the mandible (C).

	Superior		Inferior		Mandible	
	r^2	p	r^2	p	r^2	p
Male	0.0057	0.456	0.0066	0.479	0.0028	0.634
Female	0.0088	0.505	0.056	0.131	0.0494	0.163

Table 5.1. OLS regression results of log(Centroid Size) against log(Island Size) of *M. fascicularis* for each set of landmarks.

PCA. For the superior region of the skull, the first five PCs explained 51.7% of the variance in shape, with PC1 explaining 19.4% and PC2 explaining 10.2% (Figure 5.4). PC1 is mostly weighted by variation in the protrusion of the maxilla, elevation at the dorsal end of the skull, and variation in landmarks 17 and 18. PC2 explains variation in the elevation of the maxilla, width of the zygomatic arches, and location of landmark 38, which lies on a ridge at the dorsal posterior end of the skull (Figure 5.5). The PC plots do not appear to differentiate specimens by island type or island size (Figures 5.9A and 5.10A), but there is separation according to sex (Figure 5.8A) in PC1. The shape differences in PC1 are mostly due to sexual dimorphism. Specimens from continental islands, oceanic islands, and the mainland occupy the same morphospace, and so do specimens from islands of various sizes.

The first five PCs for the inferior region of the skull explained 55.9% of the variance in shape, with PC1 explaining 20.7% and PC2 explaining 15.13% (Figure 5.4). PC1 explains most of the variation in length and width of the tooth row, and length of the dorsal half of the skull. PC2 appears to explain variation in width of the molars, and how anterior or posterior the location of the zygomatic arches is (Figure 5.6). Again the PC plots do not differentiate specimens by island type or island size (Figures 5.9B and 5.10B), but they are separated by sex (Figure 5.8B) in PC1. So the shape differences in PC1 are mostly due to sexual dimorphism.

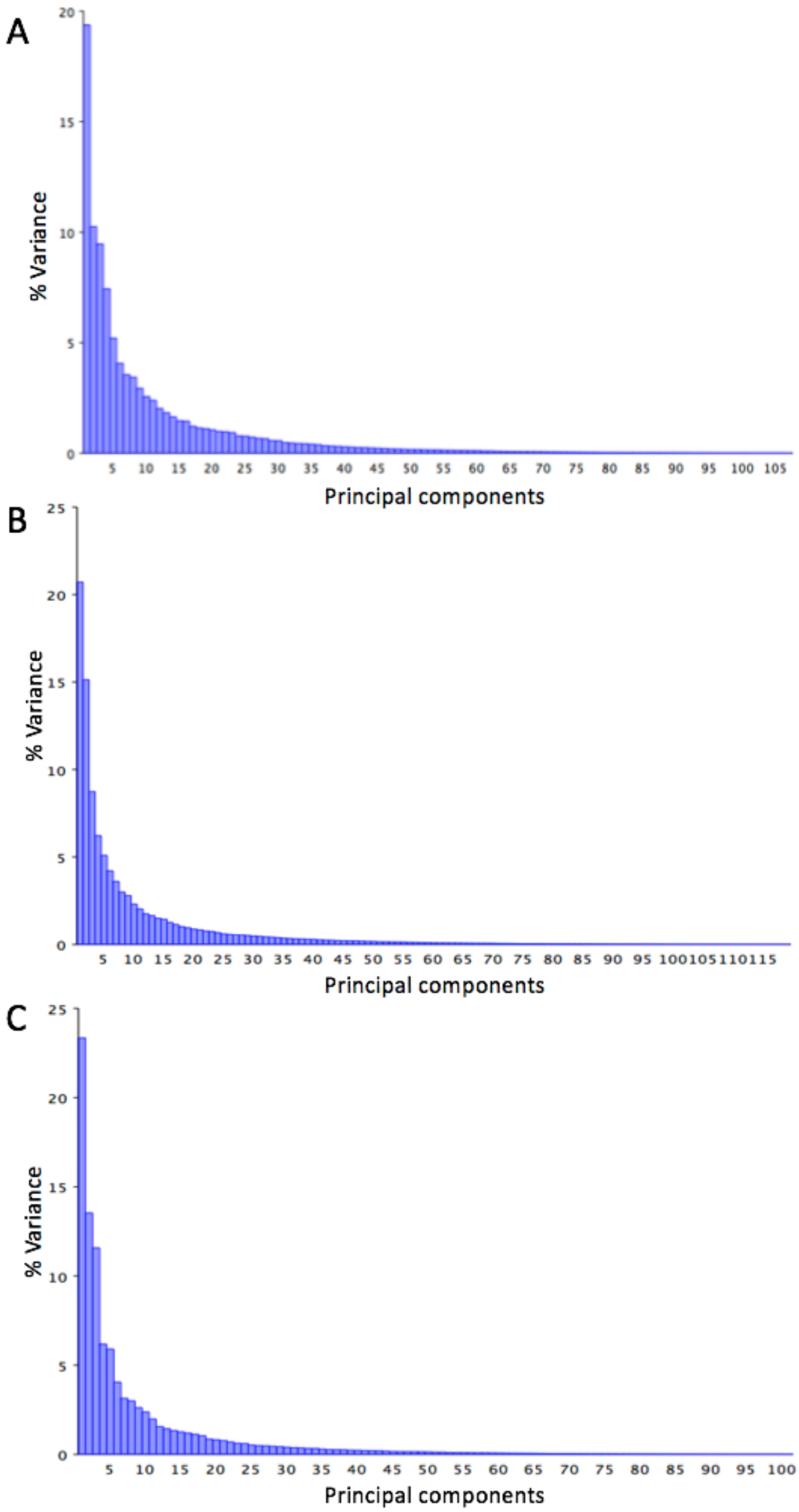

Figure 5.4. Plot indicating the % variance explained by each principal component for the superior region of the skull (A), inferior region of the skull (B), and the mandible (C) in *M. fascicularis*.

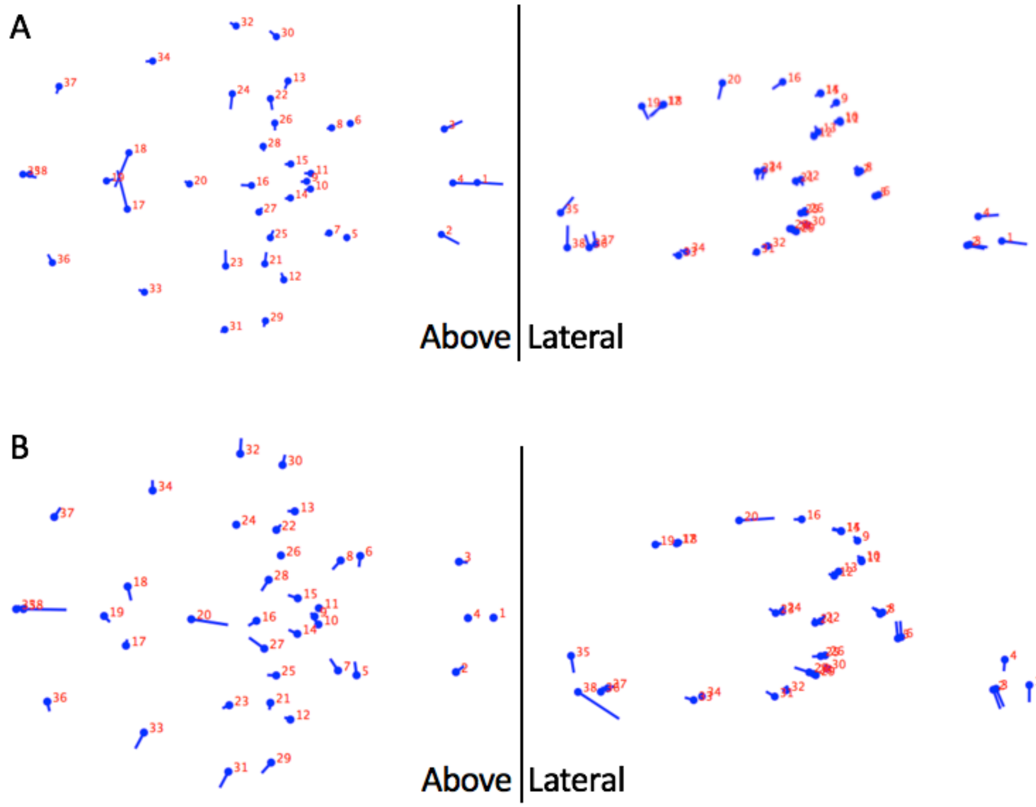

Figure 5.5. Shape changes for the superior region of the skull of *M. fascicularis*. A: PC1 shape changes; B: PC2 shape changes.

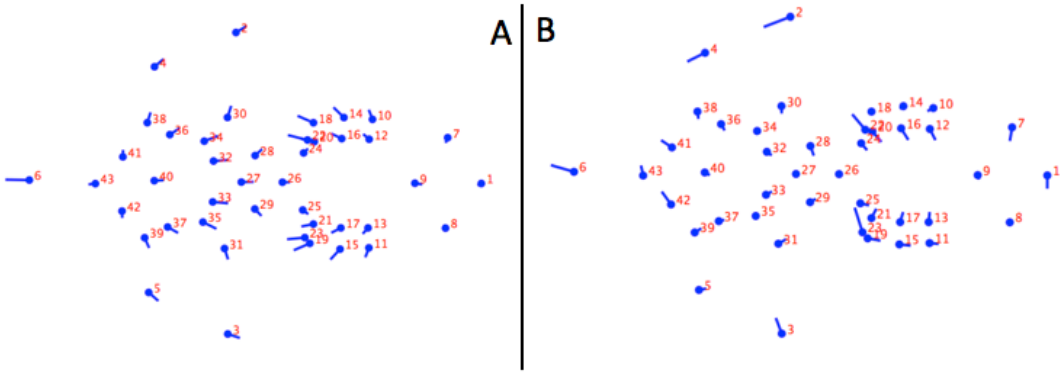

Figure 5.6. Shape changes for the inferior region of the skull of *M. fascicularis*. A: PC1 shape changes; B: PC2 shape changes.

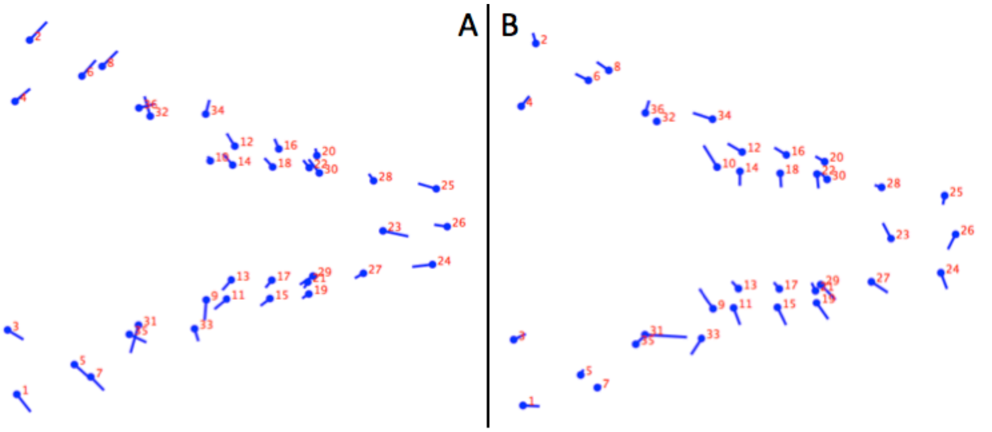

Figure 5.7. Shape changes for the mandible of *M. fascicularis*. A: PC1 shape changes; B: PC2 shape changes.

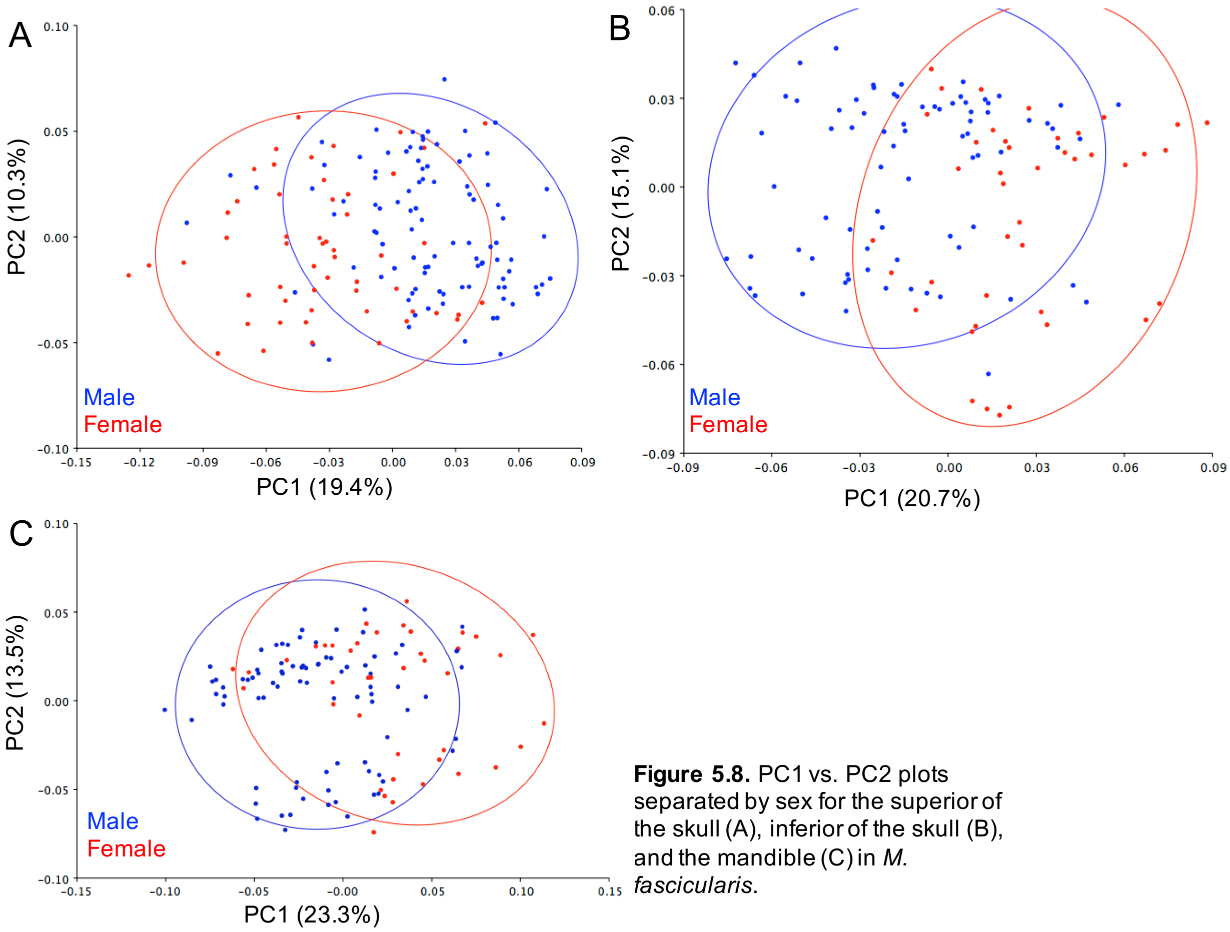

Figure 5.8. PC1 vs. PC2 plots separated by sex for the superior of the skull (A), inferior of the skull (B), and the mandible (C) in *M. fascicularis*.

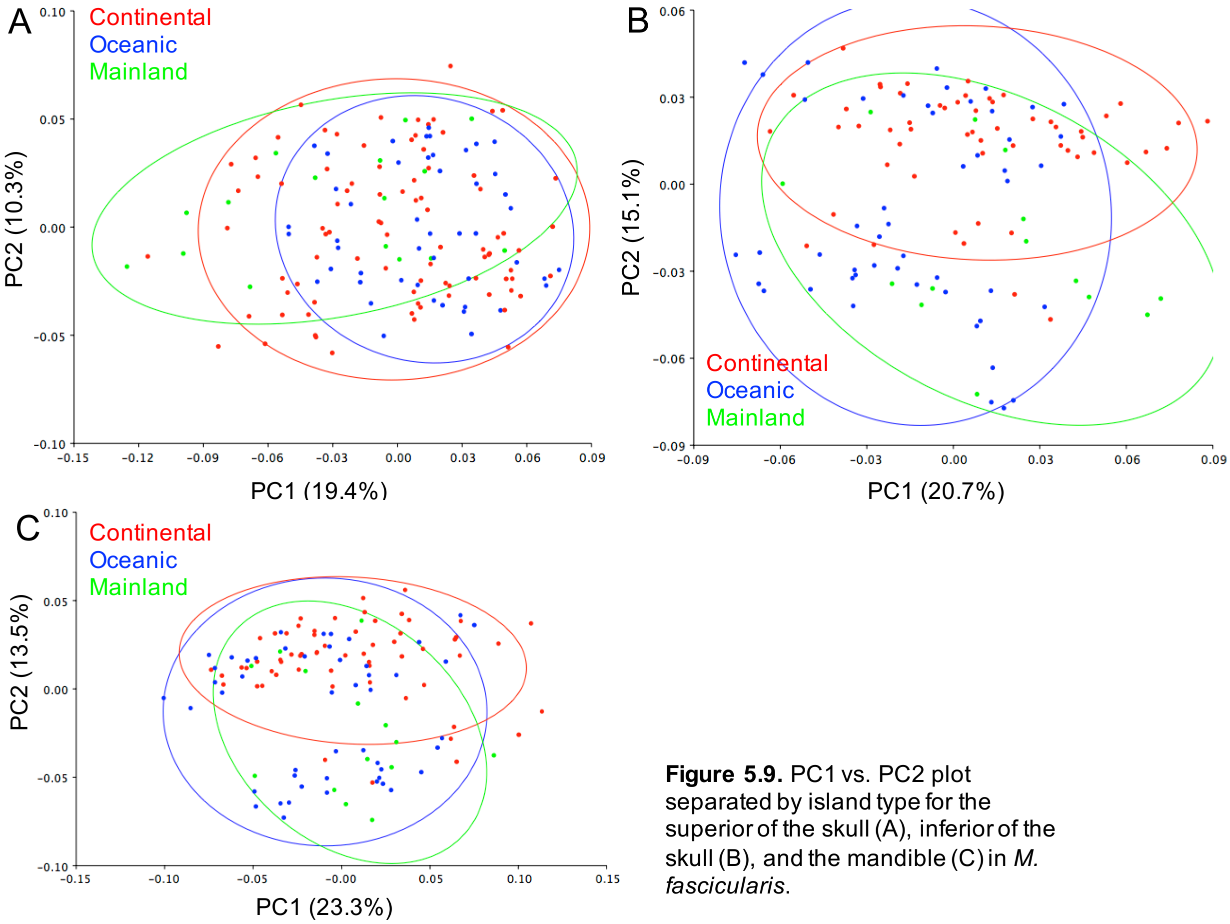

Figure 5.9. PC1 vs. PC2 plot separated by island type for the superior of the skull (A), inferior of the skull (B), and the mandible (C) in *M. fascicularis*.

The first five PCs for the mandible explained 60.5% of the variance in shape, with PC1 explaining 23.3% and PC2 explaining 13.5% (Figure 5.4). PC1 explains most of the variation in length and width of the mandible, particularly at the anterior and posterior ends. PC2 may be explaining variation in the width of the molars (Figure 5.7). Again the PC plots do not differentiate specimens by island type or island size (Figures 5.9C and 5.10C), but they are separated by sex, although to a lesser degree than in the PC plots for the superior and inferior regions of the skull (Figure 5.8C) in PC1. So, in this case too, the shape differences in PC1 are mostly due to sexual dimorphism. For the superior region of the skull, the inferior region of the skull and the mandible, all other combinations of PC plots do not show a separation of the specimens by island type or island size. Loading and eigenvalue data will be available upon publication or upon request from Lu Yao or Robert D. Martin.

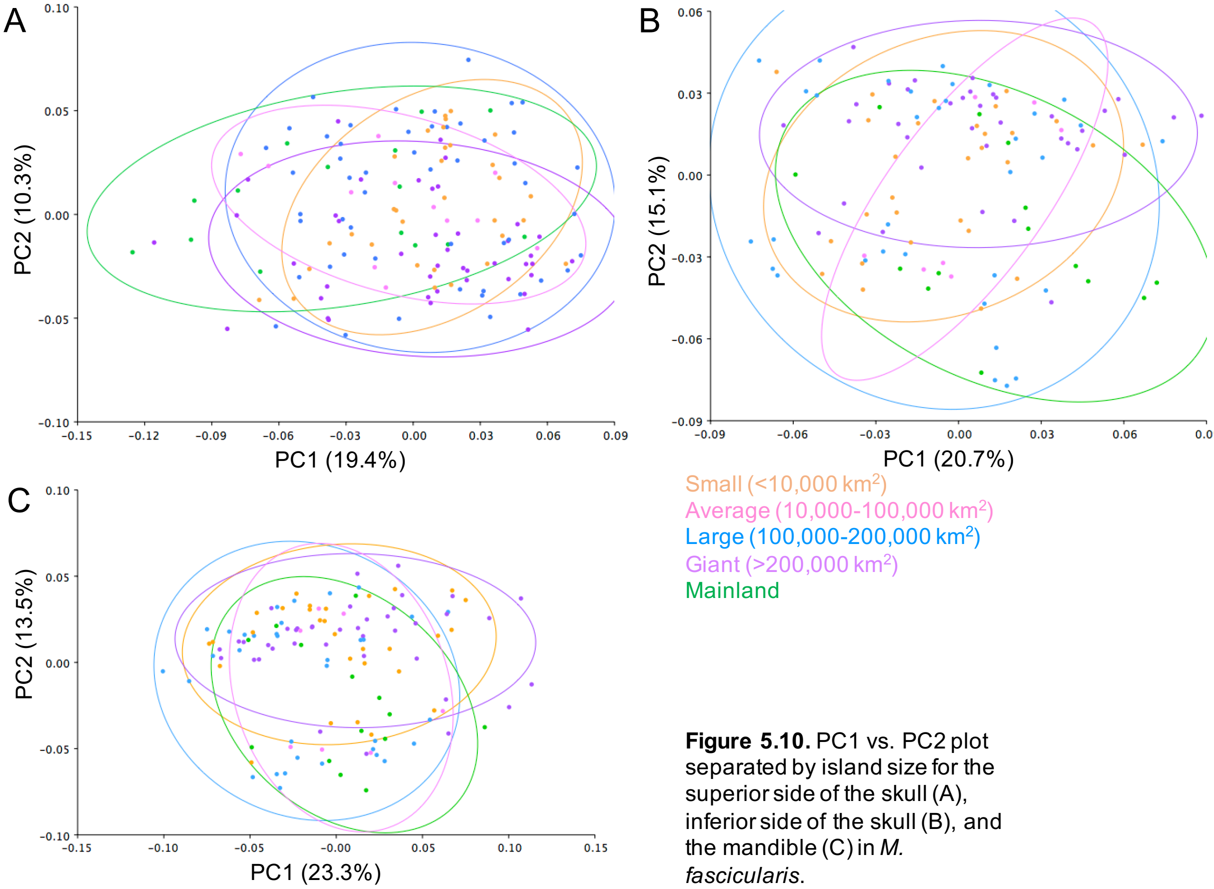

Figure 5.10. PC1 vs. PC2 plot separated by island size for the superior side of the skull (A), inferior side of the skull (B), and the mandible (C) in *M. fascicularis*.

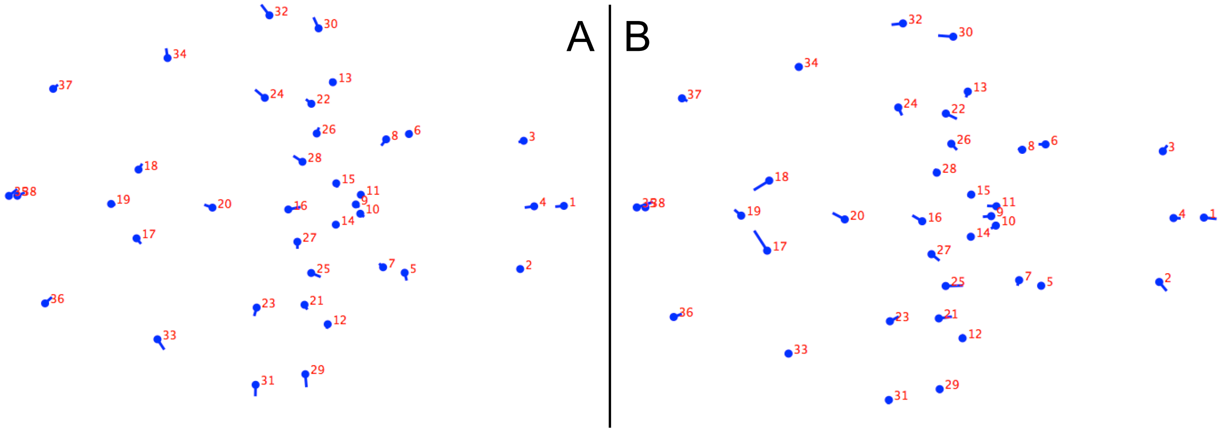

Figure 5.11. Shape changes for the superior side of the skull with island type as the classifier in *M. fascicularis*. A: CV1 shape changes; B: CV2 shape changes.

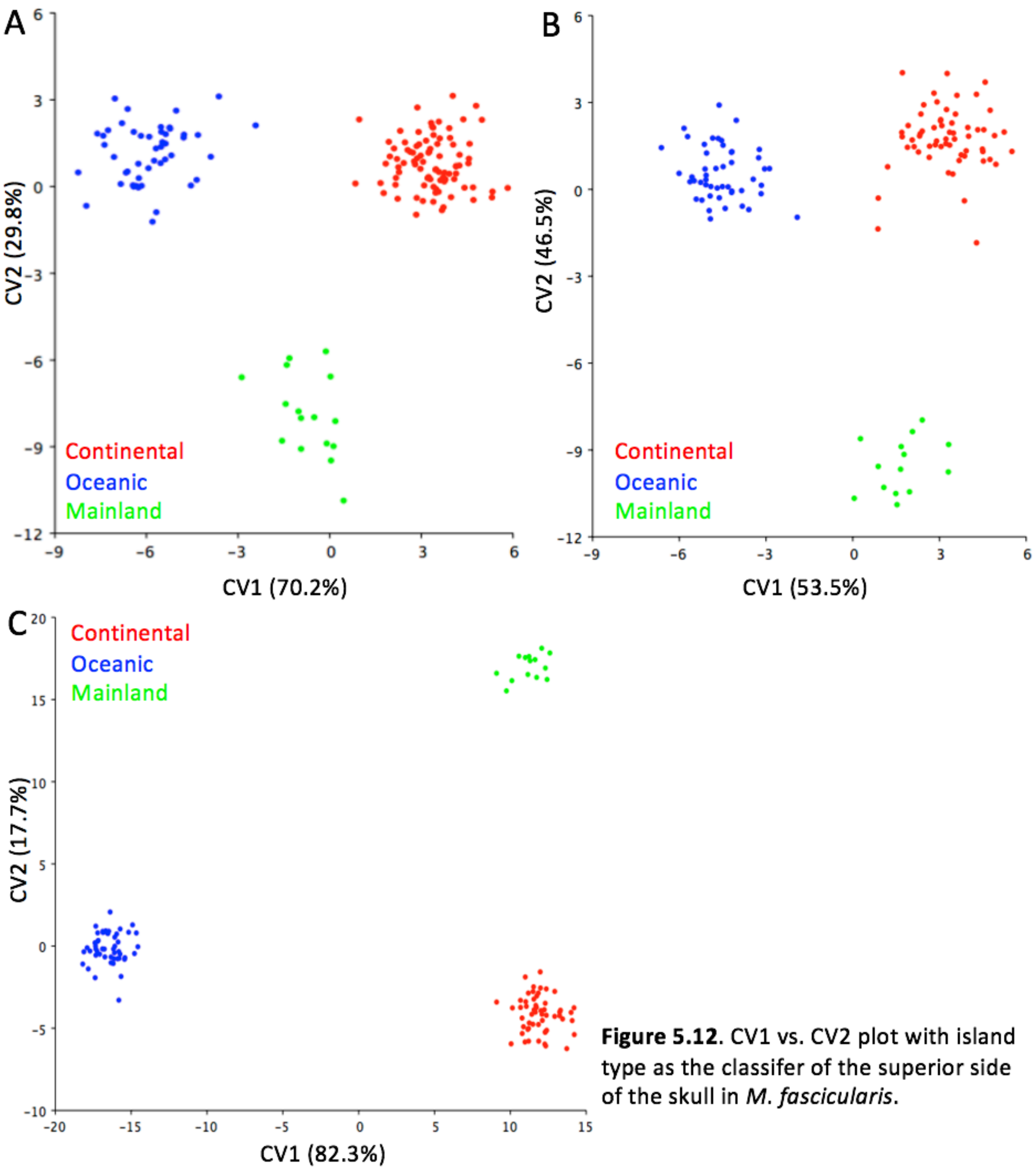

Figure 5.12. CV1 vs. CV2 plot with island type as the classifer of the superior side of the skull in *M. fascicularis*.

CVA. The canonical variate analyses for the superior regions of the skull show a separation into the respective groups for island type (continental or oceanic) and mainland. This stands in direct contrast to the principal components analyses, which indicated no obvious separation. The CV plot of the superior region of the skull with island type as the classifier (Figure 5.12A) differentiates the specimens into the three groups, which appear to differ mostly in width of the skull at the zygomatic arches and in the protrusion of the maxilla (Figure 5.11). All three groups are statistically significantly different from one another (p<.0002 for all pairwise permutation tests for Procrustes distances among groups). The canonical variate analysis based on island size shows that there are two subsets. The first set is exemplified by CV1 and CV2 (Figure 5.13A), with which specimens from small islands cluster with those from giant islands, while the large and average-sized island groups cluster together. The second set is exemplified by CV1 and CV3 (Figure 5.13B), with which the average-sized, large and mainland groups cluster together, while the giant islands and small islands are separated into their own clusters.

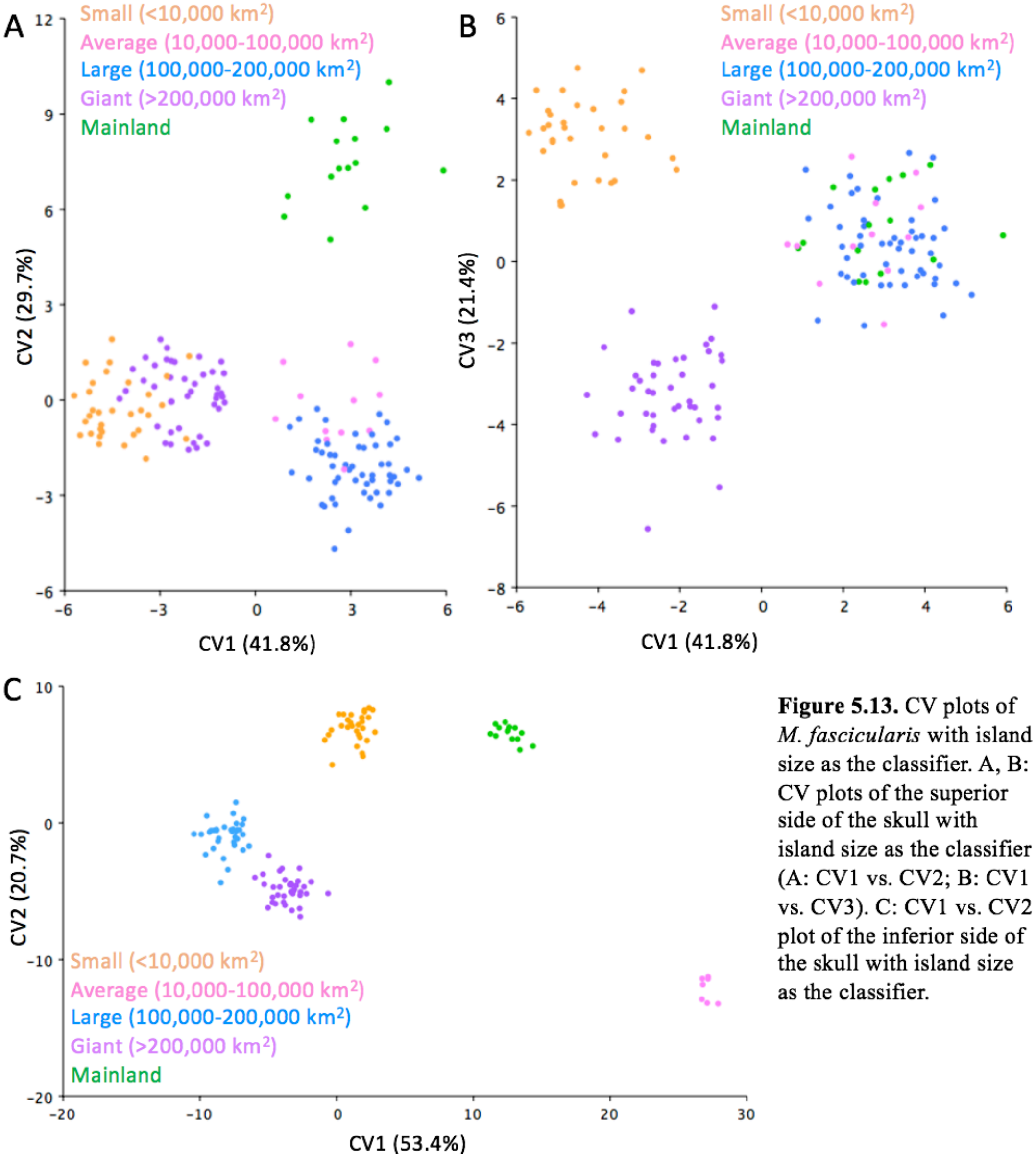

Figure 5.13. CV plots of *M. fascicularis* with island size as the classifier. A, B: CV plots of the superior side of the skull with island size as the classifier (A: CV1 vs. CV2; B: CV1 vs. CV3). C: CV1 vs. CV2 plot of the inferior side of the skull with island size as the classifier.

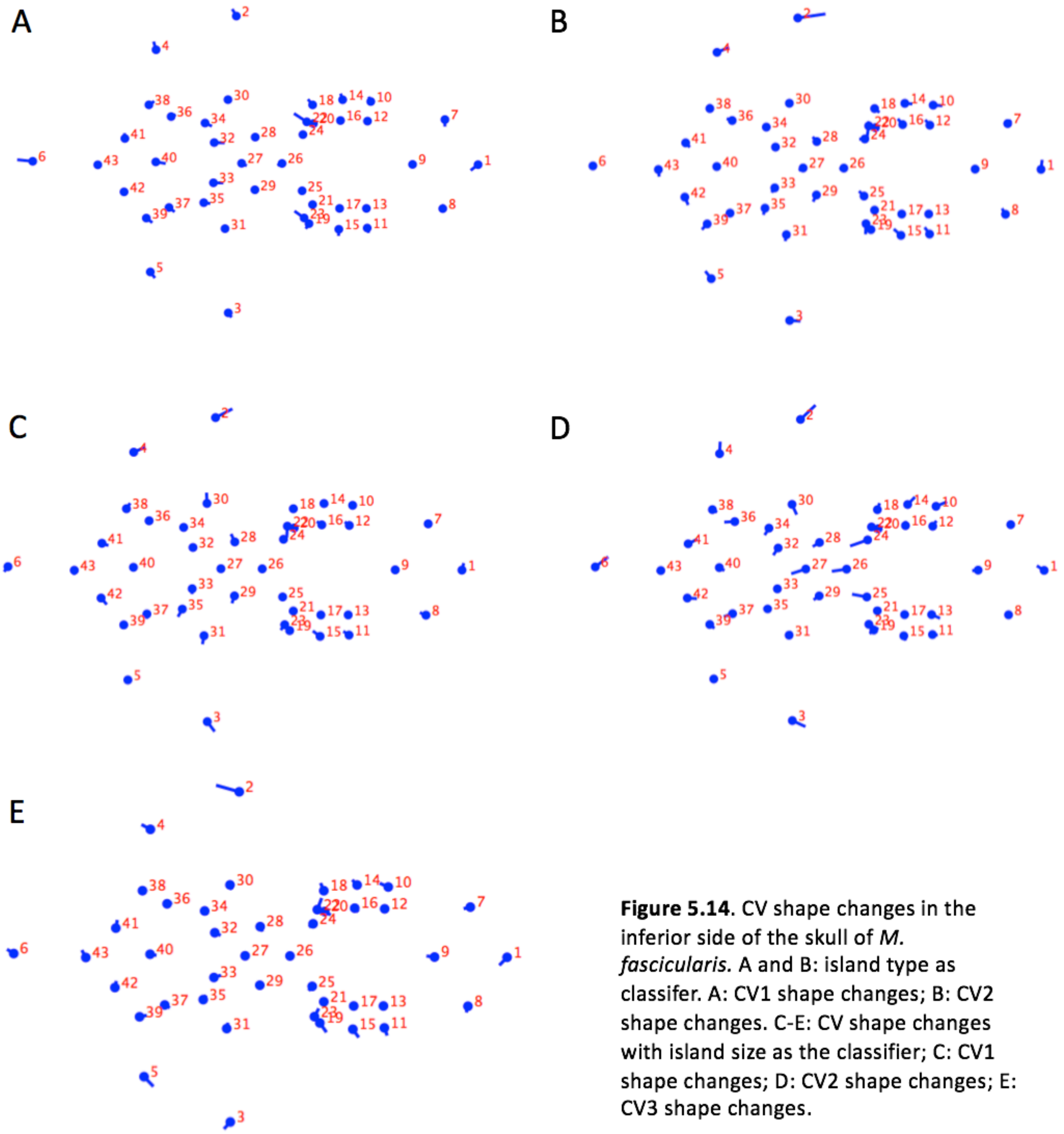

Figure 5.14. CV shape changes in the inferior side of the skull of *M. fascicularis.* A and B: island type as classifer. A: CV1 shape changes; B: CV2 shape changes. C-E: CV shape changes with island size as the classifier; C: CV1 shape changes; D: CV2 shape changes; E: CV3 shape changes.

As with the results for the superior region of the skull, the canonical variate analyses for the inferior region of the skull with island type as the classifier clearly reveals a separation into the respective groups. The three groups differ mostly in protrusion of the posterior region of the cranium, and length of the zygomatic arch (Figure 5.14A, B). However, when island size is designated as the classifier (Figure 5.14C-E), there is a difference among the island size groups with both sets of classifiers, island type and island size (Figure 5.13C). The other CV plots with island size as the classifier also show all island size bins separated into their own clusters. All groups are statistically significant from one another ($p<0.0008$). The specimens from the island size bins differ in width and length of the zygomatic arches along with the position of landmarks along the midline of the skull (landmarks 17-19). The canonical variate analyses for the mandible show results similar to those for the inferior region of the skull (Figure 5.15). Again, the CV plots with island size or island type as classifiers show the various groups in their own clusters, with no overlap between groups. And all groups are statistically different from one another ($p<0.0291$). Based on island type, the mandibles of specimens from different island types tend to differ in the width of the molars. There are very subtle differences in the position of the condyles and the width of the molars amongst specimens from various sized islands.

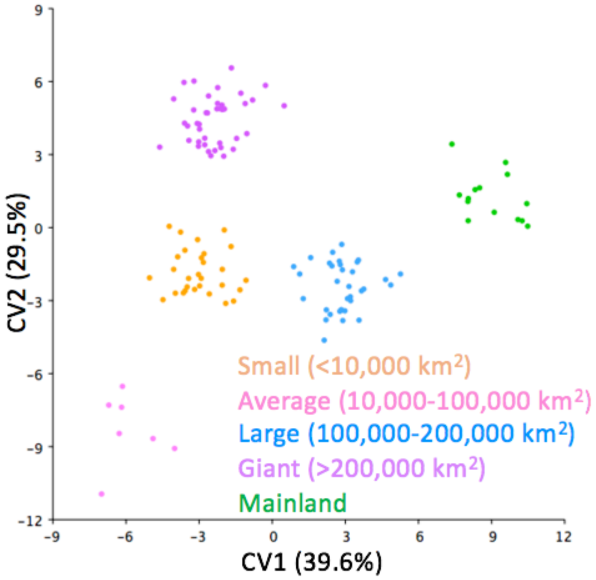

Figure 5.15. CV plot of the mandible of *M. fascicularis* with island size as the classifier.

Two-way MANOVA. Two-way MANOVA tests were performed for the superior and inferior regions of the skull. For both regions, the two-way MANOVA shows that the interaction effect between island type and sex is statistically significant (p=0.003 for superior side; p=0.048 for inferior side; Tables 5.2 and 5.3)., It is therefore crucial to control for sex when analyzing shape and island type. And there is a statistically significant difference in shape based on island type when sex is controlled for.

When looking at shape against island size based on the five bins I had assigned, the interaction effect between island size and sex is not statistically significant (p=0.216 for superior region; p=0.558 for inferior region; Tables 5.2 and 5.3). Accordingly, it is not necessary to take that interaction effect into account when analyzing cranial shape and island size. So there is a statistically significant difference in shape based on island size. However, cranial shape does vary across island types and island sizes in *M. fascicularis*.

Table 5.2a

	Df	**Sums of squares**	**Mean squares**	**F.Model**	**r^2**	**Pr(>F)**
Island type	2	0.0665	0.0333	5.6048	0.0686	0.001***
Sex	1	0.0188	0.0188	3.1711	0.0194	0.002**
Island type:Sex	2	0.0185	0.0092	1.5581	0.0191	0.003*
Residuals	146	0.8664	0.0059		0.8930	
Total	151	0.9702			1	

Table 5.2b

	Df	**Sums of squares**	**Mean squares**	**F.Model**	**r^2**	**Pr(>F)**
Island size	21	0.2363	0.0113	2.0416	0.2435	0.001***
Sex	1	0.0181	0.1805	3.2759	0.0186	0.001***
Island size:Sex	10	0.0601	0.0060	1.0907	0.0620	0.216
Residuals	119	0.6558	0.0055		0.6759	
Total	151	0.9702			1	

Table 5.2. Two-way MANOVA results analyzing shape of the superior region of the skull against island size and sex (A) and island type and sex (B) in *M. fascicularis*.

Table 5.3a

	Df	Sums of squares	Mean squares	F.Model	r^2	Pr(>F)
Island type	2	0.0204	0.0102	2.7027	0.0435	0.001***
Sex	1	0.0081	0.0081	2.1456	0.0173	0.005**
Island type:Sex	2	0.0104	0.0052	1.3800	0.0222	0.048*
Residuals	114	0.4295	0.0038		0.9171	
Total	119	0.4684			1	

Table 5.3b

	Df	Sums of squares	Mean squares	F.Model	r^2	Pr(>F)
Island size	17	0.1204	0.0071	2.0988	0.2571	0.001***
Sex	1	0.0079	0.0079	2.3442	0.0169	0.003**
Island size:Sex	9	0.0295	0.0033	0.9716	0.0630	0.558
Residuals	92	0.3105	0.0034		0.6630	
Total	119	0.4684			1	

Table 5.3. Two-way MANOVA results analyzing shape of the inferior region of skull against island size and sex (A) and island type and sex (B) in *M. fascicularis*.

DISCUSSION.

The morphometric data led to results that confirm previous conclusions regarding body size and reveal 3D shape differences in the cranium. Because I was able to use centroid size as an estimate for body size using the geometric morphometric data, a short discussion of body size and island size in macaques is warranted. In Chapter 3, I used skull length x skull width as a proxy for body size, which had previously been shown to be a suitable predictor for body mass in primates (Martin, 1990). With those data, I showed that there is no statistically significant decrease in body size on islands in long-tailed macaques. However, Villano et al. (2009) concluded from linear morphometric parameters that long-tailed macaques do have a tendency to show dwarfism in body size on islands. This discrepancy may have been due to the measurements used to estimate body size. In this study, I used centroid size, which is based on

3D geometric morphometric data, as an estimator for body size. Additionally, these data include landmarks from the inferior side of the skull and the mandible, which are not captured in the estimation based only on skull length x skull width. No statistically significant dwarfing in body size on islands is observed using the superior, inferior or mandibular landmarks. Additionally, the coefficient of determination is extremely low, showing that island size barely explains the variation in estimated body size. However, this low r^2 value is partially due to variation in the populations on different islands. Because there are many samples from each island, the data captures the large variation in body size for this species on each island, thus increasing the r^2 value.

Visually, male macaques do not appear to vary in body size across islands and the mainland, but female macaques do show a trend towards smaller body sizes on the mainland and on large Southeast Asian islands, especially when centroid size is calculated based on mandibular and inferior cranial landmarks. There has been some debate as to whether or not there is an ideal body size towards which large and small organisms converge on islands, and this optimal size has been explicitly defined as either 1 kg (Damuth, 1993) or 100 g (Brown et al., 1993; Kelt and Van Vuren, 1999). Both male and female long-tailed macaques have much larger body sizes than these "optimal" insular body sizes (Smith and Jungers, 1997), so there is no reason to expect insular gigantism in one sex. One explanation for this trend toward larger body sizes on small islands could be the small sample size available for macaques on smaller islands. With data from just two specimens, they could be driving the negative slope in the regression lines.

Additionally, in Chapter 3 the OLS regression showed that macaques on Borneo may have smaller body sizes than macaques on the mainland or other islands of smaller size. This pattern is once again apparent when using centroid size as a proxy for body size (Figure 5.3). On the other hand, the results also showed that Sumatran macaques had smaller body sizes as well. But here, using three different calculations for centroid size, I do not see any marked decrease in body size in Sumatran macaques. Thus, using centroid size, only Bornean macaques appear to display smaller body sizes, and this may be a result of the less fertile Bornean soils that in turn decreases forest productivity and organismal biomass (MacKinnon et al., 1996; Waterman et al., 1988). It is unclear as to why using centroid size would lead to different results from skull length x skull width.

Although I found that long-tailed macaques generally do not display dwarfing in body size on islands, there do appear to be differences in cranial shape amongst groups from various types of islands and various island sizes. There does not appear to be any clear separation in the principal components analyses based on sex, island type or island size, but it is notable that there is a separation in the data along PC2 for the inferior and mandibular landmarks, while the superior landmarks do not display this separation. The separation seen in both sets of landmarks separates the same specimens into two groups along PC2. It is unlikely this separation is due to measurement error for a number of reasons. First, all landmarks for each specimen were microscribed consecutively. Therefore, this separation in the principal components analysis of the superior region of the skull would be seen as well if it were due to measurement error. Next, I had microscribed each set of landmarks from each specimen twice. The second set of landmarks also displays this separation in the inferior region of the skull and the mandible. Finally, I checked to see whether the microscribe was not functioning properly at a single museum by checking if one group of specimens in the separation was from a single museum. This is not the case. Both groups along PC2 contain specimens from multiple museums.

After establishing that this separation in specimens along PC2 is not a result of measurement error, the only other explanation is that there is a biological separation in long-tailed macaques. In Chapter 2, I reconstructed an intraspecific phylogeny of long-tailed macaques, which separated the species into two major clades, one containing mostly mainland specimens, and another containing only insular specimens. However, the separation along PC2 does not match the two separate clades in the phylogeny. It does appear that specimens from continental islands tend to occupy one of the groups while oceanic island and mainland specimens occupy either group. A possible explanation for this separation is the preference for chewing side in these specimens. The differences are only apparent in the inferior side of the skull and the mandible, which are the regions associated with mastication. Studies analyzing whether or not there is a dominant side for chewing in humans yield inconsistent results (Christensen and Radue, 1985; Kazazoglu et al., 1994; Weiner, 2001; Varela et al., 2003; McDonnell et al., 2004; Martinez-Gomis et al., 2009; Gomes et al., 2011; Zamanlu et al., 2012). Few studies test for preference of chewing side in non-human primates, but one study on *Macaca radiata* by Mangalam et al. (2014) found that macaques prefer to use the cheek pouch that corresponds to the hand that is preferred for picking up foods. This study shows that chewing laterality, the preference for a chewing side, is a possibility in macaques; but more studies focusing on preferential side in non-human primates and morphological shape differences due to this are needed. Although I am not able to pinpoint why there is this separation when the data from the inferior region of the skull or the mandible are analyzed, it is clear that different patterns can be revealed depending on which set of landmarks is used.

In the canonical variate analysis to test for shape differences amongst island size groups, there is again a difference in the results for the superior region of the skull in comparison to those for the inferior region of the skull and the mandible. Along CV2 for the superior region of the skull, specimens from small islands cluster with those from giant islands, which is completely unexpected. This may be related to the smaller body sizes seen on the island of Borneo, which is categorized as a giant island. The body sizes of macaques on Borneo are similar to those of individuals from small islands, so there appears to be congruence in shape for small-bodied specimens, particularly in the width of the zygomatics and the protrusion of the maxilla. These morphological differences may be a result of different food resources found on small islands and on Borneo. Although Borneo is a giant island, as stated before it is unique in that the soils on the island are less fertile than on other large islands and this may have led to available food resources that are different from those on other large islands (MacKinnon et al., 1996; Waterman et al., 1988).

It is notable that in the PC analyses specimens from islands of different sizes and types occupy the same morphospace, whereas there is a clear separation of groups in most CV analyses. This is because a PC analysis describes individual specimens within groups by finding their greatest dimensions while a CV analysis analyzes the groups rather than the individual specimens (Zelditch et al., 2012). The CV analysis is thus capturing differences among groups and is more sensitive to these differences than the PC analysis.

Most shape changes seen in PC1 and PC2 for each set of landmarks are those associated with mastication. For example, the first two PCs based on the superior region of the skull show that some of the most prominent shape change is in the protrusion and elevation of the maxilla, along with the width of the skull. The inferior region of the skull and the mandible of long-tailed macaques show shape differences in the width of the molars, the position of anatomical parts towards the middle of the skull, and the width of the mandibular condyles, all of which are associated with feeding. This suggests that mastication may differ to a level that results in morphological cranial changes in macaques, especially between males and females. However, these skull differences that are seemingly associated with mastication do not appear to be related specifically to living on islands.

CONCLUSION.

Much more research still needs to be performed to fully determine the impact that island-living has on cranial morphology. My research here is a starting point that uses 3D geometric morphometrics to explore shape differences in mainland and insular *Macaca fascicularis.* The morphometric data show that this species does not display insular dwarfing in body size, and 3D shape analyses demonstrate that most shape differences among specimens are those associated with mastication. Additionally, when inferior and mandibular landmarks are analyzed, there appears to be a separation among long-tailed macaques that does not yet have a biological explanation. The canonical variate analyses indicate that there are shape differences among groups for island type and island size, although these differences are subtle. But this pattern varies according to which set of landmarks is used. Overall, this investigation elucidates some of the cranial morphological differences in mainland and insular *M. fascicularis* and establishes the need for further research in this direction.

CHAPTER 6

Concluding remarks

Evolution on islands has intrigued scientists for more than a century, yet so many questions are still left unanswered (e.g. Forsyth Major, 1902; Bate, 1903; Foster, 1964; MacArthur and Wilson, 1967; Van Valen, 1973; Heaney, 1984; Lomolino, 1985; Lomolino, 2005; Meiri et al., 2005a; Meiri et al., 2008a). The Island Rule is seemingly simple, indicating that large organisms become smaller and small organisms become larger on islands over evolutionary time. However, it refers to a very complex process that is affected by numerous variables, including — but not limited to — amount of food resources, number of predators or competitors, island size, time of isolation, distance from the mainland, and island type (Hessee et al., 1951; MacArthur and Wilson, 1967; Sondaar, 1977; Heaney, 1978; Lawlor, 1982; Harcourt, 1999; Burness et al., 2001; Lomolino, 1985, 2005; Lomolino et al., 2013; Meiri et al., 2005a, 2005b; White and Searle, 2007). So it is hardly surprising that studies concerning the Island Rule often result in conflicting conclusions.

Some of those conflicts, as explained in Chapter 3, depend upon whether a phylogeny is used to control for relatedness in the comparative analyses of insular and mainland organisms. Because past research has established the necessity to compensate for any phylogenetic non-independence in comparative studies (Felsenstein, 1985; Nunn, 2011), studies should at least test for the need to control for relationships when comparing organisms on islands and the mainland. Yet relatively few studies have allowed for effects of relatedness when testing the Island Rule (Meiri et al., 2006, 2008, 2011; Bromham and Cardillo, 2007; Welch, 2009; Itescu et al., 2014).

Various studies have revealed that the Island Rule occurs within species or very closely related species (Lomolino, 1985; Boback and Guyer, 2003; Meiri, 2007), but so far few studies have actually analyzed the Island Rule within a single species, and even fewer have controlled for intraspecific phylogenetic relatedness. My dissertation research was specifically designed to address this major gap.

I selected a species of primate, the long-tailed macaque (*M. fascicularis*), because it is a relatively large-bodied mammal that is widespread throughout a region littered with thousands of islands of varying sizes. Southeast Asia is also diverse in that mainland is present along with both continental and oceanic islands. Once I plunged into my research, I realized that I could not have chosen a better model organism or region to work on for this project focusing on island dwarfing.

The foremost goal of this research was to reconstruct an intraspecific phylogeny of *M. fascicularis* in order to determine relationships amongst populations on various islands and on the mainland and to have a phylogenetic framework to control for non-independence in the scaling analyses in Chapters 3 and 4. As samples I used dried tissue fragments collected from museum specimens, which were successfully analyzed at a much higher rate using next-generation sequencing than I had expected from results of prior studies. In fact, I would recommend that future research using museum specimens should focus on residual dried tissue fragments collected from the skull rather than cutting off pieces of skin. Although it was not a direct goal, my research in Chapter 2 provided insight into successful sequencing of DNA across museums and shotgun sequencing of DNA specimens as a method to target the mitogenome.

The final intraspecific phylogeny that I obtained, in which the sample size from previous studies was more than tripled, yielded exciting results including some unexpected findings. The most noteworthy novel feature is the separation between populations on the island of Sumatra, with a common ancestor for northern and southern Sumatran specimens at ~2.26 Ma. The northern Sumatran specimens stemmed from the mainland while the southern Sumatran specimens stemmed from Borneo. This disjunction is evident in a few other primate taxa, but not in other non-primate vertebrates. But perhaps not enough studies have been conducted within species to tease apart any such separation in other taxa. It is therefore important that future studies focus more on intraspecific relationships, as they could uncover unforeseen phylogeographic patterns.

The relationships I established in Chapter 2 have been particularly helpful for interpreting results in the subsequent research chapters, demonstrating the general usefulness of having an intraspecific phylogeny available for studying the Island Rule in addition to using it to control for relatedness. For example, in Chapter 3 I found that, although *M. fascicularis* does not display dwarfing in body size on islands in general, populations on Borneo and Sumatra appear to have smaller body sizes than those living on the mainland or on islands of lesser size (although in Chapter 5 I showed that this is not the case for Sumatran specimens when centroid size is used as an estimator of body size). Small body sizes of long-tailed macaques on Borneo may be driven by low soil quality on the island (Waterman et al., 1988; MacKinnon et al., 1996). Because it is now evident from the phylogeny that southern Sumatran specimens stemmed from Borneo, the colonization of Sumatra by certain Bornean lineages may have led to small body size in populations on that island as well. Because of such findings, I would particularly stress the importance of using an intraspecific phylogeny to infer ancestral colonization patterns.

In Chapter 3, I also pointed out that certain problems arise when using traditional methods to analyze body size and the Island Rule. Because those methods do not determine definite relationships between species nor take variation within populations or species into account when performing analyses, I suggest that future studies analyze body size and island size by plotting body size against island size for a single species. This avoids the short-cut approach of pairing insular and mainland species or populations, and it allows for visualization of body size variation on a single island. This may not be an entirely satisfactory way to determine whether the Island Rule applies to a species, because the large variation in body size on each island results in low correlation coefficients. But I would argue that is is certainly better than the pairwise comparison method that most previous studies have used.

In Chapters 4 and 5, I aimed to focus on an aspect of the Island Rule that has been relatively ignored by the scientific community: Do specific anatomical parts differ in insular organisms in comparison to their mainland counterparts? Brain size is a well-studied topic, especially in mammals (Marsh, 1874; Jerison, 1973; Martin, 1990; Marino *et al.*, 2004; Finarelli & Flynn, 2007; Rowe et al., 2011; Yao et al., 2012). Yet research into the evolution of brain size on islands has been a relatively recent development and was mostly spurred by the discovery of the Late Pleistocene LB1 hominin specimen known as *Homo floresiensis*, which is unique in its comparatively short stature and remarkably small endocranial volume relative to other members of the genus. The proposed explanation invoked to account for these unique characteristics is the "Island Rule", according to which large mammals evolve to become smaller on islands to reduce resource needs. However, the Island Rule as originally formulated applied only to body size. Since the Flores hominid was discovered, several investigators have sought examples of brain size reduction among island species and even mainland species, but the assumptions and analyses in many of these studies are questionable (Safi et al., 2005, Niven, 2005; Köhler and Moyà-Solà, 2004; Weston and Lister, 2009). So I set out to analyze brain size in *M. fascicularis* from islands and the mainland in Southeast Asia using both raw and phylogenetically corrected data. Regression analysis of endocranial volume relative to body size revealed no difference between island-living mammals and mainland relatives, but females showed statistically significantly larger relative brain sizes than males. When relative brain size is analyzed against island size, both female macaques and macaques of both sexes living on smaller islands have significantly but only slightly smaller relative brain sizes, with a slope very close to zero. Because only certain groups of long-tailed macaques appear to have marginally smaller relative brain size on islands, and given that this slight reduction may be driven by the small sample sizes available for specimens on very small islands, the results do not convincingly show that the Island Rule applies to brains and cast considerable doubt on island dwarfism as an explanation for the tiny absolute and relative brain size of *H. floresiensis*.

The findings in Chapter 4 (and Chapter 3) also demonstrate the importance of separating sexes in comparative analyses of insular and mainland organisms. Logically, it is a step that should be performed in any comparative analysis, but in the traditional pairwise method of analyzing body size and the Island Rule, the data for each species or each population are typically averaged with no regard to sex. This is especially problematic when the taxon or taxa in question are sexually dimorphic in body size, as is the case with *M. fascicularis*. In Chapter 3, I confirmed that males have larger body sizes than females, and in Chapter 4 I showed that females have larger relative brain sizes than males. These patterns would have passed unnoticed had I disregarded sex as a variable.

In Chapter 5, I used 3D landmark data to analyze cranial shape variation in *M. fascicularis* using museum specimens. 38 landmarks from the superior region of the skull, 43 landmarks from the inferior region of the skull, and 36 landmarks from the mandible were defined and analyzed separately. Because I could calculate centroid size, a global estimate of body size, from the morphometric data, I was able to test whether the Island Rule applies to body size in this species using this alternative and potentially more reliable estimator. Again, it emerged quite clearly that *M. fascicularis* does not display insular dwarfing in body size. The 3D shape analyses demonstrate that most shape differences amongst specimens are those associated with mastication. Additionally, when inferior and mandibular landmarks are analyzed there appears to be a separation among long-tailed macaques that does not yet have a biological explanation. Canonical variate analyses indicate that there are shape differences amongst groups for island type and island size, although these differences are subtle. And when I tested whether the interaction of sex affects the regression of skull shape against island size or island type, I found that interactions between sex and island type are statistically significant whereas interactions between sex and island size are not.

Most studies that use 3D landmark data have considered just a single set of landmarks for the entire skull or for only one section thereof. In my analyses, I separated the landmarks into three sets, which allowed me to recognize differences amongst them. It appears that the inferior side of the skull and the mandible tend to have similar separations in the landmark data whereas such separation is nonexistent in the superior side. So future studies should analyze the shape of different parts of the skull separately in addition to analyzing cranial shape as a whole.

My cross-disciplinary dissertation research utilized a combination of molecular phylogenetics and classical morphological and morphometric analyses to conduct the most penetrating analysis of island biology in a single species to date. It sets the stage for future studies of the Island Rule to focus research efforts *within* species rather than *between* species and to employ an intraspecific phylogeny if possible. There are multiple avenues to pursue for future research in regards to the Island Rule. For example, establishing relationships among populations on various islands and the mainland in more detail through broad-based genetic studies would be extremely useful. In particular, it would be worthwhile to explore the divergence on Sumatra for other species. Additionally, it would be beneficial to develop a statistical method that confidently tests whether the Island Rule applies to body size while also taking variation into account. Or, if one is inclined to work in the field of morphometrics, the analyses presented here are just a starting point, and investigators studying the Island Rule should develop the foundation for island ecology and masticatory morphology, perhaps by collaborating with scientists in other fields. By no means do we fully understand evolution on islands as yet. My work on *M. fascicularis* has broad implications for future research concerning evolution on islands, and I hope scientists will continue eliminating the gaps that I have only begun to fill with this dissertation.

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