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# Exploring Patterns of Genetic Diversity of a Malagasy Ant Species: Anochetus Madagascarensis

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EXPLORING PATTERNS OF GENETIC DIVERSITY OF A MALAGASY ANT SPECIES:

*ANOCHETUS MADAGASCARENSIS*

A Thesis
presented in partial fulfillment of requirements
for the degree of Doctor of Philosophy
in the Department of Biology
The University of Mississippi

by

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ABSTRACT

Madagascar is extremely diverse and imperiled. Close to 90% of all land dwelling species are endemic to Madagascar (plants, reptiles, mammals and amphibians). Understanding patterns of genetic diversity for species can aid in better conservation efforts. In this study, I focus on the endemic Malagasy ant species, *Anochetus madagascarensis*. By employing a broad geographic sample of this species from throughout its distribution and a multilocus genetic data set, I explored population structure and historical factors that affected these patterns. I tested hypotheses proposed to be responsible for generating population structure, and by extension the process of speciation in Madagascar, including geologically based models such as the Riverine and Watershed hypotheses and employed ecological niche modeling to test for evidence of ecologically driven speciation. Four genetic clusters were recovered using GENELAND; one found on Mayotte of the Comoros Islands, one restricted to the eastern coast of Madagascar, one on the northern tip of Madagascar and one along the western dry forests of Madagascar. I found no association between the position of watersheds and the population structure of this species. Rivers do appear to function as barriers to gene flow between the clusters, as major rivers (Sofia in the northwest, Antainambalana in the northeast and Mandrare in the southeast) were found to demarcate the boundaries of the three Malagasy genetic clusters. The persistence of interpopulation migration on the mainland confirms these entities do indeed represent a single species, but the magnitude and pattern of this migration reveals much about the migratory capabilities of this species and the factors that influence interpopulation connectivity. I found that the ecological niche of the four clusters are not identical, but are no less similar than

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would be expected by chance.  Together, these data provide strong support for geographic

(allopatric) diversification and the absence of significant ecological divergence despite the

occupation of very dissimilar habitat.

LIST OF ABBREVIATIONS AND SYMBOLS

BLAST:  Basic Alignment Search Tool

bp:  Base pair

COI:  Cytochrome oxidase I gene

DNA:  Deoxyribonucleic acid

ENM:  Environmental Niche Model

kb:  Kilobase

kyr:  Thousand years

km:  Kilometers

LGM:  Last glacial maximum

M/μ:  Migration per mutation

MCMC:  Markov Chain Monte Carlo

mtDNA:  Mitochondrial DNA

mya:  Millon years ago

NCA:  Nested Clade Analysis

NCBI:  National Center for Biotechnology Information

$^o$C:  Degrees Celsius

PCR:  Polymerase Chain Reaction

UV:  Ultraviolet light

ACKNOWLEDGEMENTS

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INTRODUCTION

The tropics are the most biologically diverse regions in the world (Solomon, 2008), and as such are ideal for testing models of speciation. The island of Madagascar lies just off the southeast coast of Africa and at 587,040 km$^2$, makes up less than 0.4% of the Earth's land surface. Despite this, species richness and diversity are incredibly high on the island. Madagascar has more endemic species than any other place of equivalent size on Earth (Yoder and Nowak, 2006). For example, more than 15% of all living primates are endemic to Madagascar (Yoder and Nowak, 2006). Regions, like Madagascar, with such high levels of biodiversity, are among the highest priority for terrestrial conservation.

Much of Madagascar's landscape has been altered by humans, specifically the forests (Richard and O'Connor, 1997). Only a small portion of original forest cover remains, and it is estimated that ~90% of the island's unique organisms are forest dwelling (Dufils, 2003). Humans have degraded the forests for fuel and products for building materials and have also cleared land for grazing animals and crops such as butter beans, cotton and corn (Durbin *et al.*, 2003 and Dewar, 2003). Madagascar has 46 legally protected areas located in 44 sites, covering 1,698,639 ha as of 2003 (Randrianandianina *et al.*, 2003).

Categorizing spatial patterns of species richness and endemism will allow the proper allocation of conservation funds (Kremen *et al.*, 2008). It is important to categorize areas of species richness in Madagascar because the government of Madagascar plans to increase the protected areas to include 10% of the country. Currently, 6.3% of Madagascar is protected in the

form of reserves and parks (Kremen *et al.*, 2008).  A better understanding of patterns of species richness will help managers decide which areas are top priorities (Smith *et al.*, 2005).  If scientists can determine where species are located, then they can try to protect areas where the most endemic species are found.

In order to understand the biogeography of Madagascar, one must understand its origins. Madagascar was part of the supercontinent, Gondwana, during the early Jurassic (~184 mya) (Figure 1).  Gondwana was composed of what are now South America, Africa, Madagascar, India, Australia and Antarctica.  Gondwana began to split into eastern Gondwana (Madagascar, India, Australia and Antarctica) and western Gondwana (South America and Africa) in the middle Jurassic (~166 mya).  Eastern Gondwana drifted south from Africa, however, this process was gradual and there was a possibility for biotic exchange between Africa and Madagascar until the end of the early Cretaceous (~130-118 mya).  As eastern Gondwana continued to drift south, Madagascar and India remained connected as the IndoMadagascar subcontinent.  Antarctica and Australia separated from IndoMadagascar shortly after eastern and western Gondwana split apart.  Recently, fossil studies from the late Cretaceous suggest that there may have been a land bridge that connected Antarctica to South America and Antarctica to IndoMadagascar that lasted until ~80 mya (Krause, 2001).  Madagascar separated from India ~88 mya and like Madagascar's separation from Africa, this too, was gradual (Yoder and Nowak, 2006).  Lying more than 400 km from the nearest landmass (Africa) Madagascar has been isolated for the last 80-90 mya (Vences, 2009).  This temporal and geographic isolation (Madagascar is also 4000, 5000 and 6000 km from India, Antarctica and Australia respectively) has led to a remarkably unique and diverse biota (Yoder and Nowak, 2006).
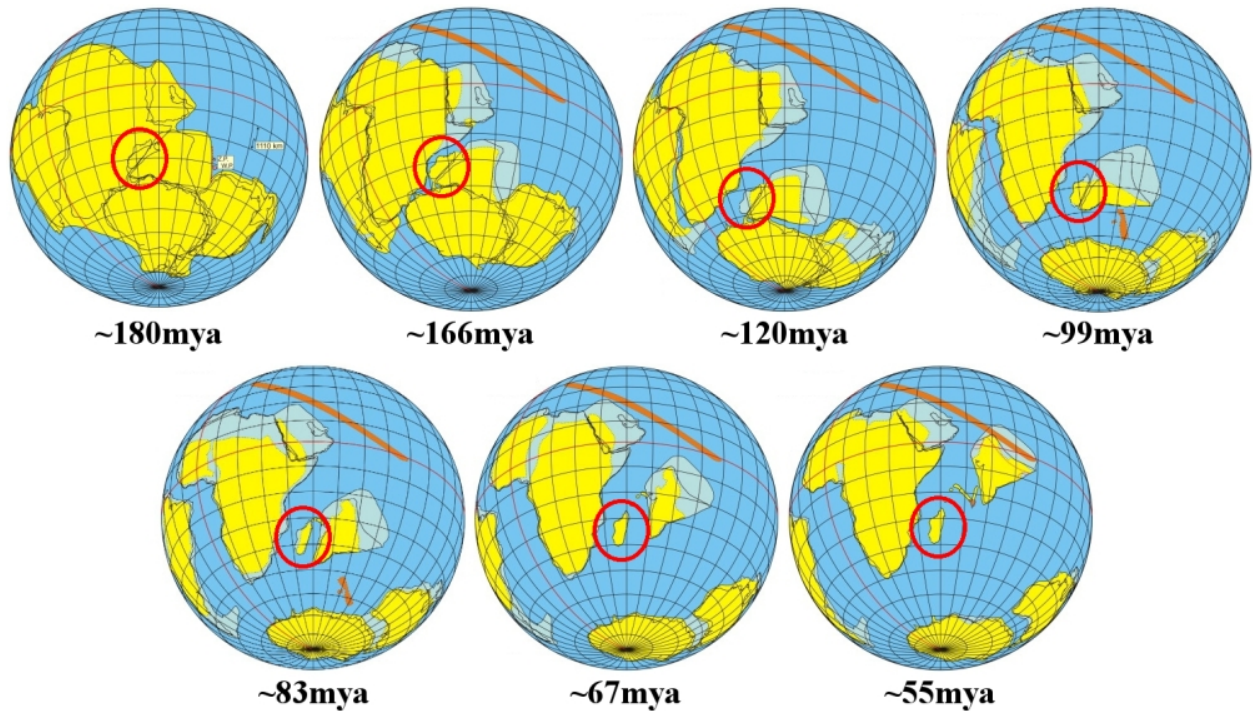
Figure 1: The position of Madagascar throughout the breakup of Gondwana. The gradual breakup of Gondwana (over ~ 125my) into what is now known as South America, Africa, Madagascar, India, Australia and Antarctica. Madagascar is located in the red circle (Ali and Aitchison, 2008).

The long separation from other land-masses has led to endemism on the island (Pearson and Raxworthy, 2008). More than 90% of all the plant species found on Madagascar occur nowhere else on earth. Approximately 44% of birds, 74% of lepidopterans, 92% of reptiles and 100% of amphibians and terrestrial mammal species on Madagascar are endemic to the island (Vences *et al.*, 2009). The extreme antiquity of Madagascar and relatively long isolation time raise questions about the origins of the high diversity and endemism on the island. There have been several hypotheses put forward to explain how organisms colonized Madagascar. Vicariance has been the major mechanism used to explain trans-oceanic distributions of organisms to continental islands (Bocxlaer *et al.*, 2006). Vicariance is an actual change in the

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geography of the region, including such phenomena as mountain building, sea level fluctuation (which can expose land bridges) and tectonic movement (such as the breakup of Gondwana) (Vences *et* al., 2001 and Haffer, 1996). It has been proposed that some organisms were able to cross into Madagascar via land bridge connections (Noonan and Chippindale, 2006). For example, Noonan and Chippindale (2006) found that the presence of some Malagasy reptiles (boid snakes, podocnemid turtles and iguanid lizards) was due to a land bridge connection to Antarctica approximately 80 mya.

Most studies have found that trans-oceanic migration has led to some of the colonizations of Madagascar. Some organisms have migrated to Madagascar since its isolation in the Late Cretaceous (see Monaghan *et al.*, 2005; Raxworthy *et al.*, 2002). Mayflies colonized Madagascar from Africa through multiple dispersals (Monoghan *et al.* 2005). Raxworthy *et al.* (2002) found that chameleons originated in Madagascar and have dispersed multiple times to the African mainland and other islands in the Indian Ocean. Some organisms have ties to taxa found in Africa, supporting Cenezoic origins and subsequent dispersal to Madagascar and other continents connected in Gondwana (Yoder and Nowak, 2006).

Madagascar is divided longitudinally by a north-south chain of mountains that run down the eastern side of the island. Combined with the complex pattern of eastern trade winds, this topography produces highly variable climates across the island. The northern tip of Madagascar and the eastern side of the mountains tend to be tropical, with humid forests, owing in part to the Eastern trade winds that provide a substantial amount of rainfall. There is very little rainfall in the west and south of Madagascar causing it to be more arid (Boumans *et al.*, 2007). Biomes of Madagascar are extremely diverse, ranging from the tropical humid northeast and east to the subarid southwest (Vences, 2009). These conditions can lead to microendemism in areas of the

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island where individual species can become specialized to certain types of environments. For example, Wilme *et al.* (2006) found that species confined to low elevational watersheds had more endemic species than watersheds located at higher elevation. Also, almost all of the leaf chameleons in the genus *Brookesia* occupy a relatively narrow elevational range restricted to northern rainforests (Raxworthy and Nussbaum, 1995).

In allopatric speciation, geographical changes can effectively separate one population into several isolated populations. Geographic barriers are defined as a barrier that the species can no longer cross. These barriers, however, are not limited to actual geographic barriers such as mountain ranges, and rivers. Environmental changes, such as climate change, can also separate a population if it occurs more rapidly than the species can adapt/evolve. These species can become isolated in ecological niches that are now divided by unfavorable conditions (Haffer 2008).

There have been several hypotheses proposed relating to factors that drive speciation on the island of Madagascar. Each of these hypotheses describe some sort of barrier (e.g. rivers, mountains and even unsuitable habitat due to unsuitable climates and other ecological barriers) that the species can no longer cross that give rise to allopatric speciation if the barrier remains.

Forested and non-forested regions have changed continuously in distribution over time, fragmenting and expanding due to climate change (Haffer, 1996). These changes have occurred several times over the last 60 million years. When these changes occurred, small patches of suitable habitat that remained acted as a refuge for species. If the species are isolated long enough, there may be opportunity for them to speciate. This is known as the refuge hypothesis (Haffer, 1996). For example, several species of reptiles (geckos and boid snakes) and several

amphibian species (treefrogs) that were widespread throughout Madagascar adapted to either humid (eastern side of Madagascar) or dry (western side of Madagascar) regions when the climate fluctuated (Nussbaum and Raxworthy, 1998, Nussbaum *et al.*, 1998, Glaw and Vences, 1994, Andreone *et al.*, 2002, Vences and Glaw, 2002 and 2003,).  There have also been several studies that show a north-south split in some vertebrates including mouse lemurs (Yoder *et al.,* 2000 and Yoder and Heckman, 2006) and dwarf chameleons (Raxworthy *et al*., 2002).  Boumans *et al.* (2007) found a similar north-south pattern for several reptile species including chameleons and geckos.

The riverine hypothesis suggests that rivers form a barrier to interpopulation migration (Goodman and Ganzhorn, 2004).  Individuals of a population are separated when a river is formed and they are unable to traverse the river, especially in the lower reaches where the rivers are the widest (Figure 2) (Vences *et al.*, 2009).  There are several problems with this hypothesis. First, one must assume the individuals that have been separated by the river are unable to cross the river easily.  Second, the headwaters of the rivers tend to be less of a barrier to gene flow. And lastly, animals can be "passively" transported across the river on debris or floats (Haffer, 1996).  Studies of vertebrate phylogeographic patterns in Madagascar (Pearson, 2009; Goodman and Ganzhorn, 2004) have reported some evidence supporting a role for rivers in structuring biodiversity (e.g. lemurs; Goodman and Ganzhorn, 2004).
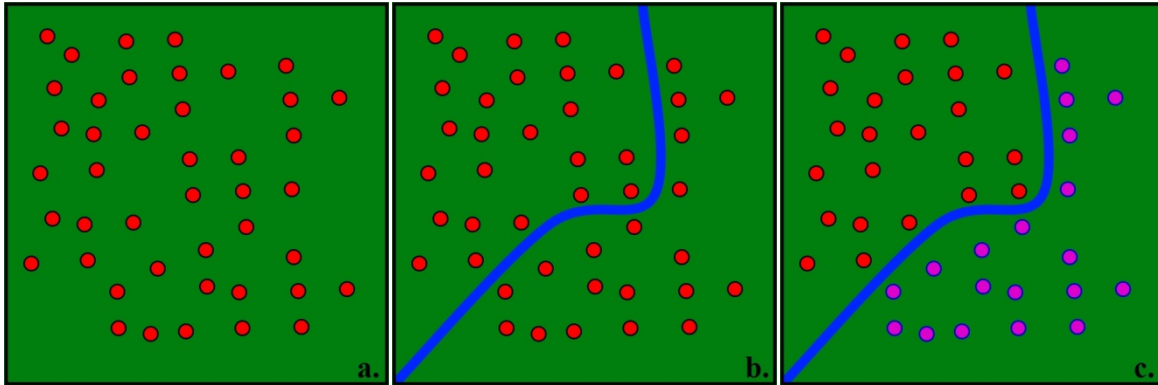
Figure 2: Riverine barrier hypothesis, where a continuous distributional range (a) is separated by a river (b) which leads to vicariance (c). (Vences *et al.*, 2009)

More recently, a role for high altitude watersheds has been invoked as a causal factor in the origination of such high levels of Malagasy biodiversity. This hypothesis states that fluctuations in the climate over time have shaped the population structure by cyclically altering connectivity among watersheds. During times of cooler and drier climates, watersheds with sources at high altitudes could act as a refuge for species adapted to the more mesic conditions, giving them the ability to move around within that watershed. Watersheds with sources at lower elevations are predicted to have been drier than higher elevation habitats (shaded regions of figure 3), and species found in those areas would be trapped within that region separated from mesic areas by arid regions that act as a barrier to gene flow (Wilme, 2006). This hypothesis encompasses more than just the rivers in a given area, unlike the riverine hypothesis. When climates changed in the past from more mesic conditions to drier conditions, associated with glacial maximums, species that were located in higher elevation watersheds were buffered from the drier conditions because of the connection to high elevation water sources (Townsend *et al.*, 2009). The last glacial maximum (LGM) was 23,000-18,000 years ago affecting the present day

distribution of species and was less extreme in equatorial regions (Proven and Bennett, 2008). When there is a glacial maximum, most of the fresh water is frozen and unavailable to organisms. In equatorial regions, the effect is less severe because the water doesn't freeze; the conditions become more arid (Proven and Bennett, 2008). The orographic precipitation allows for the perpetuation of mesic conditions along the courses of rivers with sources at high elevations during climatic cycles of low rainfall. This allows species adapted to those conditions the ability to move within the watershed because there is more water available than in a watershed associated with lower altitudes. Species that are unable to track these forested corridors and move among these high altitude watersheds are then isolated, which would lead to diversification in isolation. In a study of 41 vertebrate species, Pearson and Raxworthy (2008) found that 20 exhibited population structure associated with watersheds. For these, the assumption is that the watersheds acted as refugia during periods when precipitation was greatly reduced. When precipitation subsequently increased, the species were again able to move across a less fragmented landscape. One of the limitations of the watershed hypothesis is the temporal extent of glacial maxima and subsequent reconnection of isolates. The average extent of any glacial period was less than 30 kyr, presumably insufficient time for species differentiation. So far, this pattern has been supported by patterns observed in some groups of reptiles and lemurs and is difficult to distinguish from the riverine barrier hypothesis (Vences *et al.*, 2009).
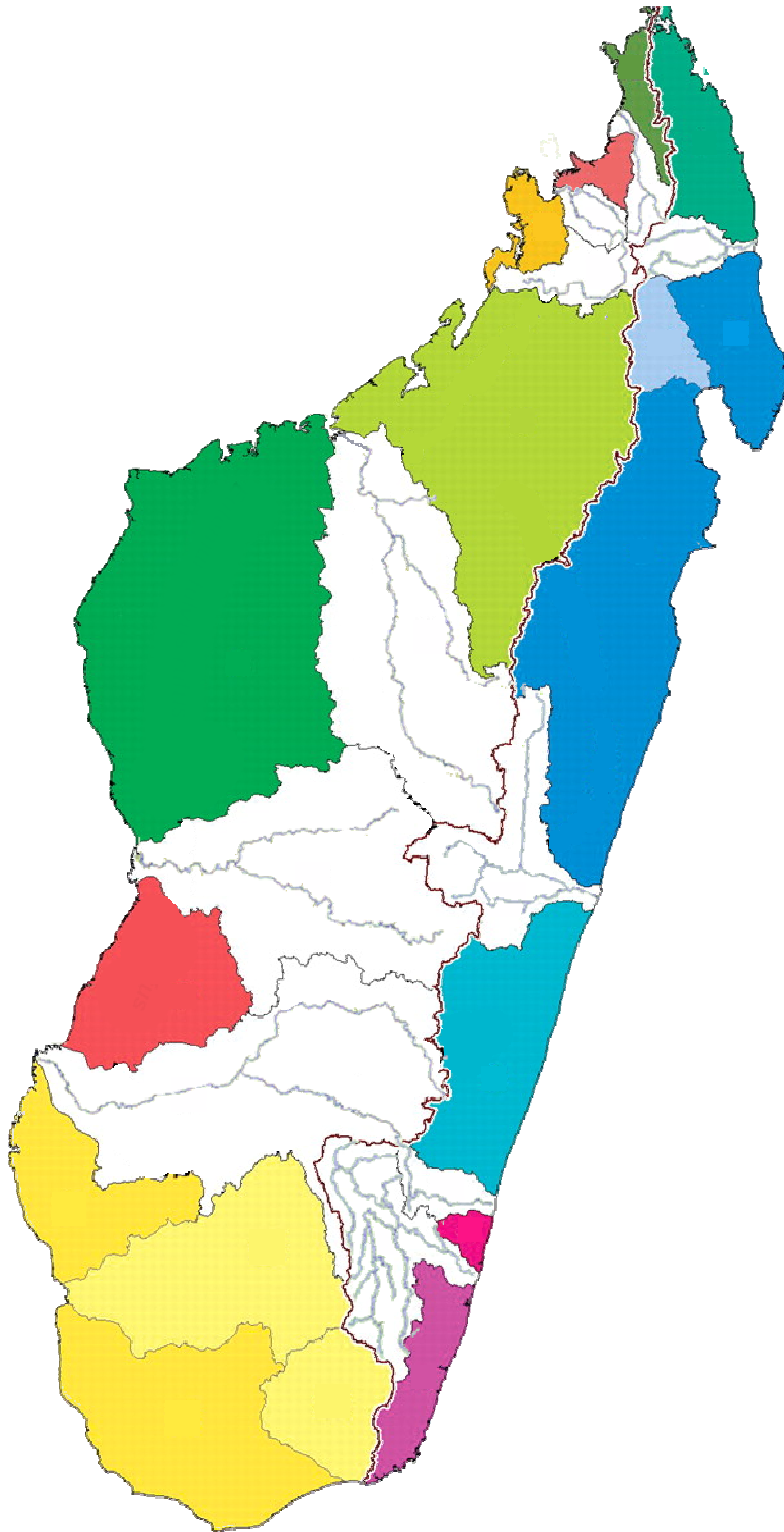
Figure 3: Watershed map of Madagascar.  The white areas are regions associated with high elevation watersheds and drainage basins and are likely to remain wet even during dry periods.

The colored areas are regions associated with low elevation watersheds and where endemics are expected to occur (Wilme *et al.,* 2006)

There have also been studies that show that species adapt to certain elevations (Wollenberg *et al.*, 2008). Montane endemism tends to be very high in tropical regions, where species are confined to a very narrow elevational zone at or near the summit of a mountain (Raxworthy *et al.*, 2008). Wollenberg *et al*. (2008) examined patterns of spatial niche conservation in cophyline frogs, finding that mountain massifs have functioned as refugia for these taxa. Wiens and Graham (2005) define niche conservation as the tendency of a species to maintain ancestral ecological characteristics. As a result, ancestral ecological characteristics may be retained within a speciating lineage. If a species is limited to a specific climatic optimum, then this limits that species' ability to geographically change its range, potentially leading to allopatric speciation. Here, the same climate change that may have caused species isolation in watersheds may have changed species distribution and interpopulation continuity on mountains.

To better understand the roles of these mechanisms driving diversification, I explored historical phylogeography of a widespread species of ant endemic to Madagascar and the nearby Comoros Islands. As invertebrates form the bulk of terrestrial diversity and are important in ecosystem function (Fisher, 1999), evolutionary patterns of these oft neglected taxa can be particularly informative in understanding the history of other components of the biota. Invertebrates, especially insects, are the most abundant and diverse animal species in tropical areas (Solomon *et al.*, 2007). Because ants are ectotherms, this makes them ideal subjects for studying the effects of global climate change as ectotherms, may be more sensitive to changes in

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temperature and precipitation (Dunn *et al.*, 2009). Ectotherms that live in the tropics have a very narrow temperature range and most are already living at the upper limit of their optimal temperatures (Deutsch *et al.*, 2008).

Madagascar has a diverse ant fauna with 48 of the 52 ant genera estimated to be indigenous to the Malagasy region (Fisher, 1997). There are thought to be as many as 1000 species, and of that number, ~96% are endemic to Madagascar (Smith *et al.*, 2005). In this work, I focus on *Anochetus madagascarenesis*, a widespread species found throughout Madagascar and the Comoros Islands in forests or shrubland habitats below 1100m elevation. By employing a broad geographic sample of this species from throughout its distribution and a multilocus genetic data set, I explored population structure and historical factors affecting these patterns. Specifically, I tested hypotheses proposed to explain biotic diversification in Madagascar using methods that examine the genetic structure of populations and their ecological tolerance/differentiation. I did not consider the montane endemism hypothesis (Wollenberg *et al.*, 2008) because *A. madagascarensis* is not located above 1100 meters.

I considered the riverine hypothesis, which as stated above, suggests that rivers form barriers to gene flow. If this hypothesis is contributing to the distributional patterns of *A. madagascarensis,* groups would be found on opposite sides of major rivers in the areas where this species is found. If genetic diversity is shaped by the rivers of Madagascar, the expectation would be significant genetic differentiation among populations on opposite sides of major rivers. Distinct from this is the watershed hypothesis, which predicts evolutionary divergence among low elevation watersheds. If the elevation of watersheds explains population structure, I would expect to see a genetic difference between populations that is associated with lower elevation watersheds and an absence of structure among watersheds with high elevation sources.

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Finally, I will use niche modeling to determine if populations that are divided by some sort of physical or ecological barrier have diverged in their niche requirements. If they have, I would expect that tests of niche identity/equivalency would show statistically significant ecological differences indicating that the niche for one group is not identical to the niche of another group. If ecologically differentiated, populations may be effectively isolated and no longer able to exchange alleles and may represent divergent species. However, if niche similarity/background tests fail to show statistically significant differences, then this suggests that a barrier is isolating the two groups, that if removed, the two would freely exchange alleles.

MATERIALS AND METHODS

*Samples and DNA sequencing*

Collections of *Anochetus madagascarensis* have been made by Brian Fisher and colleagues throughout Madagascar and the Comoros islands over the last fourteen years (1997-2011).  Once collected, specimens were preserved in 100% Ethanol and deposited in the California Academy of Sciences entomology collection.  A total of 71 individuals representative of 71 total collections (collection events from different localities) were used for this study of island-wide population structure.  Specimen data can be found in Table 1.

Table 1:  Specimen codes and locality of each individual of *Anochetus madagascarenesis.*

| Specimen Code | Collection Code | Country | Latitude | Longitude |
| --- | --- | --- | --- | --- |
| CASENT0454527-D20 | BLF03252 | Madagascar | -13.79861 | 48.16167 |
| CASENT0460281-D20 | BLF03338 | Madagascar | -14.30889 | 47.91433 |
| CASENT0007166-D20 | BLF01908 | Madagascar | -13.97667 | 48.42333 |
| CASENT0071786-D20 | BLF14343 | Madagascar | -22.14817 | 48.02267 |
| CASENT0004382-D20 | BLF02072 | Madagascar | -25.06167 | 46.87 |
| CASENT0007168-D20 | BLF01486 | Madagascar | -23.65 | 44.63333 |
| CASENT0004381-D20 | BLF02102 | Madagascar | -24.77167 | 47.17167 |
| CASENT0416405-D20 | BLF03200 | Madagascar | -12.25889 | 49.37467 |
| CASENT0006746-D20 | BLF03128 | Madagascar | -12.32278 | 49.33817 |
| CASENT0120032-D20 | BLF15305 | Madagascar | -24.9815 | 46.92567 |
| CASENT0416427-D20 | BLF03034 | Madagascar | -12.86361 | 49.22583 |

| | | | | |
|---|---|---|---|---|
| CASENT0042377-D20 | BLF05420 | Madagascar | -25.00778 | 46.306 |
| CASENT0443500-D20 | BLF05452 | Madagascar | -24.92972 | 46.20967 |
| CASENT0048278-D20 | BLF10745 | Madagascar | -17.28333 | 49.43333 |
| CASENT0461550-D20 | BLF03422 | Madagascar | -13.41944 | 48.33117 |
| BLF1996(18)-D20 | BLF01996 | Madagascar | -13.96167 | 48.43333 |
| CASENT0066826-D20 | BLF12721 | Madagascar | -16.67233 | 49.70117 |
| CASENT0120033-D20 | BLF15466 | Madagascar | -24.569 | 47.204 |
| CASENT0120040-D20 | BLF15419 | Madagascar | -25.03883 | 46.996 |
| CASENT0043819-D20 | BLF09872 | Madagascar | -13.16667 | 49.71 |
| CASENT0041768-D20 | BLF10116 | Madagascar | -13.21167 | 49.55667 |
| CASENT0048218-D20 | BLF10787 | Madagascar | -17.175 | 49.268 |
| CASENT0071206-D20 | BLF13833 | Madagascar | -23.19383 | 47.723 |
| CASENT0486367-D20 | BLF06635 | Madagascar | -16.40667 | 45.31 |
| CASENT0107422-D20 | BLF11287 | Madagascar | -12.80467 | 49.37383 |
| CASENT0466025-D20 | BLF04340 | Madagascar | -19.13222 | 44.81467 |
| CASENT0491074-D20 | BLF06750 | Madagascar | -16.925 | 44.36833 |
| CASENT0006309-D20 | BLF02043 | Madagascar | -24.95167 | 47.00167 |
| CASENT0416391-D20 | BLF02654 | Madagascar | -12.46889 | 49.24217 |
| CASENT0061067-D20 | BLF12296 | Madagascar | -21.4 | 47.94 |
| CASENT0487653-D20 | BLF10300 | Madagascar | -13.11833 | 49.23 |
| CASENT0489665-D20 | BLF07427 | Madagascar | -22.59167 | 45.12833 |
| CASENT0491969-D20 | BLF06475 | Madagascar | -16.46667 | 45.35 |
| CASENT0067011-D20 | BLF13091 | Madagascar | -17.7095 | 49.454 |
| CASENT0120036-D20 | BLF15105 | Madagascar | -24.7585 | 46.85367 |
| CASENT0120335-D20 | BLF15672 | Madagascar | -24.95267 | 47.0025 |
| CASENT0054031-D20 | BLF11672 | Madagascar | -15.96267 | 47.43817 |
| CASENT0040872-D20 | BLF09426 | Madagascar | -13.08333 | 49.90833 |
| CASENT0041186-D20 | BLF09556 | Madagascar | -13.255 | 49.61667 |
| CASENT0053798-D20 | BLF10879 | Madagascar | -13.26333 | 49.60333 |

| | | | | |
|---|---|---|---|---|
| CASENT0107696-D20 | BLF11554 | Madagascar | -13.4645 | 48.55167 |
| CASENT0071691-D20 | BLF13972 | Madagascar | -23.01583 | 47.719 |
| CASENT0109556-D20 | BLF11245 | Madagascar | -13.71533 | 50.10167 |
| CASENT0109757-D20 | BLF10996 | Madagascar | -14.668 | 50.18667 |
| CASENT0109521-D20 | BLF11044 | Madagascar | -14.67933 | 50.18367 |
| CASENT0498416-D20 | BLF09981 | Madagascar | -12.97 | 49.7 |
| CASENT0017631-D20 | BLF05316 | Madagascar | -24.95694 | 46.2715 |
| CASENT0432920-D20 | BLF04232 | Madagascar | -19.14194 | 44.828 |
| CASENT0490650-D20 | BLF07693 | Madagascar | -22.31333 | 45.29167 |
| CASENT0133006-D20 | BLF19060 | Mayotte | -12.96279 | 45.15037 |
| CASENT0132392-D20 | BLF19049 | Mayotte | -12.85492 | 45.19889 |
| CASENT0132939-D20 | BLF19098 | Mayotte | -12.76796 | 45.18615 |
| CASENT0132387-D20 | BLF19047 | Mayotte | -12.85492 | 45.19889 |
| CASENT0132459-D20 | BLF18959 | Mayotte | -12.80586 | 45.10054 |
| CASENT0132557-D20 | BLF18915 | Mayotte | -12.95903 | 45.13411 |
| CASENT0071786-D30 | BLF14343 | Madagascar | -22.14817 | 48.02267 |
| CASENT0134927-D20 | BLF18814 | Mayotte | -12.7926 | 45.10764 |
| CASENT0132276-D20 | BLF18639 | Mayotte | -12.80632 | 45.15314 |
| CASENT0132524-D20 | BLF18882 | Mayotte | -12.95776 | 45.13403 |
| CASENT0132743-D20 | BLF19070 | Mayotte | -12.76894 | 45.19021 |
| CASENT0132937-D20 | BLF19084 | Mayotte | -12.76796 | 45.18615 |
| CASENT0132833-D20 | BLF19035 | Mayotte | -12.86735 | 45.20827 |
| CASENT0132554-D20 | BLF18906 | Mayotte | -12.95903 | 45.13411 |
| CASENT0134888-D20 | BLF18976 | Mayotte | -12.87585 | 45.15672 |
| CASENT0132749-D20 | BLF19065 | Mayotte | -12.76894 | 45.19021 |
| CASENT0133230-D20 | BLF18962 | Mayotte | -12.87585 | 45.15672 |
| CASENT0133841-D20 | BLF18636 | Mayotte | -12.80632 | 45.15314 |
| CASENT0132530-D20 | BLF18888 | Mayotte | -12.95776 | 45.13403 |
| CASENT0134966-D20 | BLF18811 | Mayotte | -12.7926 | 45.10764 |

| CASENT0134839-D20 | BLF18758 | Mayotte | -12.75754 | 45.0678 |
| CASENT0505297-D20 | BLF04297 | Madagascar | -19.142 | 44.828 |

DNA was extracted using a modified salt extraction method (Teoh *et al*., in prep) on a single ant from each collection. Each extraction was then tested for the presence of high quality DNA by PCR amplification using conserved primers for the ribosomal, 18s gene. The rDNA 18s gene is a ribosomal RNA sequence found in all eukaryotic cells. Amplification success was tested by running the PCR products on a 1% agarose gel containing GelGreen DNA stain (Phenix Research), which binds to DNA and fluoresces under ultraviolet (UV) light, and photographing the gel while illuminated with UV. For samples in which the modified salt method failed, a second extraction was performed using the Wizard SV Genomic DNA purification system (Promega).

Highly variable molecular markers are needed to explore evolutionary history and demographic patterns within species. For the purposes of this study I employed anonymous nuclear loci to test evolutionary hypotheses and explore population structure and genetic diversity. An anonymous locus is an unknown region of the nuclear genome with no known function, but at least two allelic states that can be scored through DNA sequencing. Twenty-four anonymous loci were created for the *Anochetus* study using the method of Noonan and Yoder (2009). Total genomic DNA was extracted from single specimens of *Anochetus madagascarensis* using the high-salt precipitation method of Crandall *et al.* (1999). This extraction was amplified using the whole genome amplification kit, QIAGEN Repli-G, to increase yield. Amplified genomic material from multiple individuals was then combined into a

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concentrated solution (~550 ng/ul) assayed using a NanoDrop ND-1000. To prepare the

genomic DNA for the construction of DNA library, this genomic DNA was fragmented via

restriction enzyme digestion with *Rsa*1, which generates blunt ended fragments. Digested DNA

was then visualized on an agarose gel (1%), and size selected to remove fragments too large

(>3kb) or too small (<1kb) for marker development. Fragments within this size range were

excised from the agarose gel and purified using a QIAGEN Gel Extraction kit and eluted with

water to facilitate concentration. The DNA in the gel extraction elution was then quantified via

NanoDrop and concentrated via vacufuge to 25 ng/ul giving a 10:1 molar ratio of insert:vector in

the subsequent cloning reactions. Approximately 100 ng of the size-selected DNA was ligated

into 25 ng of pCR Blunt vector, which was then transformed into competent *Escherichia coli*

One Shot TOP10 cells (Invitrogen) and plated on agar plates containing 50 µg/mL kanamycin

and 60 µg/mL X-Gal and grown overnight at $37^o$ C. PCR was performed on positive

transformants (clones containing a fragment of the *Anochetus* genome) using M13 primers, by

transferring bacteria directly from the plate to the reaction mixture. All fragments in the size

range of 600-1500 bps were sequenced in both directions. Sequences were then examined for

undesirable characteristics (high AT content, lack of suitable priming sites, presence of repetitive

elements) and compared to the NCBI database using a BLAST search to determine whether the

locus demonstrated similarity (and thus potential homology) to known functional genes, in which

case the fragment was excluded from marker development. Primers were designed to amplify a

small region (400-600 bp) of the cloned fragments using the PRIMER3 algorithm in Geneious

(v. 4.7.4).

    The 24 anonymous loci were then tested on a panel of seven individuals representative of

the geographical distribution of the species. PCR was performed using the following conditions:

initial denaturation at 94 °C for 90 seconds; 10 cycles of 94 °C for 35 seconds, annealing at 63

°C (with a -0.5 °C per cycle) for 35 seconds, extension at 72 °C for 60 seconds; 10 cycles of 94

°C for 35 seconds, annealing at 58 °C for 35 seconds, extension at 72 °C for 60 seconds; 15

cycles of 94 °C for 35 seconds, annealing at 52 °C for 35 seconds, extension at 72 °C for 60

seconds, and a final extension of 72 °C for 10 minutes. PCR products were then visualized on

an agarose gel (1%) that contained GelGreen (Phenix Research) and visualized via UV light, and

five loci were chosen for this study based on amplification success from this seven individual

panel (Table 2). The criteria for whether or not a locus was chosen were based upon how well

the locus amplified and whether the primer pair was specific enough to produce only a single

band; the presence of multiple amplified fragments revealed some primer pairs to be non-specific

and thus unsuitable for Sanger sequencing. Once the target loci were chosen, PCR was

performed on all individuals (71 total) for all 5 loci under the following conditions: initial

denaturation at 94 °C for 90 seconds; 30-35 cycles of 94 °C for 45 seconds, annealing

temperature, (varies by locus; see Table 2), for 45 seconds, extension at 72 °C for 45 seconds to

one minute and a final extension of 72 °C for 10 minutes. PCR products were then run on 1%

agarose gels containing GelGreen (Phenix Research) and visualized via UV light. Successful

PCR products were purified with ExoSap-IT (GE Healthcare) prior to sequencing. ExoSap-IT is

a combination of two hydrolytic enzymes: Exonuclease I and Shrimp Alkaline Phospatase, each

of which performs a specific function in the cleanup of PCR products. Exonuclease I removes

residual single-stranded primers and extraneous single-stranded DNA produced in the PCR.

Shrimp Alkaline Phosphatase removes unincorporated dNTPs from the PCR mix. Products were

then sequenced on an ABI 3130 genetic analyzer (Applied Biosystems) at the University of

Mississippi using a BigDye Terminator Cycle Sequencing Kit (Applied Biosystems).

Table 2: Primer sequences and annealing temperatures for anonymous loci used in this study.

| Primers | sequence | annealing temperature in °C |
|---------|----------|------------------------------|

206

| F | AATTTCCCAGAAATGCATCG | 50 |
|---|----------------------|----|
| R | GTTCTCGACGCCTACAAAGC |    |

242

| F | TGTAACGTCCCAAGTGGTCA | 56* |
|---|----------------------|-----|
| R | CCGTAACACCTCCCCCTATT |     |

247

| F | TCACCAAAACCTCGGGATAG | 57 |
|---|----------------------|----|
| R | ACTCCAAGATGCTTGCTCGT |    |

* = Touchdown of -0.2/cycle

*Editing and Alignment*

Sequences were edited and aligned with Geneious Pro (v. 4.7.4). The default settings for Geneious Alignment were used to align the sequences (Cost Matrix: 65% similarity (5.0/-4.0); Gap open penalty: 12; Gap extension penalty: 3; Alignment type: Global alignment with free end gaps; Refinement iterations: 2). The IUPAC nucleotide ambiguity codes were used in cases where individuals appeared to be heterozygous for a particular nucleotide or where sequence signal was ambiguous. PHASE (v. 2.1), a program that reconstructs haplotypes using Bayesian statistical methods, was used to determine the sequence of alleles in heterozygous individuals.

*Phylogenetic Analyses*

Mitochondrial DNA (mtDNA) of the Cytochrome oxidase I (COI) gene for *A. madagascarensis* was used to construct a haplotype network and perform nested clade analysis. To reconstruct phylogenetic relationships among populations of *A. madagascarensis*, mtDNA used in the study Fisher and Smith (2008) was obtained. A phylogenetic tree of unique haplotypes was estimated using an algorithm from Templeton *et al.* (1992) (Figure 5). A haplotype network is similar to a gene tree, except that a network shows haplotypes at nodes (interior) or tips and each step found in the network represents one mutational step of a haplotype. TCS (v. 1.21) was used to identify mitochondrial haplotypes present in the dataset, calculate their frequencies, and generate a haplotype network (figure 6). Following Templeton *et al.* (1987), haplotypes were identified as either tip (those that are only connected to one other haplotype) or interior (those that are connected to two or more haplotypes). Starting with a tip haplotype and proceeding toward the center of the map to the next change constituted 1-step. This process was repeated until all 1-step clades were identified (this is the connection between the tip haplotype and the interior haplotype with which it is connected by $j + 1$ mutational steps). Once the 1-step clades were identified, then the 2-step clades were determined by in a similar fashion. This process continued until all clades were combined into a single 5-step clade. The haplotype network was then used to perform a Nested Clade Analysis (NCA) of *A. madagascarensis* in order to detect the presence of population structure within this widespread species. Nested Clade Analysis is useful for analyzing haplotype networks and testing for associations between haplotypes and geography to infer processes that could have led to the current population structure of the species (Templeton *et al.*, 1992). *GEODIS* (v. 2.5) was used to test hypotheses of population structure by calculating the clade distance ($D_c$) and the nested clade difference ($D_n$). The clade distance measures the geographical spread of the clade and the

20

nested clade distance measures how each clade is distributed relative to other clades in the same

higher-level nesting category.  The clade comparisons are calculated as the average pairwise

geographic distance between members of the same focal clade and the average pairwise distance

between members of the focal clade and all members of the nesting clade.  The significance of

these values were then determined using a dichotomous key (v. 2.5, Clement *et al.,* 2000)

devised on the expected patterns of geographical association based on three types of historical

events: restricted gene flow, range expansion and allopatric fragmentation.  If it is found that

these values are not significant, then there is no support for geographic patterns structuring

haplotypes.  The interior-tip statistic (I-T) was then used with *Anochetus grandidieri* as an out-

group to specify which haplotype is the oldest (interior) and which are younger (tips).  This

information was useful to establish patterns of ancestry for the haplotypes and a framework for

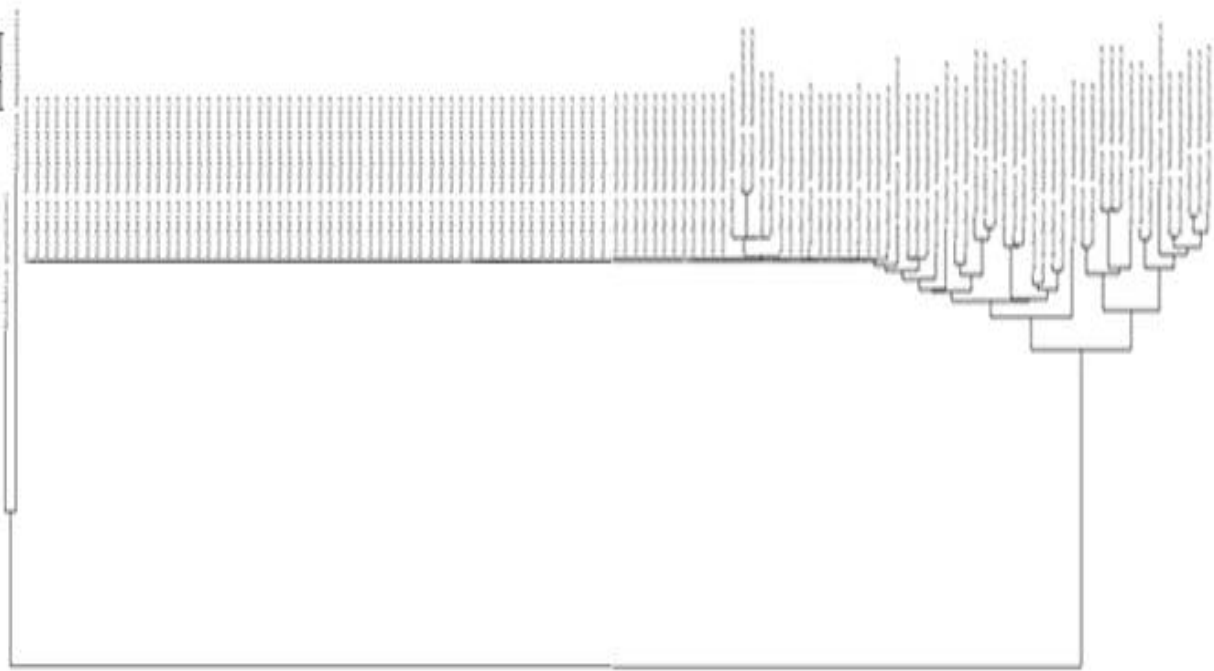exploring patterns with nuclear sequence data.

Figure 5:  Mitochondrial gene tree of the Cytochrome oxidase I (COI) gene for *Anochetus madagascarensis.  Anochetus grandidieri* was used as the outgroup to root the tree.
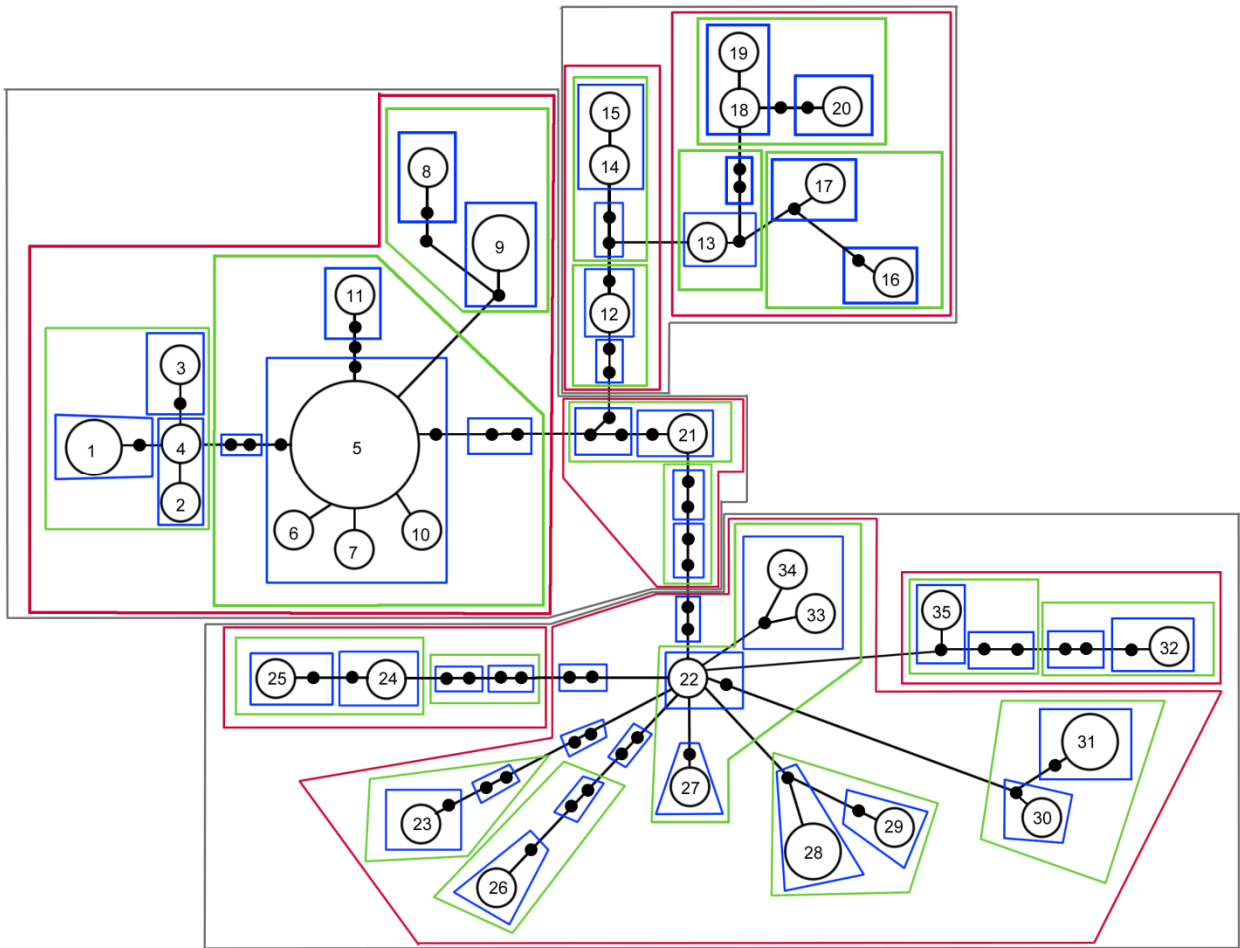


Figure 6:  Nested clade analysis using the mitochondrial DNA gene Cytochrome oxidase I (COI) on the 71 individuals collected.  Blue boxes represent one-step clades.  Green boxes represent two-step clades.  Red boxes represent three-step clades and the gray boxes represent four-step clades.

Sequences of anonymous loci required the resolution of heterozygous sites to identify the distinct alleles present.  DnaSP (v. 5) was used to calculate DNA sequence statistics between and among populations, and incorporated the heterozygote site resolution analyses of PHASE (v.

2.1). With these applications I inferred the distinct alleles present in the dataset via Bayesian

methods and assigned alleles to individuals. Once the allelic phase for each individual was

determined, the data was analyzed with GENELAND (v.3.2.4), a program that combines

geospatial data for each sampled allele to determine population structure and the number of

genetic populations (Guillot *et al.*, 2005a, 2005b, 2008 and Guillot, 2008). GENELAND

employs a Markov Chain Monte-Carlo (MCMC) algorithm to estimate the parameters *K* (the

number of population clusters) and assigns a probability of assignment of each individual to each

cluster. GENELAND was run under two different models. The first model, the correlated allele

frequency model, was run under the following parameters: ploidy set to diploid, number of

populations (K) set to vary from 1-10, $10^4$ MCMC iterations with thinning set to save 100

iterations, maximum rate of Poisson process fixed at 100, maximum number of nuclei in the

Poisson-Voronoi tessellation fixed to 300 and an uncertainty associated with the spatial

coordinates of 0 km and the allele frequency model set to Correlated. Ten multiple independent

runs were conducted. When these runs were complete, *K* was then estimated from the modal

values. $F_{ST}$ and $F_{IS}$ values were calculated for each of the ten independent runs. The second run

employed the uncorrelated model under the same conditions as above. The runs were then

processed with a burn-in of 200 iterations to obtain probabilities of individual membership into

proposed clusters.

Once clusters were identified by GENELAND, IMa2 (Isolation with migration v. 6.3.10)

was used to explore demographic history of the clusters: interpopulation migration (m), time of

divergence (t) and population size (q) (Hey and Neilsen, 2007). Several trial runs were done in

order to estimate the suitability of various priors for these parameters. Once conservative priors

for m, q and t were determined empirically, a prior file was constructed. IMa2 was then run

using the prior file with medium heating (-hfg –hn40 –ha0.975 –hb0.75) and a burn-in duration of $10^4$.

*Niche Modeling*

Niche modeling was used to explore the influence of niche conservatism on diversification within *A. madagascarensis.* The null hypothesis of this analysis is that genetically distinct populations are isolated from one another by intervening habitat unsuitable for persistence and resistant to dispersal. MAXENT (employing the maximum entropy model) is an effective modeling program that uses presence-only data to estimate species distributions by finding the closest to uniform distribution within the environmental variable constraints (Elith *et al.* 2006). MAXENT creates ecological niche models (ENMs) by combining the GIS data of the species locations with environmental data (Warren *et al.* 2008). MAXENT (v. 3.3.3), was used to estimate the distribution of *A. madagascarensis* based on the 71 localities sampled in this analysis and constrained by ecological/environmental variables that might prevent the population from reaching maximum entropy. GIS layers at 30 arc seconds spatial resolution (~1 km$^2$) of altitude and bioclimatic variables (BIOCLIM) for Madagascar were obtained from WORLDCLIM (http://www.worldclim.org/, Hijmans *et al.,* 2005). The bioclimatic variables were derived from monthly temperature and rainfall data and are believed to represent more biologically significant variables than raw meteorological data (see table 3 for explanation of biological variables). Additionally, a high-resolution vegetation layer, developed by Kew Gardens' "Mapping the Vegetation of Madagascar" project (http://www.kewgardens.org, Du Puy and Moat 1996, 1997, 1998 and 1999), classified the entire area of Madagascar in one of 15 distinct vegetation zones (Figure 4). MAXENT was run under auto features, response curves, pictures of predictions and jackknife measurements with the logistic output format. All

environmental layers were continuous except the vegetation layer, which was categorical. The resulting niche predictions were projected into a map of Madagascar using DIVA-GIS, and the 10 percentile training presence criterion used as the binary point for delimiting predicted presence/absence. Environmental Niche Model (ENM) analyses were run on each cluster indentified by MAXENT and output for each genetic cluster (see results, Table 5) was compared to identify differences between clusters and which environmental variables contributed the most to the niche of any given cluster.

Table 3: Codes for bioclimatic variables obtained from www.worldclim.org (Hijmans *et al.,* 2005).

| Bio1 | Annual Mean Temperature |
|---|---|
| Bio2 | Mean Diurnal Range (Mean of monthly (max temp-min temp)) |
| Bio3 | Isothermality (Bio2/Bio7)(*100) |
| Bio4 | Temperature Seasonality (standard deviation *100) |
| Bio5 | Max Temperature of Warmest Month |
| Bio6 | Min Temperature of Coldest Month |
| Bio7 | Temperature Annual Range (Bio5-Bio6) |
| Bio8 | Mean Temperature of Wettest Quarter |
| Bio9 | Mean Temperature of Driest Quarter |
| Bio10 | Mean Temperature of Warmest Quarter |
| Bio11 | Mean Temperature of Coldest Quarter |
| Bio12 | Annual Precipitation |
| Bio13 | Precipitation of Wettest Month |
| Bio14 | Precipitation of Driest Month |
| Bio15 | Precipitation Seasonality (Coefficient of Variation) |

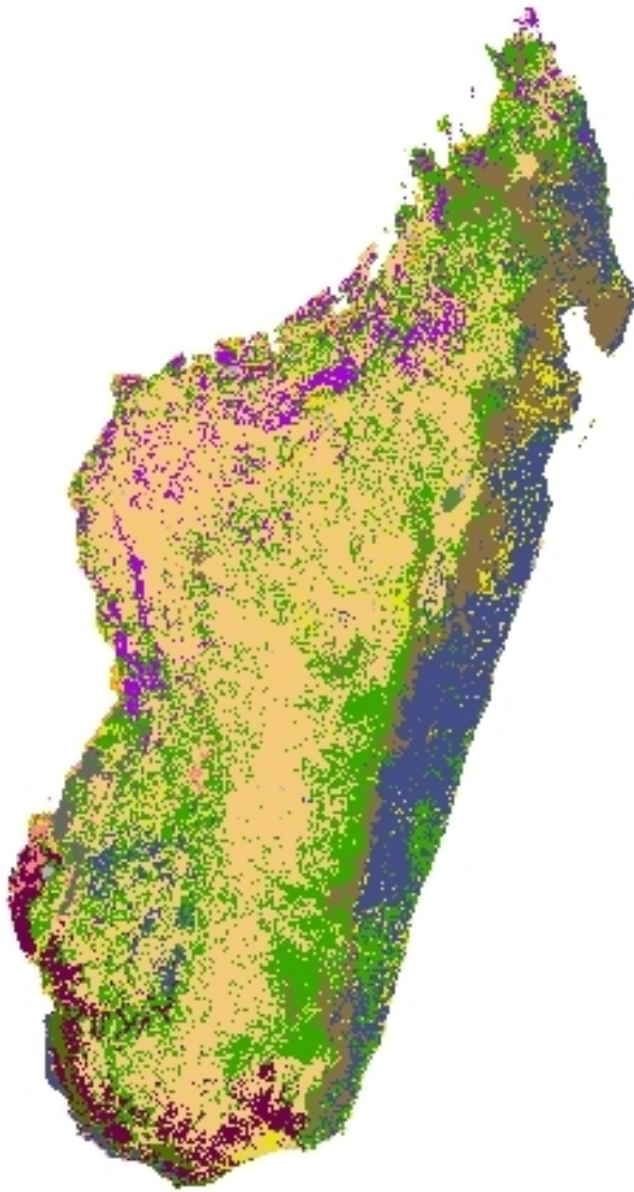| Bio16 | Precipitation of Wettest Quarter |
|-------|--------------------------------|
| Bio17 | Precipitation of Driest Quarter |
| Bio18 | Precipitation of Warmest Quarter |
| Bio19 | Precipitation of Coldest Quarter |

Figure 4: Vegetation map of Madagascar. This map shows the different vegetation layers used for niche modeling. This map is part of the Vegetation Mapping Project of the Royal Botanic Gardens, Kew (Du Puy and Moat, 1996, 1997, 1998 and 1999).

Table 5: Contributions of specific environmental variables to environmental niche models (ENM's) for each cluster. Highlighted cells indicate the variable that contributed most to the model for each of the four clusters. Maps of clusters can be seen in figure 12.

**Percent contribution**

| Environmental variable | Cluster 1 | Cluster2 | Cluster3 | Cluster4 |
|---|---|---|---|---|
| Vegetation layer (vegkew) | 0 | 18.2 | 30.4 | 21.3 |
| Bio1 | 0 | 0 | 3 | 0 |
| Bio2 | 0 | 0 | 0 | 4.5 |
| Bio3 | 0 | 5.3 | 4.8 | 3.1 |
| Bio4 | 0 | 2.8 | 13.2 | 0 |
| Bio5 | 0 | 0 | 0 | 0.6 |
| Bio6 | 0.1 | 0 | 0.8 | 0 |
| Bio7 | 86.8 | 0 | 32.8 | 0 |
| Bio8 | 0 | 0.1 | 0 | 46.7 |
| Bio9 | 0.1 | 0 | 0.5 | 0 |
| Bio10 | 0 | 0 | 0.5 | 0 |
| Bio11 | 0 | 0 | 0 | 0 |
| Bio12 | 0.4 | 0 | 0 | 3.5 |
| Bio13 | 0.1 | 1.2 | 0 | 0 |
| Bio14 | 9.9 | 53.8 | 11.9 | 0 |
| Bio15 | 0 | 18.7 | 0.8 | 0.1 |
| Bio16 | 0 | 0 | 0 | 0 |
| Bio17 | 0.4 | 0 | 0.1 | 20.1 |
| Bio18 | 2.1 | 0 | 1.3 | 0 |

| | | | | |
|---|---|---|---|---|
| Bio19 | 0 | 0 | 0 | 0 |

*Niche Differentiation*

In addition to using niche models to project suitable habitats for each cluster, niche identity tests were performed using ENMTools. Niche differentiation was assessed using the niche equivalency and niche similarity methods of Warren *et al.* (2008) using ENMTools (v. 1.1) to measure the degree of ecological overlap between clusters. ENMTools uses two statistical measures: Schoener's $D$ ($D$ ($p_x$, $p_y$) = $1 - ½ \Sigma | p_{x.i} - p_{y.i} |$) and Warren's $I$ ($I$ ($p_x$, $p_y$) = $1 - ½H$ ($p_x$, $p_y$)) to compare ENM predictions. Tests of equivalency and similarity determine whether one 'taxon's' niche is identical to that of another 'taxon' and whether it predicts that of another 'taxon' better than expected by chance alone. The niche identity test was used to determine if the environmental niche models (ENMs) created by ENM analyses are more dissimilar than if they were sampled from the same underlying distribution. For this test, files containing the occurrence of each cluster were imported and the number of replicates was set to 100. This allowed for every possible pairwise comparison between the clusters. Subsequently the background similarity test was used to determine if any of the clusters predicted ENM's could predict the occurrence of another cluster better than expected by chance alone. For this test, a file containing the occurrence data for one cluster (the focal 'taxon') and a file containing a mask of the ENM of another cluster were used to randomly generate background samples and determine whether two species are more ecologically divergent than if they were randomly sampled from within their respective habitats (see Warren *et al*'s ENMTools User Manual, v1.0) again with 100 replicates.

RESULTS

*Phylogenetic Analyses*

A gene tree was constructed using mitochondrial sequence data from the CO I gene with

*Anochetus grandidieri* as outgroup to root the tree (Figure 5). Following construction of the

gene tree, a haplotype network was constructed (Figure 6)

The Nested Clade Analysis recovered evidence of range expansion onto the island Nosy

Be (clade 2-2) and two instances of allopatric fragmentations (clade 4-1 in the North and clade 4-

2 in the South) (Figures 7 & 8). NCA recovered four step clades shown as gray boxes in figure
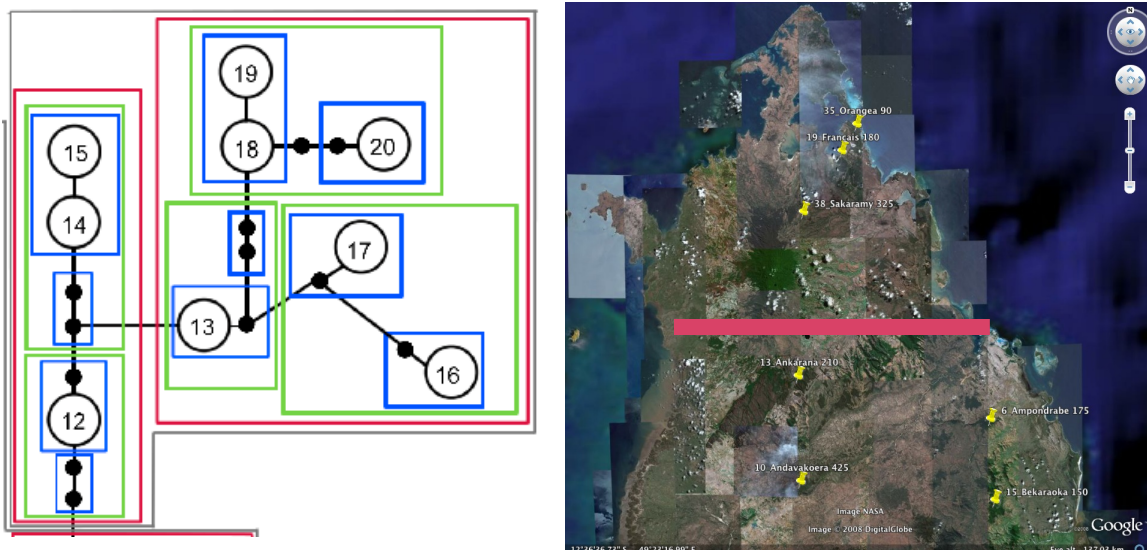
6.

Figure 7:  Nested Clade Analysis showing possible allopatric fragmentation in the northern tip of Madagascar.  The pink line denotes the split between Clade 3-3 in the north and 3-2 in the south.
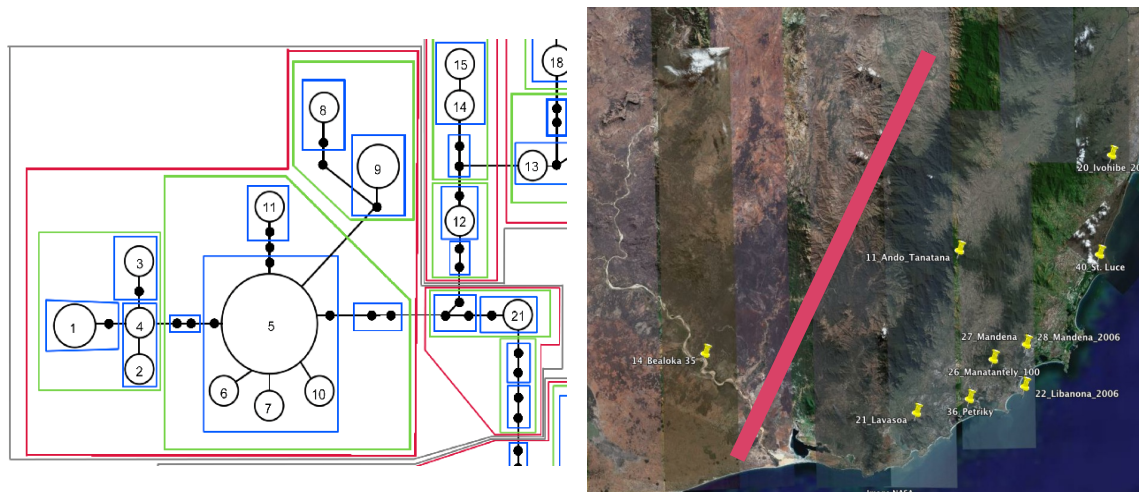


Figure 8:  Nested Clade Analysis showing possible allopatric fragmentation in the southern tip of Madagascar.  The pink line denotes the split between Clade 2-1 in the west and 3-1 in the east.

Sequence data from the three anonymous loci averaged 404 base pairs and contained an average of seven variable sites.  GENELAND was used to infer the number of distinct genetic clusters (K) within *A. madagascarensis* and assigned individuals to clusters based on posterior probability of membership.  GENELAND's correlated run recovered six clusters (Figure 9a) whereas the uncorrelated run recovered four clusters (Figure 9b).  Because the correlated model seems to have algorithm instabilities and can have a tendency to depart from the model assumptions, I only considered the results from the uncorrelated run (Guillot *et al.,* 2009).  The geographic distribution of the individuals assigned to each of the genetic clusters was

incorporated into the GENELAND analysis and a map depicting probability of presence for each

cluster was generated. There appears to be no correlation between the recovered clusters and

watersheds located at high altitude or low altitude on mainland Madagascar (Figure 10). Rather,

the clusters seem instead to be separated by major rivers. Of the four clusters indentified from

the uncorrelated analyses, one (#1) is restricted to the Comoros Islands off the northwest coast of

Madagascar (Fig. 10a), cluster #2 is found along the eastern portion of Madagascar, south of the

Antainambalana River but not south of the Mandrare River (Fig. 10b), cluster #3 is restricted to

the northern tip of Madagascar, north of the Sofia River to the west and the Antainambalana

River on the east (Fig. 10c), cluster #4 is restricted to the western side of Madagascar, south of

the Sofia River in the north but not east of the Mandrare River (Fig. 10d). $F_{ST}$ values indicated a

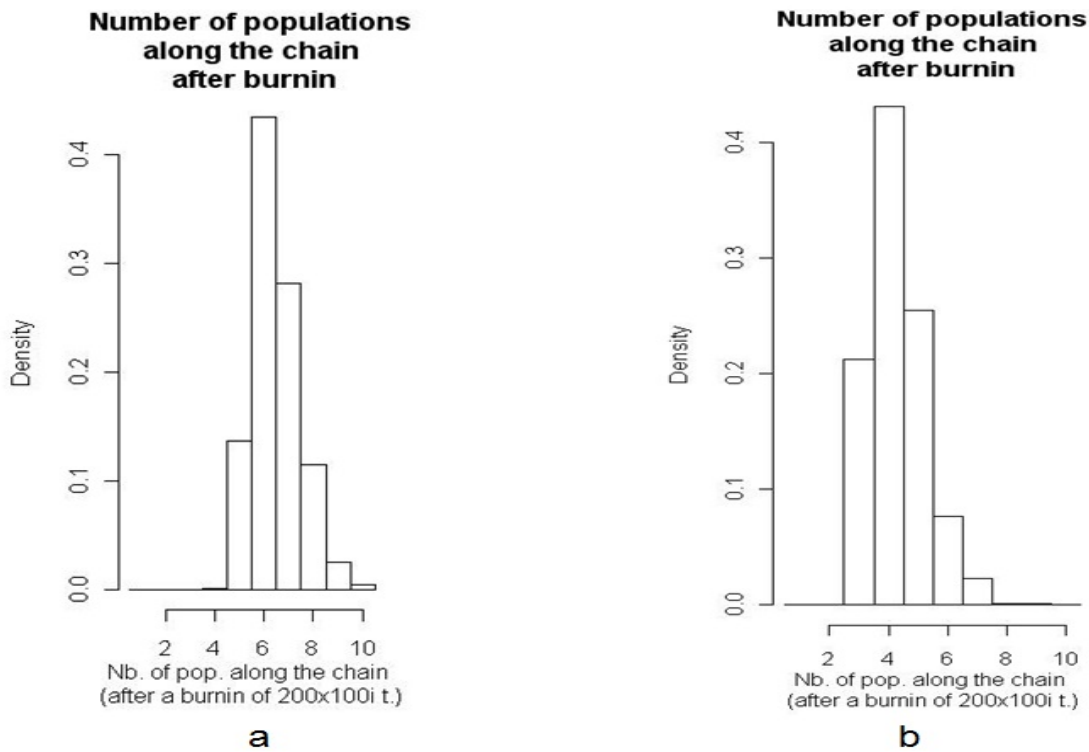high level of genetic differentiation among clusters under the uncorrelated model.



Figure 9: Estimated number of population clusters from GENELAND analyses. Pooled results
of posterior density distribution of the number of clusters estimated by GENELAND analysis in

10 out of 10 replicates for correlated (a) and uncorrelated (b).  Correlated and uncorrelated runs recovered 6 and 4 clusters, respectively.
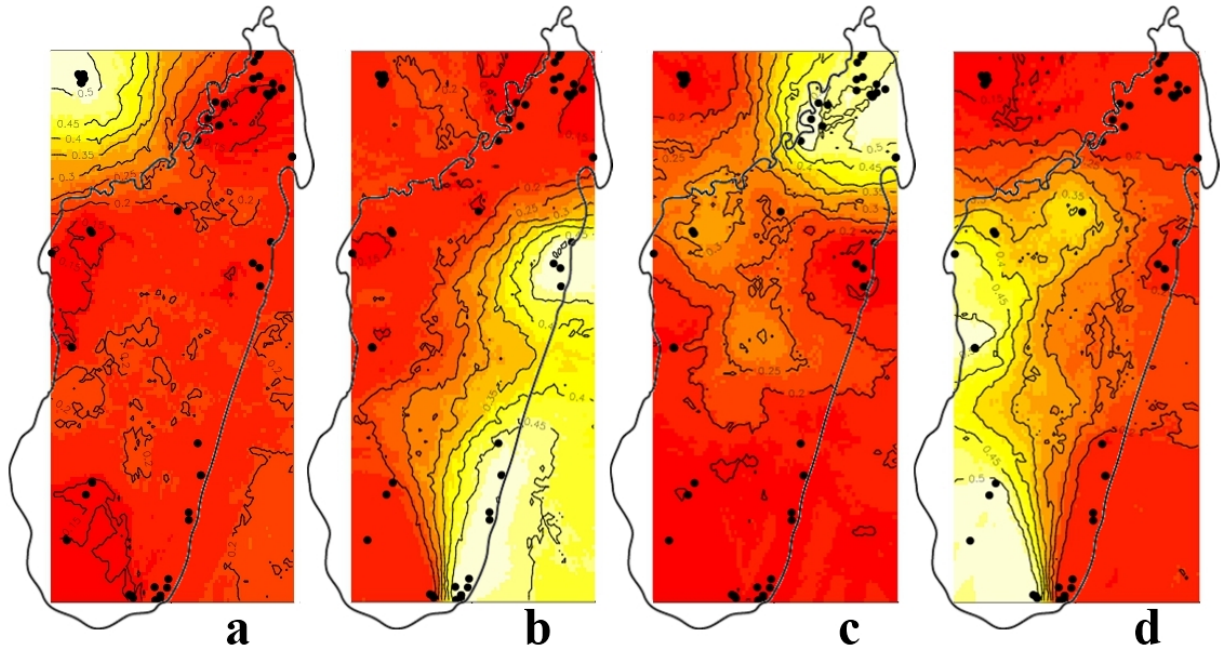


Figure 10:  Map of GENELAND population assignments to clusters for the uncorrelated run. The four plots represent the probability of assignment of pixels to each cluster: (a, cluster 1) Comoros Island cluster, (b, cluster 2) eastern portion of Madagascar, south of the Antainambalana River but north of the Mandrare River, (c, cluster 3) northern tip of Madagascar, north of the Sofia River to the west and the Antainambalana River on the east and (d, cluster 4) western side of Madagascar south of the Sofia River in the north but not east of the Mandrare River.  Individual assignment ranges from highest probability (light yellow) to lowest probability (dark red).

The results of the uncorrelated GENELAND runs were used to structure the demographic analysis using IMa2.  A simplified version of the same mtDNA tree was used to determine the relationships between the four proposed clusters (Figure 23).  The results of the four-population IM analysis can be found in table 4.  The greatest amount of migration observed was from cluster 3 to cluster 2 at 19.99 M/μ (migration per mutation) (Table 4, Figure 11).  Low levels of migration were found from the ancestral populations of clusters 2, 3 and 4 (A and B of Figure 23) to cluster 1 (1.595 and 4.737 M/μ respectively).  However, it appears that there is only a

small amount of migration between most of the clusters on the mainland of Madagascar which

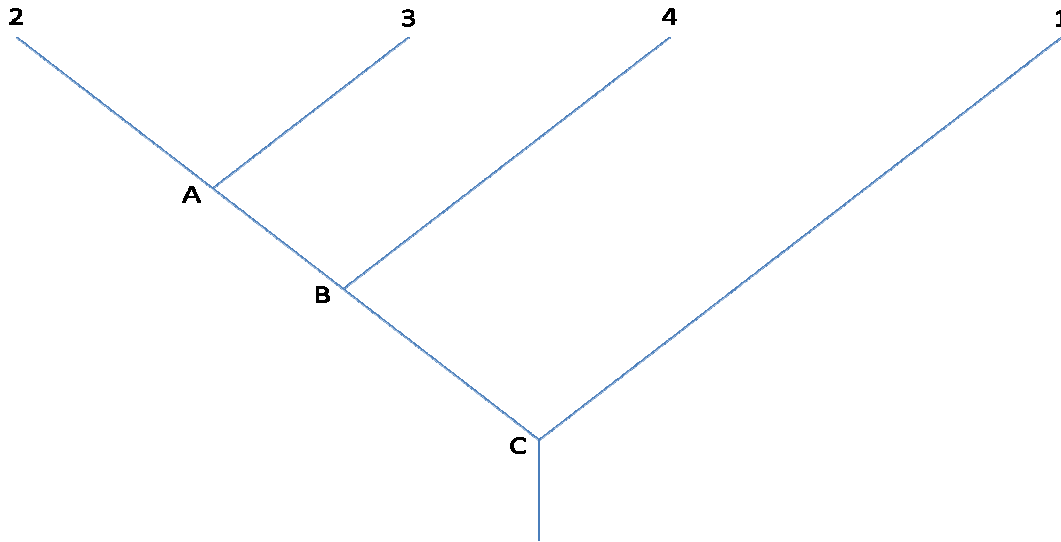suggests that there is a barrier to migration and the clusters are indeed isolated from one another.



Figure 23:  Simplified version of the mitochondrial gene tree of the Cytochrome oxidase I (COI)
gene for *Anochetus madagascarensis*.  The tips show the four proposed clusters and the nodes
show three ancestral populations.  *Anochetus grandidieri* was used as the outgroup to root the
tree.

Table 4:  Estimates of migration between genetic clusters from IMa2.  Directionality of
migration is from the horizontal axis to the vertical axis.

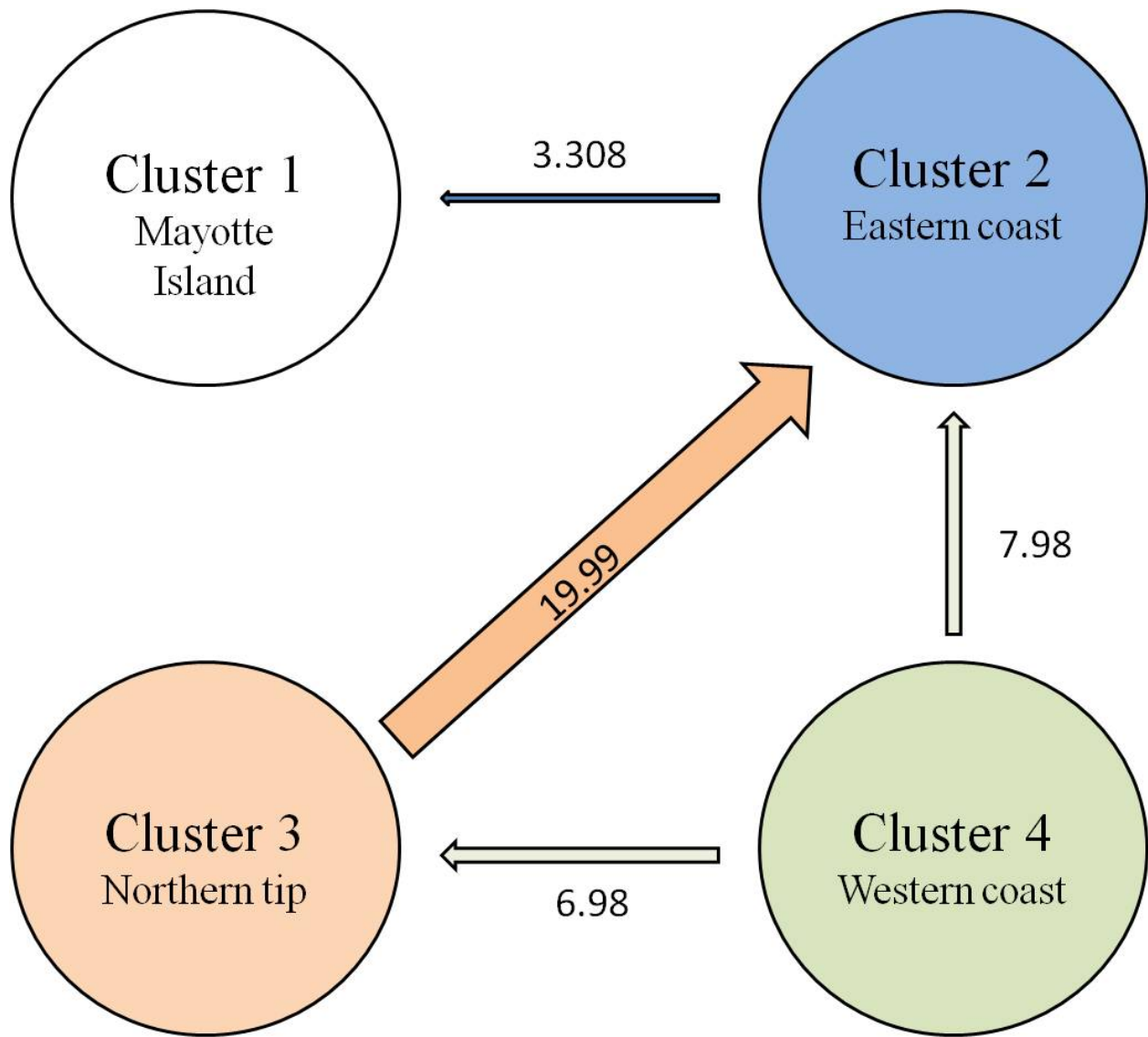|   | 1 | 2 | 3 | 4 | A | B |
|---|---|---|---|---|---|---|
| **1** | 0 | 3.308 | 0.4275 | 0.075 | 1.595 | 4.737 |
| **2** | 0.0025 | 0 | 19.99 | 7.98 | 0 | 0 |
| **3** | 0.0125 | 0.0025 | 0 | 6.98 | 0 | 0 |
| **4** | 0.0025 | 0.0025 | 0.01 | 0 | 0.025 | 0 |
| **A** | 0.0025 | 0 | 0 | 0.01 | 0 | 0 |
| **B** | 0.0025 | 0 | 0 | 0 | 0 | 0 |

Figure 11: Isolation with migration analysis. The four circles are the four GENELAND proposed clusters. The arrows correspond to migration per mutation (M/μ) values from each cluster to the cluster where migration is occurring. The largest amount of migration is occurring from cluster 3 (the northern cluster) to cluster 2 (the eastern cluster) at 19.99 M/μ. Cluster 4 (the western cluster) has migration occurring to both cluster 2 and 3, at 7.98 and 6.98 M/μ respectively. Cluster 2 has a small amount of migration occurring to the Comoros Islands (Mayotte Island) cluster (cluster 1) at 3.308 M/μ.

*Niche Modeling*

Environmental niche models (ENMs) for all four clusters have high AUC (area under the receiver-operating characteristic curve) statistics, 0.999, 0.984, 0.995 and 0.868 respectively. These numbers indicate the "fit" of the model to the testing data, or the suitability scores. Cluster 1's predicted distribution has a low suitability score for any region on Madagascar with the entirety of the predicted area limited to Mayotte Island of the Comoros Islands located off the northwestern coast of Madagascar (Figure 12a). Mayotte Island is the closest island to Mainland Madagascar (~452.28 km from Antisiranana on the northern tip of Madagascar). Cluster 2, 3 and 4 are predicted to have mainland Madagascar distributions limited to the eastern coastline, fragmented patches of the northern tip of the island, and the west respectively (Figure 12b, c, d). These ENMs correspond well to the areas predicted by GENELAND analysis (Figure 10).
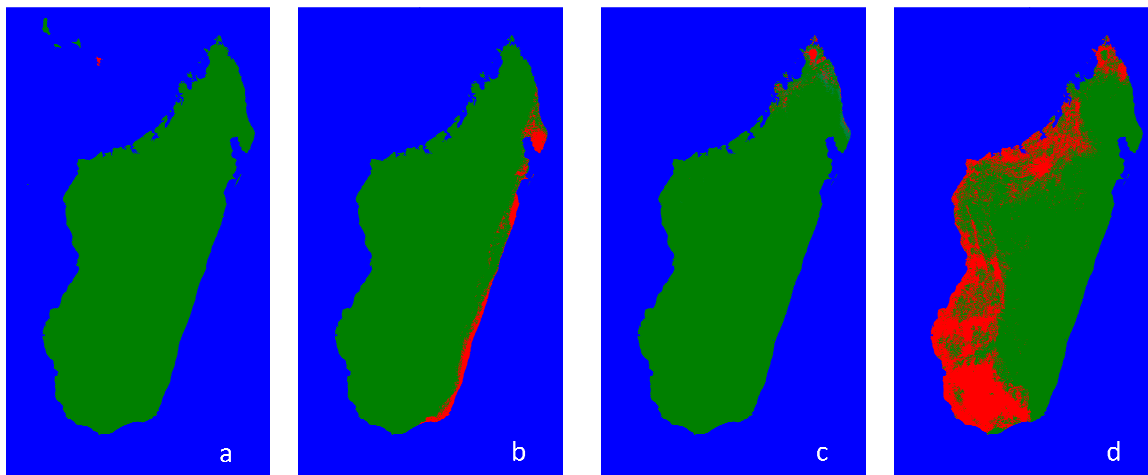


Figure 12: Ecological niche model predictions for population clusters of *Anochetus madagascarensis*. Maps (a-d) correspond to predicted geographic distributions of cluster 1, cluster 2, cluster 3 and cluster 4 respectively. The areas shaded in red indicate suitable habitat at the ten percentile training presence (0.480, 0.082, 0.300 and 0.417 respectively).

Each cluster varied in which parameters were ecologically important, i.e. contributed to the ENM. Table 5 shows the relative contribution of each variable to the niche model for the four identified clusters.

Two variables contributed the most to delimiting the predicted range for the Comoros Island population (#1); annual temperature range contributed 86.8% while precipitation of the driest month contributed 9.9% to the model. Notably, the Kew vegetation layer was unavailable for the Comoros Islands and was not included in the analysis for this cluster.

Three variables contributed significantly to delimiting the range of the mainland eastern cluster (#2); precipitation of the driest month contributed the most with 53.8%, precipitation seasonality and the vegetation layer also contributed (18.7 and 18.2% respectively). With regard to the vegetation layer, it appears that the distribution of this species in the east is in part delimited by the presence of humid forest (Figure 4).

For the cluster restricted to the northern tip of Madagascar (#3), annual temperature range contributed and vegetation, again humid forest, contributed 32.8 and 30.4% to the model respectively. Temperature seasonality (13.2%) and precipitation of the driest month (11.9%) also contributed to the delimitation of the range of this species in the north.

The western cluster (#4) was largely limited by mean temperature of the wettest quarter (46.7%) with vegetation layer (21.3%) and precipitation in the direst quarter (20.1%). Notably, this cluster appears to occur in vegetation classified as "western dry forest", a very different biome from the humid forest supporting clusters 2 and 3.

When niche identity tests were run, all four clusters were found to be unique (i.e. no cluster's ENM could be used to predict the occurrence of another cluster). Using both *I* and D

statistics (Table 6), the null hypothesis of niche equivalency can be rejected for all pairwise comparisons.  When the eastern cluster's ENM was used to predict the occurrence of the northern and western populations, the values for the null distribution ranged from 0.73-0.87 and 0.72-0.95 (north and west respectively) for the Warren's *I* statistic and 0.59-0.80 (N) and 0.54-0.92 (W) for the Schoener's *D* statistic, whereas the niche overlap values were 0.3896 (N) and 0.3377 (W) for *I* and 0.1111 (N) and 0.03321 (W) for *D* (Figures 14 and 15).  When the northern cluster's ENM was used to predict the occurrence of the western population, the values for the null distribution ranged from 0.62-0.88 for the Warren *et al*.'s *I* statistic and 0.44-0.81 for the Schoener's *D* statistic, whereas the niche overlap values were 0.4487 for *I* and 0.1607 for *D* (Figure 16).  Niche identity tests could not be run on the Comoros Island cluster because the vegetation layer was not available for the Comoros Island.

Table 6:  Niche Overlap values.

Warren *et al.*'s I

|  | Cluster 2 | Cluster 3 | Cluster 4 |
|---|---|---|---|
| Cluster 2 | 0 | 0.389613 | 0.337675 |
| Cluster 3 | 0.389613 | 0 | 0.44867 |
| Cluster 4 | 0.337675 | 0.44867 | 0 |

Schoener's D

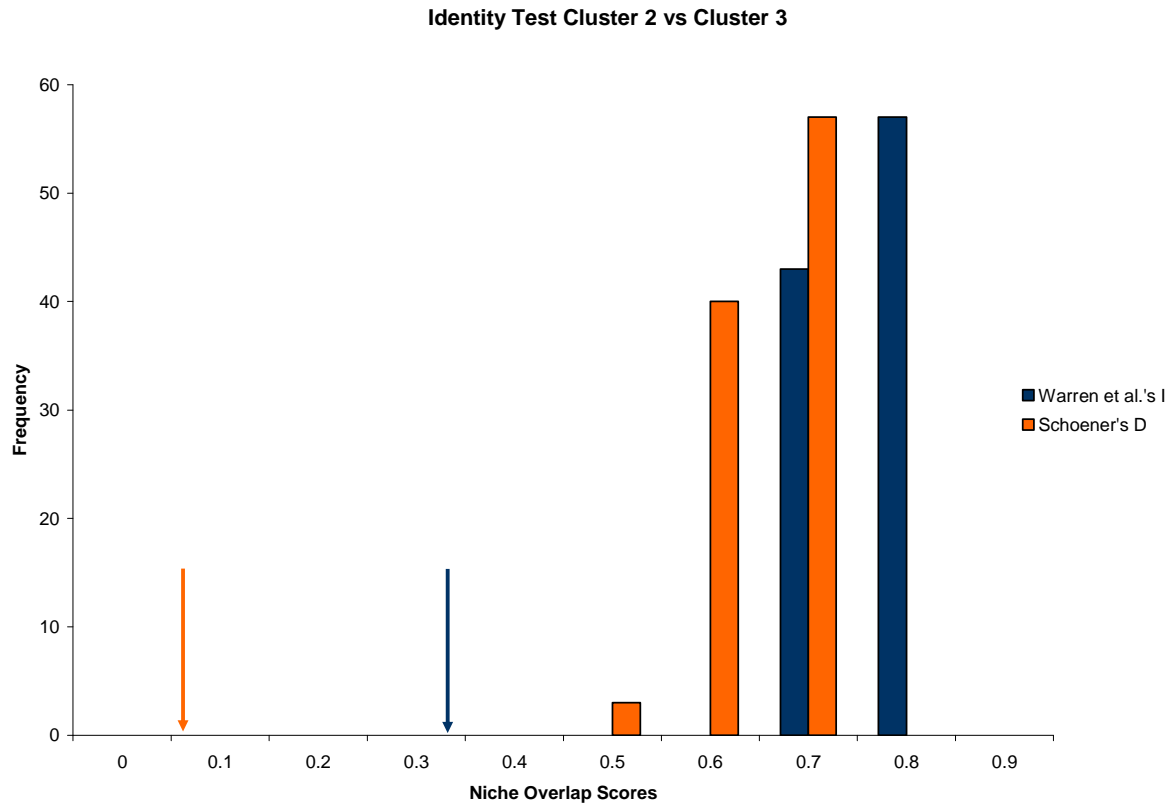|  | Cluster 2 | Cluster 3 | Cluster 4 |
|---|---|---|---|
| Cluster 2 | 0 | 0.111106 | 0.033207 |
| Cluster 3 | 0.111106 | 0 | 0.160716 |
| Cluster 4 | 0.033207 | 0.160716 | 0 |

Figure 14: Niche Identity Test of cluster 2 and cluster 3. Bars represent the null distribution obtained using the identity test on ENMTools for the statistics Schoener's D (0.59-0.80) and Warren *et al.*'s I (0.73-0.87). Arrows correspond to the measured niche overlap between species using Schoener's D (0.1111) and Warren *et al.*'s I (0.3896).
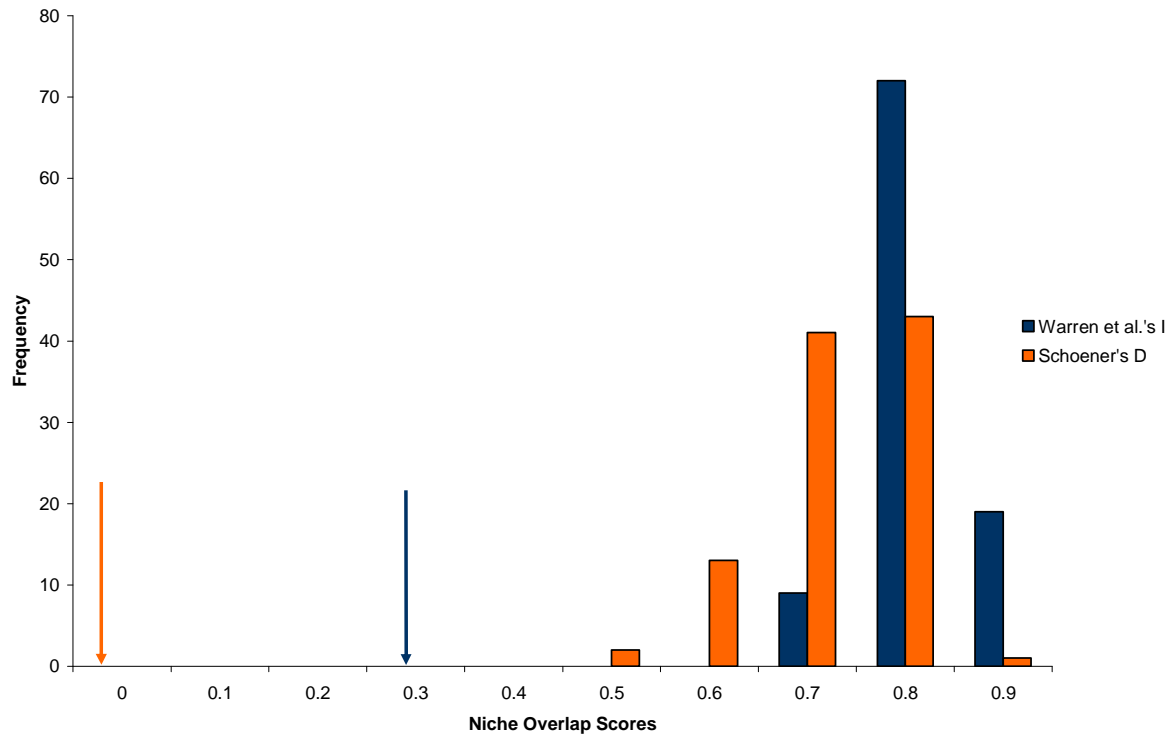
Figure 15: Niche Identity Test of cluster 2 and cluster 4. Bars represent the null distribution obtained using the identity test on ENMTools for the statistics Schoener's D (0.54-0.92) and Warren *et al.*'s I (0.72-0.95). Arrows correspond to the measured niche overlap between species using Schoener's D (0.0332) and Warren *et al.*'s I (0.3376).
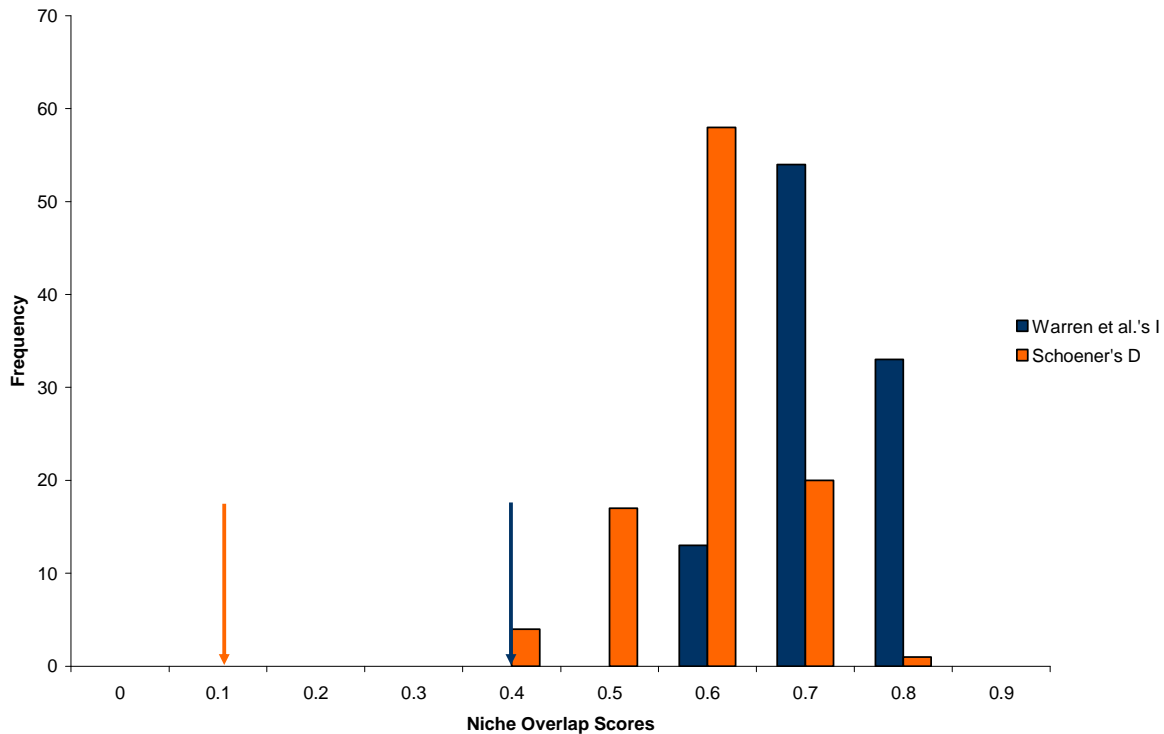
**Identity Test Cluster 3 vs Cluster 4**



Figure 16:  Niche Identity Test of cluster 3 and cluster 4.  Bars represent the null distribution obtained using the identity test on ENMTools for the statistics Schoener's D (0.44-0.81) and Warren *et al.*'s I (0.62-0.88).  Arrows correspond to the measured niche overlap between species using Schoener's D (0.1607) and Warren *et al.*'s I (0.4487).

When niche background tests were run, all three mainland clusters were found to be similar (i.e. the three clusters were not more different than expected by chance given the different areas in which they occur).  Using both *I* and D statistics (Table 6), the null hypothesis of niche similarity cannot be rejected for all pairwise comparisons.  When the background area for the eastern cluster's ENM was used to predict the occurrence of the northern and western populations, the values for the null distribution ranged from 0.30-0.42 and 0.31-0.50 (north and west respectively) for the Warren's *I* statistic and 0.00-0.17 (N) and 0.02-0.24 (W) for the Schoener's *D* statistic, whereas the niche overlap values were 0.3896 (N) and 0.3377 (W) for *I*

and 0.1111 (N) and 0.03321 (W) for *D* (Figures 17, 18).  When the background area for the northern cluster's ENM was used to predict the occurrence of the eastern and western populations, the values for the null distribution ranged from 0.37-0.42 (E) and 0.41-0.56 (W) for the Warren's *I* statistic and 0.07-0.18 (E) and 0.12-0.27 (W) for the Schoener's *D* statistic, whereas the niche overlap values were 0.3896 (E) and 0.4487 (W) for *I* and 0.1111 (N) and 0.1607 (W) for *D* (Figures 19, 20).  Finally, when the background area for the western cluster's ENM was used to predict the occurrence of the eastern and northern populations, the values for the null distribution ranged from 0.32-0.36 (E) and 0.39-0.47 (N) for the Warren's *I* statistic and 0.01-0.06 (E) and 0.08-0.19 (N) for the Schoener's *D* statistic, whereas the niche overlap values were 0.3377 (E) and 0.4487 (N) for *I* and 0.0332 (E) and 0.1607 (N) for *D* (Figures 21, 22).
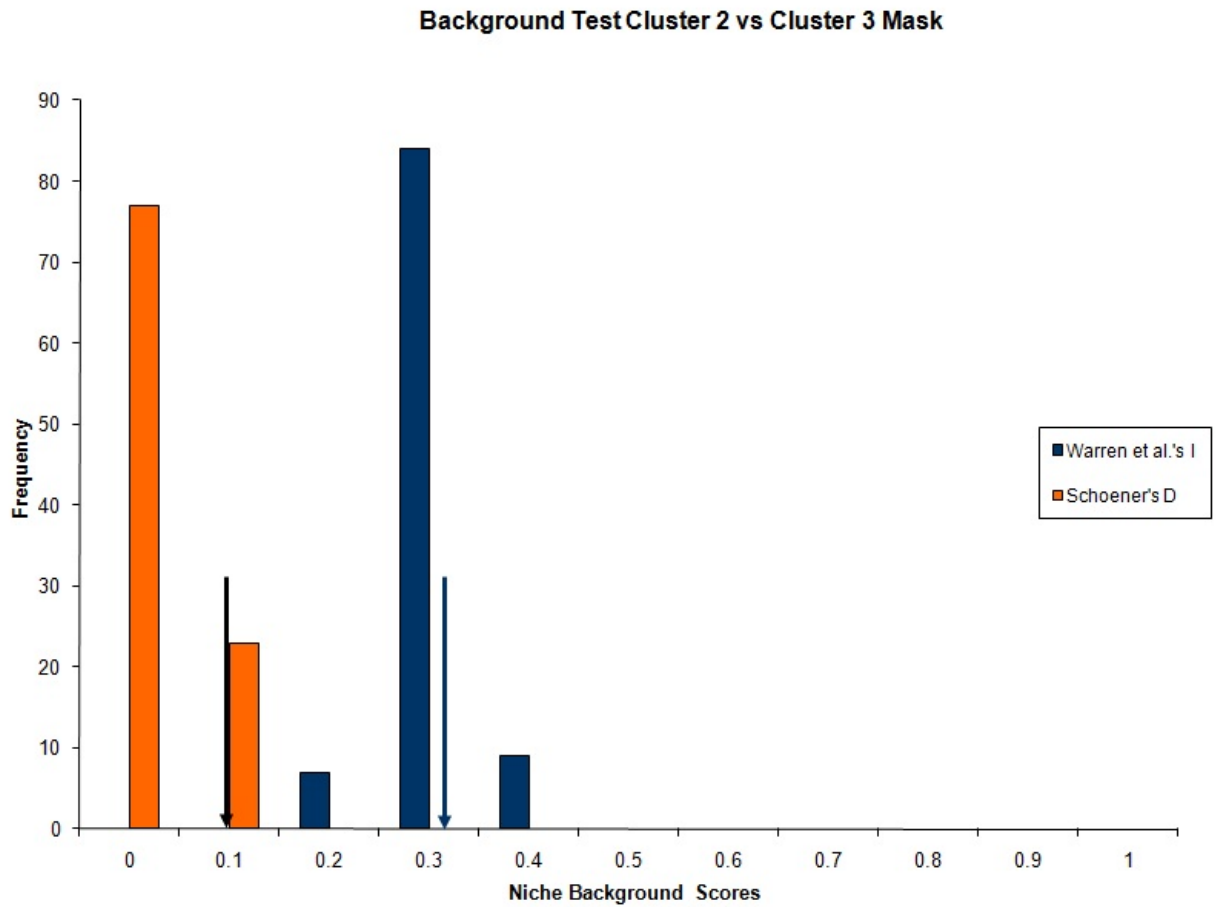
Figure 17:  Niche Background Test of cluster 2 and cluster 3.  Bars represent the null distribution obtained using the background test on ENMTools for the statistics Schoener's D (0.00-0.17) and Warren *et al.*'s I (0.30-0.42).  Arrows correspond to the measured niche overlap between species using Schoener's D (0.1111) and Warren *et al.*'s I (0.3896).
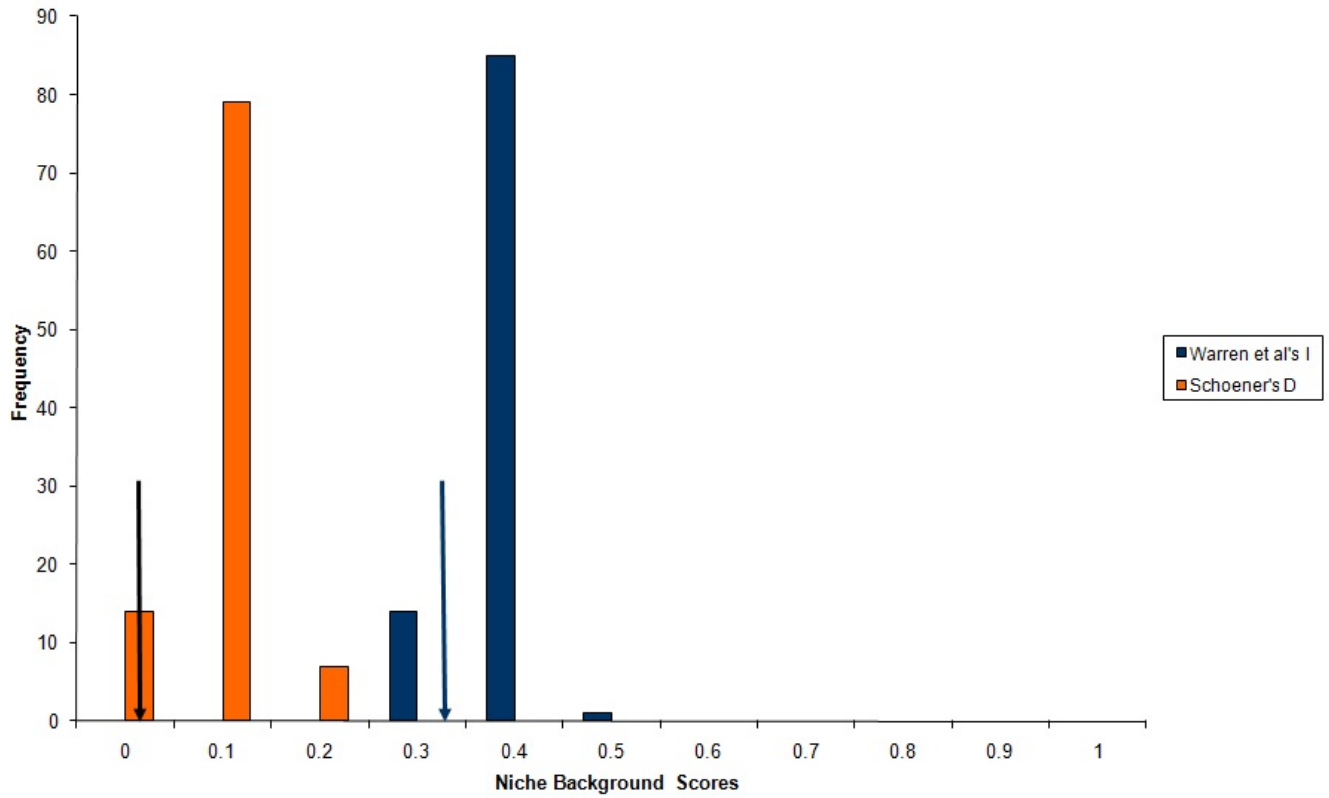
**Background Test Cluster 2 vs Cluster 4 Mask**

Figure 18: Niche Background Test of cluster 2 and cluster 4. Bars represent the null distribution obtained using the background test on ENMTools for the statistics Schoener's D (0.02-0.24) and Warren *et al.*'s I (0.31-0.50). Arrows correspond to the measured niche overlap between species using Schoener's D (0.0332) and Warren *et al.*'s I (0.3376).

Figure 19: Niche Background Test of cluster 3 and cluster 2. Bars represent the null distribution obtained using the background test on ENMTools for the statistics Schoener's D (0.07-0.18) and Warren *et al.*'s I (0.37-0.42). Arrows correspond to the measured niche overlap between species using Schoener's D (0.1111) and Warren *et al.*'s I (0.3896).
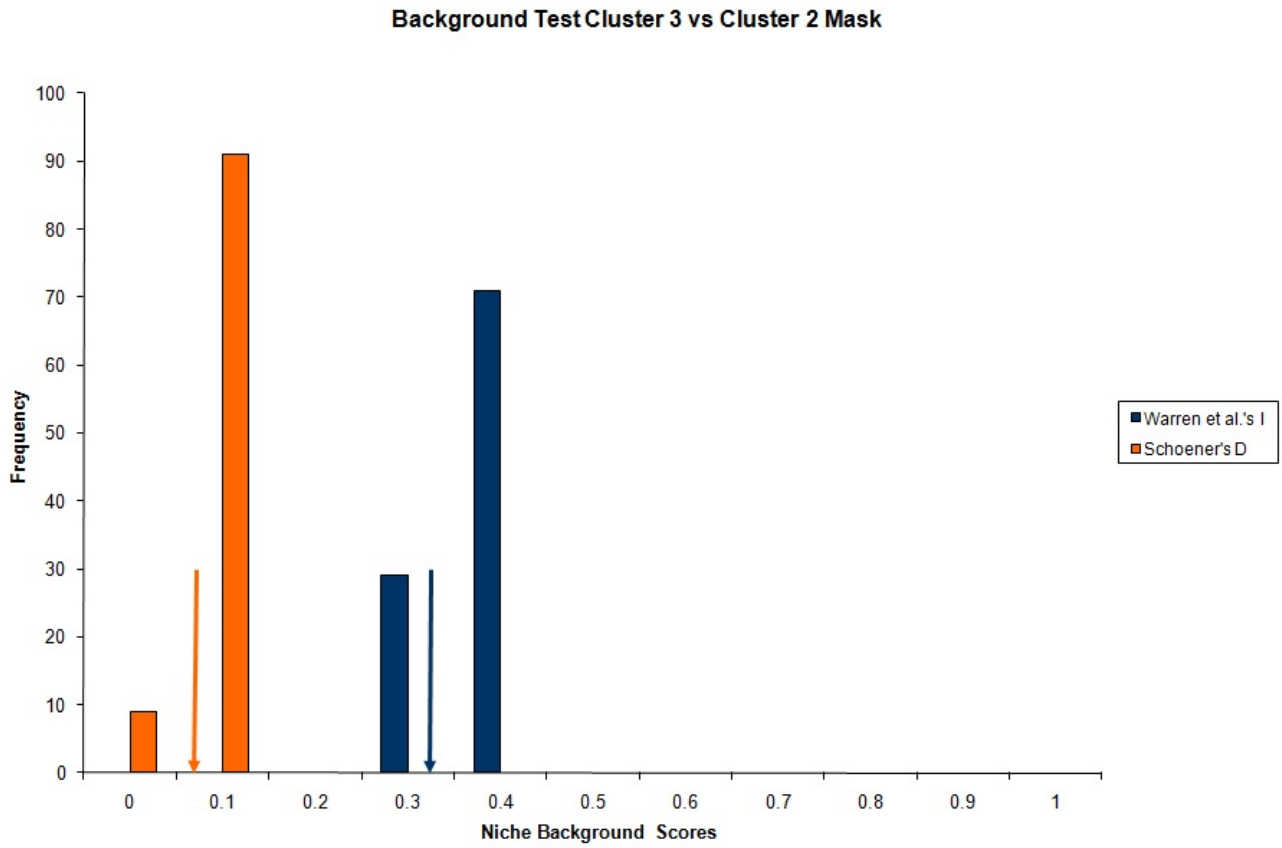
Figure 20: Niche Background Test of cluster 3 and cluster 4. Bars represent the null distribution obtained using the background test on ENMTools for the statistics Schoener's D (0.12-0.27) and Warren *et al.*'s I (0.41-0.56). Arrows correspond to the measured niche overlap between species using Schoener's D (0.1607) and Warren *et al.*'s I (0.4487).

Figure 21: Niche Background Test of cluster 4 and cluster 2. Bars represent the null distribution obtained using the background test on ENMTools for the statistics Schoener's D (0.01-0.06) and Warren *et al.*'s I (0.32-0.36). Arrows correspond to the measured niche overlap between species using Schoener's D (0.0332) and Warren *et al.*'s I (0.3376).
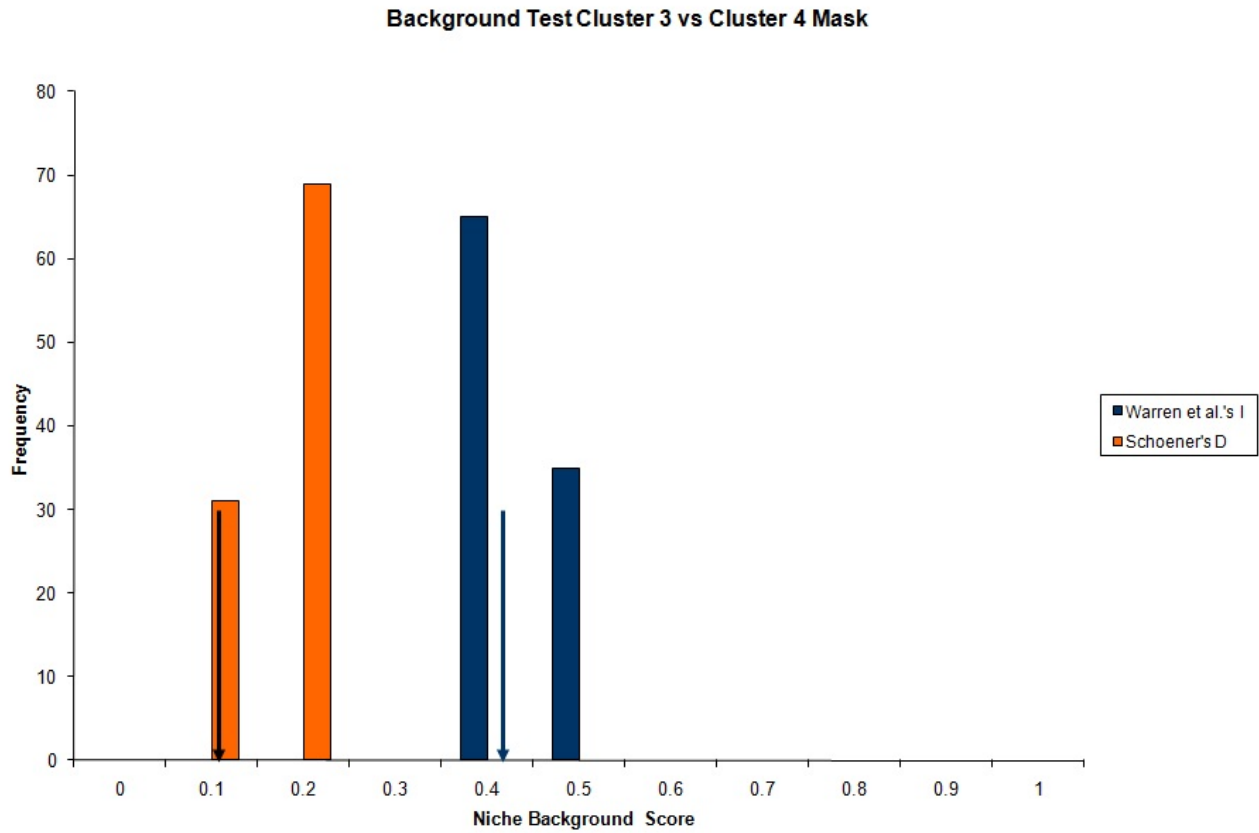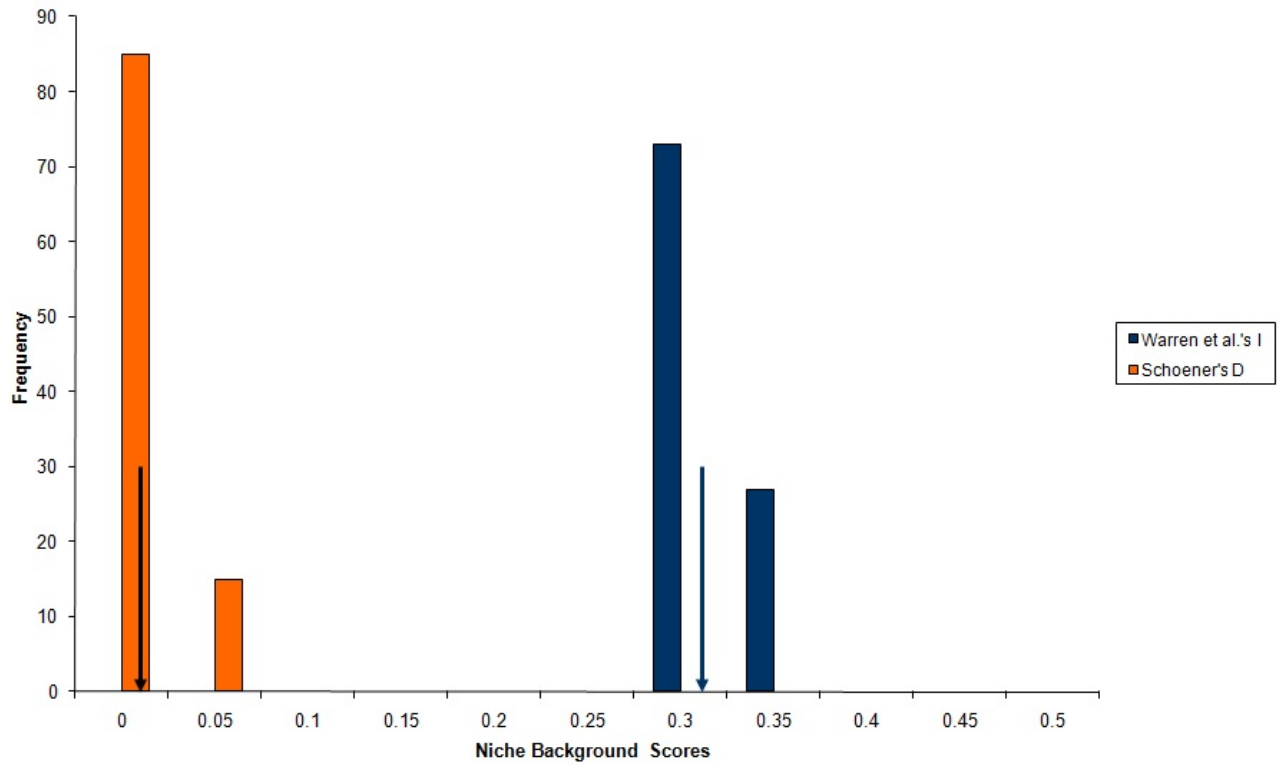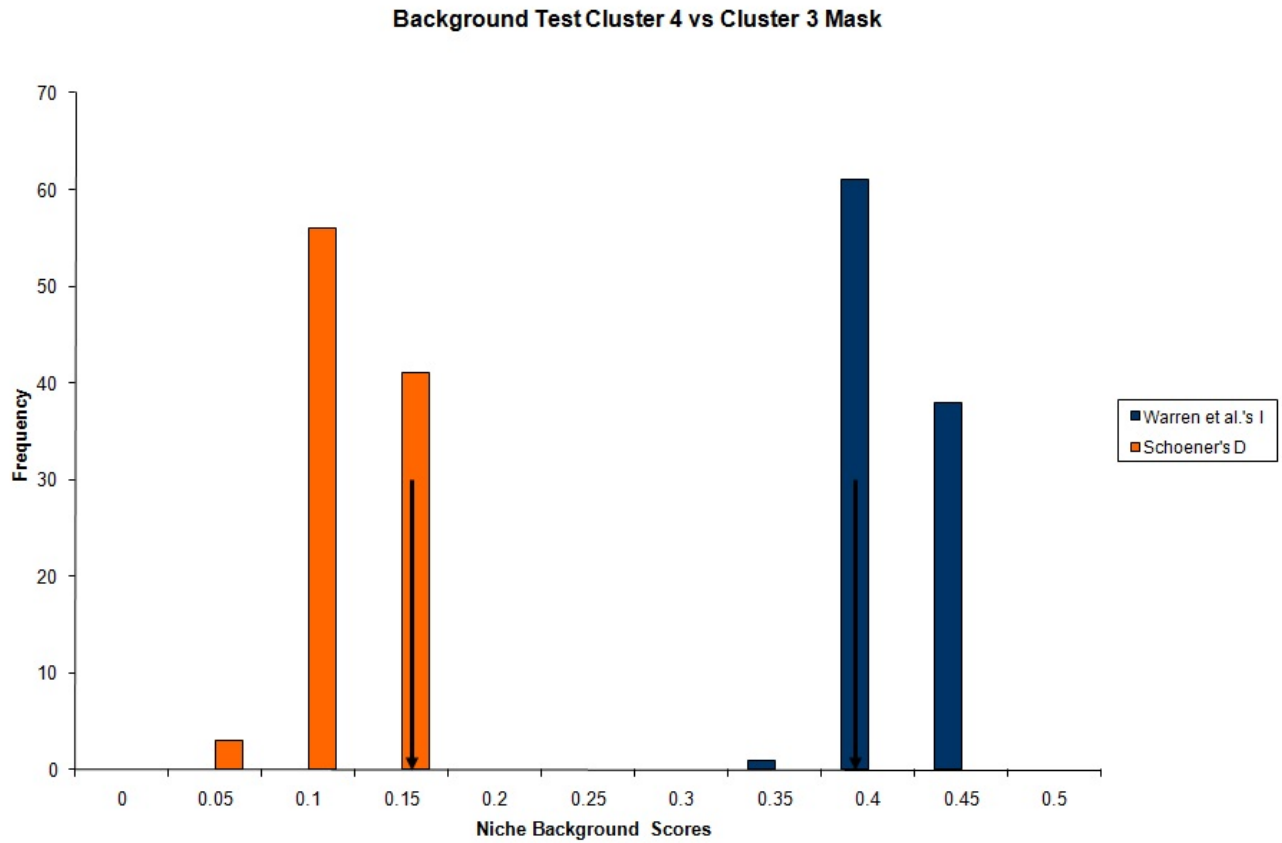
Figure 22: Niche Background Test of cluster 4 and cluster 3. Bars represent the null distribution obtained using the background test on ENMTools for the statistics Schoener's D (0.08-0.19) and Warren *et al.*'s I (0..39-0.47). Arrows correspond to the measured niche overlap between species using Schoener's D (0.1607) and Warren *et al.*'s I (0.4487).

DISCUSSION

*What factors are influencing population structure in* Anochetus madagascarensis*?*

Montane endemism has been found to contribute to diversification of species confined to a narrow elevational range. Wollenberg *et al.* (2008) found that Cophyline frogs have conserved niches in mountain massifs. Because *Anochetus madagascarensis* is not found above 1100 meters (Fisher-Griswold Arthropod Team and the Malagasy Ant Team), this mechanism is not expected to contribute to population structure of this species. Furthermore, IMa2 indicates a high rate of migration between clusters 2 and 4 (7.980, Table 4, Figure 11) which are found on either side of the Ankaratra Massif.

Watersheds have been proposed as causal factors for the diversification of several vertebrate lineages found on Madagascar, including lemurs, geckos and chameleons (Pearson and Raxworthy 2008). However, watersheds do not seem to be associated with population structure across the distribution of *Anochetus madagascarensis*. None of the four clusters recovered by GENELAND analyses (Figure 10a-d) can be attributed to any one watershed. Rather, the geographic clusters recovered for this species by GENELAND appear to be shaped by rivers (Figure 13). For this reason I discount the influence of this mechanism on generating divergence and driving speciation.
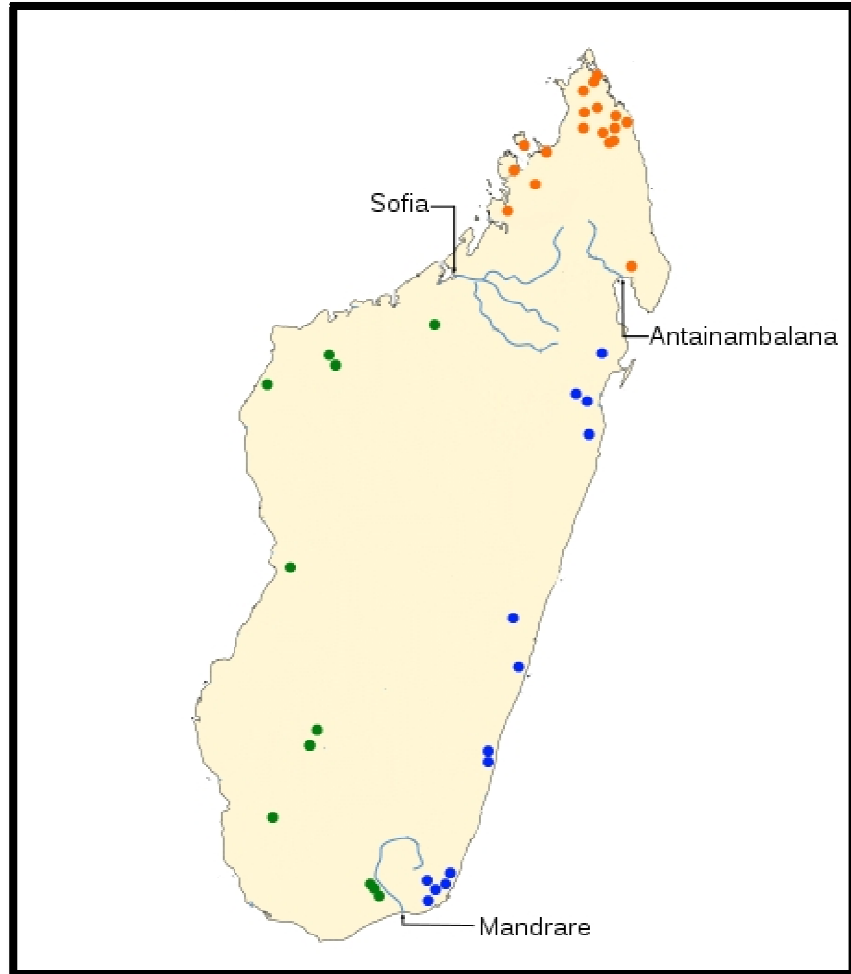
Figure 13: Map of localities for the three mainland clusters. The eastern cluster (cluster 2, blue) is distributed south of the Antainambalana River and north of the Mandrare River. The northern cluster (cluster 3, orange) is distributed north of the Sofia in the West and the Antainamblana River in the east. The western cluster (cluster 4, green) is distributed south of the Sofia River, but not east of the Mandrare River.

Cluster 2, found on the eastern side of Madagascar, is not found north of the

Antainambalana River and is not south of the Mandrare River (Figure 12b). Cluster 3, found on

the northern tip of Madagascar, is north of the Maevarano River on the west and the

Antainambalana River on the east (Figure 12c). Cluster 4, found on the western side of

Madagascar, is south of the Sofia River in the north but not east of the Mandrare River on the

eastern side of Madagascar (Figure 12d). That being said, IMa2 results indicate that migration

has occurred from cluster 3 to cluster 2 (19.99 M/μ) and from cluster 4 to cluster 3 (6.980 M/μ) (Table 4, Figure 11). Migration would not be possible between these clusters if the rivers were absolute barriers to gene flow. There have been several other studies of Malagasy fauna that have found that rivers do not form barriers to gene flow. Townsend *et al*. (2009) found that the watershed, riverine and Pliocene/Pleistocene refugia hypotheses did not contribute to the diversification of the *Brookesia* Leaf Chameleons. See also Goodman and Ganzhorn (2004) where they found that several lemur species have elevational ranges that allow them to exchange alleles across rivers at the headwaters. Solomon *et al*. (2008) also found that the Amazon River is not a barrier to gene flow for leafcutter ants (*Atta* spp), and that in fact it could be marine incursions in the Miocene or climate changes in the Pleistocene or both that led to the diversification of the leafcutter ants. However, populations of *A. madagascarensis* seem to be structured around these rivers. In evolution of a species, conservatism of the ecological niche is expected during diversification (Webb *et al.*, 2002). This stems from active stabilizing selection from ancestral or fixed traits limiting the potential variety of outcomes during evolution of niches (Lord *et al.,* 1995). Very early ENM work exploring niche overlap in clusters separated by a geographic barrier, such as a river, supports evolutionary diversification characterized by niche conservatism (Peterson 2001). When considering the niche models for each of the four recovered clusters of *A. madagascarensis*, the three mainland clusters do not occupy identical niches (we can reject the null hypothesis of niche equivalency). However, their niches are similar enough that if there was no barrier, such as a river, between them, the clusters could exist in the same areas (we are unable to reject the null hypothesis of niche similarity). Thus we conclude that there exists within *Anochetus madagascarensis* to be distinct evolutionary clusters that are separated by a physical barrier that have not yet diverged ecologically. These findings

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suggest a strong role in the fragmentation of populations by river courses for forest inhabiting species in invertebrates and illustrates the utility of using these species to explore evolutionary patterns and the process of speciation in Madagascar.

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# VITA

Nicole Annette Lewis (nee Davison) was born in Pusan, South Korea on May 18, 1977.  She spent most of her childhood outdoors, exploring her hometown of Orange City, Florida.  After graduating from DeLand High School, she attended the University of Florida, Gainesville.  In 2006, she graduated *cum laude* with a baccalaureate degree in Zoology.  While working on her undergraduate degree, she pursued a research project working in the Photosynthesis lab under Drs. Julia Reiskind and George Bowes.  Here, she received a grant to isolate the PPDK gene found in *Hydrilla.*

After graduation, she worked as a scientist for the cancer/genetics research center at the University of Florida.  During that time, she gained experience using several molecular techniques. Wishing to further her education, she enrolled as a master's student in the Biology Department at the University of Mississippi.